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Purification and Characterization of a Transcription Factor That Confers Promoter Specificity to Human RNA Polymerase I

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A whole-cell HeLa extract was fractionated into two components required for accurate in vitro transcription of human rRNA. One fraction contained endogenous RNA polymerase I, and the second component contained a factor (SL1) that confers promoter selectivity to RNA polymerase I. Analysis of mutant templates suggests that the core control element of the rRNA promoter is required for activation of transcription by SL1. We purified SL1 approximately 100,000-fold by column chromatography and have shown that the addition of SL1 can reprogram the otherwise nonpermissive mouse transcription system to recognize and initiate accurate RNA synthesis from human rDNA. Antibodies raised against SL1 bind preferentially to a protein localized in the nucleolus of primate cells and specifically inhibit in vitro transcription initiating from the human rRNA promoter. By contrast, anti-SL1 does not react with the nucleolus of rodent cells and has no effect on the in vitro synthesis of mouse rRNA by a transcription system derived from mouse cells. These findings suggest that SL1 is a selectivity factor present in the nucleolus that imparts promoter recognition to RNA polymerase I and that can discriminate between rRNA promoters from different species.

To understand the mechanism of transcription initiation, it is necessary both to define the *cis*-acting promoter sequences and to characterize the *trans*-acting proteins that interact with the promoter to carry out the synthesis of RNA. The development of an extract that efficiently catalyzes the de novo initiation of RNA synthesis in vitro has proved to be very useful not only for mapping promoter elements but also for subsequent biochemical analysis of the transcription machinery. These in vitro studies have revealed that each of the three distinct classes of RNA polymerase in eucaryotes displays its own specificity and mechanism for promoter utilization (30 and references contained therein).

The genes encoding the major rRNA species are actively transcribed in the nucleoli of animal cells and account for most of the RNA synthesized in growing cells. Unlike RNA polymerases II and III, RNA polymerase I acts on only one type of promoter sequence that is reiterated about 200 times per haploid mammalian genome. The transcription of rRNA is regulated and responds to a wide range of stimuli, including the growth state of the cell, nutrient starvation, cell cycle regulation, and viral infection (1, 10, 13, 15, 22, 26, 28, 29, 31). Thus, the synthesis of rRNA by RNA polymerase I offers an opportunity to study a unique regulatory system.

Several laboratories used in vitro transcription systems to map the sequences that constitute the promoter region recognized by mammalian RNA polymerase I (11, 17). These studies also revealed an unexpected and interesting specificity intrinsic to rRNA transcription. Extracts that are prepared from human cells and that actively transcribe the human rRNA promoter fail to initiate transcription from the mouse rRNA promoter and vice versa (12, 18). A comparison of the regulatory sequences for transcription initiation from mouse and human rDNA confirmed that the rRNA promoter regions have diverged significantly, whereas the structural gene sequences are highly conserved (9). The biochemical basis of this apparent species-specific promoter recognition has been studied by fractionating cell extracts. When such an extract was fractionated into four protein pools by phosphocellulose chromatography (24, 25), all four pools were needed for reconstituting specific and efficient transcription activity. From these initial studies it appears that multiple factors are required for transcription and that in addition to RNA polymerase I there are specific transcription factors required for promoter selectivity and species specificity of the RNA polymerase I transcription system. To understand the mechanism of transcription initiation in detail, it ultimately is necessary not only to identify such factors but also to purify individual components and characterize their biochemical properties.

In this study we addressed the general question of what transcription factors are needed to confer promoter selectivity to human RNA polymerase I. In particular, we attempted to purify a component that is responsible for species-specific transcription in vitro. Using a new fractionation procedure, we separated a whole-cell HeLa extract into two components. The first fraction contains endogenous RNA polymerase I, whereas the second fraction includes a factor SL1 that is required to direct transcription from the human rRNA promoter. We investigated the ability of highly purified SL1 to discriminate between promoters from different species and to program the specificity of RNA polymerase I. Antibodies raised against SL1 were used to determine the intracellular localization of this RNA polymerase I transcription factor and to help characterize its promoter specificity. Finally, we discuss how SL1 may function to impart promoter recognition to RNA polymerase I.

MATERIALS AND METHODS

Whole-cell extract preparation. Extract was prepared from HeLa cells as described by Manley et al. (20). Cells were routinely harvested at densities between 5×10^5 and 7×10^5 cells per ml by centrifugation and subsequently stored at −20°C.

SL1 purification: buffers and resins. The standard buffer used in column chromatography was TM buffer, which consists of 50 mM Tris-hydrochloride (pH 7.9), 1 mM

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EDTA, 12.5 mM $MgCl_2$, 20% glycerol, 1 mM dithiothreitol, and KCl in concentrations indicated below (6).

Heparin-agarose was prepared as described by Davison et al. (5). Bio-Gel P10 (50 to 100 mesh; Bio-Rad Laboratories), Ultrogel ACA34 (LKB Instruments, Inc.), DEAE-Sepharose CL6B (Pharmacia Fine Chemicals), and Bio-Rex 70 (Bio-Rad) were prepared and run according to the recommendations of the manufacturers.

Chromatographic procedures. A HeLa whole-cell extract (80 ml; prepared from 40 liters of cells) was applied to a heparin-agarose column (10 cm by 8 cm^2) equilibrated with TM buffer containing 0.1 M KCl. The column was washed with TM buffer containing 0.1 M KCl (40 ml) and then eluted stepwise with TM buffer containing 0.2 M KCl (200 ml), followed by TM buffer containing 0.4 M KCl (200 ml). Finally, a 400-ml gradient elution of 0.5 to 1.0 M KCl was performed. The transcription activity of individual fractions was determined by the runoff assay.

Fractions containing SL1 activity were pooled (approximately 700 μg of protein), diluted fourfold with TM buffer to reduce the salt concentration, and chromatographed on a small heparin-agarose column (0.5 ml). After loading the sample, the heparin column was washed with TM buffer containing 0.1 M KCl (5 ml) and then step-eluted with TM buffer containing 1.0 M KCl (5 ml). Fractions containing peak concentrations of protein were pooled and then further concentrated by precipitation with 70% ammonium sulfate. The protein pellet was then suspended in 200 μl of TM buffer containing 0.5 M KCl.

The concentrated pool of proteins from the heparin-agarose column was chromatographed on an Ultrogel ACA34 column (25 cm by 0.8 cm^2) equilibrated and run in TM buffer containing 0.5 M KCl. Fractions were tested for transcription activity, and active fractions were pooled (approximately 15 μg of protein), divided into portions, and stored at −70°C.

The 0.4 M KCl eluate from the heparin-agarose column contained RNA polymerase I devoid of SL1 activity. The protein pool (approximately 200 mg of protein) was dialyzed for 3 h against TM buffer containing 0.1 M KCl and then applied to a DEAE-Sepharose CL6B column (7.5 cm by 7 cm^2) equilibrated with TM buffer containing 0.1 M KCl. This column was washed with TM buffer containing 0.1 M KCl (120 ml) and then step-eluted with 120 ml of TM buffer containing 0.225 M KCl. Fractions containing protein eluted by 0.225 M KCl were pooled, dialyzed against TM buffer containing 0.1 M KCl, and frozen in portions at −70°C to be used as a source of endogenous RNA polymerase I in transcription assays (6).

In vitro transcription assays: runoff transcription assays. Specific initiation of rRNA synthesis was assayed by runoff transcription of truncated DNA templates. The concentration of template in the reconstituted transcription reaction was 4 μg/ml. The human rRNA gene was cleaved with either *Bam*HI or *Xho*I, producing a runoff transcript of 1,500 or 1,360 nucleotides, respectively (18). The plasmid carrying a portion of the mouse rRNA gene (prMo3) was truncated with *Pst*I, producing a 2,400-nucleotide in vitro transcript.

