Presence of Multiple mRNA Cycling Sequence Element-binding Proteins in Crithidia fasciculata*

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Bidyottam Mitra, Krishna M. Sinha, Jane C. Hines, and Dan S. Ray‡
From the Molecular Biology Institute and Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, California 90095

A consensus sequence present in the 5′- or 3′-untranslated regions of several Crithidia fasciculata messenger RNAs encoding proteins involved in DNA metabolism has been shown to be necessary for the periodic accumulation of these mRNAs during the cell cycle. A protein complex termed cycling sequence-binding protein (CSBP) has two subunits, CSBPA and CSBPB, and binds the consensus sequence with high specificity. The binding activity of CSBP was shown to vary during the cell cycle in parallel with the levels of putative target mRNAs. Although disruption of the CSBPA gene resulted in loss of both CSBPA and CSBPB, the putative target message levels still continued to vary during the cell cycle. The presence of an additional and distinct binding activity was revealed in these CSBPA null mutant cells. This activity, termed CSBP II, was also expressed in wild-type Crithidia cells. CSBP II has higher binding specificity for the cycling sequence element than the earlier described CSBP complex. Three polypeptides associated with purified CSBP II show specific binding to the cycling sequence. These proteins may represent a family of sequence-specific RNA-binding proteins involved in post-transcriptional regulation.

Trypanosomes contain a novel mitochondrial DNA network termed kinetoplast DNA consisting of thousands of minicircle DNA molecules and a small number of maxicircle DNA molecules interlocked to form a huge catenated structure (1). Kinetoplast DNA replication occurs through a unique process in which the minicircles are released from the network for replication, and the newly replicated minicircles still containing nicks and gaps are reattached to the network periphery (for recent reviews see Refs. 2 and 3). However, the maxicircles replicate while remaining attached to the network. Unlike in higher eukaryotes where mitochondrial DNA replication occurs throughout the cell cycle, kinetoplast DNA replication in trypanosomes occurs in apparent synchrony with nuclear DNA replication (4). Studies have been undertaken to understand the possible role that cell cycle-dependent coordinated expression of DNA replication genes may play in coordinating nuclear and kinetoplast DNA replication.

Regulation of gene expression in trypanosomatids is predominantly post-transcriptional (reviewed in Ref. 5). Polycistronic messages are generated through constitutive transcription of protein-coding genes by RNA polymerase II, which then undergo 5′-trans-splicing and 3′-polyadenylation to produce the mature mRNA. Multiple points of regulation controlling expression of specific transcripts have been investigated. cis-acting factors that affect mRNA stability have been identified within the 3′-untranslated region (UTR) of specific transcripts. AU-rich cis-regulatory elements present in the 3′-UTR have been shown to regulate the stability of transcripts of procyctic acidic repetitive proteins (EP and GPEET) or procyclins (6, 7) and the variable surface glycoprotein gene transcripts in Trypanosoma brucei (2, 8) and those of mucin (9), amastin (10), and H2A histone (11) genes in Trypanosoma cruzi. Trans-acting factor, a developmentally regulated U-rich RNA-binding protein involved in selective mRNA destabilization, has recently been identified in T. cruzi (12). The trans-acting factor protein recognizes 44-nucleotide instability elements in the 3′-UTR region of mucin SMUG mRNA (13) as well as GU-rich sequences. Homologs of the poly(A)-binding protein 1 have been cloned from T. brucei (14) and Leishmania major (15). In higher eukaryotes, the poly(A)-binding protein 1 binding to the poly(A) tail of matured transcripts has been shown to enhance message stability.

