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Association of an RNA polymerase III transcription factor with a ribonucleoprotein complex recognized by autoimmune sera

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ABSTRACT

RNA polymerase III transcription can be inhibited in vitro by two sera from patients with autoimmune diseases. The first serum, designated anti-SS-B (or La), has antibodies directed against a 50,000 dalton polypeptide that is part of a larger ribonucleoprotein complex. The second serum, designated anti-SpNo, recognizes a target antigen polypeptide of greater than 100,000 daltons as well as the SS-B antigen. Both sera selectively remove required transcription factors from the transcription extract, and inhibition can be rescued by the addition of a HeLa S100 extract to the depleted transcription system. The HeLa S100 extract was sequentially fractionated by ion-exchange chromatography on DEAE-cellulose and phosphocellulose. The high salt eluate from the latter column was also able to rescue the anti-SS-B inhibition as was the immunoaffinity-purified SS-B ribonucleoprotein complex isolated from HeLa, Xenopus or rabbit thymus. Immunoblots of the active fractions indicated that all contained the SS-B immunoreactive polypeptide, but probes of replica filters for DNA-binding suggested that the transcription factor is not the SS-B antigen but a 64,000 dalton polypeptide component of the antigen ribonucleoprotein complex. SS-B is itself an RNA-binding protein and could be shown to bind nascent 5S RNA transcripts in vitro. Differential ammonium sulfate precipitation and DNA cellulose chromatography has confirmed that a group of 64-68 K dalton polypeptides are components of the SS-B ribonucleoprotein complex associated with transcription factor activity.

INTRODUCTION

Antinuclear antibodies are a characteristic feature of rheumatic diseases and are directed against a varied spectrum of target antigens (for reviews see references 1 and 2). These antisera are rapidly becoming a valuable resource as probes of nuclear structure and function, and have been used to particular advantage in the study of the snRNP complexes (3-4). One such target protein is the soluble antigen designated SS-B (also known as La and Ha) associated with the diseases Sjogren's Syndrome and systemic lupus erythematosus (5-6). The antigen has been identified as one or more polypeptide species of varying molecular weights but recent evidence suggests that it is readily susceptible to proteolysis (7-8). SS-B can be isolated as a single immunoreactive species with a molecular weight of approximately 50,000 daltons in association with snRNAs and a set of other polypeptides (7-9). We refer to this complex as the SS-B snRNP. The SS-B antisera have been utilized to date to characterize the

RNA components of these complexes to include a heterogeneous group of snRNA species of 5S size and smaller in uninfected cells and various viral-derived RNA species in infected cells (3-4). The common element joining these diverse RNA species is their synthesis by RNA polymerase III (9-12). The SS-B antigen is itself an RNA-binding protein (9-10), but its role relative to polymerase III transcription has not been established.

For assaying the class III RNA polymerases, soluble, cell-free systems have been developed by several investigators (13-16). The development of such in vitro transcription systems has facilitated the search for factors critical to the transcriptional process. Recent studies using protein fractions prepared by conventional ion-exchange chromatographic procedures have indicated a multiplicity of general and specific factors required for accurate transcription (17-18). Using the genes for 5S RNA and tRNA as templates, the results would suggest a minimum of two common polymerase III factors but the degree of purification precludes any maximum estimate. Only one gene specific factor, the 5S-associated TFIIIA species, has been isolated to homogeneity (19). TFIIIA interacts directly with a control region within the 5S RNA gene and is required for the accurate initiation of transcription (20). TFIIIA participates in the formation of stable pre-initiation complexes (21) and is required for the assembly of active 5S gene chromatin templates (22). The actual mechanism by whereby TFIIIA facilitates transcription is obscure. Recent evidence suggests that it alters the helical configuration of 5S DNA (23) but the consequences of this activity relative to other transcription factors and to the interaction of TFIIIA with these same factors awaits their isolation.

In the work reported here, we have used autoimmune sera to develop a rapid, sensitive assay to survey protein factors required for transcription. These same sera can be used for the subsequent isolation of functionally active complexes containing such factors. We present evidence that the SS-B antigen-associated snRNP complex contains a polymerase III transcription factor. The factor is a DNA-binding constituent of the snRNP that can be unequivocably separated from the 50,000 dalton SS-B antigen.

MATERIALS AND METHODS

Cellular Extracts and Human Sera. HeLa cells were grown in spinner culture in RPMI media. Soluble extracts (S100) were prepared by the method of Weil et al. (14). All extracts were stored at -70° and thawed once prior to use. Sera from patients with rheumatic diseases were obtained from Dr. Eng Tan of this institution. Normal human sera were obtained from individuals at this institution. Sera were characterized for antinuclear antibodies by E. Tan and C. Peebles.

