Characterization of Two Protein Activities That Interact at the Promoter of the Trypanosomatid Spliced Leader RNA*

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All trypanosome mRNAs have a spliced leader (SL). The SL RNA gene in Leptomonas seymouri is a member of the small nuclear RNA gene family. However, the SL RNA is required in stoichiometric amounts for splicing during mRNA formation. Expression of the SL RNA gene requires sequence elements at bp −60 to −70 and bp −30 to −40 upstream from the transcription initiation site. Using conventional and affinity chromatography, we have identified and characterized an 122-kDa protein, promoter-binding protein (PBP) −1, that binds to double-strand DNA. The PBP-1-binding site is within the bp −60 to −70 element determined by DNase I footprinting. Therefore, PBP-1 is the first characterized double-strand DNA binding activity that interacts with a trypanosome gene promoter. A second protein, PBP-2, interacts with the PBP-1:DNA complex and its DNase I footprint extends to include the second promoter element (bp −30 to −40). An alteration of the spacing between the two promoter elements or mutation of the second element decreases PBP-2/PBP-1:DNA stability. Taken together, these data suggest that PBP-1 and PBP-2 are components of a transcription initiation complex that assembles within the SL RNA gene promoter.

The Trypanosomatidae are an important family of flagellated, unicellular organisms that parasitize a diverse array of multicellular organisms. The disease-causing trypanosomes, found primarily in developing countries, inflict debilitating symptoms and eventual death on many thousands of people annually.

A distinguishing feature of the protozoan family Trypanosomatidae is the presence of a capped, 39-nucleotide (nt) RNA preceding the translational start site of every mRNA (reviewed in Ref. 1). This short RNA is derived from a 3–4-fold longer RNA, called the spliced leader (SL) RNA or Mini Exon Donor RNA. The 5′-end of the SL RNA fuses to a primary mRNA and the 3′-end is rapidly degraded. Although the trans-splicing of mRNA by an SL RNA occurs in several trematodes, nematodes, and echinoderms, only in trypanosomatids is addition of an SL RNA essential for the formation of every mRNA (2).

The SL RNA genes are members of the small nuclear (sn) RNA class of eukaryotic genes (3–5). These genes encode short, nonpolyadenylated RNAs that participate in mRNA-processing reactions. In higher eukaryotes, in which snRNA genes have been extensively studied, snRNAs are synthesized from independent transcription units that contain promoter elements specific to this gene class (6, and reviewed in Refs. 7–9). The presence of common promoter elements was unexpected since some snRNA genes are transcribed by RNA polymerase II and others by RNA polymerase III. In trypanosomatids, the SL RNA genes appear to be transcribed by RNA polymerase II, whereas other snRNA genes, including the U2 snRNA, U6 snRNA, and U3 snRNA homolog (U-snRNA B) genes, are transcribed by RNA polymerase III (5, 10–12).

To characterize transcription of the SL RNA, we began by assessing the effects of mutations on a marked copy of an SL RNA gene. The marked gene was reintroduced into Leptomonas seymouri, an easily manipulated trypanosomatid, using a stably maintained extrachromosomal vector (13, 14). Three upstream cis-acting elements, positioned between bp −1 to −10, −30 to −40, and −60 to −70 upstream from the transcription initiation site (+1) were identified (4) (see Fig. 1). Analysis of the SL RNA gene promoter from two other trypanosomatids, Leishmania tarentolae and L. amazonensis, revealed a similar SL RNA gene promoter structure (3, 5). It has been suggested that the bp −60 to −70 region of the SL RNA gene promoter in trypanosomes is analogous to the proximal sequence element (PSE) found within the snRNA genes of other eukaryotes (3–5). PSEs, identified in vertebrates and sea urchins, are located −55 bp upstream of the snRNA gene transcription initiation site in these eukaryotes (8, 9, 15). The trypanosomatid SL RNA gene PSE sequence contains a trypanosome-specific 5′-GAC-3′ core region (5) (and see below) but is divergent from higher eukaryotic PSE sequences, as expected.

In preliminary biochemical experiments, we detected two specific protein-DNA complexes that required wild type (WT) DNA sequences in two promoter elements defined in vivo (4). In this study, we have characterized these complexes (now called I and II) and identified two proteins that assemble at the SL RNA gene promoter.

