Efficient Extraction and Partial Purification of the Polyribosome-Associated Stem−Loop Binding Protein Bound to the 3′ End of Histone mRNA†

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ABSTRACT: Replication-dependent histone mRNAs end in a highly conserved stem−loop sequence rather than a polyA sequence. A 45-kDa stem−loop binding protein (SLBP), which specifically binds the stem−loop of histone mRNA, is present in both polyribosomes and nuclei. An identical 45-kDa protein, as determined by partial protease digestion, is cross-linked to a 30 nt RNA containing the 3′ stem−loop from both nuclei and polyribosomes. The SLBP can also be detected by a Northwestern blot procedure using the 30 nt RNA as a probe. As judged from the Northwestern assay, more than 90% of the SLBP in the cell is found in the polyribosomes with the remaining SLBP localized to the nucleus. Only 5−10% of the SLBP could be extracted from the polyribosomes with salt. Treatment of the polyribosomes with micrococcal nuclease prior to salt extraction solubilized 5−10 times more SLBP as an RNA−protein complex. The SLBP could be subsequently partially purified from this complex.

Replication-dependent histone mRNAs are the only class of metazoan mRNAs that do not have polyA sequences at their 3′ end. Instead replication-dependent histone mRNAs end in a stem−loop structure that has been highly conserved in evolution (Marzluff, 1992; Birnstiel et al., 1985). Histone mRNA levels are tightly coupled to DNA replication. Much of this regulation is postranscriptional (Schümperli, 1986; Marzluff & Pandey, 1988), and a critical aspect of this regulation is the control of the histone mRNA half-life. Histone mRNA is rapidly degraded at the end of S-phase and when cells are treated with inhibitors of DNA replication to simulate the end of S-phase (Sittman et al., 1983; Gallwitz, 1975). The stem−loop sequence at the 3′ end of histone mRNA is necessary and sufficient for the cell to identify a histone mRNA and direct the cytoplasmic degradation (Pandey & Marzluff, 1987; Marzluff & Pandey, 1988). Histone mRNA is degraded 3′ to 5′ by a polyribosome-associated exonuclease (Ross & Kobs, 1986; Ross et al., 1986).

The polyA sequence present at the 3′ end of most mRNAs has a number of functions in mRNA metabolism, which are mediated by interaction with the polyA binding protein (Sachs et al., 1987; Sachs & Davis, 1989; Bernstein & Ross, 1989; Jackson & Standart, 1990), including participating in translation (Gallie, 1991; McGrew & Richter, 1990; Munroe & Jacobson, 1990) and contributing to mRNA stability. Similarly, the 3′ end of histone mRNA is involved in mRNA processing (Melin et al., 1992; Vasserot et al., 1989; Mowry et al., 1989), transport (Williams et al., 1994), translation (Sun et al., 1992) and regulation of stability (Sun et al., 1992; Marzluff, 1992). These functions are likely mediated by interaction with specific protein(s). We have identified a factor (termed the SLBP)[1] that binds to the stem−loop at the 3′ end of histone mRNA and is present both in polyribosomes and in nuclei (Williams & Marzluff, 1995; Pandey et al., 1991). The SLBP is likely to be a component of the histone mRNP (Pandey et al., 1991). Here, we report that the SLBP binds the 3′ end of histone mRNA as a 45-kDa monomer and can be extracted efficiently from polyribosomes only after treatment with micrococcal nuclease. The SLBP can then be partially purified using a Northwestern blot procedure as a binding assay.

EXPERIMENTAL PROCEDURES

Cell Culture. Mouse myeloma cells were grown in Dulbecco's modified eagle's media supplemented with 10% horse serum and 100 units/mL penicillin−streptomycin. For large scale studies, cells were grown in 35- and 15-L spinner flasks (Bellco), and exponentially growing cells ($4-6 \times 10^5$/mL, 16 h generation time) were harvested.

Isolation of Polyribosomes. Cells (15−20 g) were harvested by centrifugation and washed two times with phosphate-buffered saline. The cells were washed once in 5 packed cell vol of hypotonic buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT)] and resuspended in the same amount of hypotonic buffer. The cells were allowed to swell for 25 min (monitored by phase contrast microscopy). The cells were broken by homogenization in a Dounce homogenizer (20−25 strokes with the tight pestle). Cell lysis was monitored by phase contrast microscopy. One-tenth volume of restore buffer (final

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[1] Abbreviations: DTT, dithiothreitol; GuHCl, guanidine hydrochloride; nt, nucleotide; PMSF, phenylmethanesulfonyl fluoride; RSB, reticulocyte standard buffer 1 mM KOAc, 1.5 mM $Mg(OAc)_2$, 10 mM Tris-HCl, pH 7.4; SDS, sodium dodecyl sulfate; SLBP, stem−loop binding protein; TEGD buffer, 20 mM triethanolamine, pH 7.5, 1 mM EDTA, 1% glycerol, 0.1 mM DTT; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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concentration 66% sucrose, 50 mM HEPES, pH 7.9, 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT) was added to prevent nuclear lysis (Shapiro et al., 1988). The nuclei were pelleted by centrifugation at 1000*g* for 3 min. The nuclear pellet was suspended in storage buffer (25% glycerol, 50 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, and 5 mM DTT) at a concentration of 5×10^8 nuclei/mL, quick-frozen in liquid N_2, and stored at −80 °C. The nuclei could be used for subsequent preparation of nuclear extracts, which were prepared as previously described (Pandey et al., 1991). The supernatant was centrifuged at 11000*g* for 10 min to remove cell membranes and mitochondria. The resulting supernatant was adjusted to 0.1% Triton X-100, and 25 mL was layered over a 5-mL sucrose pad (30% sucrose in RSB: 1 mM KOAc, 1.5 mM $Mg(OAc)_2$, and 10 mM Tris, pH 7.4) and centrifuged at 100000*g* in a T865 rotor for 150 min. The translucent pellets were rinsed twice with RSB and resuspended in 0.2 mL/5×10^8 cells of RSB containing 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.01 mM leupeptin, 0.01 mM TPCK, 10 μg/mL aprotinin, 0.75 mM spermidine, and 0.15 mM spermine. The polyribosomal suspensions were frozen in liquid N_2 and stored at −80 °C.

Extraction with Micrococcal Nuclease. Treatment with micrococcal nuclease followed the procedure of Pelham and Jackson (1976) for treatment of reticulocyte lysates. The polyribosomes were thawed, freshly prepared micrococcal nuclease (15000 units/mg) was added to a final concentration of between 1 and 100 units/mL, and the concentration of $CaCl_2$ was adjusted to 1 mM. The samples were incubated at 25 °C for 15 min with constant agitation on a Labquake (Lab Industries Inc., Berkeley, CA). The micrococcal nuclease was inactivated by adjusting the solution to 2 mM EGTA and then cooling on ice for 5 min. Following the nuclease treatment, 4 M KCl was added to a final concentration of 0.8 M, and the polyribosomes were extracted by shaking at 4 °C for 30 min. The samples were centrifuged at 100000*g* for 84 min in a Beckman tabletop ultracentrifuge. The supernatant was dialyzed against buffer A (20 mM HEPES, pH 7.2, 1% glycerol, and 1 mM DTT) containing 0.1 M NaCl, quick-frozen in liquid N_2 and stored at −80 °C. For extraction without micrococcal nuclease, the identical procedure was used except the micrococcal nuclease was omitted.

Mobility-Shift Assay. Oligoribonucleotide probes labeled with [α-^{32}P]UTP were synthesized using T7 RNA polymerase by the method of Milligan et al. (1987) as described previously (Pandey et al., 1991). The mobility-shift assay was performed as previously described (Pandey et al., 1991). The same 30 nt transcript (the wild-type probe shown in Figure 1) was used as a probe in the Northwestern assay.