In Vitro Transcription Reactions. Transcription reactions were carried out in a final volume of 50 µl with 20 µl of the HeLa S100. The transcription reactions contained 10 mM Hepes (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.6 mM unlabeled nucleoside triphosphates and 0.02 mM

α^{32}P-GTP (NEN, final specific activity = 10 Ci/mmole). Incubations were at 22° for 2 h. RNA was purified by phenol/chloroform extractions and analyzed on 8% polyacrylamide gels containing 8.3 M urea. Gel autoradiograms were exposed at -70° with Kodak XAR film and DuPont Cronex Lighting-Plus intensifying screens. Template DNAs (0.5-2 µg per reaction) were a Xenopus leavis $tRNA^{met}$ gene (obtained from Dr. M. Birnsteil), Xenopus laevis oocyte-type 5S RNA genes (clone pX1o8 obtained from Dr. D. D. Brown), Adenovirus 2 DNA (purchased from Bethesda Research Laboratories) and a rat cDNA clone p2A120 (obtained from Dr. G. Sutcliffe). Assays for inhibition of transcription by human sera were carried out as described in the legend to Figure 1. Pansorbin (10% suspension) was obtained from Calbiochem and washed three times with transcription buffer (above) prior to resuspension at 20% (w/v) in transcription buffer. All transcription reactions included controls for the effect of normal human serum and Pansorbin. With commercially available Pansorbin this control generally gave a level of transcription of 40-60% of that of an untreated HeLa extract. This diminution was due to Pansorbin and not to the addition of normal human serum. Recent results have demonstrated less control inhibition and variability with freshly prepared formalin-fixed S. aureus cells maintained at -70° and thawed once prior to use. Rescue experiments reflect restoration of transcription levels to that of the normal human serum-Pansorbin control level.

Chromatography of HeLa Soluble Extracts. The HeLa S100 was chromatographed on DEAE cellulose (Whatman DE52) in 20 mM Hepes, pH 7.9, 20% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (Buffer A). Samples (1 ml S100 containing 5-10 mg protein per 1 ml packed resin) were applied to the column in buffer A containing 130 mM ammonium sulfate and the column was washed with 5-10 column volumes of this buffer prior to step elution with buffer A containing 350 mM ammonium sulfate. Fractions were collected and those representing the peak of absorbance at 280 nm were dialyzed extensively against Buffer B (equivalent to buffer A with 0.5 mM spermine in place of 5 mM $MgCl_2$) containing 100 mM ammonium sulfate. This material was applied to a phosphocellulose (Whatman P11) column (1-2 mg protein/ml packed resin) equilibrated with buffer B containing 100 mM ammonium sulfate. After extensive washing with this buffer, the column was step eluted with buffer B containing 600 mM ammonium sulfate. The peak of absorbance at 280 nm was pooled and dialyzed against buffer A containing 100 mM KCl and aliquots were stored at -70°. The HeLa S100 was also chromatographed directly on P11 as described by Segall et al. (17) except buffer B (above) was used. All chromatographic steps were performed at 4°.

Protein Gel Electrophoresis and Blot Transfers. Prior to electrophoresis, individual samples were precipitated overnight at -20° by the addition of ethanol. Samples were fractionated by SDS-polyacrylamide slab gel electrophoresis using the system of Laemmli (24). The following proteins were used as molecular weight standards: conalbumin (77 K); bovine serum albumin (66 K) human IgG heavy chain (50 K); ovalbumin (43 K); carbonic anhydrase (29

K); human IgG light chain (22.5 K); bovine pancreatic RNase (13.7 K); cytochrome c (12.4 K). Proteins separated on the SDS gels were electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell) at 60 V for 2 hr in 12.5 mM Tris-OH/96 mM glycine buffer with 20% methanol (25). The filter replicas to be analyzed for immunoreactive species were saturated for 30 min on a rocker platform in phosphate-buffered saline (PBS) containing 5% (w/v) each bovine serum albumin (BSA) and ovalbumin (OVA). The filters were washed briefly with PBS, and then incubated for 1 h with an appropriate dilution of the test antibody in 2% BSA/OVA, followed by a second incubation with ^{125}I-labeled rabbit antihuman IgG. Antigenic polypeptides were identified by autoradiography using Kodak XRP or XAR film at -70° with an intensifying screen.

Equivalent protein blots were washed overnight in a solution containing 0.2% (w/v) Ficoll, bovine serum albumin and polyvinyl pyrrolidinone (av. mol. wt. 40,000). After extensive washing in chromatographic buffer A containing 100 mM KCl, 1 µg of ^{32}P-labeled DNA (pX1o8 or pBR322, labeled by the fill-in procedure of Smith and Thomas [26]) or 10 µg of ^{32}P-Xenopus oocyte RNA (labeled with γ-^{32}P-ATP and polynucleotide kinase by the method of Maxam and Gilbert [27] following incubation in 100 mM Tris-Cl, pH 9.5, at 100°C for 10 min.) was added. Interaction of the labeled nucleic acid and filter-bound proteins was allowed to take place at 20° for 2-4 h. After extensive washing in buffer A containing 100 mM KCl, filters were air dried and exposed to X-ray film (Kodak XAR). Bound nucleic acids (greater than 90%) can be removed by washing in buffer A containing 1 M ammonium sulfate. After extensive washing in buffer A plus 100 mM KCl, the filters can be challenged with a new probe.