**EXPERIMENTAL PROCEDURES**

**Parasites**

*Leptomonas seymouri* (ATCC 30220) was maintained in logarithmic phase at room temperature with gentle stirring in *Crithidia* medium described previously (16) and supplemented with 13 mM sodium phosphate adjusted to pH 7.4.

**Protein Purification**

All steps were carried out at 4 °C. All buffers contained a protease inhibitor mixture of 0.1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, and 1 mM leupeptin. Complex I and II forming activities were monitored by an electrophoretic mobility shift assay (EMSA, see below) (4).
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Phosphocellulose Chromatography—Complex I and II forming activities were separated by chromatography on phosphocellulose (P-11, Whatman). 1.5 × 10^12 cells were harvested, washed with phosphate-buffered saline, resuspended in 200 ml of Buffer I (20 mM Hepes-KOH, pH 7.6, 60 mM KCl, 4 mM EDTA, and 0.5 mM dithiothreitol), and sheared by passage through a StanstedTM model 516 Cell Disrupter (4). Nuclei were collected by centrifugation, 3000 × g for 10 min, resuspended in 200 ml of Buffer I (20 mM Hepes-KOH, pH 7.6, 60 mM KCl, 4 mM EDTA, and 0.5 mM dithiothreitol, and 90 mM NaCl). The supernatant was dialyzed overnight against Buffer I containing the basal SL RNA gene promoter. To obtain only complex I, 25 µg/ml poly(dI-dC)-poly(dI-dC) was added to the dialysate and the column was loaded and run at 0.1 ml/min. To obtain complexes I and II, 4 mM MgCl2 was also added to the dialysate and the column was loaded and run at 0.05 ml/min. In addition, loaded protein was retained on the affinity column for 10 min before the wash step. The column was washed extensively with affinity buffer and developed using a linear 0.09–0.5 mM KCl gradient.

Specific DNA Affinity Chromatography—The activity peak (0.96 mg of protein) obtained from the nonspecific double-strand DNA-cellulose chromatographic step was applied to a specific DNA affinity column containing the basal SL RNA gene promoter. To obtain only complex I, 25 µg/ml poly(dI-dC)-poly(dI-dC) was added to the dialysate and the 1.5-ml column was loaded and run at 0.1 ml/min. To obtain complexes I and II, 4 mM MgCl2 was also added to the dialysate and the column was loaded and run at 0.05 ml/min. In addition, loaded protein was retained on the affinity column for 10 min before the wash step. The column was washed extensively with affinity buffer and developed using a linear 0.09–0.5 mM KCl gradient.

Preparation of a Specific DNA Affinity Column—Plasmid pHIL7, which contains two head-to-head copies of the SL RNA gene promoter sequence, from bp –10 to –86 (4) (see Fig. 1),2 cloned into the polylinker region of pBluescript (pBS/SK II; Stratagene Co.), was digested using SacI and BamHI. The BamHI 3’ ends were selectively biotinylated using dATP, dGTP, and biotinylated dUTP (Boehringer Mannheim) and the Klenow fragment of DNA polymerase I. The promoter-containing DNA fragment was separated from the vector sequences after A-15 chromatography and digestion with HindIII. The 0.19-kilobase fragment that contained the SL promoter DNA was bound to 4 ml of immobilized streptavidin (Boehringer Mannheim) and used to make a 1.5-ml specific DNA affinity column (containing 108 pmol of DNA) (17).

2 H. Luo and V. Bellofatto, unpublished data.
Gel Filtration Chromatography—A Superdex 200 HR 10/30 column (S-200, Pharmacia Biotech.) was calibrated at a flow rate of 0.1 ml/min, using affinity buffer containing 0.3 M KCl and the following standards (total protein, 150 μg in 0.1 ml): ferritin, 61 Å; rabbit muscle aldolase, 48.1 Å; bovine serum albumin (BSA), 35.5 Å; and carbonic anhydrase, 20.1 Å. Affinity-purified protein, containing either complex I or both complex I and II forming activity, was concentrated in a Centricon™ 10 (Amicon) to 0.1 ml (~35 μg) and run under the conditions used for the standards.