Standard transcription reactions containing a total volume of 25 μl included 25 mM Tris-hydrochloride (pH 7.9), 0.5 mM EDTA, 0.5 mM dithiothreitol, 6.25 mM $MgCl_2$, 50 to 80 mM KCl, 250 μM each of ATP, CTP, and UTP, 25 μM GTP, 3 μCi of [α-^{32}P]GTP (Amersham Corp.), 2% polyvinyl alcohol, (P8136; Sigma Chemical Co.), 100 μg of α-amanitin per ml, and 10% glycerol. Reactions were carried out for 30 min at 30°C. After isolation and glyoxalation of the RNA, the in vitro transcripts were analyzed by electrophoresis on a 1.4% agarose gel.

Protein assays. Protein concentrations were measured by the procedure of Bradford with bovine serum albumin as the standard (2). The protein composition of individual and pooled column fractions was determined by electrophoresis on an 8% sodium dodecyl sulfate-polyacrylamide gel (16).

Immunization and preparation of antibody. BALB/c mice were injected intraperitoneally with partially purified SL1 (100 μg of total protein obtained as a pool eluted from heparin-agarose by a 0.5 to 1.0 M KCl step) emulsified in complete Freund adjuvant. Booster injections of 50 μg of protein in incomplete Freund adjuvant were administered 3 and 6 weeks after the primary innoculation. Serum of the mice was tested 7 weeks after the initial immunization. Immunoglobulin G (IgG) was purified from serum by ammonium sulfate precipitation, followed by chromatography on DEAE-Sepharose (14, 19). Protein flowing through the resin was collected and used as a source of antibody for transcription experiments.

Indirect immunofluorescence. COS7 or 3T6 cells were grown on tissue culture plates until they were 40 to 60% confluent. Cells were washed with phosphate-buffered saline and fixed to the plastic with a cold solution of acetone-methanol (1:1 [vol/vol]). Cells were washed with phosphate-buffered saline, allowed to air dry, and then probed with immune or preimmune serum for 15 min at room temperature. After the cells were washed extensively, fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories) was incubated with the cells as before. Cells were viewed with a Nikon UV photomicroscope.

RESULTS

Fractionation of a whole-cell HeLa extract. The preparation of a whole-cell extract from HeLa cells that accurately and efficiently transcribes cloned human rDNA previously was described (18). To isolate and study specific cellular components required for transcription from rDNA, the HeLa cell extract was fractionated by heparin-agarose chromatography (Fig. 1A). All of the endogenous RNA polymerase I activity was eluted from the column by a 0.2 to 0.4 M KCl salt step, but this fraction alone was unable to accurately initiate RNA synthesis from the rRNA promoter (Fig. 1C, h.4). However, the ability of the endogenous RNA polymerase I to initiate transcription at the proper site was restored when proteins that were eluted with high salt (SL1) were combined with the polymerase fraction (Fig. 1C, h.4/h1.0). Thus, accurate rRNA transcription was reconstituted by mixing RNA polymerase I and SL1 but was not observed in the presence of either fraction alone, nor was the efficiency or quality of transcription significantly affected by the addition of other protein fractions (Fig. 1C).

Purification of an RNA polymerase I transcription factor. Extensive purification of SL1 was carried out by the scheme shown in Fig. 2. The first step involved chromatography of the HeLa whole-cell extract on heparin-agarose as described above, except that a linear gradient of KCl was used to elute SL1 from the column. The bulk of the proteins in the extract was eluted between 0.1 and 0.6 M KCl (Fig. 1B), whereas SL1 activity was retained on the resin until 0.65 M KCl (Fig. 3A). Individual fractions were assayed for their ability to restore promoter-specific transcription activity to partially purified RNA polymerase I. A peak of SL1 activity was detected in fractions 36 to 48 (Fig. 3B), and these fractions were then combined to give a 60-ml pool of SL1 ($h\nabla$). The protein concentration was determined by the Bradford dye-

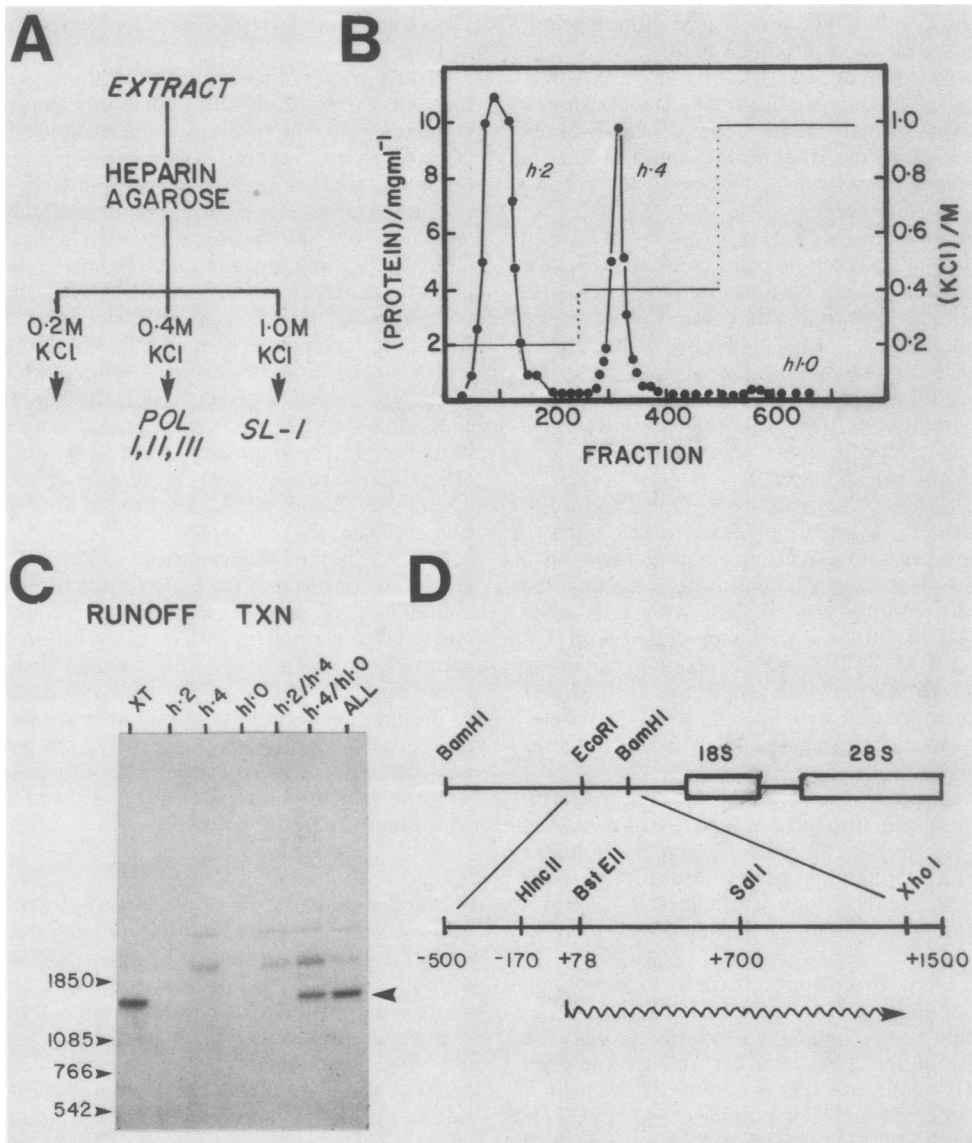

FIG. 1. Fractionation of RNA polymerase I transcription factors by heparin-agarose chromatography. (A) Fractionation scheme of HeLa whole-cell extract on heparin-agarose. (B) Heparin-agarose step elution chromatography. Column fractions were assayed for protein content by the method of Bradford. (C) Runoff transcription. RNA was synthesized in vitro from truncated, cloned rDNA templates, with crude extract (XT) or h.2, h.4, or h1.0 protein pools in the reconstituted transcription system. The template used in these assays was *Bam*HI-cleaved prHu3 (100 ng). Individual enzyme fractions (5 μl) were added as indicated. The level of cryptic RNA products in the reconstituted transcription reaction seen as high-molecular-weight transcripts depends on the reaction conditions, varies between different protein preparations, and is not a general feature of transcription in the fractionated system. The molecular weights indicated at the left were determined with ^{32}P-labeled DNA fragments treated with glyoxal and run in parallel with the in vitro RNA transcripts. (D) Cloned rDNA region containing the RNA polymerase I promoter, shown diagrammatically.

binding assay (Fig. 3A). We estimated that the specific activity of SL1 was increased 3,000-fold relative to that of the crude extract (Table 1) in this single heparin-agarose chromatographic step. The pattern of proteins present in the gradient fractions was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and multiple polypeptides were detected by silver staining (Fig. 3C).