SS-B snRNP Purification. The SS-B or La snRNP used in these experiments was isolated by immunoaffinity chromatography of sonicated whole cell extracts from viable frozen HeLa cells or rabbit thymus acetone powder (7, 28) and by similar chromatography of the Xenopus ovary S100 extract. The extracts in 10 mM Tris-HCl (pH 7.4) containing 0.35 M NaCl and 0.2 mM phenylmethylsulfonyl fluoride were passed through immunoaffinity columns with specificity for the SS-B antigen. By an immunodiffusion assay in the Scripps Clinical Pathology Laboratory, this serum also had anti-SS-A reactivity. A serum with only anti-SS-B and no anti-SS-A specificity is rare (29). The bound fraction was eluted with 10 mM Tris (pH 7.4) containing 0.1 M NaCl and 6 M urea. The urea was removed by dialysis against a buffer compatible with the transcription assay, namely 10 mM Hepes (pH 7.9) containing 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA and 10% glycerol (v/v). The SS-B snRNP purified by immunoaffinity chromatography was further fractionated by ammonium sulfate precipitation and DNA cellulose chromatography. The eluate from the SS-B antibody column was made 30% (w/v) in ammonium sulfate. After 30 min on ice the sample was centrifuged at 10,000 g for 10 min. The pellet was resuspended in 500 µl buffer A (above) containing 50 mM KCl and applied to 0.4 gm of DNA cellulose (10.2 mg calf thymus DNA/gm resin) equilibrated in the same buffer. After incubation at 0° for 1 h with occasional shaking the resin was washed thoroughly with buffer A containing 50 mM KCl. Bound proteins were eluted with 2 M KCl in 10 mM Hepes,

pH 7.9, 1 mM EDTA. All samples were dialyzed against buffer A plus 50 mM KCl and tested for rescue of SS-B antibody inhibition of transcription. Protein content of the fractions was analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie blue or silver nitrate staining.

RESULTS

Inhibition of in vitro transcription of RNA polymerase III genes by sera of autoimmune patients. We have taken advantage of the cell free transcription system developed by Weil et al. (14) and the availability of a number of cloned class III genes to analyze the effect of various autoimmune sera on RNA polymerase III transcription. The transcription extract was preincubated with serum and antibody-antigen complexes were removed from solution with Pansorbin (formalin-fixed S. aureus cells). We have examined the effect of the sera listed in Table 1 on the transcription of a cloned Xenopus laevis oocyte-type 5S RNA gene.(30) Two classes of sera of differing specificities (anti-SS-B/La and anti-SpNo) exhibited strong inhibition of transcription. Figure 1 shows that these sera also inhibit the transcription of three additional cloned genes transcribed by RNA polymerase III. These templates are a Xenopus $tRNA^{met}$ gene (31), the adenovirus VA I gene (17) and a rat repetitive DNA sequence (ID, ref. 32). The experiment was structured in such a way that the antisera were used to selectively precipitate their target antigens (and any associated molecules) from the transcription extract prior to the addition of the DNA template. Thus on the basis of this experiment we can not differentiate which component(s) of the antigen-associated ribonucleoprotein complex are critical to the transcription process. The simultaneous addition of the DNA template and the antisera did not result in complete inhibition, presumably because of the greater avidity of the transcription factor(s) for the DNA over the antibody molecules, as has been suggested recently in a somewhat analogous study of RNA polymerase II (33). Similarly, addition of the DNA template to the transcription extract prior to the addition of serum did not result in complete inhibition. Inhibition can also be achieved by immunodepletion with SS-B IgG coupled to protein A (S. aureus cells) and by immunodepletion with protein A Sepharose (data not shown). One of the SS-B sera we have used (Ze, Table 1) has been extensively characterized by C. Peebles and E. Tan (personal communication). The target antigen is nuclear in location and the serum exhibits no anti-DNA or anti-histone activity. Further, we find that this serum does not exhibit significant ribonuclease activity (data not shown, but see Table 3). We have used this serum in most of the experiments which follow; however, equivalent results have been obtained with other SS-B sera (Table 1). Our prototype SS-B antiserum (Ze) did not inhibit in vitro RNA polymerase II transcription (34) of a human γ-globin gene (D. Bazett-Jones, S. Hoch and J. Gottesfeld, unpublished). The second inhibitory antiserum (SpNo) was relatively uncharacterized but was known to give a speckled nucleolar pattern upon immunofluorescent staining (2). Another patient's serum also characterized as

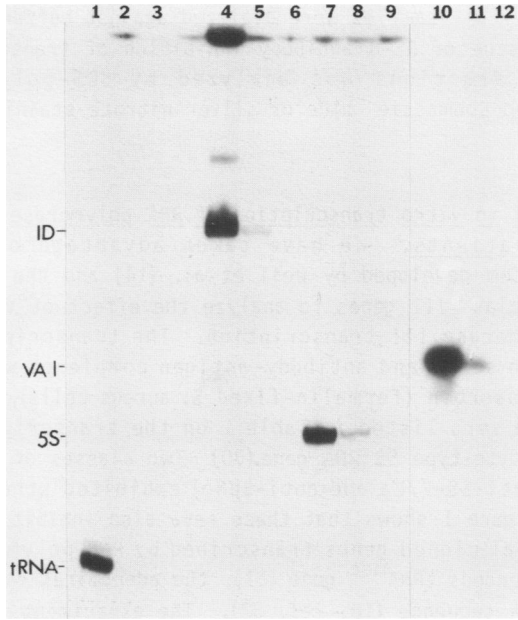

Fig. 1. SS-B and SpNo antisera inhibit in vitro transcription of RNA polymerase III genes.
HeLa cell-free transcription extracts (20 µl) (14) were pretreated with 5 µl of normal human serum (lanes 1,4,7 and 10), 5 µl SS-B serum (lanes 2,5,8 and 11) or 5 µl SpNo serum (lanes 3,6,9 and 12) and 5 µl of 20% Pansorbin for 30 min at 0°. After centrifugation at 10,000 rpm for 3 min, DNA and nucleoside triphosphates were added to the supernatants. Products of transcription were analyzed on denaturing polyacrylamide gels. The DNA templates (0.5 µg) were: lanes 1-3, X. laevis $tRNA^{met}$ gene (31); lanes 4-6, rat "identifier" sequence p2A120 (32); lanes 7-9, X. laevis oocyte-type 5S RNA gene pXlo8 (30); lanes 10-12, adenovirus 2 VA I gene (17). The gel autoradiogram is shown.

anti-SpNo (patient Ge, Table 1) did not inhibit 5S RNA transcription. Both inhibitory sera (SS-B and SpNo) recognize the 50 K dalton SS-B antigen on protein blots (data not shown, but see Fig. 4). The SpNo serum (patient Tr) also recognized a target antigen with a molecular weight of greater than 100,000 daltons (not shown).