Glycerol Gradient Sedimentation—Triplicate 5-ml 15–30% (v/v) linear glycerol gradients in affinity buffer containing 0.3 M KCl were set overnight at 4 °C. The gradients were overlaid with 10–30 mg/0.1-ml S-200-purified proteins or 30 mg/0.1-ml standards (bovine liver catalase (11.30 S), rabbit muscle aldolase (7.35 S), BSA (4.30 S), hen egg ovalbumin (3.66 S) (Pharmacia Biotech). Protein eluted from the S-200 column was concentrated in a Centricon™ 10 to 0.1 ml and loaded onto the gradient. The gradients were centrifuged at 42,000 rpm in a Beckman SW 55 rotor, for 28 h at 4 °C. The gradients were fractionated from top to bottom and 0.125-ml fractions were collected.

EMSA Analysis

Substrates—The 66-bp WT promoter (bp 217 to 283 region) was made by annealing two 66-nt complementary oligonucleotides (see Fig. 1). The 95-bp WT substrate (bp 21 to 295 region) was made by polymerase chain reaction amplification of a cloned copy of the SL RNA gene and appropriate primers (4).

The “37-bp” and “29-bp” probes were made by digesting the 66-bp WT promoter with Tru9 I. The 37-bp probe contained the bp 246 to 283 region, the 29-bp region contained the bp 245 to 217 region.

The “d-43/46” probe was made by annealing an oligonucleotide that contained the sequence from nt 283 to 265 of the coding strand (5’-GGCTACTATATATACATAGA-3’) to a 64-nt oligonucleotide that contained the noncoding strand from nt 17 to 88, but without nt 43 to 46. The coding strand was extended using the Klenow fragment of DNA polymerase I to produce a double-strand DNA.

The “A-31/40” probe was made by annealing two complementary 66 nt oligonucleotides, from nt 17 to 83, with a 10-bp mutation of nt 31 to 40 (5’-(C)8CT-3’ on the coding strand) to 5’-GAGGTTAACG-3’.

**TABLE I**

| Fraction | Protein\(^a\) | Volume | Total activity\(^b\) (10^8) | Specific activity\(^c\) |
|----------|--------------|--------|--------------------------|------------------------|
| Nuclear extract | 760.0 | 190.0 | 133.0 | 0.17 |
| DEAE\(^d\) | 72.0 | 46.0 | 156.0 | 2.17 |
| Heparin | 9.60 | 48.0 | 84.6 | 8.75 |
| dsDNA-cellulose | 0.96 | 1.2 | 31.7 | 33.0 |
| DNA affinity | 0.07 | 1.0 | 3.80 | 54.3 |

\(^a\) Protein was determined by the bicinchoninic acid method (Pierce).
\(^b\) One unit is equal to an arbitrary number of PhosphorImager (Molecular Dynamics) density values present in complex I after EMSA analysis.
\(^c\) Specific activity is total activity per mg of protein.
\(^d\) Peak activity fractions were pooled and diluted 2-fold prior to loading onto heparin.

**FIG. 3.** Partial purification of PBP-1 using specific DNA affinity chromatography. A, EMSA analysis of the PBP-1 activity peak. Fractions 11–37 are shown. Complex I is indicated by an arrow. Free DNA is indicated at the bottom of the gel. B, aliquots (8 μl) of the overlined fractions were analyzed by 8% SDS-PAGE. The three polypeptides, molecular masses of 57, 46, and 36 kDa, that co-fractionate with the peak of complex I-forming activity are indicated by dots. The trichloroacetic acid (TCA) lane contains a similarly fractionated extract from an independent purification. Lane M contains molecular weight markers.

**FIG. 4.** Determination of the Stokes’ radius and sedimentation coefficient of PBP-1 and PBP-2. Analytical gel filtration (A) and sedimentation (B) were performed at 0.3 M KCl. Standards (open boxes): PBP-1 or PBP-2 (closed boxes). CA, carbonic anhydrase (20.1 Å); BSA, (35.5 Å, 4.30 S); ALD, aldolase (48.1 Å, 7.35 S); FER, ferritin (61 Å); OVA, ovalbumin (3.66 S); CAT, catalase (11.30 S).
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**Fig. 5.** Density sedimentation fractionation of the PBP-1 fraction. EMSA analysis of fractions obtained from density centrifugation. A, protein from S-200 chromatography (load) was applied to a 15–30% glycerol gradient. The probe was the WT 95-bp DNA. B, the binding specificity of fractionated PBP-1 was assessed using competitor DNAs in an EMSA. Fractions 20–23 were pooled, incubated with WT 95-bp probe in the presence of a 50-fold molar excess of either unlabeled WT 95-bp DNA ("S") or a pBS DNA fragment ("N"). Complex I and free DNA are indicated by arrows.