UV Cross-Linking and Partial Protease Digestion. UV cross-linking was performed as described previously using the 30 nt stem−loop RNA labeled with [^{32}P]UTP as a probe (Pandey et al., 1991). Nuclear and polyribosomal extracts containing similar amounts of SLBP activity as judged by a mobility-shift assay were incubated with equal amounts of either wild-type or mutant 30 nt RNAs and cross-linked with UV light. The reaction was divided and subjected to protease digestion with V8 protease. The undigested and digested products were separated on 12% polyacrylamide gels

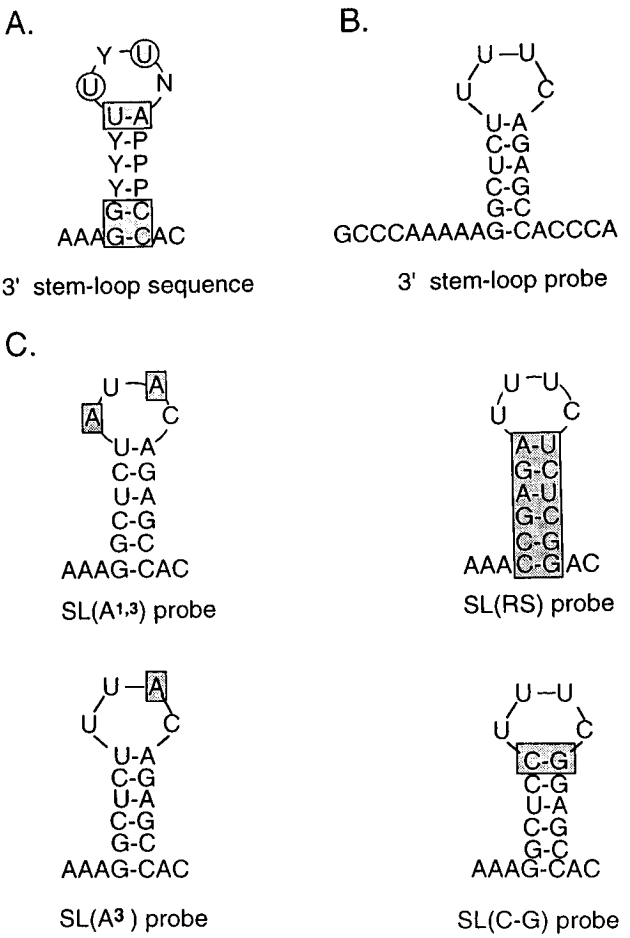

FIGURE 1: 3′ end of histone mRNA. (A) The structure of the 3′ end of a typical replication-dependent mammalian histone mRNA is shown. The boxed and circled nucleotides have been conserved in all metazoans. (B) The sequence of the wild-type stem−loop probe is shown. (C) The four mutant probes, SL(RS), SL($A^{1,3}$), SL(A^3), and SL(C-G), are shown. These probes contained the same nucleotides 5′ and 3′ of the stem−loop as the wild-type probe shown in panel B, but the flanking nucleotides have been omitted from the figure.

(Laemmli, 1970), and the labeled proteins were detected by autoradiography.

Northwestern Assay. Protein samples were resolved by electrophoresis on 12% polyacrylamide gels. The proteins were transferred to nitrocellulose (Amersham Hybond) using the Hoefer Transphor (TE 52X or TE 22) apparatus (1.5 h at 1 amp in Towbin buffer (Towbin & Gordon, 1984): 25 mM Tris, 193 mM glycine, 10% methanol, and 0.1% SDS) at 4 °C. The filter (6×9 cm) was washed with denaturation buffer (50 mM Tris-HCl, pH 8.0, 6 M GuHCl, 6 mM DTT, 2 mM EDTA, and 0.5% BLOTTO) (Johnson et al., 1984) for 1 h at 4 °C with continuous agitation. To achieve uniform wetting of the filter, it was placed in a 50-mL conical centrifuge tube and rotated at room temperature on a SEPCO tube rotator (Scientific Equipment Products, Baltimore, MD). The filter was incubated with renaturation buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.1% NP-40, and 0.25% BLOTTO) with constant agitation for 16−20 h at 4 °C.

The filter was incubated with the radiolabeled probe (6700 cpm/mL; specific activity 1.5×10^9 cpm/ μg) in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 50 mM NaCl for 1 h at 25 °C.

The filters were washed three times each in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA at 25 °C for 10 min, exposed to a PhosphorImager screen, and then subsequently exposed to X-ray film for 16−72 h.

RNA Affinity Chromatography. Biotinylated RNA was synthesized on an Applied Biosystems synthesizer with biotin attached to the CPG matrix (Bioteg, Glen Research) with a 15 atom linker between the biotin and the last nucleotide. The RNA sequence was 5′-ACCCAAAGGCUCUUUUCA-GAGCCACCAC-biotin. The RNA was deblocked and purified by preparative gel electrophoresis, and 1 μg was labeled with [γ-^{32}P]ATP. The labeled RNA was mixed with 50 μg of unlabeled RNA and then incubated with the protein sample at room temperature for 20 min. Heparin (1 mg/mL) was added to reduce nonspecific binding. Streptavidin agarose (Gibco-BRL) was thoroughly washed with 0.2 M NaCl in TEGD buffer (20 mM triethanolamine, pH 7.5, 1 mM EDTA, 1% glycerol, and 0.1 mM DTT), RNAsin (Pharmacia, 500 units/mL), and protease inhibitors (0.01 mM TPCK, 10 μg/mL aprotinin, 1 mM PMSF, and 0.01 mM leupeptin). The RNA−protein complex was added to the streptavidin agarose and incubated for 2 h at 4 °C with constant agitation. The matrix was washed extensively with 0.2 M NaCl and 2.0 M NaCl in TEGD buffer. The bound proteins were eluted with 3.0 M GuHCl in TEGD buffer.

Materials. Cibacron Blue 3-G-A-agarose type 3000-CL and heparin agarose (H-5380, type II) were obtained from Sigma. Streptavidin agarose was obtained from Gibco-BRL. Micrococcal nuclease was purchased from Pharmacia or from Sigma, and identical results were obtained with both preparations. Leupeptin and PMSF were purchased from Sigma. Aprotinin and TPCK were purchased from Boehringer Mannheim. Hybond 100% nitrocellulose was obtained from Amersham. Recombinant La protein and anti-La sera (Chambers et al., 1988) were a generous gift of Drs. Dan Kenan and Jack Keene (Duke University) and were used as controls in several experiments.

RESULTS

Messenger RNAs exist as ribonucleoprotein complexes (mRNPs) while they are being translated on polyribosomes. One protein that is a component of most mRNPs is the polyA binding protein (PABP), which is bound to the polyA tail of mRNAs (Jackson & Standart, 1990; Sachs et al., 1986; Adam et al., 1986). The PABP binds polyA tightly and is removed efficiently only under denaturing conditions (Sachs & Kornberg, 1990). Histone mRNAs are the only metazoan mRNAs that lack polyA, but instead end in a conserved 26 nt sequence which contains a 6-base stem and a 4-base loop (Marzluff, 1992), which is shown in Figure 1A. We have previously identified a 45-kDa protein, which specifically binds to the stem−loop (Pandey et al., 1991). This protein, which we call the SLBP, is present in polyribosomes as assayed by a mobility-shift assay (Pandey et al., 1991) and UV cross-linking. A similar polypeptide is also present in the nuclear fraction (Pandey et al., 1991).

The Nuclear and Polysomal SLBP Are Identical. Using UV cross-linking followed by partial protease digestion, we detected the protein(s) that interact(s) with the stem−loop at the 3′ end of histone mRNA. Previously, we showed that the nuclear and polyribosomal SLBP have similar binding specificities as judged by their affinities for a number of mutant stem−loop sequences in a mobility-shift assay (Williams & Marzluff, 1995). To demonstrate that the polypeptide detected by UV cross-linking had similar binding specificity as observed in the mobility-shift assay, we cross-linked the wild-type sequence and two mutants, SL(CG) with a CG base pair at the top of the stem, and SL(A^3) with an A replacing the conserved U at the third position of the loop (see Figure 1). All three probes were cross-linked to the nuclear and cytoplasmic extracts. As previously reported, the wild-type RNA was cross-linked to a 45-kDa polypeptide (which often was resolved as a doublet) in both the nuclear and polyribosomal extracts (Figure 2A, lanes 3 and 8). Although the mutant RNAs were cross-linked to the same polypeptides, the efficiency of cross-linking was much lower. The SL(CG) RNA (Figure 2A, lanes 2 and 7) bound more efficiently than the SL(A^3) RNA (Figure 2A, lanes 1 and 5) to the 45-kDa protein. The mutants had similar relative affinities in the mobility shift experiments (Williams & Marzluff, 1995).