Rescue of anti-SS-B inhibition of 5S RNA transcription. To begin to define the elements critical to the transcription process, the S100 extract from HeLa was sequentially fractionated by ion-exchange chromatography on DEAE-cellulose and phosphocellulose as outlined in Materials and Methods. The starting material (S100) and fractions from both columns were tested for their ability to rescue transcriptional activity after prior treatment of the HeLa transcription system with anti-SS-B sera. Transcription of the Xenopus 5S gene was then examined. Both the S100 extract and the high salt eluate of the DEAE-cellulose column restored activity. This material was dialyzed to 100 mM

Table 1. Inhibition of 5S RNA Transcription In Vitro with Autoimmune Sera

Patient Serum	Specificity[a]	5S RNA Transcription[b]
St	α-centromere	+
Ru	α-R-RNP	+
Ma	α-no	+
Ha	α-Scl	+
Eb	α-PCNA	+
Ze	α-SS-B	inhibition
Re	α-no	+
Co	α-RNP	+
We	α-Sm	+
Fa	α-no	+
Tr	α-SpNo	inhibition
Gf	α-SS-A	+
Ca	α-SS-A/SS-B	inhibition
Go	α-SS-A/SS-B	inhibition
Ge	α-SpNo	+
Le	α-SS-B	inhibition
Fl	α-SS-A	+

[a]Specificities as characterized by Tan. (2)
[b]2 µl of serum were mixed with 20 µl of the HeLa S100 transcription extract and antibody-antigen complexes were removed from solution with 5 µl of 20% Pansorbin as described in the legend to Figure 1. The depleted extract was then tested for its ability to support the synthesis of 5S RNA from 1 µg of cloned 5S DNA. We define inhibition as greater than 90% diminution of the 5S RNA signal measured on a denaturing polyacrylamide gel relative to a control reaction on the same gel where the HeLa transcription extract was treated with normal human serum and Pansorbin. This control generally gave a level of transcription of 40-60% that of an untreated HeLa extract.

ammonium sulfate and applied to phosphocellulose. The rescue activity was found to bind tightly to this resin and to elute at high ionic strength (600 mM, Figure 2A). Tight binding to DEAE cellulose and phosphocellulose suggests both acidic and basic moieties in the rescue activity. We have also tested SS-B snRNPs that had been purified by immunoaffinity chromatography for their ability to rescue transcription in SS-B depleted extracts. These RNA-protein complexes contain a reproducible set of polypeptides (Fig. 4A, and ref. 7) and the usual set of small RNAs of 5S size and smaller (3-4). Rechromatography of these particles yields the same set of abundant polypeptides. Since the sera used to construct the immunoaffinity columns are not monospecific, proof of the association of each of these polypeptides in the same snRNP must await the availability of monoclonal antibodies. However, several SS-B sera have been used to construct columns and equivalent protein profies have been obtained. This argues strongly that the SS-B antigen (50 K dalton polypeptide) exists in a complex with the snRNAs and other polypeptides. We have purified SS-B snRNPs from HeLa, Xenopus and rabbit thymus. Each rescued SS-B inhibited transcription (Fig. 2B). These rescue experiments argue that inhibition is not due to the addition of some deleterious substance found in autoimmune patients' sera to the transcription system.

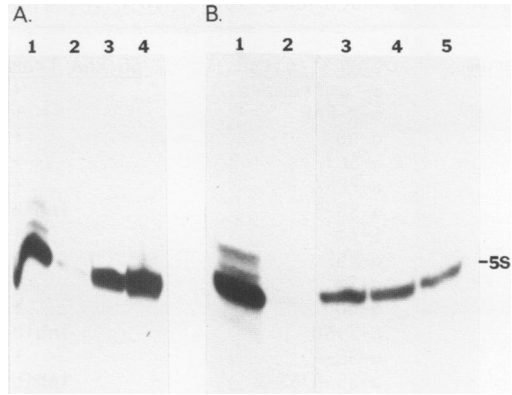

Fig. 2. Rescue of SS-B inhibition of 5S RNA transcription.
Panel A. HeLa S100 components rescue SS-B inhibition. The HeLa S100 was pretreated with 5 µl of normal (lane 1) or SS-B serum (lanes 2-4) as described in Figure 1. 0.5 µg pX1o8 DNA (lanes 1 and 2) or DNA preincubated for 15 min. with 15 µl HeLa P11 fraction (containing 5 µg protein) (lane 3) or 10 µl HeLa S100 (lane 4) was added to the supernatants after immunoprecipitation. After 2 h incubation in the presence of nucleoside triphosphates, RNA was extracted and analyzed on a denaturing polyacrylamide gel.
Panel B. SS-B snRNPs rescue SS-B inhibition. The HeLa S100 was pretreated with 5 µl of normal (lane 1) or 5 µl of SS-B serum (lanes 2-5). 0.5 µg pX1o8 DNA or DNA preincubated with SS-B snRNP was added to the depleted extract and transcription was monitored. RNP additions were from Xenopus (lane 3), HeLa (lane 4) and rabbit thymus (lane 5). In lanes 1 and 2 DNA was preincubated in the same buffer without added RNPs or protein.