The most active fractions from the heparin-agarose column were pooled and concentrated as described above and then subjected to further purification by gel filtration chromatography. Fractions eluted from the ACA34 column were analyzed as before (Fig. 4), with the peak of SL1 activity contained in fractions 21 to 33. Because the SL1 transcription factor was highly purified at this stage, only small amounts of protein could be detected by silver staining (Fig. 4C). There is a cluster of polypeptides that copurifies with SL1 activity, but the very low levels of protein remaining at this stage preclude definitive identification of SL1. When these fractions were pooled, we calculated that an additional 30-fold purification of SL1 was achieved with good recovery of transcription activity.

The net purification and yield of SL1 are summarized in Table 1. Because SL1 is only one factor in a multi-component transcription system, it is difficult to accurately quantitate the yield of SL1 activity through the various steps. Therefore, we assayed for promoter-dependent transcription by supplementing partially purified RNA polymerase I with the SL1 factor present in the crude extract or in the purified

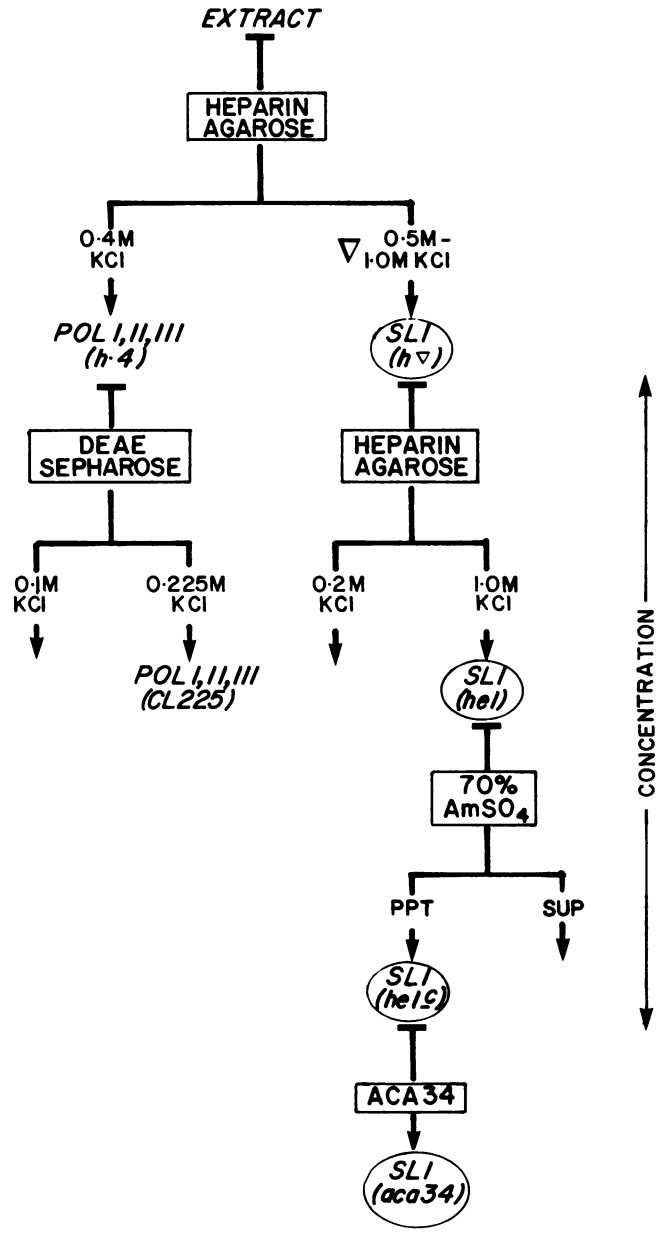

FIG. 2. Purification scheme for the RNA polymerase I transcription factor SL1.

protein pools. By using this assay, SL1 activity is effectively uncoupled from the other transcription factors and can be more accurately measured. Of course, we cannot eliminate the possibility that inhibitors of transcription were removed during the purification and that this loss contributes to the apparent purification of SL1. Estimates for the specific activity of SL1 at the various stages of purification (Table 1) are based on a titration of SL1 in the reconstituted transcription assay.

In addition, we used both primer extension analysis (23) and dinucleotide initiation assays (18, 33) to ensure that RNA synthesis is faithfully initiated by the isolated factors. The results of these assays confirm that transcription is being accurately initiated in the reconstituted system and that purification of SL1 does not reduce the fidelity of RNA chain initiation (data not shown).

Transcription of promoter mutants. Ribosomal DNA (rDNA) templates containing promoter mutations were assayed for their ability to direct RNA synthesis in the fractionated system, and a comparison was made between transcription by purified factors and transcription by crude extract. The construction of rRNA promoter deletion mutants and linker scanning mutants has been described previously (13a, 17). The transcription reactions (Fig. 5) contained both mutant and wild-type rDNA templates plus either whole-cell extract or the endogenous RNA polymerase I fraction, both in the presence and absence of SL1. RNA synthesis was measured by the runoff transcription assay.

TABLE 1. Purification of human SL1

Fraction	Protein concn (per ml)	Total vol (ml)	Total protein	Total transcription units[a]	Sp act[b] (U/mg)	% Yield
Hela whole-cell extract	25 mg	85	2,100 mg	1×10^6	450	100
Heparin-agarose (pooled)	10 μg	65	700 μg	9.1×10^5	1.3×10^6	90
aca34 (pooled)	15 μg	1.0	15 μg	6×10^5	4×10^7	60

[a] One transcription unit equals the number of transcripts per gene in a 1-h reaction. This was determined by determining the titer of the protein being assayed and quantitated by measuring the incorporation of [^{32}P]GTP into the runoff transcript.

[b] Specific activity equals the number of transcription units per milligram of protein assayed.

Mutations in the core promoter element, extending from nucleotides −45 to +8, had a dramatic effect on the level of transcription both in the extract and in the reconstituted system. For example, mutant LS -33/-24 was transcribed at a markedly lower efficiency than was wild-type template, whereas mutant Δ5′-26 was essentially incapable of directing any detectable levels of RNA synthesis in the in vitro reactions. The effect of these mutations on the level of transcription was the same whether purified factors or extract was used to drive transcription. In contrast, removing the upstream control element drastically reduced transcription from the mutant template (Δ5′-83) in reactions containing extract, whereas the effect was less dramatic in the reconstituted transcription reaction with RNA polymerase I and purified SL1 (cf. lanes 1 and 4 with lanes 2 and 5 [Fig. 5]). These findings suggest that SL1 activation of transcription is correlated with core promoter sequences. However, mutations in either the upstream control element or the core sequences did not alleviate the requirement for SL1 for specific initiation of transcription.