Crithidia fasciculata, a member of the family trypanosomatidae that includes many human pathogens like T. brucei causing sleeping sickness and the Leishmania parasites causing a range of disease forms including the fatal visceral leishmaniasis, is infective to insect cells. Most biochemical studies of kinetoplast DNA replication have been carried out in Crithidia as it can be easily grown in large scale cultures that can also be synchronized by hydroxyurea treatment. In synchronized cultures, the transcript levels of the genes encoding the large and middle subunits of the nuclear protein RPA (RPA1 and RPA2, a homolog of the human replication protein A), dihydrofolate reductase-thymidylate synthase (DHFR-TS), the kinetoplast-specific topoisomerase II (TOP2), and the histone-like kinetoplast-associated protein 3 have been shown to cycle in parallel, reaching maximum levels during the late G2 and S phases and then declining rapidly during the G1 and M phases (16). Transcripts of these genes were found to possess one or more copies of a consensus octamer sequence (C/A)AUAGAA(G/A) with a highly conserved hexameric core in either their 5′- or 3′-UTR (16, 17). The sequence has been proposed to have a destabilizing role as mutations in the octamer sequence lead to accumulation of the messages to the highest level (16). Introduction of six copies of the octamer sequence (6× octamer DNA) in the 5′-UTR of a gene that does not cycle under normal conditions resulted in cycling of the message (18).

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‡ To whom correspondence should be addressed: 301A Paul D. Boyer Hall, 611 Charles Young Dr., E., UCLA, Los Angeles, CA 90095-1570. Tel.: 310-825-4178; Fax: 310-206-7286; E-mail: danray@ucla.edu.

The abbreviations used are: UTR, untranslated region; RPA, replication protein A; DHFR-TS, dihydrofolate reductase-thymidylate synthase; TOP, topoisomerase; CSBP, cycling sequence-binding protein; DTT, dithiothreitol; BSA, bovine serum albumin.
In our effort to understand the mechanism of this message cycling, we identified and purified a high molecular mass complex, the cycling sequence-binding protein (CSBP) that binds to the cycling sequence with high sequence specificity (18–20). The binding of CSBP to the TOP2 5'-UTR RNA and to the 6× octamer RNA probe varied during the cell cycle in parallel with the levels of the putative target mRNAs (18). The putative target mRNAs are found to be most stable at times when the cycling sequence binding activity is high, and the message levels decline sharply in parallel with the decrease in binding activity. Based on these observations we had proposed that the binding of the CSBP protein complex to the octamer sequence might confer a cell cycle-dependent stabilization of transcripts containing these sequence elements.

To study its functional role, the gene encoding CSBPA, one of the two subunits of the CSBP complex (20), has been disrupted by gene replacement. Although the knock-out of the gene resulted in a loss of both CSBPA and CSBPP proteins, the putative target message levels were found to still cycle in the CSBPA null mutant cells. This mutant cell line has allowed us to detect the presence of an alternate cycling sequence binding activity. This activity termed CSBP II is also a high molecular mass complex comparable to the earlier described CSBP complex. CSBP II also shows high sequence specificity in binding to RNA probes. We report here the identification, purification, and biochemical characterization of this CSBP II protein complex.