The nuclear and polyribosomal SLBP gave similar digestion products with V8 protease. The nuclear and polyribosomal SLBP were cross-linked to a labeled stem−loop RNA with UV light (Figure 2B, lanes 1 and 2). The labeled polypeptides were treated with low concentrations of V8 protease, and the partial protease digestion products were resolved by electrophoresis. The two protease digestion products (18 and 14 kDa) of the polyribosomal and nuclear SLBP had identical mobilities, suggesting that the nuclear and polyribosomal 45-kDa proteins are identical. Longer digestion with V8 protease converted all the labeled polypeptides to the 14-kDa polypeptide (not shown).

Northwestern Assay for the SLBP. Some of the polyribosomal associated SLBP was solubilized by extraction with 0.8 M KCl. However after salt extraction, the amounts of SLBP found in the polyribosomal fraction and the nuclear fraction were similar, as measured by mobility-shift assay (Pandey et al., 1991), even though more than 95% of the histone mRNA was present in the polyribosomal fraction. Clearly, if the SLBP is a component of the histone mRNP, then it was probably not efficiently solubilized from the polyribosomes simply by treatment with salt.

Since we had observed that the SLBP bound tightly to the stem−loop even in the presence of high salt (Pandey et al., 1991) and that once bound the SLBP was not readily displaced from the stem−loop by unlabeled RNA (Williams & Marzluff, 1995), we reasoned that the extraction of polyribosomes with salt was likely to be inefficient. Most of the SLBP in polyribosomes is tightly associated with histone mRNA and might not be readily solubilized. Therefore, digestion of the mRNA might be necessary to release the SLBP. Micrococcal nuclease treatment of the polyribosomes, which is known to preferentially cleave mRNAs (Pelham & Jackson, 1976), was used to release an RNP complex. Since the SLBP in this complex is tightly bound to the 3′ end of histone mRNA, it will not be detected by the mobility-shift assay. Hence, we developed a binding assay for the SLBP using a Northwestern blot procedure. This assay allows detection of the SLBP after separation of the polypeptides from the RNA by SDS−polyacrylamide gel electrophoresis.

To detect the SLBP, the polyribosomal proteins were denatured in the presence of SDS, separated by SDS−PAGE and subsequently transferred to nitrocellulose. The proteins

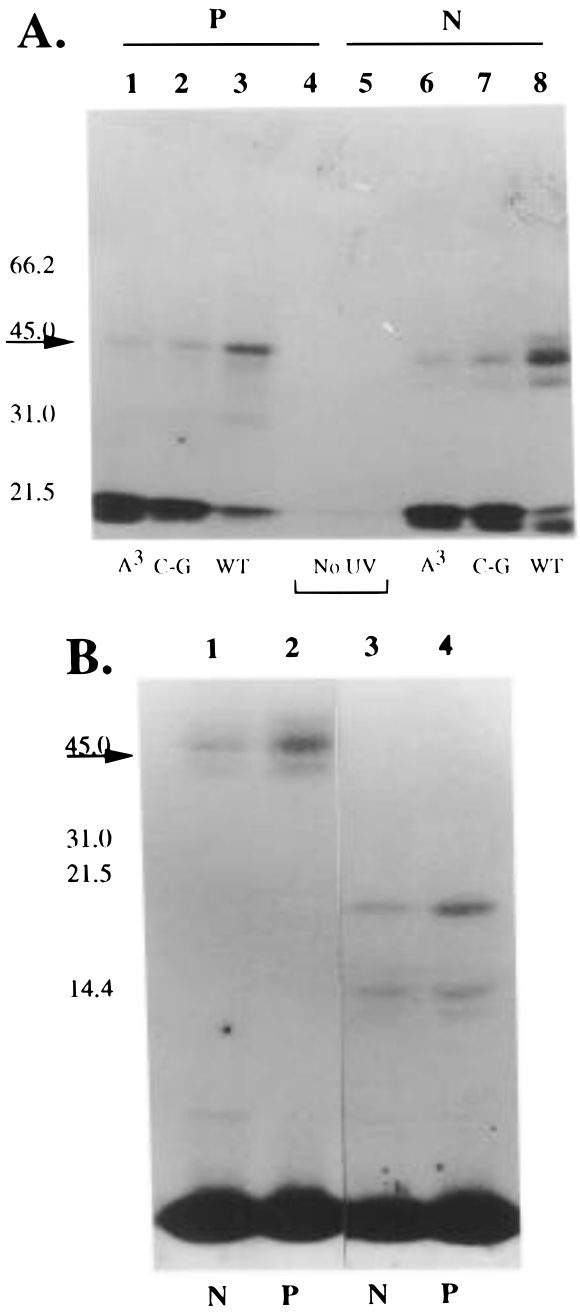

FIGURE 2: Identification of the SLBP by UV cross-linking. (A) 30 nt RNA labeled with [^{32}P]UTP encoding the wild-type stem−loop (lanes 3 and 8), the SL(A^3) mutant (lanes 1 and 6), and the SL-(C-G) mutant (lanes 2 and 7) were synthesized from oligonucleotide templates using T7 RNA polymerase. A total of 10 fmol of the probes was incubated with either a 0.6 M KCl extract of polyribosomes or a 0.6 M KCl extract of nuclei in the presence of 30 μg of yeast tRNA; the reaction was irradiated with UV light and then treated with RNase A. The proteins were resolved by electrophoresis on 12% SDS−polyacrylamide gels, and the cross-linked polypeptides were detected by autoradiography. Lanes 4 and 5 are the analyses of a reaction with the wild-type probe, which was not irradiated. The position of molecular weight markers is indicated. (B) The nuclear and polyribosomal extracts were incubated with the wild-type probe and irradiated with UV light as described in panel A. The samples were divided in half, and one half was treated with V8 protease for 10 min. The samples were then analyzed by electrophoresis on 12% SDS−polyacrylamide gels, and the cross-linked polypeptides were detected by autoradiography. Lanes 1 and 2: UV cross-linked samples of the nuclear and polyribosomal extracts; lanes 3 and 4: V8 protease treated samples of the same reactions.

on the filter were denatured with guanidine hydrochloride and then renatured. The filter was then incubated with the radiolabeled stem−loop RNA. There is a major 45-kDa protein present in polyribosomes that binds to the radiolabeled stem−loop (Figure 3A, lanes 1 and 2), which was the same size as the polypeptide detected by UV cross-linking. The signal was proportional to protein concentration (Figure 3B). In addition, there were some lower molecular weight proteins that were detected in some experiments. These likely represent the abundant ribosomal proteins. However, the amount of the probe bound to the ribosomal proteins was variable in different experiments, even with the same extract, relative to the binding to the 45-kDa polypeptide. The smaller polypeptides could be a result of proteolytic digestion of the 45-kDa polypeptide, but the variability of the binding to these make it more likely that this is nonspecific binding to the abundant ribosomal proteins in the total polyribosomes. After initial fractionation by column chromatography, only a single 45-kDa protein was detected with the SLBP probe, even in complex mixtures of proteins (see below).

Most of the 45-kDa SLBP Is Present in the Polyribosomes. Although the mobility shift (Pandey et al., 1991) and UV cross-linking (Figure 2A) of the nuclear and polysomal extracts was quantitatively similar, the Northwestern analysis of total SLBP gave significantly different results. There was slightly more SLBP present in the polyribosomal extract than in the nuclear extract (Figure 3A, lanes 3 and 4) as determined by the Northwestern assay, in reasonable agreement with the results obtained with the mobility-shift assay. However, most of the SLBP present in polyribosomes was not extracted by the salt treatment alone (Figure 3A, lane 3; see below).

Two different preparations of nuclei and polyribosomes were analyzed to determine the relative amounts of the SLBP in the nucleus and cytoplasm. The polyribosomes and the nuclei were prepared from the same number of cells, and the proteins were resolved by gel electrophoresis. There was very little SLBP detected in the nuclear fraction by the Northwestern blot (Figure 3A, lanes 4 and 5), and about 20 times as much SLBP was detected in the polyribosomes (Figure 3A, lane 6). Thus, the majority of the SLBP is present in polyribosomes.

A 0.8 M KCl extract of the polyribosomes released very little of the SLBP (Figure 3A, cf. lanes 1 and 3, which have equivalent amounts of polyribosomes). We also did not detect significant amounts of SLBP in the cytosol fraction (Pandey et al., 1991; <5% of the amount present in polyribosomes, not shown). The Northwestern assay gave a linear response for the SLBP with increasing amounts of polyribosomal protein (Figure 3B) as well as with partially purified preparations of the SLBP (not shown).