**Fig. 6.** Complex I and II bind within essential promoter elements. PhosphorImager analyses of a 6% denaturing gels are shown. A, DNase I protection of promoter DNA using affinity-purified PBP-1 (see fraction 12, Fig. 10, A). Lane "I" is DNA extracted from complex I after EMSA. "F" and "F*" are DNA extracted from the unbound substrate; DNA in "F" migrated ahead of the majority of free substrate (F) and was the more extensively digested substrate. The vertical bars show the protected DNA region. A, DNase I-hypersensitive site lies near the middle of the binding site, possibly due to localized DNA distortion by PBP-1. Numbers on the right indicate distance (in bp) from the transcription initiation site (see Fig. 1). B, DNase I protection of promoter DNA using affinity-purified complex II forming activity (fraction 22, see Fig. 10, A). "II" is DNA extracted from complex II. I and F are as in A.

The "Δ60/74" substrate was made by annealing an oligonucleotide (5'-CTATATACGGCTGTACCAGCTGGAGCGGGTGCATTAACTC-3') to a primer complementary to nt -17 to -36. The Klenow fragment of DNA polymerase I was used to complete the double-strand molecule. The product was cloned into pBS/SK II and a standard polymerase chain reaction using the SK and KS primers (Stratagene), produced a 133-bp fragment that contained the polylinker region of pBS/SK II. Each substrate DNA was polyacrylamide gel-purified before use.

**Assay Conditions—**Reaction mixtures (20 μl), containing affinity buffer, 0.5 μg of poly(dI-dC)-poly(dI-dC), 100 mM KCl, 4 mM MgCl2, and 20 fmol of the γ-32P-labeled substrates were incubated with titrated amounts of protein for 30 min at room temperature (4). The reaction products were applied to a neutral 4% polyacrylamide gel in 0.5 × Tris borate-EDTA buffer and electrophoresed at 13 mA for 2 h at room temperature. Protein-DNA complexes were quantitated by PhosphorImager analysis of dried gels.

**DNase I Protection Assays**

A 130-bp HindIII/BamHI fragment from pLL 95-6 (a derivative of pBS/SK II containing the bp -1 to -95 SL RNA gene promoter region) was gel purified and uniquely 5'-end labeled at the recessed 3' HindIII site using the Klenow fragment of DNA polymerase I and [α-32P]dATP. 10 ng of probe (106 cpm) was incubated with 8 μl (~1 μg) of affinity-purified protein in a 20-μl reaction under standard EMSA conditions. DNase I (25 ng) and CaCl2 (1 mM) were added, and the reaction was terminated with 5 mM EDTA after 1 min at room temperature (18). DNase I-nicked free and bound substrate was separated by EMSA and visualized by autoradiography. The DNA fragments were excised from the gel and electrophoresed on a 6% polyacrylamide, 7 M urea gel in 1 × Tris borate-EDTA buffer. The corresponding dideoxy-sequenced SL RNA gene promoter region was run on the same gel.

**Photocross-linking**

Uniformly-labeled DNA was prepared by annealing an oligonucleotide (nt -83 to -63, top strand) to the bottom strand (66 nt oligonucleotide) WT SL promoter template and filling in with dATP, dCTP, 8'-bromouracil, and [α-32P]dGTP. The product was digested with Pvu 9

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**Fig. 7.** PBP-1 and PBP-2 binding to mutated promoters. A, WT 95-bp probe or a mutated promoter (Δ60/74; see Fig. 1) and affinity-purified PBP-1 were combined in an EMSA. B, WT 66-bp probe or a truncated probe which contained only the bp -46 to -83 bp sequence (37 bp) and affinity-purified PBP-1/PBP-2 were combined in an EMSA. The unbound 37-bp probe migrated ahead of the 66-bp probe. Complex I, II, and free DNA are indicated by arrows.
Identification of Nuclear Proteins Interacting Specifically with the SL Promoter—Nuclear extracts contained proteins that generated two specific protein-promoter complexes. The more slowly migrating complex, complex II, was in greater abundance. Fractionation of extract on phosphocellulose resulted in the loss of complex II forming activity and a substantial increase in complex I forming activity. Fig. 2 shows that complex II could be restored by mixing the complex I forming fraction (fraction 42) with a fraction (fraction 64) that alone did not produce a significant gel shift. When a fixed amount of fraction 42 was mixed with increasing amounts of fraction 64, more complex II was produced. As was the case for unfraccionated extract, both complexes could be competed only with WT promoter-containing unlabeled DNA (data not shown). These data suggest that complex I and II share a common specific DNA-binding protein. This protein is named promoter binding protein (PBP)-1. The protein that supershifts complex I to complex II is referred to as PBP-2.