MATERIALS AND METHODS

Knock-out of CSBPA Gene by Gene Replacement—The CSBPA gene was targeted by homologous recombination. Targeting constructs were created to replace both alleles of the gene. Homologous sequences that were used in the targeting constructs were PCR-amplified from the plasmid pHi3748 (20) with Taq DNA polymerase (Invitrogen) following conditions given by the supplier. The targeting sequences flanking the CSBPA gene were amplified with the primer pairs G17 (5'-GATATCT-GCCGCGGCGTGGAGA-3') and F164 (5'-CTCGGGATCCACACCTGCGA-GTGTTGCTGAGCTTGC-3') and F65 (5'-CTCGGGATCCATGC-CTCGGGAGGACACT3') and F66 (5'-GATATCCTGCCGACTCGGCT-3') respectively. The amplified products were cloned separately in plasmid pCRII-TOPO using a TOPO TA cloning kit (Invitrogen), yielding the plasmids pMS38.1 and pMS38.2, respectively. The hygromycin phosphotransferase expression cassette was released from the plasmid pXH5HY (21) as a SalI/BglII fragment and ligated under conditions recommended by the supplier. The linearized fragments were used as templates for preparation of the 6× wild-type (CAUAGAAG) and mutant (CAUAGcAG) octamer probes. The 5'-flanking region of the CSBPA gene was PCR-amplified using the primers G17 and F104, whereas the 3'-flanking sequence was amplified using the primer pair F105 (5'-GATATCTCTCCTCTCTCTG-3') and RV-Bam (5'-CTCGGGATCCGCTTCGCTTCACGCTTCA-3') used for electroporation of C. fasciculata. The amplified 5'-flanking region of the CSBPA gene was PCR-amplified using the primers G17 and F104, whereas the 3'-flanking sequence was amplified using the primer pair F105 (5'-GATATCTCTCCTCTCTCTG-3') and RV-Bam (5'-CTCGGGATCCGCTTCGCTTCACGCTTCA-3'). The amplified 5'-flanking sequence was digested with XhoI and the 3'-flanking sequence was digested with BamHI and gel-purified. The purified products were ligated with a XhoI-BamHI neo cassette derived from pX2KO (15). The linear ligation product was then cloned using a TOPO TA cloning kit to give pMS38.7. Digestion of pMS38.7 with EcoRV produced a fragment of 5 kbp, which was used for disruption of the remaining allele of CSBPA. Plasmid DNA (10 μg) containing the wild-type or mutant 5'- and 3'-flanking regions was ligated to the targeting constructs digested with appropriate restriction enzymes to release the target sequences. The digested DNA was electroporated in C. fasciculata cells as described previously (15). Electroporated cells were selected for drug resistance on agar plates containing brain heart infusion medium (Invitrogen) supplemented with hemin (20 μg/ml) and streptomycin (100 μg/ml) in the presence of the appropriate drug. Genomic DNA was isolated from putative mutant strains and analyzed by Southern blot analysis to confirm the targeting constructs were digested with appropriate restriction enzymes to release the target sequences. The digested DNA was electroporated in C. fasciculata cells as described previously (15).

Preparation of RNase Protection Blots—C. fasciculata cells grown in 106 cells was fractionated by 10% SDS-PAGE and immunoblotted as described previously (15). Blots were probed with polyclonal anti-CSBPA and anti-CSBPPB serum at 1:5000 dilution.

Preparation of RNA Probes—Radioactive 32P-labeled RNA probes were prepared by in vitro transcription reactions from the T7 polymerase promoter as described previously (18) using the Maxicrypt kit (Ambion). Plasmids pRM16 and pRM25 (18) linearized with NotI were used as templates for preparation of the 6× wild-type (CAUAGAAG) and mutant (CAUAGcAG) octamer probes. TOP2 wild-type or mutant probes were prepared as described previously (19). The two octamer sequences present in the TOP2 5'-UTR were mutated singly or both from CAUAGAAG to CAUAGcAG. Plasmids containing the wild-type or mutant 5'- and 3'-flanking sequences were linearized with HindIII and used as templates for PCR reactions to introduce a T7 promoter upstream of the sequence to be transcribed. The PCR product was then used as template for synthesizing 32P-labeled RNA using the Ambion Maxiscript Kit. The RNA probes were gel-purified, heated at 65 °C for 15 min, and then allowed to cool to room temperature before being used in assays.

Gel Retardation Assays—Cycling sequence binding activity was monitored by performing binding assays (19) using RNA probes in reactions containing 10 mg/ml heparin and RNase inhibitor (10 units/reaction).

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plasmid pGL3TD (22) to give pMS38.10. A 235-bp fragment containing the N terminus of the CSBPP gene was deleted from the construct by PCR mutagenesis to yield the CSBPA expression plasmid pMS38.20. This plasmid was electroporated into C. fasciculata CSBPA null mutant cells as described previously (15) and plated on brain heart infusion medium (Invitrogen) plus hemin (20 μg/ml) and streptomycin (100 μg/ml) in the presence of blastidicin S HCl (Invitrogen) at 100 μg/ml.