Inclusion of excess nonradioactive stem−loop RNA abolished the binding of the SLBP (Figure 3C). Duplicate samples of total polyribosomal protein (Figure 3C, lanes 1 and 3) or extracted polyribosomal proteins (Figure 3C, lanes 2 and 4) were separated by electrophoresis and then transferred to nitrocellulose. The filter was then cut in half. One half of the filter was incubated with the stem−loop probe (Figure 3C, lanes 1 and 2). The other half of the filter was incubated with 100 ng/mL unlabeled stem−loop probe for 30 min before the addition of the labeled probe (Figure 3C, lanes 3 and 4).

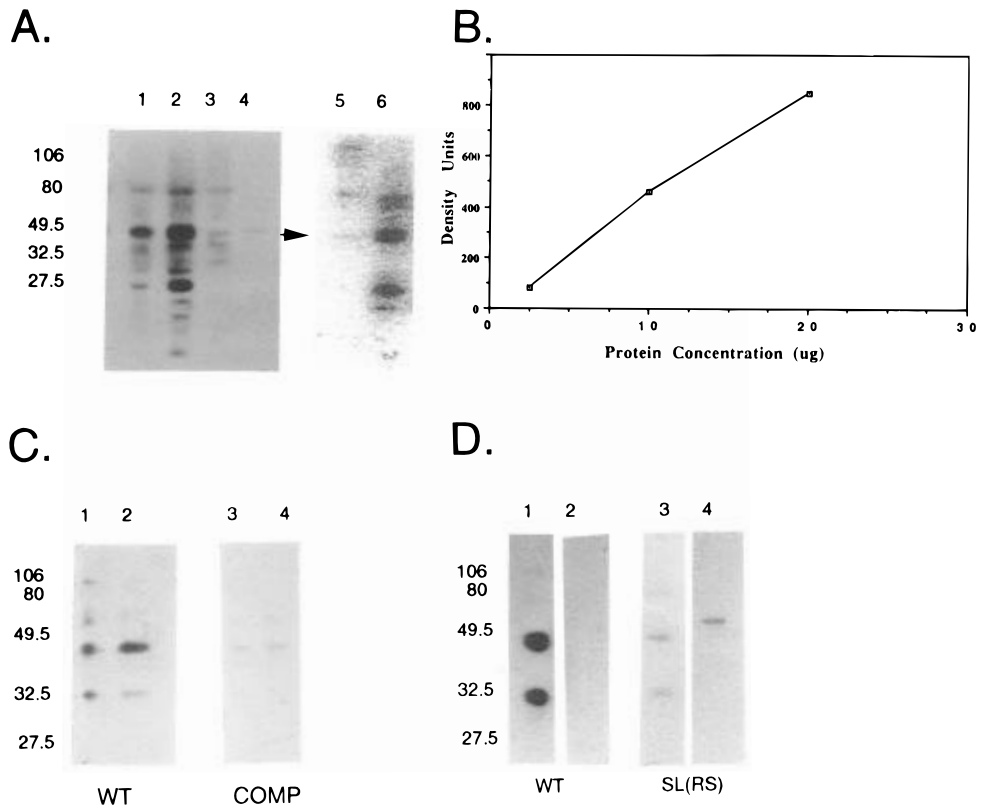

FIGURE 3: Northwestern analysis of SLBP. (A) Total polyribosomal proteins were resolved by electrophoresis on a 12% polyacrylamide gel (lanes 1 and 2, 5 and 25 μg of protein, respectively). A 0.8 M KCl extract of polyribosomes from the same amount of polyribosomes analyzed in lane 1 was analyzed in lane 3. A 0.6 M KCl nuclear extract (25 μg of protein) was analyzed in lane 4. After electrophoresis, the proteins were transferred to nitrocellulose, denatured, renatured, and then incubated with the radiolabeled stem−loop probe (6700 cpm/mL). Lanes 5 and 6 are the analyses of an independent preparation of nuclear extract (lane 5) or polyribosomes (lane 6) from the same number of cells. (B) Varying amounts of total polyribosomes were analyzed by SDS−polyacrylamide gel electrophoresis and the SLBP detected by a Northwestern blot. The intensity of the 45-kDa band was measured using a PhosphorImager. The amounts of protein applied to the gel are plotted against the amount of probe bound (arbitrary density units) to the 45-kDa protein. (C) Duplicate samples of polyribosomal proteins extracted with 0.8 M KCl were analyzed by gel electrophoresis and transferred to nitrocellulose. The filter was cut in half, and one half was incubated with the labeled wild-type probe for 1 h (lanes 1 and 2). The other half (lanes 3 and 4) was first incubated with 100 ng of unlabeled wild-type stem−loop for 30 min, and then the labeled probe was added and incubated for 1 h. (D) Duplicate samples of 25 μg of total polyribosomal protein (lanes 1 and 3) or 0.5 μg of recombinant La protein (lanes 2 and 4) were resolved by gel electrophoresis and transferred to nitrocellulose. The filter was cut in half and incubated with 6700 cpm/mL of either the wild-type (lanes 1 and 2) or the mutant reverse stem (lanes 3 and 4) probe.

The 45-kDa protein binds poorly to a mutant stem−loop, SL(RS), which has the stem sequence reversed but the loop and flanking sequences unchanged. Duplicate filters containing polyribosomal protein or recombinant La protein were incubated with the stem−loop probe (Figure 3D, lanes 1 and 2) or with the SL(RS) probe (Figure 3D, lanes 3 and 4). The wild-type probe again reacted strongly with a 45-kDa polypeptide and did not react with the La protein, which was present in much larger amounts (Figure 3D, compare lanes 1 and 2). In this preparation, we observed binding to a 32-kDa protein also. This could be a proteolytic product of the SLBP or another protein, such as the hnRNP A protein, which is known to be transiently associated with some mRNPs (Piñol-Roma & Dreyfuss, 1992). In comparison, the mutant RS probe reacted weakly with the polyribosomal proteins as opposed to the wild-type probe but clearly reacts with recombinant La (Figure 3D, cf. lanes 3 and 4). In addition, another mutant stem−loop sequence, SL($A^{1,3}$), which has the uridines at positions 1 and 3 in the loop replaced by adenosines, bound the SLBP <5% as well as the wild-type probe using the Northwestern assay (not shown). We have previously shown that the SLBP does not bind either the SL(RS) or SL($A^{1,3}$) probe in the mobility-shift assay (Pandey et al., 1991), although they do bind 1−5% as well as the wild-type sequence as assayed in competition assays (Williams & Marzluff, 1995).

Similar Polypeptides Are Detected in the Mobility Shift and in the Northwestern Assay. To demonstrate that similar polypeptides were detected in the mobility shift, UV cross-linking, and the Northwestern assays, a radiolabeled stem−loop probe was incubated with an extract from polyribosomes, and the SLBP−RNA complex was resolved by mobility-shift analysis (Figure 4A). As a control, an aliquot of the extract was analyzed on the native gel without any RNA, and the material in the same region of the gel was excised and eluted. These proteins were resolved by gel electrophoresis by SDS−PAGE. The proteins were transferred to nitrocellulose, and the filter was incubated with the labeled probe. A protein band with the identical mobility to the major band present in polyribosomes was detected (Figure 4B, lane 3) in the complex, and this band was not present in samples incubated without RNA (Figure 4B, lane 2).