Species specificity of SL1. To investigate the relationship of SL1 to the species specificity of rRNA promoter activation, a nuclear extract was prepared from mouse L1210 cells that is capable of initiating accurate transcription from cloned mouse rDNA but not from human rDNA (Fig. 6A, lane 1). We tested the ability of human SL1 to reprogram mouse RNA polymerase I to allow transcription from both human and mouse rDNA. In the presence of increasing amounts of highly purified human SL1, the ability of the mouse extract to transcribe the human template increased, whereas the level of mouse rRNA synthesis diminished (Fig. 6A, lanes 2 to 5). S1 nuclease analysis confirmed that the RNA synthesized in the mouse extract supplemented with human SL1 had the same 5′ end as did authentic human precursor rRNA. In the absence of SL1, no discrete fragment of the

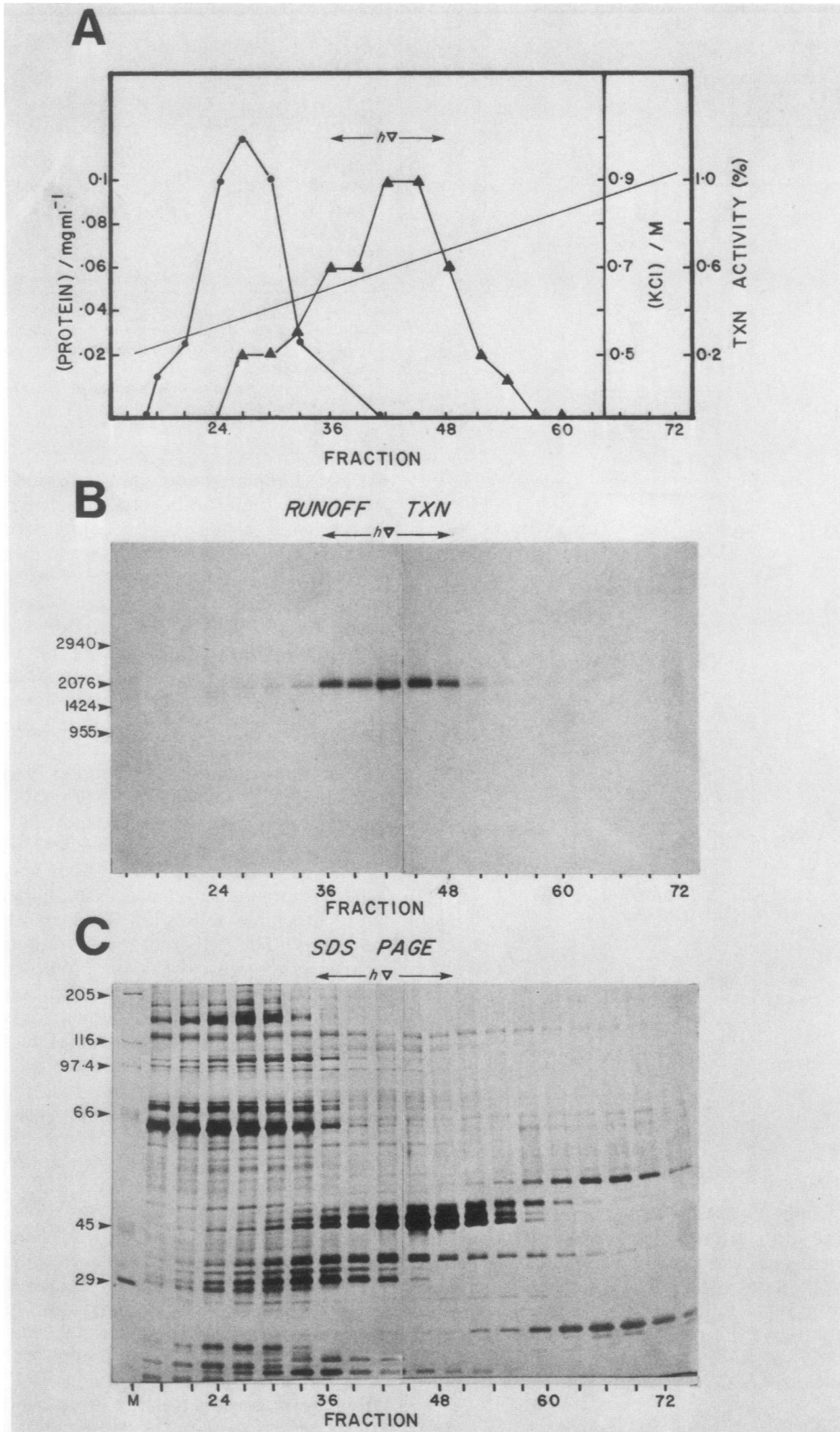

FIG. 3. Purification of SL1 by heparin-agarose chromatography. (A) Profile of gradient elution from heparin-agarose. Fractions (5.0 ml) were assayed for protein (●) by the method of Bradford, for salt concentration (——), or for enzymatic activity by a partially reconstituted RNA polymerase I transcription assay (△). Relative levels of transcription are described as a fraction of the maximum signal. (B) Agarose (1.4%) gel electrophoresis of reaction products from runoff transcription assays of individual fractions. Each assay included partially purified RNA polymerase I (50 μg) and 1 μl of the fraction indicated. End-labeled molecular weight standards were run in parallel to the runoff transcripts. (C) Sodium dodecyl sulfate (8%)-polyacrylamide gel electrophoresis (SDS PAGE) of proteins in gradient fractions. Heparin-agarose column fractions (50 μl) were precipitated at 0°C with 20% trichloroacetic acid and loaded onto the gel. Molecular weight markers were (in daltons): myosin, 205,000; β-galactosidase, 116,000; phosphorylase, 97,400; bovine serum albumin, 66,000; egg albumin, 45,000; carbonic anhydrase, 29,000. The gel was stained with silver.