Table 2. SS-B snRNP Rescue of 5S Transcription

µg SS-B snRNP Protein Added	% Transcription[a] Exp. 1	Exp. 2
0	18.2	0
0.12	27.0	8.9
0.24	30.6	23.0
0.63	48.0	35.8
1.24	89.3	87.0
2.5	100	100

[a]%RNA transcription relative to an S100 extract (20 µl) treated with 4 µl normal human serum and 5 µl 20% S. aureus cells. 1 µg pX1o8 DNA was preincubated for 15 min. with the listed amounts of SS-B snRNP prepared by immunaffinity chromatography of a HeLa whole cell extract. The S100 (20 µl) was treated with 4 µl of SS-B sera (exp. 1) or 5 µl serum (exp. 2) and 5 µl of Pansorbin and nucleoside triphosphates were added. After incubation at 22° for 2h, RNA was purified and analyzed by gel electrophoresis. To obtain % 5S RNA transcription, the gel autoradiogram was scanned with a densitometer.

We have determined the amount of SS-B snRNP (expressed as µg of protein) needed to restore transcriptional activity to an SS-B depleted extract to the level of transcription obtained with a control extract treated with Pansorbin and normal human serum (Table 2). Between 1 and 2 µg of SS-B snRNPs added to the depleted extract gave the same level of transcription as an extract treated with normal human serum. This corresponds to 1-2 X 10^6 HeLa cell equivalents of SS-B snRNP. Since 20 µl of transcription extract is obtained from 3.5 X 10^6 HeLa cells this means that the addition of 30-50% of SS-B snRNP contained in an undepleted extract restores activity to a depleted extract. Similar rescue of $tRNA^{met}$ transcription has also been demonstrated (data not shown).

Direct substitution of the SS-B snRNP in the in vitro RNA polymerase III transcription system. The experiments described above were based on the inhibition of transcription by the selective removal of critical factors. An alternative approach is to initially fractionate the components of the transcription system (as outlined in Figure 3A), and by "mix and match" reconstitution experiments directly determine the fractions containing transcription factors. Such an approach was taken by Segall et al. (17) using phosphocellulose to fractionate an S100 extract into three fractions, A, B and C (Figure 3A). When any one of these fractions is absent there is little or no transcriptional activity as seen in Figure 3B (lanes 2-4), but activity can be restored by mixing all three fractions (lane 1). Of interest to these results was the effective substitution of the HeLa SS-B snRNP for fraction C (lane 5). Again approximately 1 µg of SS-B snRNP is required to substitute for the C fraction. Substitution of the SS-B snRNP for either fraction A or fraction B did not restore transcriptional activity (lanes 6 and 7).

Characterization of protein fractions active in in vitro transcription. We arrived independently at the isolation of two protein fractions active in RNA polymerase III-directed transcription, one by conventional ion-exchange chromatography and one by immunoaffinity chromatography using anti-SS-B IgG. As the two fractions appeared interchangeable, a direct comparison of protein composition was undertaken as illustrated in Figure 4. The Coomassie stained protein profiles indicated far fewer polypeptide species in the purified SS-B snRNPs than in a corresponding phosphocellulose P11 high salt eluate. However, when replica filters of these fractions were probed with an anti-SS-B serum by the protein blot technique (Figure 4B), all contained the characteristic 50,000 dalton immunoreactive SS-B polypeptide (7-9) suggesting the presence of the SS-B snRNP in the P11 fraction. Occasionally we have noted a second minor reactivity at approximately 64,000 daltons with HeLa SS-B snRNPs. Since none of the fractions contained a single polypeptide species, we incubated replica filters with a labeled DNA probe to determine which, if any, of the polypeptide species were DNA-binding proteins, and by inference, could be the putative transcription factor(s). We noted above that addition of template DNA to the transcription extract before the addition of serum did not result in complete inhibition. This could suggest that the transcription factor is a DNA binding protein. Again similar sized DNA-binding species