When the proteins present in the complex were precipitated with TCA, separated by SDS−PAGE, and then silver-stained, three polypeptides were detected. One of these proteins had the same mobility as the 45-kDa protein detected by UV cross-linking and Northwestern assay (Figure 4C,

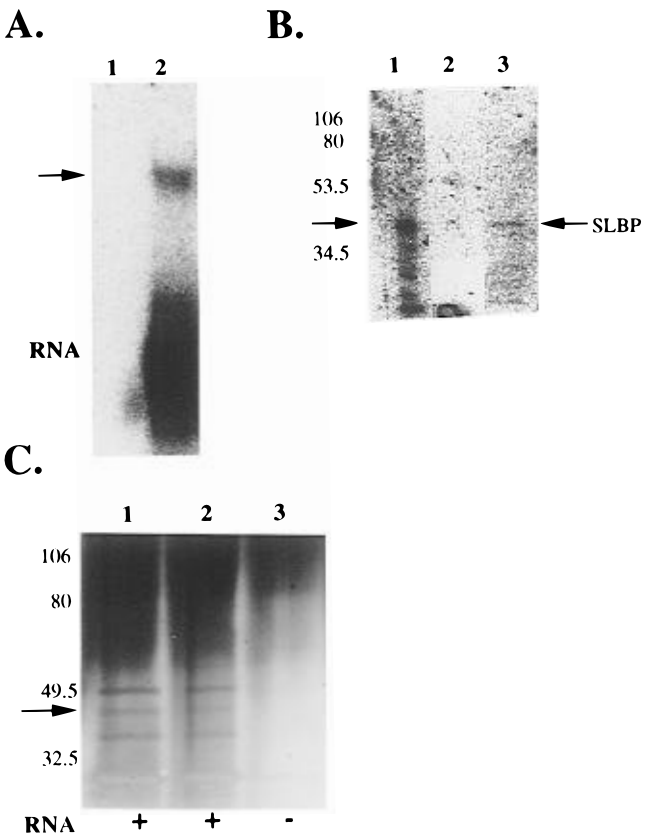

FIGURE 4: Identification of the 45-kDa protein in the SLBP−RNA complex. An extract of polyribosomes treated with micrococcal nuclease (10 units/mL) and then with 0.8 M KCl was incubated with an excess of wild-type RNA labeled with [α-^{32}P]UTP, and the complex was resolved by electrophoresis on a 6% polyacrylamide gel under native conditions. A parallel sample of extract was resolved by electrophoresis in the absence of labeled RNA. The complex was detected by autoradiography (lane 2). (B) The gel slice containing the complex was excised, and the proteins were eluted from the gel and resolved on a 12% SDS−polyacrylamide gel (lane 3). In lane 1, the proteins from polyribosomes were analyzed, and in lane 2 the proteins from a parallel portion of the gel were analyzed. The proteins were transferred to nitrocellulose, the filter was incubated with the labeled wild-type probe, and the labeled polypeptides were detected using a PhosphorImager. The arrow indicates the position of the SLBP. (C) The experiment in panel A was repeated, and the proteins recovered from the gel slice were analyzed on 10% SDS−polyacrylamide gels and detected by staining with silver. Lanes 1 and 2 are samples incubated with the labeled RNA, and lane 3 is from an equivalent part of the same gel where the extract was fractionated without the addition of RNA. The arrow indicates the position of the SLBP detected by the Northwestern assay.

lanes 1 and 2). These proteins were absent in the control sample treated identically except that the RNA was omitted (Figure 4C, lane 3).

Extraction of the SLBP from Polyribosomes with Micrococcal Nuclease. We compared the amount of SLBP extracted from polyribosomes by 0.8 M KCl with and without treatment with micrococcal nuclease. The proteins extracted from polyribosomes were analyzed by the Northwestern blot and the mobility-shift assay. Micrococcal nuclease treatment (either 20 or 100 units/mL) resulted in a 10-fold increase in the amount of the 45-kDa protein released from polyribosomes (Figure 5A, cf. lane 1 with lanes 2 and 3), as judged by the Northwestern assay. In this experiment, there was also some binding of the probe with higher molecular weight proteins, and these also were extracted more efficiently after treatment with micrococcal nuclease. The polypeptides solubilized from polyribosomes with 0.8 M KCl and 0.8 M KCl plus micrococcal nuclease were similar (Figure 5B, cf. lane 2 with lanes 3−5), although there were some additional high molecular weight proteins solubilized by micrococcal nuclease. About 20% of the total polyribosomal protein was extracted with salt, with or without micrococcal nuclease treatment.

There is still residual SLBP activity remaining in the polyribosomal pellet after extraction, but this pellet was very difficult to solubilize, and hence an accurate estimate of the SLBP activity remaining could not be obtained (not shown). However, we compared the amount of SLBP activity in the initial polyribosome preparation with the amount of SLBP extracted with micrococcal nuclease plus salt. About 50% of the total SLBP was extracted from polyribosomes with micrococcal nuclease plus salt (Figure 5A, lanes 4 and 5) compared with less than 10% of SLBP extracted with salt alone (Figure 5A, lane 1; Figure 3A, lanes 1 and 3). In contrast, treatment of the nuclear fraction with RNase (micrococcal nuclease solubilized the chromatin, making analysis impractical) did not increase the amount of SLBP released (not shown).

The efficiency of solubilizing the SLBP was dependent on the amount of micrococcal nuclease used. Increasing the concentration of micrococcal nuclease from 1.5 to 15 units/mL increased the amount of SLBP solubilized, but further digestion with higher concentrations of micrococcal nuclease did not increase the amount of the SLBP extracted (Figure 5A, lanes 6−8, and 10). Even the low levels of micrococcal nuclease increased the release of SLBP significantly (Figure 5A, lanes 6 vs 9). Additional experiments showed that 4−6 units micrococcal nuclease/mL gave maximal release. The 3′ end was removed from all the histone mRNAs at this concentration as measured by an S1 nuclease protection assay (not shown).

We also analyzed the SLBP activity released from polyribosomes by salt in the presence and absence of micrococcal nuclease by the mobility-shift assay (Figure 5C). As judged by this assay, similar SLBP activity was present in the 0.8 M KCl extracts prepared without (Figure 5C, lane 3) or with (Figure 5C, lane 4) micrococcal nuclease. The unextracted polyribosomal sample was also analyzed and showed similar amounts of binding to the extracted samples as measured by the mobility-shift assay (Figure 5C, lane 2). There were fewer "polyribosome equivalents" (one-fifth the amount) analyzed in lane 2 than in lanes 3 and 4, accounting for the apparently lower binding activity in this sample. Thus, the SLBP is present in two forms in the polyribosomes; a minor population that can be solubilized with salt and is available to bind directly with a labeled RNA probe and the major population that is tightly bound to histone mRNA.

There was no difference in the amount of La protein solubilized by treating the polyribosomes with micrococcal nuclease; similar amounts of La were extracted with salt with or without micrococcal nuclease as judged by a Western blot using anti-La antibody (Figure 5D), demonstrating that micrococcal nuclease was not necessary for solubilizing all RNA binding proteins.

The SLBP Extracted from Polyribosomes Is a Monomer. To determine the size of the SLBP−RNA complex released from polyribosomes with micrococcal nuclease, the extract was chromatographed on an S-200 column and the SLBP