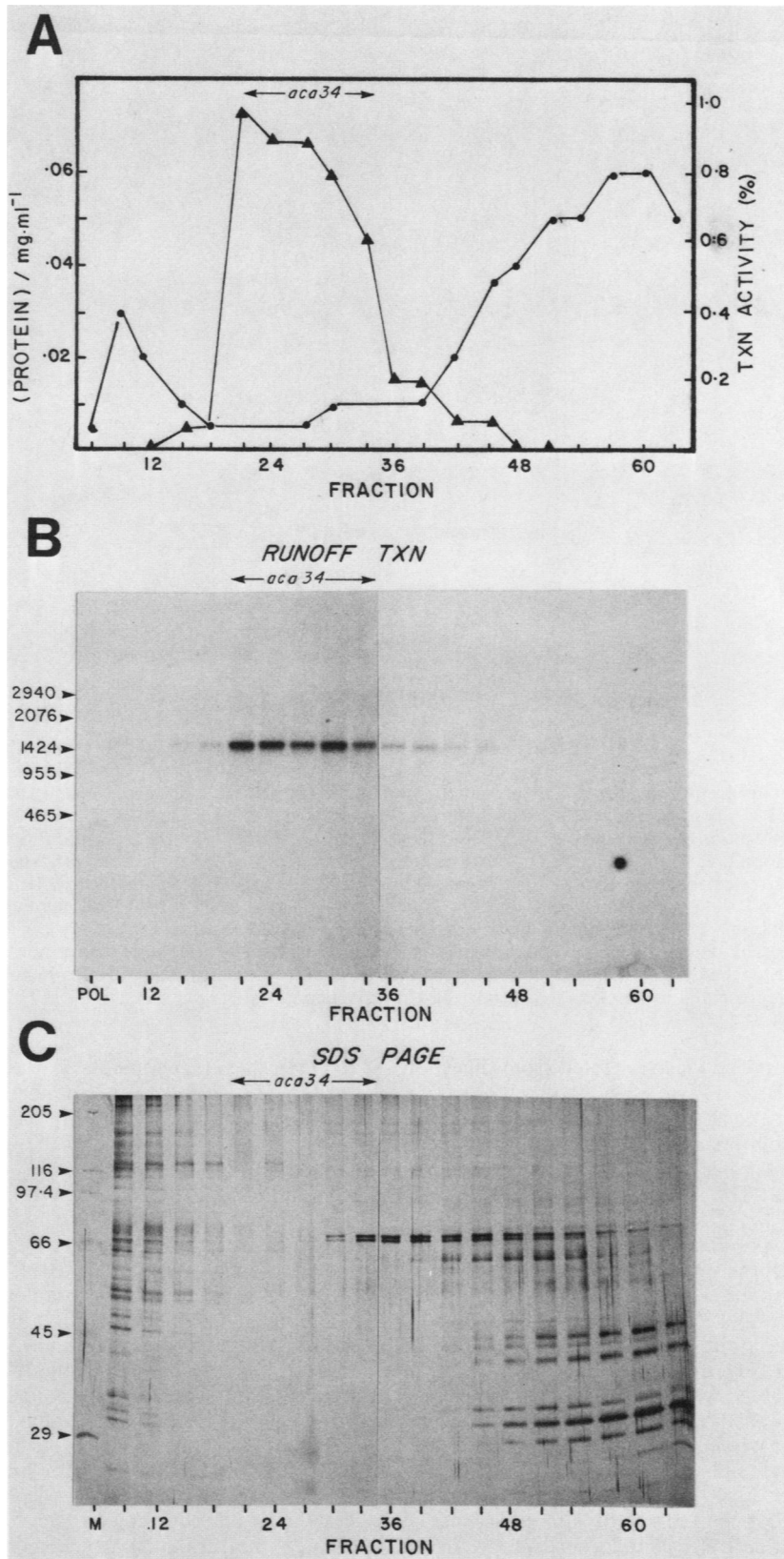

FIG. 4. Purification of SL1 by Ultrogel ACA34 chromatography. (A) Profile of elution from ACA34. Fractions (100 μl) were assayed for protein (●) by the Bradford dye-binding assay (2) and for SL1 activity by a partially reconstituted RNA polymerase I transcription assay (△). Relative levels of RNA synthesis are expressed as a fraction of the maximal signal. (B) Agarose (1.4%) gel electrophoresis of RNA synthesized in runoff transcription assays of individual fractions. Each assay included RNA polymerase I (50 μg; cl 225) and 1 μl of the fraction indicated. End-labeled molecular weight standards were run in parallel to the runoff transcripts. (C) Sodium dodecyl sulfate (8%)-polyacrylamide gel electrophoresis (SDS PAGE) of proteins in ACA34 column fractions. Individual fractions (25 μl) as indicated were precipitated at 0°C with 20% trichloroacetic acid and loaded onto the gel. Molecular weight standards were included as described previously. The gel was stained with silver.

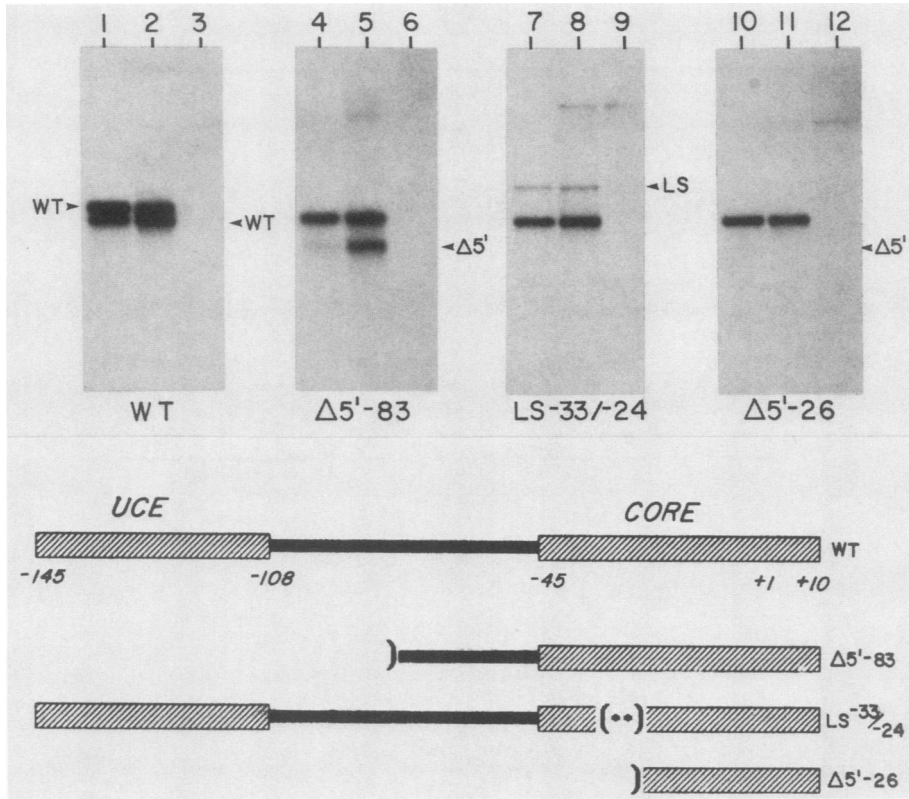

FIG. 5. Analysis of in vitro transcripts directed by promoter mutants. (A) Runoff transcription. Human extract (150 μg; lanes 1, 4, 7, and 10) or endogenous human RNA polymerase I (50 μg; remaining assays) was used to transcribe an equimolar mixture of wild-type and mutant human rDNA templates. Reactions were carried out in the presence of 0.5 ng of SL1 from the aca34 pool (lanes 2, 5, 8, and 11) or in the absence of human SL1 (lanes 3, 6, 9, and 12). Each reaction included wild-type prHu3 DNA cleaved with *Xho* as an internal standard. The following truncated rDNA templates were used to direct transcription: wild-type prHu3 cleaved with *Bam*HI (lanes 1 to 3); Δ5′-83 (lanes 4 to 6) and Δ5′-26 (lanes 10 to 12) cleaved with *Bam*HI, and LS −33/−24 (lanes 7 to 9) cleaved with *Pst*I. Arrows denote the predicted size of the runoff RNA from wild-type (WT) and mutant templates (Δ5′ and LS). Note that these assays were adjusted so that the level of transcription from the internal standard was the same in each reaction. (B) Diagram of rRNA promoter mutants. The 5′-flanking DNA is represented by lines. The hatched boxes indicate the sequences that have been previously identified as important for rRNA transcription initiation. Mutations are represented as brackets that define the altered sequences.

single-stranded human probe was protected from digestion by S1 nuclease (Fig. 6B).

In a complementary set of experiments, the mouse extract was fractionated by chromatography on Bio-Rex 70 to separate RNA polymerase I activity from the corresponding mouse selectivity factor. The fraction containing the mouse selectivity factor then was used to program the partially purified human RNA polymerase I in the reconstituted transcription system. In the presence of human RNA polymerase I alone, no discrete RNA products were detected when both human and mouse rDNA templates were used. Specific transcription from the human template required the presence of human SL1 (Fig. 6A, lane 6), whereas transcription of the mouse rDNA was observed only when the reconstituted system was supplemented with mouse SL1 (Fig. 6A, lane 7). In the presence of both human and mouse selectivity factors, both templates were transcribed (Fig. 6A, lane 8).

Functional characterization of SL1 with immune serum. To further examine the structural and functional properties of SL1, we immunized mice with partially purified SL1 and prepared IgG that was directed against the transcription factor. First, we determined whether in vitro rRNA transcription would be affected by adding anti-SL1 to either the reconstituted human transcription system or the crude extract. In these experiments, various amounts of preimmune or anti-SL1 IgG were preincubated with the transcription factor (in the form of partially purified SL1 or extract), and transcription was started by the addition of substrates and template.