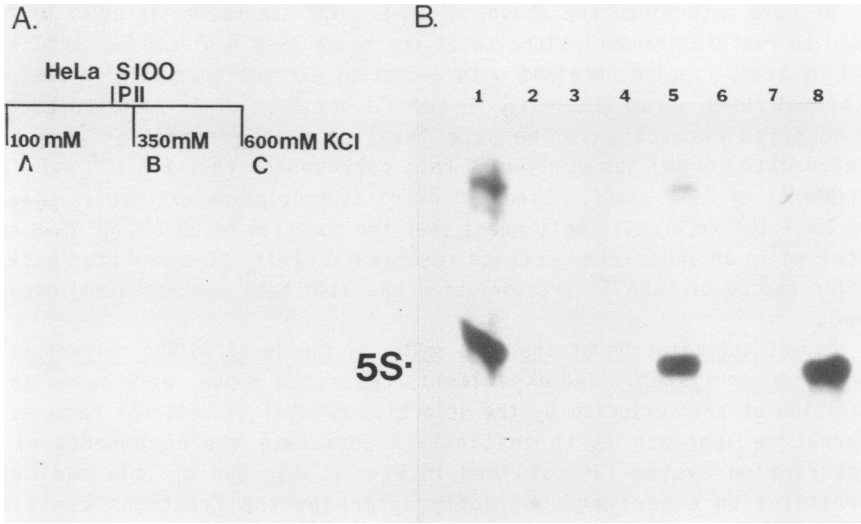

Fig. 3. The SS-B snRNP can substitute for a P11 column fraction required for in vitro transcription of 5S RNA genes.
Panel A. The HeLa S100 was subjected to chromatography on phosphocellulose P11 in 100 mM KCl (17). Flow through ("A"), 350 mM KCl step ("B") and 600 mM KCl step ("C") fractions were obtained and dialyzed.
Panel B. 5S gene transcription with A + B + C fractions (lane 1); A + B fractions (lane 2); B + C fractions (lane 3); A + C fractions (lane 4); A + B fractions plus 15 µl of HeLa SS-B snRNP (lane 5); A + C fractions plus 15 µl of HeLa SS-B snRNP (lane 6); B + C fractions plus 15 µl of HeLa SS-B snRNP (lane 7); A + B fraction plus 15 µl of DNA-cellulose bound proteins of HeLa SS-B snRNP (lane 8). 15 µl aliquots of each P11 fraction were used in a final volume of 50 µl containing 0.5 µg pXlo8 DNA and nucleoside triphosphates. The protein content of each fraction (15 µl) was "A", 29 µg; "B", 5 µg; "C", 4.5 µg; SS-B snRNP, 2.3 µg; DNA cellulose bound material, 1.5 µg. The gel autoradiogram is shown.

could be seen in both the P11 and immune eluate fractions. The major DNA-binding species were not of the size of the SS-B antigen, but rather at a molecular weight of approximately 64,000 daltons (Figure 4C). If this DNA-binding protein does function as a transcription factor, the data would suggest that it co-purifies with the SS-B antigen and could in fact be an integral constituent of the SS-B snRNP. The most abundant polypeptide in the SS-B snRNP is 64-67 K daltons (Fig. 4A). A similar analysis of a replica filter using labeled RNA as a probe (Figure 4D) was in agreement with the recent characterization of the SS-B antigen as an RNA-binding protein (9). ^{32}P-labeled Xenopus oocyte RNA binds specifically to the 50K dalton SS-B antigen polypeptide. Little RNA binding is detected at 64K daltons. The second major RNA-binding polypeptide seen in lanes 2 and 3 is of the size expected for the presumed SS-B degradation product upon exposure to endogenous proteases (43K daltons) (7-8). An independent verification of the RNA-binding

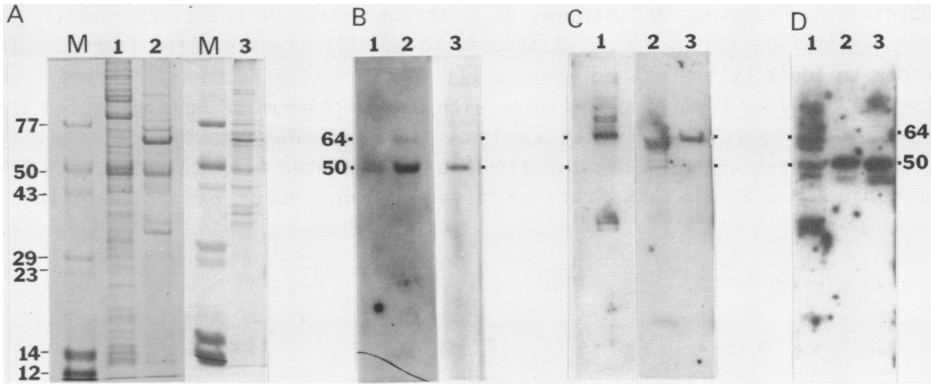

Fig. 4. Protein gel analysis of P11 column fractions and SS-B snRNPs.
Panel A shows the Coomassie stain profile of HeLa P11-bound fraction proteins (lane 1), the HeLa SS-B snRNP (lane 2) and the rabbit thymus SS-B complex (lane 3). M denotes molecular weight markers (in K daltons).
Panel B shows a protein blot of HeLa P11 fractions (lane 1), HeLa SS-B snRNP (lane 2) and rabbit thymus SS-B snRNP (lane 3) probed with the SS-B antibody and ^{125}I-anti-human IgG.
Panel C shows DNA binding proteins detected on nitrocellulose blots probed with ^{32}P-pX1o8 DNA. Lane 1, HeLa P11 proteins; lane 2, rabbit SS-B snRNP; lane 3, HeLa SS-B snRNP.
Panel D shows RNA-binding proteins detected on nitrocellulose blots. All lanes were probed with ^{32}P-Xenopus oocyte RNA. Lane 1, HeLa P11 fraction proteins; lane 2, HeLa SS-B snRNP; lane 3, rabbit thymus SS-B snRNP.

ability of the SS-B snRNP was provided by the immunoprecipitation experiment outlined in Table 3. Anti-SS-B serum was added to the in vitro transcription system after initiation of the reaction with the resulting precipitation of the antigen and the nascent 5S RNA transcripts. Similar results have been

Table 3. Immunoprecipitation of 5S RNA Transcripts Synthesized In Vitro

Serum	Cpm in Pellet	Cpm in Supernatant	% Cpm in Pellet
Normal	484	4279	10.2
SS-B	3056	611	83.4

^{32}P-5S RNA was transcribed from plasmid pX1o8 DNA in the HeLa cell-free system as described in Fig. 1 and immunoprecipitated with 5µl of the indicated serum plus 5µl of 20% Pansorbin. After 30 min the samples were centrifuged at 10,000 rpm for 5 min and RNA was extracted from the pellets and supernatants. This RNA was subjected to electrophoresis. The 5S RNA bands were excised from the gel with the aid of an autoradiogram and radioactivity was determined by scintillation counting with a toluene-based flour.

obtained by Francoeur and Mathews (9) with adenovirus VA RNA transcripts.