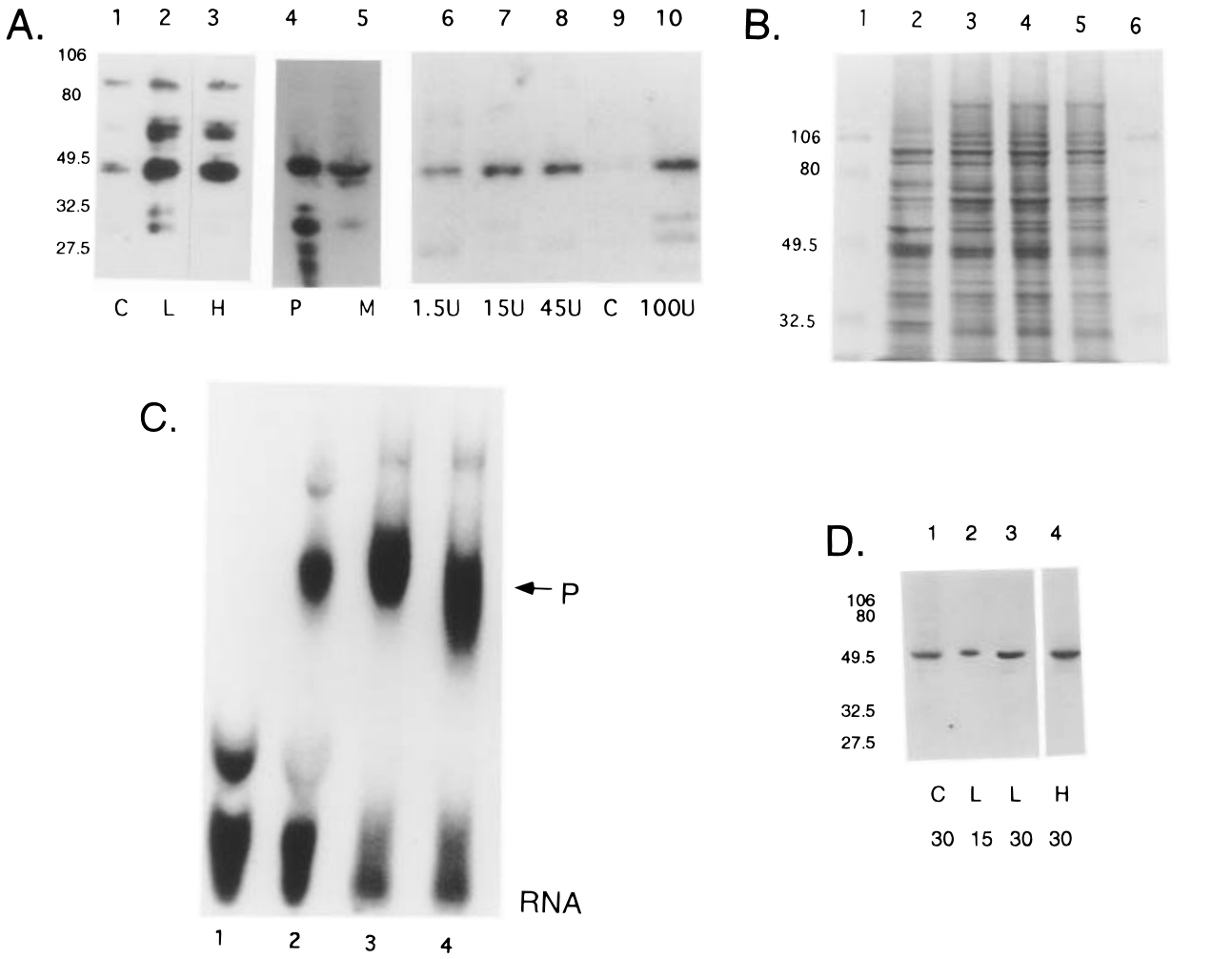

FIGURE 5: Micrococcal nuclease extraction of polyribosomes. (A) Northwestern analysis. Proteins extracted from polyribosomes with 0.8 M KCl, without (lanes 1 and 9, C) or after treatment with micrococcal nuclease (20 units/mL, lane 2; 100 units/mL, lanes 3 and 10; 1.5 units/mL, lane 6; 15 units/mL, lane 7; 45 units/mL, lane 8) were resolved by electrophoresis on 12% polyacrylamide gels. Lane 1 contained 25 μg of protein, and lanes 2 and 3 contained 10 μg of protein, while lanes 6−10 contained proteins from the same amount of polyribosomes. In lane 4, total polyribosomal proteins were analyzed, and in lane 5, the proteins extracted from the same amount of polyribosomes with micrococcal nuclease plus 0.8 M KCl were analyzed. The proteins were resolved on a 12% SDS−polyacrylamide gel and then transferred to nitrocellulose and probed with the wild-type radiolabeled RNA as described in Figure 2A. The position of the molecular weight markers (in kDa) is indicated. (B) Purified polyribosomes were extracted with 0.8 M KCl either without (lane 2) or after treatment (lanes 3−5) with increasing amounts of micrococcal nuclease (20, 60, and 100 units/mL of polyribosomes). The proteins were resolved by electrophoresis on 10% SDS−polyacrylamide gels, and the gel was stained with Coomassie Blue (R-250). Lanes 1 and 6 are molecular weight standards (Bio-Rad prestained standards). (C) A mobility shift analysis using the wild-type stem−loop sequence as a probe was performed as previously described (Pandey et al., 1991). Lane 1 shows the probe incubated in buffer. Lane 2 is the analysis of 8 μg of total polyribosomes. Lanes 3 and 4 are 8 μg of protein extracted from polyribosomes without (lane 3) or with (lane 4) 20 units/mL micrococcal nuclease. The arrow indicates the wild-type polyribosomal complex (P). (D) Extracts of polyribosomes were fractionated by SDS gel electrophoresis, transferred to nitrocellulose, and probed with a polyclonal antisera against the human La protein (a rabbit polyclonal antisera generously donated by Drs. Dan Kenan and Jack Keene) and developed using the ECL chemiluminescence (Amersham) kit. Lane 1 is 25 μg of protein extracted from polyribosomes with 0.8 M KCl. Lanes 2 and 3 are 25 μg of protein extracted from polyribosomes with 0.8 M KCl after treatment with 20 units/mL micrococcal nuclease for 15 and 30 min, respectively. Lane 4 is 25 μg of protein extracted from polyribosomes with 0.8 M KCl after treatment with 100 units/mL micrococcal nuclease for 30 min. The extracts used in lanes 1, 3, and 4 are the same extracts analyzed in lanes 2, 3, and 5 of panel B.

was detected by the Northwestern blot assay. The SLBP eluted as a single major component of about 50 kDa compared with a series of globular protein standards (Figure 6A and B).

Similar results were obtained when the SLBP activity was analyzed using a mobility-shift assay. An extract of polyribosomes treated with micrococcal nuclease was chromatographed on an S-200 column, and the SLBP activity was detected by the mobility-shift assay. The SLBP activity eluted as a single component with a size of about 50 kDa compared with the globular protein standards (Figure 6C). The polyribosomal extract was also analyzed by glycerol gradient centrifugation. As assayed by both the mobility-shift assay and the Northwestern blot, the SLBP migrated with a size of about 50 kDa (not shown). Thus, the complex released from the polyribosomes with micrococcal nuclease most likely contains a single SLBP polypeptide together with the 3′ end of the histone mRNA.

Partial Purification of the SLBP. The SLBP could be partially purified from the micrococcal nuclease treated polyribosomal extract. Since in the initial micrococcal nuclease treated polyribosomal extract much of the SLBP was bound to RNA, the initial step was chromatography on Cibacron Blue agarose, which was used successfully to

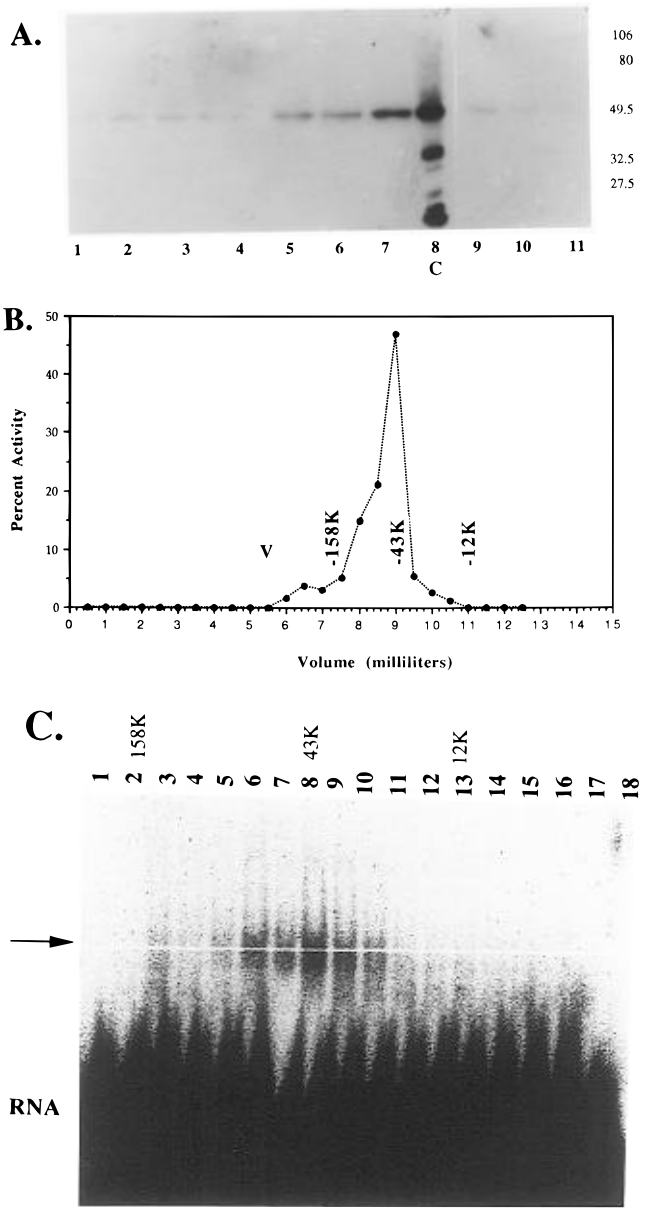

FIGURE 6: Size of the SLBP−RNA complex. (A) A 0.8 M KCl extract of micrococcal nuclease treated polyribosomes was dialyzed against 0.15 M KCl, 10 mM Tris, pH 7.5, 1% glycerol, and 1 mM DTT and chromatographed on an S-200 column in the same buffer. Fractions of 0.5 mL were collected, and equal amounts of each fraction were analyzed by a Northwestern assay. Lanes 1−7 are the analyses of fractions 12−18 (6−9 mL), and lanes 9−11 are the analyses of fractions 19−21 (9.5−11 mL). Lane 8 is the analysis of total polyribosomes. Molecular weight standards (aldolase, ovalbumin, and cytochrome *c*) were run on the same column and detected by SDS−polyacrylamide gel electrophoresis. (B) The 45-kDa band from panel A was quantified in a PhosphorImager and plotted versus elution volume. The positions of the molecular weight standards are indicated. (C) The polyribosomal extract was fractionated by chromatography on S-200 in the absence of the RNA, and the SLBP was detected by the mobility-shift assay. The fractions were assayed starting with the void volume (fraction 1). The positions of elution of the molecular weight markers are indicated.

release the PABP from polyA in the purification of the PABP (Sachs & Kornberg, 1990). All of the SLBP activity, as assayed either by mobility shift (not shown) or by Northwestern blot (Figure 7A), was quantitatively retained on the column. Elution with high salt (2 M NaCl) did not release any SLBP assayed with either assay. The SLBP activity was recovered by elution with 1.5 M GuHCl (Figure 7A).