In the presence of IgG from immune but not from preimmune serum, the level of specific runoff transcript directed by the human RNA polymerase I promoter was severely reduced (Fig. 7A, lanes 2 to 8). This inhibition could be specifically overcome by supplementing the transcription reaction with additional SL1 after the preincubation with anti-SL1 antibody (Fig. 7A, lanes 9 to 12). In contrast, runoff transcription directed by the mouse rRNA promoter in mouse extract (Fig. 7C) or by the adenovirus type 2 major late promoter in human extract (Fig. 7D) was not significantly affected by IgG from either immune or preimmune serum. To further confirm the specificity of the antibody-SL1 interaction, transcription was carried out under conditions in which both human and mouse rDNA templates are actively transcribed. Extracts from human and mouse cells were combined and then incubated with anti-SL1 IgG. Transcription was initiated by adding substrates and both human and mouse templates. In the absence of IgG, both templates were efficiently transcribed (Fig. 7B, lane 1). However, in the presence of increasing amounts of anti-SL1,

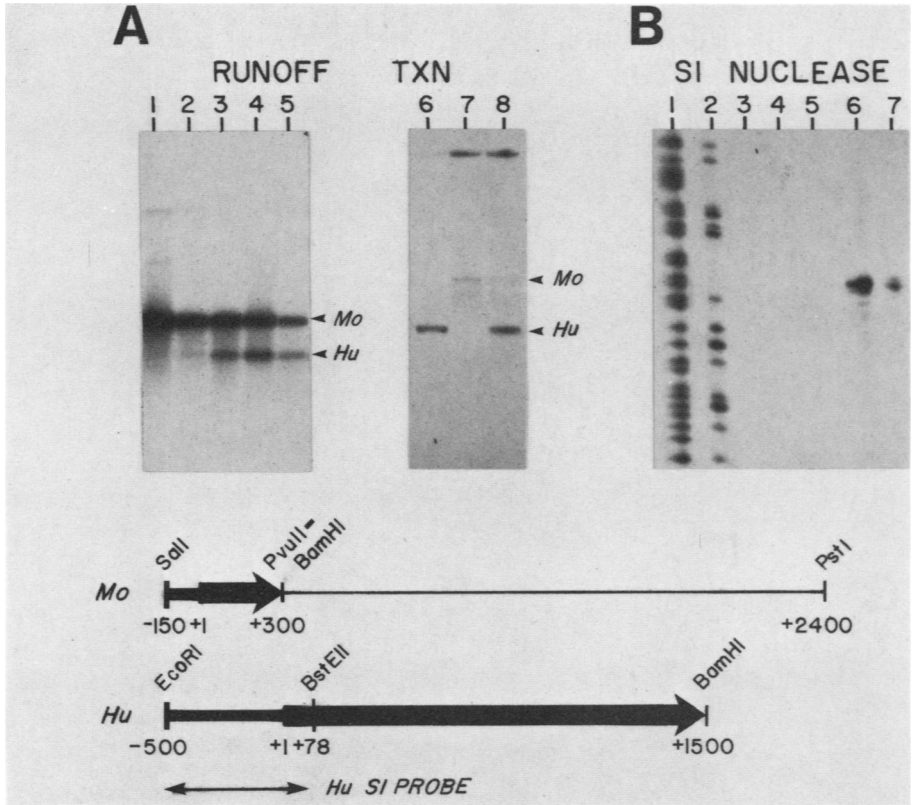

FIG. 6. Species specificity of SL1 action. (A) Runoff transcription. RNA synthesis was directed by 100 μg of nuclear mouse extract (lanes 1 to 5) with DNA templates derived from mouse rDNA (*Mo*) or human rDNA (*Hu*). Templates are shown diagrammatically at the bottom. Reactions were supplemented with human SL1 (h▽) (lane 2, 4.0 ng; lane 3, 6.5 ng; lane 4, 10 ng; lane 5, 20 ng). Transcription reactions including 50 μg of human RNA polymerase I (CL225, lanes 6 to 8) were supplemented with human SL1 (lanes 6 and 8; 0.5 ng from aca34 pool) or mouse SL1 (lanes 7 and 8: 100 ng from Bio-Rex 70 pool). (B) S1 nuclease analysis. Nuclear mouse extract (100 μg; lanes 5 and 7) or endogenous human RNA polymerase I (50 μg; lanes 4 and 6) was used to transcribe human rDNA. Reactions were carried out in the absence (lanes 4 and 5) or in the presence (lanes 6 and 7) of 0.5 ng of SL1 from the aca34 pool. A single-stranded 5'-labeled DNA probe derived from human rDNA (see lower panel) was hybridized to RNA isolated from reactions described above, the RNA-DNA hybrid was digested with S1 nuclease (32), and the products were subjected to electrophoresis on an 8% polyacrylamide gel in urea (21). Lane 3 contains a digest of probe in the absence of RNA. DNA sequence markers (A and G, lane 1; G, lane 2) were prepared from end-labeled probe DNA.

transcription from the human template but not the mouse template was specifically inhibited (Fig. 7B, lanes 2 to 4).

These results indicate that the serum from mice immunized with partially purified SL1 contained antibodies directed against the species-specific transcription activity. Using the anti-SL1 serum as a probe, we then used indirect immunofluorescence to examine the intracellular localization of the antigen. Because active transcription of rDNA by RNA polymerase I is confined to the nucleolus, we expected that serum raised against a bona fide RNA polymerase I transcription factor might display nucleolar specific immunofluorescence. The nucleoli in primate cells were brightly stained when anti-SL1 was used but not when control serum was used (Fig. 8). Although anti-SL1 interacted with a nucleolar antigen present in both monkey (Fig. 8) and human (data not shown) cells, the nucleoli in mouse cells were not stained by either control or immune serum. Thus, antibodies raised against a species-specific transcription factor are able to detect a nucleolar antigen in a species-dependent manner.

DISCUSSION

In the study reported here, we made use of a cell-free transcription extract that accurately initiates RNA synthesis from the human rRNA promoter to study the components that impart promoter specificity to RNA polymerase I. The data presented here, as well as previous observations, indicate that multiple components are required to reconstitute accurate in vitro transcription of the major rRNA genes. As a first step in identifying and characterizing the auxiliary transcription factors associated with rRNA transcription, we fractionated the crude extract into two components, one containing the endogenous RNA polymerase I enzyme activity and a second consisting of a transcription factor SL1 that is required for accurate and efficient synthesis of rRNA. The RNA polymerase I preparations have not been extensively purified, and therefore it is possible that this fraction contains other accessory factors associated with RNA polymerase I that are involved in rRNA transcription. By contrast, SL1 has been highly purified and likely represents a single factor that is required for transcription. Its behavior on various chromatographic columns, its sensitivity to heat (data not shown), and its antigenic properties all suggest that SL1 is a transcription factor that contains a protein component. In addition, inhibition of human rRNA transcription by anti-SL1 antibodies confirms that species-specific selection and recognition of rRNA promoters is at least in part due to an accessory transcription factor SL1 and not to RNA polymerase itself.