Partial Purification of PBP-1—We fractionated nuclear extract through several chromatographic steps and obtained a 320-fold purification (Table I). The essential features of the fractionation included a high salt extract of protein from a crude preparation of nuclei (19); the use of an ion-exchange specific double-strand DNA-cellulose column, and a specific DNA affinity column. EMSA of fractions obtained from the final chromatography step showed that the peak of PBP-1 activity eluted at 0.26 M KCl (Fig. 3, panel A). Although the recovery of PBP-1 from this final column was low (10%) this step was key in removing a majority of the protein that co-purified with PBP-1 through the nonspecific DNA column. We found that three polypeptides co-fractionated with the peak of complex I forming activity (Fig. 3, panels A and B). These proteins have apparent molecular masses of 57, 46, and 36 kDa. The affinity-purified PBP-1 was used to analyze the activity of this protein on DNA and for its physical characterization.

Physical Properties of PBP-1—PBP-1 was subjected to two analytical procedures. Gel filtration chromatography on Superdex-200 showed that PBP-1 had a molecular mass of approximately 140 kDa and a Stokes' radius of 41 ± 3.3 Å (Fig. 4, panel A). After concentration of the PBP-1 peak from gel filtration, PBP-1 was sedimented through a 15–30% glycerol gradient. EMSA analysis of protein fractionated in the glycerol gradient is shown in Fig. 5, panel A. The activity was present in fraction
PBP-2 with a constant amount of PBP-1. Lane 4
was calculated by incorporating the Stokes’ radius and sedi-
fications, a reasonable estimate for both native molecular weight
and frictional ratio could be made. The native molecular weight
of PBP-2 is added to PBP-1 and DNA. PBP-1 fraction was
affinity-purified protein; PBP-2 was obtained using gel filtration (S-
coefficient values into the standard hydrodynamics
100-fold molar excess in competition ex-
periments (lanes 2–7). Lane 1 shows the
binding of protein to DNA without any
competitors. Complex I, II, and free DNA
are indicated by arrows.

17–28; the peak of activity was in fraction 17–23. These fractions
contained an approximately quantitative recovery of
PBP-1 activity. Fig. 5, panel B, shows that the PBP-1 recovered
from the sedimentation procedure retained the same DNA
binding specificity as did the input material. This analysis of
PBP-1 gave an s20,w of 7.25 ± 0.8 (Fig. 4, panel B).

Since these data were obtained using nondenaturing conditions,
a reasonable estimate for both native molecular weight
and frictional ratio could be made. The native molecular weight
was calculated by incorporating the Stokes’ radius and sedi-
mentation coefficient values into the standard hydrodynamics
equation of Siegel and Monty (20). A native molecular mass of
122 ± 16 kDa (x ± 1 S.D.) was calculated for PBP-1. Using this
value, we calculated a shape factor of f/f0 of 1.2, which indicated
that PBP-1 was a relatively symmetric molecule. This predicted symmetry was qualitatively consistent with the inde-
pendent molecular weight calculations made by comparing ei-
ther the peak of PBP-1 activity that eluted from the gel
filtration column or the relative sedimentation of PBP-1 with
molecular weight standards. These data indicate that PBP-1 is
either a single polypeptide that was proteolytically cleaved into
the 57-, 46-, and 36-kDa polypeptides during purification or
that PBP-1 is a multisubunit protein.

**PBP-1 Binds to an Essential Region of the SL RNA Gene**

Promoter—DNase I footprint analysis was used to localize the
binding site of PBP-1 on the SL promoter. Fig. 6, panel A,
shows that the DNase I-resistant area was between nt −55 to
−74 on the bottom strand of the promoter (the top strand
represents the SL RNA coding region that is downstream from
the promoter). The breadth of the footprint was 18 nt, indicat-
ing that PBP-1 was a large protein, consistent with a molecular
mass of 122 kDa.