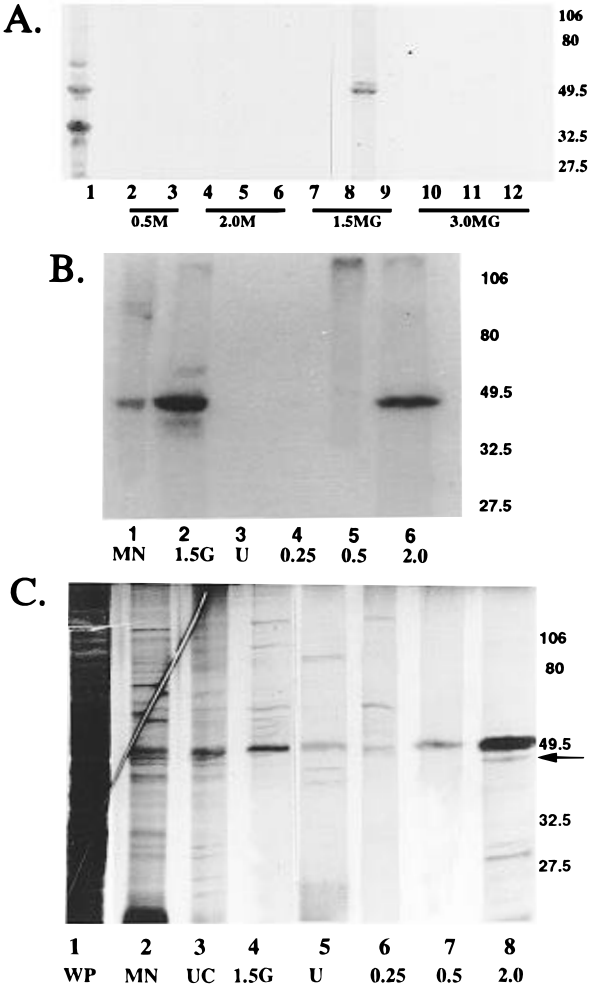

FIGURE 7: Partial purification of the SLBP by chromatography on Cibacron Blue agarose and heparin agarose. (A) The micrococcal nuclease extract of polyribosomes in 0.25 M KCl was applied to a Cibacron Blue agarose column (0.6 × 9.5 cm). The column was washed with 2 column vol of 0.25 M KCl and eluted with successive steps of 2 column vol of 0.5 M KCl, 2.0 M KCl, 1.5 M GuHCl, and 3.0 M GuHCl, all in buffer A. The fractions were dialyzed against 0.25 M KCl and concentrated. An aliquot of each step was analyzed by SDS−PAGE and transferred to nitrocellulose, and the SLBP was detected by the Northwestern procedure. The samples are as follows: micrococcal nuclease extract of polyribosomes (lane 1); elution with 0.5 M KCl (lanes 2 and 3) and 2.0 M KCl (lanes 4−6); elution with 1.5 M GuHCl (lanes 7−9); elution with 3 M GuHCl (lanes 10−12). Equivalent amounts of each fraction were analyzed. (B) The pooled fractions from the Cibacron Blue column were dialyzed against 0.25 M NaCl in buffer A and applied to a heparin agarose column. The column was washed with 2 vol of 0.5 M NaCl in buffer A, and the SLBP was eluted with 3 column vol of 2 M NaCl. A 100-μL sample of each fraction was dialyzed and concentrated and then analyzed on 12% SDS−PAGE gels by the Northwestern procedure as described in Figure 2A. Lane 1, polyribosomal extract; lane 2, 1.5 M GuHCl fraction from Cibacron Blue agarose; lane 3, unbound protein of heparin agarose; lanes 4−6, proteins eluted with 0.25, 0.5, and 2 M NaCl. (C) The fractions from the Cibacron Blue and heparin agarose columns were analyzed on 12% polyacrylamide−SDS gels, and the gel was stained with silver. Lane 1, total polyribosomal protein; lane 2, micrococcal nuclease extract of polyribosomes; lane 3, proteins not bound to Cibacron Blue; lane 4, proteins eluted from the Cibacron Blue with 1.5 M GuHCl; lanes 5−8, the unbound and 0.25, 0.5, and 2.0 M fractions from heparin agarose. A larger fraction of the proteins were loaded in lane 8. The arrow points to the position of the SLBP detected by the Northwestern blot. The position of the SLBP was determined by running two identical samples on the same gel, staining one lane and detecting the SLBP with a Northwestern assay of the other lane.

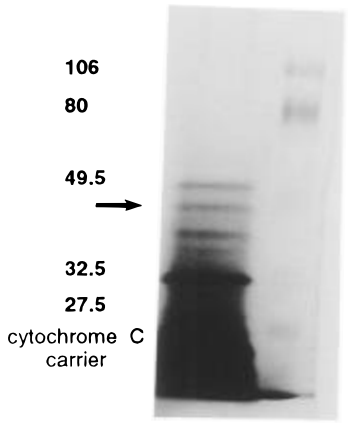

FIGURE 8: RNA affinity chromatography. A stem−loop biotinylated RNA was incubated with the SLBP after heparin agarose chromatography. The mixture was then incubated with the streptavidin matrix at 4 °C. The matrix was washed with 0.2 M and then with 2 M NaCl in TEGD buffer, and the SLBP was eluted with 3 M GuHCl. Cytochrome *c* was added to the sample prior to application to the resin and to each fraction to reduce losses due to adsorption. The sample was dialyzed, concentrated, and analyzed by electrophoresis on a 12% polyacrylamide−SDS gel, and the proteins were detected by staining with silver. The arrow indicates the position of the SLBP detected by the Northwestern blot. The bands below 32 kDa are from the cytochrome *c* carrier or the streptavidin matrix.

Exposure of the SLBP to GuHCl greatly reduced the activity in the mobility-shift assay, presumably due to partial denaturation of the protein. Small amounts of activity could be recovered by gradient dialysis to remove the GuHCl, but not enough activity was recovered to allow the use of the mobility-shift assay to monitor purification (data not shown). Hence the Northwestern assay was used to follow the SLBP activity in subsequent steps.

Following chromatography on Cibacron Blue agarose, the protein was applied to a heparin agarose column. The SLBP bound tightly to the column and was eluted only with salt concentrations higher than 1 M NaCl (Figure 7B). The SLBP was readily detected by the Northwestern assay. The fractions eluting from the heparin agarose column contained a complex mixture of polypeptides, with a band at the position of the SLBP only a minor component (Figure 7C, lane 8, arrow).

To further purify the SLBP, we used an RNA affinity procedure. A 25 nt oligoribonucleotide containing the stem−loop was synthesized with a biotin attached to the 3′ end. The biotinylated ribonucleotide was incubated with the heparin agarose fraction and then bound to streptavidin agarose. The affinity matrix was washed thoroughly, and the bound proteins were eluted with 3 M GuHCl. All of the SLBP activity was bound to the matrix (not shown). There were three major polypeptides in the size range of 40−55 kDa bound to the resin, one of which has the same mobility as the SLBP detected by the Northwestern assay (Figure 8, arrow). The pattern of polypeptides bound to the RNA affinity resin is strikingly similar to the pattern of polypeptides found in the mobility shift complex (compare Figure 8 with Figure 4C). Whether these three polypeptides all represent proteins that bind specifically to the stem−loop is not clear, since the complex is clearly a monomer under most conditions (Figure 6). However, in the Northwestern assay and in UV cross-linking, the labeled band is often resolved as a doublet. It is possible that two bands represent different forms of the same polypeptide or proteolytic products, which still retain binding activity. The band at 50 kDa is the major contaminant present prior to RNA affinity chromatography (Figure 7C). We did not obtain sufficient amounts of protein to get sequence information. A summary of a typical purification is given in Table 1. There was reasonable recovery of the SLBP through the inital chromatographic steps.