The promoter-specific activity of transcription by SL1 prompted us to identify those DNA sequences in the rRNA

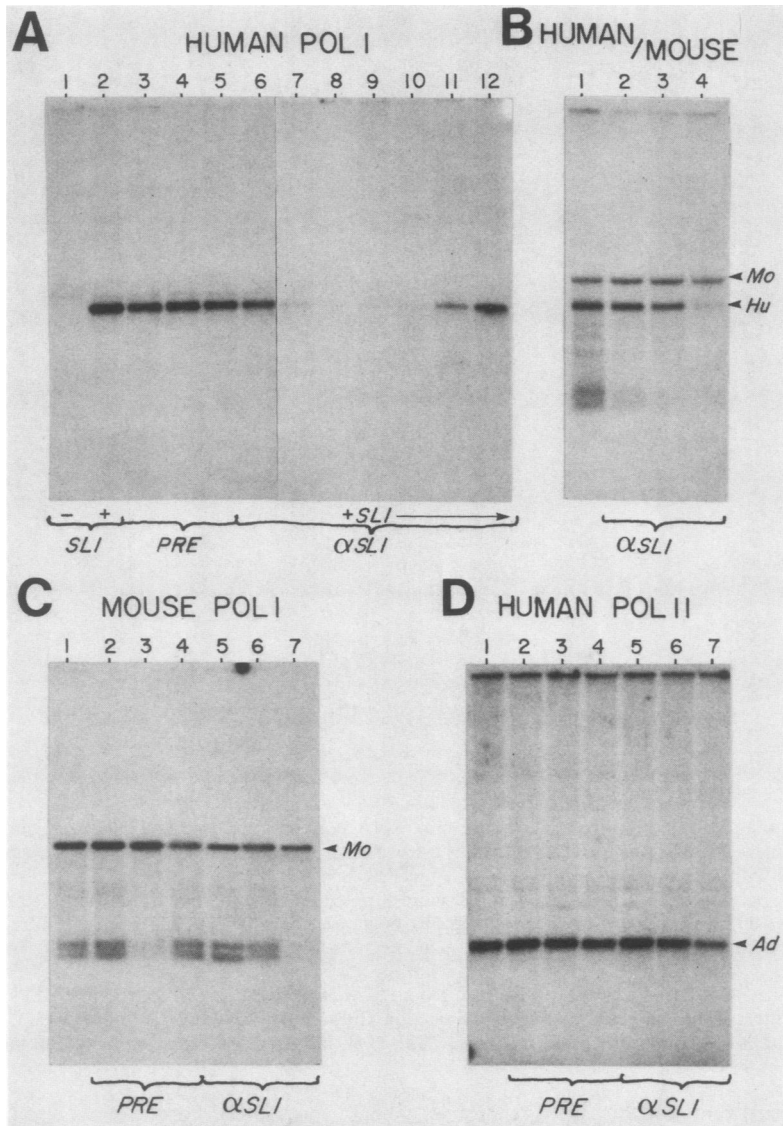

FIG. 7. Inhibition of human rRNA synthesis by anti-SL1 antibodies. (A) Human polymerase I. Inhibition reactions with purified IgG from immune and preimmune mice were performed with 50 μg of endogenous human RNA polymerase I (lanes 1 to 12), 10 ng of SL1 from the h∇ pool (lanes 2 to 12), and human rDNA template cleaved with *Bam*HI as described in the text. Lane 1 contains no human SL1. Human rRNA synthesis was assayed in the presence of 0 (lane 2), 1 (lane 3), 2 (lane 4), or 4 (lane 5) μg of preimmune IgG or in the presence of 1 (lane 6), 2 (lane 7), or 4 (lane 8) μg of αSL1 IgG. Reactions in lanes 9 to 12 were supplemented with 1 (lane 9), 2 (lane 10), 5 (lane 11), or 10 (lane 12) ng of additional SL1. (B) Human-mouse polymerase I. Transcription reactions included 100 μg of nuclear mouse extract, 120 μg of whole-cell human extract, and 200 ng each of *Pst*I-cleaved mouse rDNA and *Bam*HI-cleaved human rDNA (Fig. 7) and were carried out in the presence of 0 (lane 1), 1 (lane 2), 2 (lane 3), or 4 (lane 4) μg of anti-SL1 IgG. (C) Mouse polymerase I. Inhibition reactions with purified mouse IgG were performed with 100 μg of mouse nuclear extract as described in the text. Mouse rRNA synthesis was assayed in the presence of 0 (lane 1), 1 (lane 2), 2 (lane 3), or 4 (lane 4) μg of control IgG or in the presence of 1 (lane 5), 2 (lane 6), or 4 (lane 7) μg of anti-SL1 IgG. (D) Human polymerase II. Transcription was directed by 500 ng of pALE (a recombinant clone containing the adenovirus major late promoter) in the presence of 300 μg of whole-cell human extract. The levels of runoff RNA synthesis were monitored in the presence of 0 (lane 1), 1 (lane 2), 2 (lane 3), or 4 (lane 4) μg of control IgG or in the presence of 1 (lane 5), 2 (lane 6), or 4 (lane 7) μg of anti-SL1 IgG.

promoter that are responsive to SL1. Previous in vitro promoter mapping studies (17), coupled with more recent analyses of linker scanning mutants in vitro (13a) and of promoter deletion mutants in vivo (30a), identified two distinct blocks of sequences that play a role in directing efficient transcription of the human rRNA genes. A proximal core promoter region located approximately between nucleotides +7 and −45 appears to be absolutely required for transcription, whereas a distal upstream control region located between −140 and −108 appears to modulate the level of transcription. This multiple-element promoter structure appears to be common for genes transcribed by both RNA polymerase I and RNA polymerase II. In the case of RNA polymerase II, a number of promoter-specific transcription factors have been shown to interact directly with and act at sequences upstream of the proximal promoter element (7, 27). For example, the simian virus 40-specific transcription factor Sp1 binds to sequences within the 21-base-pair repeats of simian virus 40 (7). When this sequence element was removed from the simian virus 40 early promoter, transcrip-