Identification of the SS-B associated pol III transcription factor. In order to identify the RNA polymerase III transcription factor(s) removed from the HeLa S100 by immunoprecipitation with SS-B antiserum we have subjected the SS-B snRNP to further fractionation. We find that the factor activity (assayed by rescue of SS-B inhibition) is precipitated from solution with 30% ammonium sulfate. Such fractionation does not occur prior to antibody

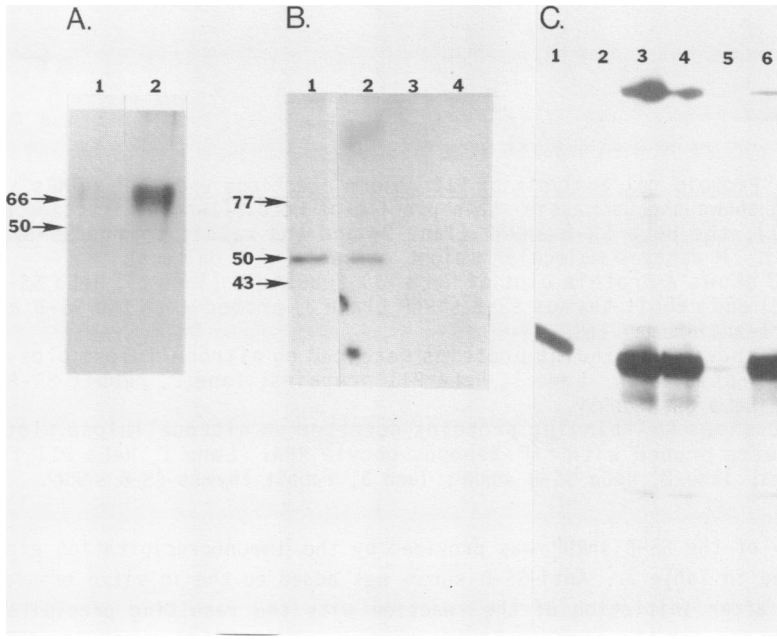

Fig. 5. A DNA binding protein rescues SS-B inhibition.
The SS-B snRNP purified by immunoaffinity chromatography was fractionated by ammonium sulfate precipitation and DNA cellulose chromatography. Panel A shows the Coomassie-stained protein profile of DNA cellulose unbound (lane 1) and bound (lane 2) proteins. Panel B shows a protein blot of (lane 1) SS-B snRNP proteins, (lane 2) 30% ammonium sulfate soluble protein, (lane 3) DNA cellulose unbound and (lane 4) DNA cellulose bound proteins. After SDS polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose and probed with SS-B serum and ^{125}I-anti-human IgG. The positions of molecular weight markers (in K daltons) are shown. Panel C shows rescue of SS-B inhibition of 5S RNA transcription. The HeLa S100 extract (20 µl per reaction) was treated with 5 µl of either normal serum (lane 1) or 4.5 µl of SS-B antiserum (lanes 2-6) and 5 µl of 20% Pansorbin. 5S template DNA (1 µg pX108 DNA per reaction) was preincubated with (lane 3) 5 µl S100; (lane 4) 10 µl rabbit thymus SS-B snRNP (1 µg protein); (lane 5) 10 µl DNA cellulose unbound fraction (0.6 µg protein); (lane 6) 10 µl DNA cellulose bound fraction (0.6 µg protein). After 15 min preincubation, the immunoprecipitated S100 and nucleoside triphosphates were added. Products of transcription were analyzed by gel electrophoresis and the autoradiogram is shown.

affinity chromatography. We subjected the ammonium sulfate precipitate to DNA-cellulose chromatography. Figure 5A shows the stained protein profile of the DNA-binding proteins, indicating a broad band at 64-68,000 daltons, consisting of at least three polypeptide species. Although precipitation of the transcription factor is not complete at 30% ammonium sulfate (as determined by rescue experiments), this concentration was empirically chosen to effectively separate the SS-B antigen from the bulk of the transcription factor as shown by the immunoblots of Figure 5B. The 50K dalton SS-B species is seen exclusively in the 30% ammonium sulfate supernatant (lane 2) but not in the DNA-cellulose fractions of the precipitated material (lanes 3-4). As seen in Figure 5C, the DNA-cellulose bound fraction retains the ability to restore transcriptional activity to an anti-SS-B depleted extract (lane 6). Similarly, this fraction substitutes for the P11 fraction C in the reconstitution experiments of Figure 3 (lane 8). These results demonstrate that the 50K dalton SS-B antigen is unlikely to be the transcription factor. Although we cannot exclude the possibility that some minor protein species is the SS-B snRNP transcription factor, it seems likely that a set of polypeptides of 64-68K daltons is associated with an activity required for transcription of class III genes.