To assess the sequence specificity of the PBP-1:DNA inter-
action, a mutated DNA probe (Δ-60/74; Fig. 1) was tested for
PBP-1 binding. Fig. 7, panel A, shows that complex I did not
assemble on this mutated DNA. In the converse experiment,
panel B shows that the 37-bp region (bp −46 to −83) was
sufficient for PBP-1 binding.

To test if PBP-1 was specific for double- or single-strand
DNA we performed the EMSA analysis shown in Fig. 8. These
data show that neither strand of the promoter nor two irrele-
vant DNA strands could compete complex I (Fig. 8, panel A,
lanes 1–8). Complex I was destabilized only when the oligonu-
cleotides that correspond to the SL promoter were annealed
prior to being used as competitor (lanes 9–12). To determine if
PBP-1 bound weakly to single-strand promoter DNA, we used
radiolabeled single-strand DNA probes in EMSA. Fig. 8, panel
B, lanes 4 and 5, shows that PBP-1 did not bind to these probes.
These data demonstrate that PBP-1 is a sequence-specific
double-strand DNA-binding protein.

Photocross-linking was used to determine which polypeptide
present in the affinity-purified PBP-1 preparation interacted
with the SL promoter. Fig. 9, panel A, shows that the 37-bp
promoter region (bp −46 to −83) cross-linked to the 46-kDa
species (see Fig. 3, panel B). The 46-kDa protein binding was
specific for the promoter sequence since this interaction could
be competed by the WT SL promoter and not by the mutated
promoter (Δ-60/74; Fig. 1). Panel B shows the complexes re-
higher salt concentration (0.3 M KCl) than did complex I (0.26 M KCl). Competition experiments (see "Experimental Procedures"), Fig. 10, panel A, lanes 1–4 contained the 6-43/46 substrate (see Fig. 1); lanes 5–8 contained WT 66-bp probe as substrate. B, lane 1 contained WT 66-bp probe; lanes 2–5 contained the mutated probe Δ31/40 as substrate. C, lane 1 contained the mutated probe Δ60/74 as substrate. Protein additions to the reactions are indicated above the lanes. Complex I and II are indicated by arrows.

Analysis of PBP-2—Complex II forming activity co-eluted with complex I forming activity through DEAE, heparin-Sepharose, and double-strand DNA cellulose. The specific DNA binding reactions are shown using affinity-purified PBP-1 and the PBP-2 fraction (fraction 64 from P-11), as indicated above each lane. A, lanes 1–4 contained the 6-43/46 substrate (see Fig. 1); lanes 5–8 contained WT 66-bp probe as substrate. B, lane 1 contained WT 66-bp probe; lanes 2–5 contained the mutated probe Δ31/40 as substrate. C, lane 1 contained the mutated probe Δ60/74 as substrate. Protein additions to the reactions are indicated above the lanes. Complex I and II are indicated by arrows.

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**FIG. 12.** Complex II formation requires two promoter elements. DNA binding reactions were shown using affinity-purified PBP-1 and the PBP-2 fraction (fraction 64 from P-11), as indicated above each lane. A, lanes 1–4 contained the 6-43/46 substrate (see Fig. 1); lanes 5–8 contained WT 66-bp probe as substrate. B, lane 1 contained WT 66-bp probe; lanes 2–5 contained the mutated probe Δ31/40 as substrate. C, lane 1 contained the mutated probe Δ60/74 as substrate. Protein additions to the reactions are indicated above the lanes. Complex I and II are indicated by arrows.

**FIG. 13.** Characterization of complex I and II formation on DNA. A, affinity-purified protein was assayed under standard EMSA conditions except that KCl concentrations were varied as indicated above each lane. B, affinity-purified protein was assayed under standard EMSA conditions except that MgCl₂ concentrations were varied as indicated above each lane. C, gel filtration-fractionated PBP-2, affinity-purified PBP-1, and/or BSA were incubated with WT 66-bp probe under standard EMSA conditions. Lane 1, PBP-1 alone (0.05 mg/ml); lane 2, PBP-2 alone (0.03 mg/ml); lane 3, BSA (0.05 mg/ml); lanes 4–6, PBP-1 and increasing amounts (0.05–0.2 mg/ml) BSA. Lanes 7–9 contained PBP-2 (0.03 mg/ml) and the increasing amounts of BSA (0.05–0.2 mg/ml). Lane 10 contained PBP-1 (0.05 mg/ml), PBP-2 (0.03 mg/ml), and BSA (0.05 mg/ml). Complexes I, II, and free DNA are indicated by arrows.