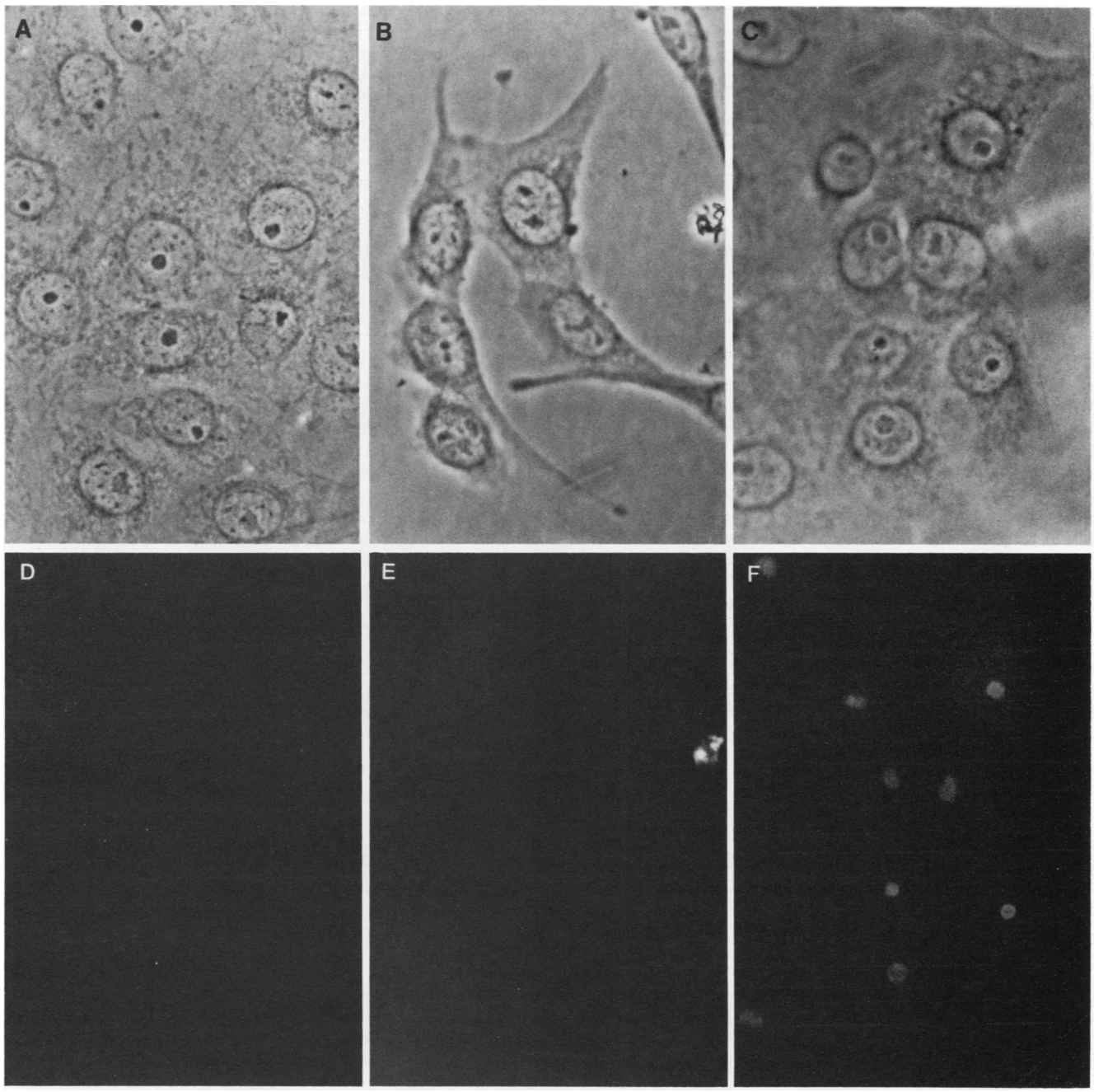

FIG. 8. Intracellular localization of SL1 by anti-SL1 antibodies. Immunofluorescence microscopy of cells (COS7 [D and F] and 3T6 [E] cells) is shown after staining with control antibodies (preimmune [D]) or antibodies to human SL1 (αSL1 [E and F]). Corresponding phase-contrast micrographs (A, COS7, preimmune; B, 3T6, αSL1; C, COS7, αSL1) are shown for comparison. Positive fluorescence is only seen in the nucleoli of COS7 cells stained with anti-SL1 antibodies.

tion was reduced to background levels of RNA synthesis even though the proximal promoter element including the AT-rich region was still intact (6). Furthermore, when the 21-base-pair repeat was positioned upstream of a heterologous transcription initiation site, synthesis from the hybrid promoter became dependent on Sp1 (D. Gidoni and R. Tjian, unpublished data). By contrast, in the reconstituted RNA polymerase I transcription system described here, removal of the distal promoter element does not greatly affect the efficiency of rRNA transcription. This finding is consistent with the idea that the core sequence rather than the 5′-upstream promoter element serves as the major target for the action of the species-specific transcription factor SL1. However, it is likely that the upstream control element also contains sequences that contribute to promoter recognition by the RNA polymerase I transcription complex but which are not absolutely required for the initiation of rRNA synthesis. To further study the relationship between promoter organization and the species-specific activation of rRNA promoters by SL1, we are currently constructing hybrid promoters in which the distal promoter element of one mammalian species is fused to the proximal core promoter

element of another species. Our preliminary results suggest that the species specificity of the rDNA template is determined by the core element.

The development of a reconstituted RNA polymerase I transcription system is the first step in studying the molecular mechanism of rRNA gene activation and the role of SL1 in transcription initiation. Based on the purification of SL1, very small amounts of the factor are present in the extract. Assuming that SL1 is a protomer of 100,000 daltons, we estimated that there are no more than 100 to 500 molecules of this transcription factor in each cell. This value is far less than the estimated number of active transcription complexes containing RNA polymerase I (10,000 to 20,000) per cell. A low stoichiometry of SL1 relative to RNA polymerase I places some restrictions on the mechanism of SL1-dependent transcription initiation and suggests that SL1 must be able to promote numerous initiation events involving different RNA polymerase I molecules.

One model for SL1 action would involve the formation of a stable protein-DNA complex with the template that could then direct multiple initiation events by RNA polymerase I. Recently, a number of workers used relatively crude enzyme fractions eluted from phosphocellulose in template commitment experiments. Results from these studies suggest that some transcription factors may mediate template selectivity by binding to specific sequences of the promoter DNA. In particular, workers in one study reported that the human transcription factor responsible for species specificity acts as a sequence-specific binding protein (24). Although it is reasonable to expect that there must be promoter-specific transcription factors for RNA polymerase I, none of the studies with the RNA polymerase I factors involved direct demonstration of promoter-specific protein-DNA interactions as has been established for the RNA polymerase III factor TFIIIA (8) or the RNA polymerase II factors Sp1 and HSTF (7, 27).

Thus far, we have been unable to detect any evidence of sequence-specific binding of highly purified and active SL1 to the rDNA promoter region by a variety of direct binding assays, including DNase footprinting, methylation protection, DNase protection, and fragment retention during electrophoresis and filter binding, either in the presence or absence of added RNA polymerase I. It is possible that the inability to detect specific SL1-DNA interactions in assays that require quantitative binding of DNA may result from technical difficulties in saturating binding sites with the small amounts of SL1 present. With each assay, however, we observed that even the highly purified preparations of SL1 contain proteins that bind strongly and nonspecifically to double-stranded DNA. This nonspecific binding activity was expected, considering the high affinity of this protein pool for cationic resins. In our hands SL1 also showed little preference in template commitment experiments for human rRNA promoter sequences over either mouse rRNA promotor sequences or nonspecific plasmid DNAs. Additionally, unlike the RNA polymerase II transcription factors required for transcription from the *Drosophila* hsp70 gene, or the simian virus 40 early promoter, whose binding requires DNA sequences upstream from the TATA box, the action of SL1 seems to require sequences in the core element, more proximal to the RNA start site. Although it is presently imprudent to eliminate all mechanisms for SL1 that involve specific DNA binding activity, at this point there is no compelling evidence for sequence-specific binding by SL1.

In the absence of direct evidence for SL1 binding, it is perhaps appropriate to consider alternative mechanisms by which SL1 may act on RNA polymerase I in a catalytic fashion to promote specific initiation of transcription. The most obvious analogy for this model is the classic action of bacterial and phage sigma factors, which interact transiently and directly with RNA polymerase to confer promoter recognition (4). Sequences in the *Escherichia coli* promoter region between −35 and −4, centered at nucleotides −33 and −8, determine the specificity of the RNA polymerase holoenzyme. Similarly, the RNA polymerase I core promoter element that includes sequences between nucleotides −45 and +7 both contains the species-specific promoter element (data not shown) and serves as the target for SL1 action. Unlike sigma, however, SL1 appears to be in low stoichiometry relative to RNA polymerase and has a high affinity for double-stranded DNA that is not characteristic of most sigma factors (3). Of course, additional experiments will have to be performed to test whether the sigma model or the direct-binding model is appropriate for the mechanism of SL1.

Despite the fact that we were able to effect a large gain in the specific activity of SL1 by a series of chromatographic steps, we have been unable to carry out an extensive biochemical characterization of this protein due to a lack of sufficient material. An important tool that could facilitate our analysis of SL1 would be antibodies directed against this transcription factor. As a preliminary step in this direction, we raised polyclonal antibodies against partially purified SL1 and found that anti-SL1 specifically inhibits human rRNA transcription and that it reacts selectively with a nucleolar antigen present in primate cells but not in mouse cells. It may be possible to further capitalize on the localization of SL1 by using this rRNA transcription factor as a nucleolar marker and by using anti-SL1 antibodies as a probe to study nucleoli structure and organization. When we used anti-SL1 as a probe for proteins in a Western blot, two polypeptides were found to interact strongly with the immune serum (unpublished data). However, when we raised monoclonal antibodies directed against these individual polypeptides, neither of these antibodies was found to recognize a nucleolar antigen in the fluorescence assay, nor could the monoclonal antibodies be used to inhibit RNA polymerase I transcription in vitro. Furthermore, we now have independent evidence based on purification that these two candidate polypeptides are not SL1. To fully exploit the use of antibodies for the characterization of SL1, we need to develop a better panel of immunological reagents. To this end, we are currently accumulating purified SL1 to raise monoclonal antibodies against this factor and thus develop a more useful reagent to carry out the necessary biochemical analysis of this interesting transcription factor.

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