Table 1: Summary of Purification of the SLBP[a]

sample	protein (mg)	total activity (%)	purification factor
polyribosomes	2050	100	1
polyribosomal extract	370	50	2.5
DE-650M	29	32	22
Cibracon Blue agarose	5.8	30	103
heparin agarose	1.08	15	300
RNA affinity	0.007		

[a] A summary of a partial purification of the SLBP is given. In this purification, the polyribosomal extract was first applied to DE-650M and the SLBP was eluted with 0.25 M NaCl. The subsequent columns are as described in Figure 7. The amount of activity was judged from the Northwestern assay compared with the amount in the total polyribosomes set at 100%. The amount of protein recovered from the RNA affinity column was estimated from the silver-stained gel. Following RNA affinity chromatography, the SLBP was judged to be about 15% pure as judged by gel electrophoresis.

We have described a method for efficiently extracting a component of the histone mRNP from the polyribosomes and partially purifying this activity. Scaling up this process should allow us to obtain sufficient amounts of SLBP protein to generate sequence information to allow us to clone the protein.

DISCUSSION

Messenger RNAs are found complexed with proteins in mRNPs in the cytoplasm, but the nature of these proteins is largely unknown (Dreyfuss et al., 1988). One well-characterized protein is the polyA binding protein (PABP), which is bound to the polyA tail found at the 3′ end of most mRNAs (Adam et al., 1986; Sachs et al., 1986). The PABP, like the SLBP, plays a central role in the metabolism of mRNAs, ranging from translation to mRNA stability (Bernstein & Ross, 1989; Jackson & Standart, 1990). Purification of the PABP was achieved initially using affinity chromatography on polyA Sepharose or oligo(dT) cellulose. However, the PABP could only be eluted from these resins with denaturing solvents (Sachs & Kornberg, 1990). An additional problem commonly faced in purification of RNA binding proteins is the propensity of many of these proteins, particularly hnRNP proteins and PABP, to aggregate.

In addition, many RNA−protein complexes are stable in high salt, which is commonly used to dissociate DNA−protein complexes (e.g., transcription factors and sequence-specific DNA binding proteins), presumably due to the hydrophobic nature of the protein−RNA binding. The binding of the PABP to polyA is salt-stable (Sachs & Kornberg, 1990) as is the binding of U2AF to the polypyrimidine region at the 3′ splice junction of eucaryotic mRNAs. Thus, when a splicing extract was chromatographed on polyU Sepharose, U2AF could only be recovered by elution with GuHCl (Zamore et al., 1992). Therefore, the properties of many RNA binding proteins make efficient recovery and subsequent purification difficult.

Extraction and Detection of the SLBP. In our previous studies, we observed that the SLBP binding activity was relatively salt-insensitive (Pandey et al., 1991). The observation that there were apparently equal amounts of SLBP binding activity in the nuclear and polyribosomal fractions as assayed by the mobility-shift assay (Pandey et al., 1991; Figure 4C) suggested that salt extraction was not sufficient to solubilize much of the SLBP bound to histone mRNA in polyribosomes. The use of micrococcal nuclease, which can be readily inactivated by chelating calcium, may be a general procedure for solubilizing RNA binding proteins from mRNPs on polyribosomes. These proteins, like the SLBP, will likely be released as RNP particles. The detection of specific binding may then require subsequent dissociation of the RNPs.

Since the RNA binding proteins may initially be obtained as stable RNP complexes, we developed an assay for detection of the SLBP after separation of the SLBP from the stem−loop RNA by electrophoresis. The Northwestern blot assay described here is based on the ability to renature DNA binding proteins after expression from bacteriophage vectors (Staudt et al., 1988; Singh et al., 1988) and after separation of DNA binding proteins by SDS−PAGE. This assay successfully detects the 45-kDa SLBP allowing us to follow its purification even after exposure to denaturing conditions. Similar assays may be useful for the detection of other sequence-specific RNA binding proteins. The recovery of activity judged by the Northwestern assay was reproducible, although it is very possible that only a small percentage of the protein on the filter is renatured. Since the initial polyribosomal protein sample as well as the chromatographic fractions are all subjected to the same denaturation with SDS prior to assay, the Northwestern assay reflects the amount of SLBP present in the sample.

Another mRNA binding protein that has been characterized, the iron response element binding protein, is present in a soluble form in the cytoplasm in the presence of iron (Klausner et al., 1993; Casey et al., 1988) and is not an integral component of the mRNP. This protein was purified using biotinylated RNA as an affinity reagent in the final step, although in this case the protein could be recovered by elution from the affinity matrix with salt (Rouault et al., 1989). Thus, some RNA binding proteins may be more readily purified by conventional chromatographic procedures.

Possible Function of the SLBP. The majority of the SLBP in the cell is present in the polyribosomes. The molecular mass of the SLBP−RNA complex released by micrococcal nuclease is about 50 kDa. Hence the SLBP must be present in a single copy per histone mRNA, and it is not tightly associated with other proteins in the mRNP. There is a small amount of SLBP in the nuclear fraction. The nuclear SLBP is detected efficiently by the mobility-shift assay (Pandey et al., 1991), suggesting that it is not bound to histone mRNA. The stem−loop at the 3′ end of histone mRNA is also important in processing of the 3′ end of histone mRNA, and there is a hairpin-binding factor involved in the 3′ end formation that recognizes the stem−loop (Melin et al., 1992; Vasserot et al., 1989; Mowry et al., 1989; Mowry & Steitz, 1987). Recently, we have shown that the polyribosomal SLBP can complement a nuclear processing extract deficient in the hairpin-binding factor (Dominski et al., 1995), suggesting that the SLBP is the hairpin-binding factor (or part of the hairpin-binding factor) required for histone 3′ end formation. The polyribosomal SLBP is likely to be present in stoichiometric amounts (1 polypeptide/histone mRNA), and hence the low amount of SLBP in the nucleus is consistent with the nuclear SLBP having a role in maturation of the histone mRNA.

One likely function of the SLBP is in transport of the histone mRNA from the nucleus to the cytoplasm. The SLBP may bind the histone pre-mRNA in the nucleus and then accompany the mature histone mRNA to the cytoplasm. Consistent with this possibility, mutations in the stem−loop which reduce SLBP affinity reduce the efficiency of transport of histone mRNA (Williams et al., 1994). It is likely that the SLBP also fulfills the "housekeeping" functions for histone mRNA that the PABP fulfills for polyadenylated mRNAs. These include a role in determining the half-life of mRNA (Pandey & Marzluff, 1987; Bernstein et al., 1989) and possibly also a role in translation (Sun et al., 1992; Jackson & Standart, 1990). Finally, it is likely that the SLBP is involved in the regulation of histone mRNA degradation, since this requires that histone mRNAs be actively translated on polyribosomes (Graves et al., 1987) and is accomplished by an initial cleavage in the stem−loop (McLaren & Ross, 1993; Ross et al., 1986) followed by degradation of the histone mRNA 3′ to 5′ by a polyribosome associated nuclease (Ross & Kobs, 1986).

General Applicability of This Approach for Characterization of mRNP Proteins. Micrococcal nuclease treatment may aid in solubilizing other proteins that are components of the polyribosomal mRNP, including, for example, the proteins that interact with AU-rich sequences present in many unstable RNAs (Brewer, 1991; You et al., 1992; Gillis & Malter, 1991) and that also bind very tightly to their target sequences (Malter, 1989). Starting with a polyribosomal extract from 150 L (6×10^{10} cells), we did not obtain enough SLBP to determine partial protein sequence. Scaling up this method (we estimate we need about 500−750 L of cells) and/or improving the recovery significantly at the RNA affinity column step should allow us to obtain enough SLBP for partial protein sequencing and production of antibodies. Alternatively, the Northwestern assay described here may allow direct selection of the SLBP cDNA from a cDNA expression library.

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