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FIG. 14. Kinetics of complex I and II association and dissociation. EMSAs are shown. A, association rate of complex I and II. An EMSA was performed under standard conditions. Aliquots were loaded onto the gel after incubation for different amounts of time, from 0.1 to 32 min. B, dissociation rate of complex I and II. An EMSA was performed under standard conditions for 10 min before a 50-fold molar excess of unlabeled WT 66-bp DNA was added as competitor. Aliquots were loaded onto the gel after incubation for different amounts of time, from 0.1 to 32 min. Lane “+” is no competitor; Lane “−” is competitor added before the 10-min preincubation.

Characterization of Complex I and II Assembly on the SL Promoter—Fig. 13 shows the effect of varying KCl and MgCl₂ concentrations on complex I and II formation in EMSA. Complex II formation was increased with increasing concentrations of KCl whereas complex I was decreased (panel A). Complex II was markedly affected by MgCl₂ concentrations; optimal binding was observed at 2–4 mM MgCl₂ (panel B). Complex I was insensitive to varying MgCl₂ concentrations. Addition of ATP did not appear to have an effect on formation of either complex (data not shown). Panel C shows that BSA mixed with either PBP-1 (lanes 4–6) or PBP-2 (lanes 7–9) did not yield complex II. Therefore, PBP-2 cannot be replaced with a nonspecific protein to assemble complex II.

We examined the association and dissociation rates of complexes I and II. PBP-1 was incubated alone or with PBP-2 and DNA under standard EMSA conditions for increasing amounts of time. Complexes were detected by EMSA (Fig. 14). PBP-1 alone bound DNA very rapidly; the rate was so rapid that by “0.1” min significant amounts of complex I were formed. Complex II formed much more slowly; initially rates were low and the amount of complex formed increased during the 32-min assay (panel A).

The dissociation rate of both complexes I and II were compared by mixing either PBP-1 alone or with PBP-2 and DNA for 10 min, to establish binding, and then challenging the complexes with unlabeled 50-fold molar excess WT promoter DNA (Fig. 14, panel B). PBP-1 alone readily dissociated from DNA; this reaction was very rapid and most of complex I was destabilized by the first time point (0.1 min). In contrast, complex II was stable and did not significantly dissociate after 32 min.

DISCUSSION

Studies of SL RNA gene expression in three organisms of the Trypanosomatidae family of protozoa have shown that the basal SL RNA gene promoter lies upstream from the transcription initiation site (+1) between bp −1 and −100. In L. seymouri, base substitution mutagenesis data indicated that sequences between bp −1 to −10, −20 to −40, and −50 to −70 were essential for gene expression in vivo (summarized in Fig. 1). PBP-1 binds in a sequence specific fashion to double-strand DNA in the bp −60 to −70 promoter element. This promoter element contains a 5′-GAC-3′ sequence that is also present in the SL promoters of two Leishmania species (3, 5). These data suggest that PBP-1 is a transcription factor within the SL initiation complex. Three polypeptides, 57, 46, and 36 kDa, co-fractionated with PBP-1 binding activity through multiple chromatographic steps and finally through sedimentation density centrifugation and gel filtration chromatography at 0.3 M KCl. The 46-kDa species was photocross-linked to the SL gene promoter. These data suggest that PBP-1 may be a tightly assembled trimeric complex which has an apparent molecular mass of 122 ± 16 kDa. Proof of these contentions awaits in vitro transcription experiments in which the role of PBP-1 can be assessed directly. We have recently developed an L. seymouri in vitro transcription system that accurately initiates SL RNA gene transcription (21). This system will be instrumental in defining PBP-1’s role in transcription.