DISCUSSION

We have shown that sera from some patients with the autoimmune diseases, Sjogren's Syndrome and systemic lupus erythematosus, effectively inhibit transcription of class III genes (5S RNA, tRNA, adenovirus VA I RNA genes, and a rat repetitive sequence) in an in vitro transcription extract (Figure 1). Inhibition of pol III transcription is observed with antisera exhibiting SS-B (or La) specificity. The SS-B antigen has been shown to be a 50,000 dalton polypeptide involved in the packaging of most, if not all pol III transcripts (11-12). We find that 5S RNA transcripts synthesized in vitro can be immunoprecipitated with anti-SS-B serum (Table 2); similar results have been obtained by Francoeur and Mathews (9) using adenovirus VA I RNA genes. Further, Rinke and Steitz (11) have shown that pol III transcripts synthesized from HeLa nuclei in vitro are precipitated with SS-B sera, and have suggested that the SS-B antigen could be involved in the processing, transport or transcription of pol III RNAs. This latter possibility was raised by the analogy of the RNA binding activity of the SS-B antigen to the 5S gene-specific transcription factor TFIIIA. This latter protein associates with both the 5S gene internal promoter region and with 5S RNA transcripts in maturing oocytes (19, 35-36). Our results, however, suggest that the 50 K dalton SS-B antigen is not a pol III transcription factor but that a DNA binding protein associated with the SS-B snRNP is a required pol III factor.

We have used a rescue or complementation assay to identify the transcription factors in the HeLa S100 extract which are removed by anti SS-B immunoprecipitation. When the HeLa S100 was subjected to sequential chromatography on DEAE cellulose and phosphocellulose, high salt elutes from

both columns had rescue activity (Figure 2). These fractions also contained the 50,000 dalton SS-B antigen (Figure 4B). The chromatographic properties of the rescue activity suggest both highly basic and acidic components, namely nucleic acid binding proteins and nucleic acids. The SS-B snRNP isolated by antibody affinity chromatography was also effective in rescuing transcriptional activity to immunodepleted extracts (Figure 2C). SS-B snRNPs from Xenopus oocytes, HeLa, and rabbit thymus were each effective. The dose response experiment of Table 2 argues that a major constituent of the SS-B snRNP is the transcription factor.

Using phosphocellulose P11 chromatography of S100 transcription extracts, Segall et al. (17) have shown that, in addition to TFIIIA, two other protein fractions are required for 5S RNA synthesis in vitro. We find that the HeLa SS-B snRNP can effectively substitute for the P11 high salt eluate fraction ("C") in a reconstitution experiment (Figure 3B). This suggests that the SS-B-associated transcription factor and TFIIIC (as defined by the chromatographic method of Segall et al.) may be the same activities.

To determine which polypeptide constituent(s) of the SS-B snRNP is a DNA binding protein(s), and hence possibly involved in transcription, we probed nitrocellulose protein blots with labeled 5S plasmid DNA. We found that the SS-B snRNP isolated by immunoaffinity chromatography contained a 64K dalton DNA binding protein (Figure 4C). This polypeptide was present in SS-B snRNPs from HeLa and rabbit as well as in the phosphocellulose P11 high salt eluate. DNA binding activity as assayed by this method is not sequence specific; both ^{32}P-labeled 5S plasmid DNA and pBR322 vector DNA gave equivalent results (data not shown). When replica protein blots were probed with ^{32}P-labeled RNA, the major RNA binding species appeared to be the 50K dalton SS-B polypeptide (Figure 4D). To demonstrate directly that the 64K dalton polypeptide is associated with transcription activity, we fractionated the SS-B antigen complex by differential ammonium sulfate precipitation and DNA cellulose chromatography. This method allowed separation of the 64K and 50K dalton species and rescue experiments demonstrated that transcription rescue activity resided in the DNA-binding fractions containing the 64-68K dalton polypeptides (Figure 5). Although we cannot rule out the possibility that some minor protein species may be the active factor it appears likely that the 64-68 K dalton polypeptides contain the transcription factor associated with the SS-B snRNP.

Several lines of evidence suggest that the 64K DNA binding proteins and the 50K SS-B antigen may be complexed to each other and snRNAs in the same class of snRNP particles. First, numerous sera of the anti-SS-B specificity inhibit pol III transcription (Table 1) suggesting that the SS-B specificity and not some minor specificity is responsible for immunoprecipitation and inhibition. Although we sometimes observe a second minor reactivity at 60-64 K daltons on protein blots (Figure 4B, lane 2) not all inhibitory sera exhibit this reactivity. (This reactivity may in fact be due to anti-SS-A [29]). Second, several SS-B sera have been used to construct immunoaffinity columns

with similar protein profiles obtained from each. Third, columns constructed with sera of other specificities (anti-Sm and anti-RNP) do not retain the transcription factor activity. This latter observation argues against an artifactual interaction of the 64K proteins with human IgG coupled to Sepharose but does not preclude an artifactual interaction of the 64K proteins with the SS-B snRNP during isolation. However, we think that this is unlikely since disruption of the 64K protein-SS-B antigen interaction requires high salt (30% ammonium sulfate) or 6 M urea. DNA cellulose chromatography of immunopurified SS-B snRNPs without the ammonium sulfate fractionation step fails to separate the 50 K SS-B antigen from the 64-68 K dalton DNA binding proteins (data not shown). Disruption of the snRNPs with urea or ammonium sulfate allows separation of the 50 K antigen from the DNA binding proteins. This suggests an association of these proteins in the same snRNPs; however, direct demonstration of this association will require the availability of monoclonal antibodies.

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