In vitro experiments showed that both the bp −30 to −40 and the bp −60 to −70 sequences were necessary for SL transcription. Both regions were necessary in their WT configuration for complex II formation. Kinetic experiments showed that complex II had a slower dissociation rate from DNA than did complex I. In addition, complex II elutes from the specific DNA affinity column at a higher salt concentration than did complex...
I. Therefore, complex II is a more stable protein-promoter interaction than is complex I. These experiments argue that complex II may facilitate the formation of the initiation complex that assembles at the SL promoter.

The data presented here support two models for complex II formation. Complex II is PBP-2 bound to complex I and to the bp −30 to −40 promoter element (model 1). Alternatively, complex II is PBP-2 bound independently or in the presence of a third protein (or a modified PBP-1) to the two promoter elements (model 2). Both models are consistent with the finding that complexes I and II have different KCl and MgCl₂ optima, different dissociation and association rates, and overlapping DNase I footprint patterns. Model 2 is supported by the finding that complex II could not be completely lost during S-200 fractionation and that complex I could not be completely supershifted to complex II with increasing amounts of S-200 purified PBP-2. However, the majority of our findings support model 1. First, PBP-2, isolated from P-11 chromatography, did not bind to DNA in an EMSA unless it was mixed with PBP-1. Second, limiting amounts of PBP-1 incubated with PBP-2 generated increased amounts of complex II (Fig. 2, lanes 2–4). S-200-purified PBP-2 only produced complex II when incubated with affinity-purified PBP-1 and not with an irrelevant protein (Fig. 11, lane 6, and Fig. 13, panel C, lanes 7–9). In addition, PBP-2 is required specifically for the supershift with PBP-1 since it could not be replaced with BSA (Fig. 13, panel C, lanes 4–6). Third, the difference in migration of complex II as compared with that of complex I in EMSA is consistent with the addition of a protein (PBP-2) added to complex I. Finally, increased stability by the addition of proteins to a promoter: DNA-binding protein complex is reminiscent of transcription factor assembly at other eukaryotic promoters (22).

Furthermore, the findings that support model 2 do not exclude model 1. S-200-fractionated PBP-2 appeared to shift a very small amount of DNA into complex II (see Fig. 13, panel C, lanes 2 and 7–9). This is likely to be due to contamination by small amounts of PBP-1 since PBP-1 and PBP-2 activity peaks were only 1.5 ml apart. Addition of PBP-1 to the S-200 PBP-2 fraction produced significant amounts of complex II, but not at the obvious expense of complex I. Although we would expect that if complex II is a supershifted form of complex I, PBP-2 should titrate all of complex I into complex II. However, this need not occur for several reasons. First, the overall amount of total probe shifted in these experiments was small, and the amount of PBP-2 activity in this final PBP-2 fraction was limiting. Second, the association and dissociation rates of PBP-1 binding to DNA are very rapid whereas the binding of PBP-2 to complex I is relatively slow (see Fig. 14). The result is that complex I cannot be easily captured by PBP-2 to form complex II. Finally, complex II may require an additional protein, or a modified form of PBP-1, that is limiting in the affinity-purified PBP-1 fraction. A detailed analysis of the PBP-2 interaction with PBP-1 awaits the production of purified recombinant proteins.

Studies of snRNA gene transcription in mammalian cells may provide useful paradigms for PBP-1 structure and function in SL RNA gene expression in trypanosomes. In mammalian cells, snRNA genes are expressed from promoters that contain an essential element, the PSE, located ∼55 bp upstream from the transcriptional start site. The PSE is bound by SNAPc (snRNA activating protein complex), also called PTF (PTF binding transcription factor), which is a stable 4-subunit complex with an apparent molecular mass of 200 kDa (23, 24). Our data suggest the Leptomonas PBP-1 protein may be analogous to SNAPc/PTF since it interacts within the PSE of the SL RNA gene promoter.

The only promoter-binding protein in trypanosomes that has been characterized is a 40-kDa single-strand DNA-binding protein from Trypanosoma brucei (25). We have now identified a sequence-specific double-strand DNA-binding protein that interacts at the SL RNA gene promoter. Further analysis of these proteins will identify their structure as well as their specific role in trypanosome gene expression. As ancient eukaryotes, trypanosomes most likely possess primordial components of the eukaryotic transcription machinery. As parasitic organisms, trypanosomes may demonstrate a flexibility in gene expression that is key to their survival. Detailed studies of the protein-DNA complexes that assemble at trypanosome gene promoters will provide insights into molecular aspects of trypanosome biology.

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