Northern Blot Analysis—RNA was prepared from 2 × 106 Crithidia fasciculata cells using an RNeasy kit (Qiagen). RNA samples (10 μg) were loaded onto 1.2% formaldehyde-agarose gels and electrophoresed for 17 h at 22 V with continuous circulation of buffer. The RNA was then transferred to Hybond-XL membrane (Amersham Biosciences), UV-cross-linked, and subsequently probed with different radioactive probes.

Radioactive 32P-labeled RNA using the Ambion Maxiscript Kit. The RNA probes were gel-purified, heated at 65 °C for 15 min, and then allowed to cool to room temperature before being used in assays.

Gel Retardation Assays—Cycling sequence binding activity was monitored by performing binding assays (19) using RNA probes in reactions containing 10 mg/ml heparin and RNase inhibitor (10 units/reaction).

Formation of RNA-protein complex was observed by relative shift in mobility of the radiolabeled probe when electrophoresed in a polyacrylamide gel. Following incubation of the binding reactions at 28 °C for 30 min, the samples were analyzed by electrophoresis in a 6% (60:1 acrylamide:biacrylamide) polyacrylamide gel (3 h at 4 °C, 150 V, in 0.5× Tris borate EDTA), which had been pre-electrophoresed for 45 min under the same running conditions. The gels were then dried and exposed to x-ray films at ~70 °C with intensifying screens. To quantitate the extent of RNA-protein complex formation, dried gels were exposed to a PhosphorImager screen and radioactive bands were quantitated with a PhosphorImager (Amersham Biosciences).

UV-cross-linking—Binding reactions were performed as described previously (18) using DE52 purified cell extracts or RNA affinity column-purified CSBP II activity using 32P-labeled RNA probes. Following incubation at 28 °C for 30 min, the reactions were chilled in ice for 5 min and then transferred onto a parafilm strip, which was again placed on ice. The binding reactions were then irradiated with UV light (Stratalinker, Stratagene) for 1 min at a distance of 9 cm from the light source. The reactions were transferred to fresh tubes to which RNase A and RNase T1 were added and further incubated at 37 °C for another 30 min. The samples were then combined with one-third volume of 4× Laemmli buffer (23) and heated at 100 °C for 3 min before being loaded onto a 0.5-mm thick, 15-cm long 10% SDS-polyacrylamide gel. Electrophoresis was carried out in TGS buffer (27 mm Tris, 187 mm glycine, 0.1% SDS) under a constant current of 20 mAmp for 3 h at 100 °C. The gels were then dried and analyzed by phosphorimaging.

Preparation of Crithidia Cell Extracts—Wild type or CSBPA null mutant C. fasciculata cells were grown in brain heart infusion medium (Invitrogen) supplemented with hemin (20 μg/ml) and streptomycin (100 μg/ml) at 28 °C with shaking. Cells were harvested at a concentration of 5–7 × 107 cells/ml in 4 liters of culture. Cell extracts were prepared from the cells essentially as described previously (19). The harvested cells were washed once with phosphate-buffered saline and then with buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride). The cells were resuspended in 25 ml of buffer B (buffer A containing 0.5% Nonidet P-40), incubated on ice for 15 min, and then centrifuged to remove debris. The cell pellet was further resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride), passed through a 20-G needle five times, and further incubated on ice.
for 15 min before being centrifuged (SS34 rotor, 12,000 rpm, 15 min, 4°C). An equal volume of buffer D (20 mM HEPES-KOH, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 20% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) was then added to the supernatant. The pellet extraction step with buffers C and D was repeated twice. The total extracts were pooled together and used for assays or were stored at −70°C until further use.

**Purification of CSBP II Activity and SDS-PAGE Analysis**—All of the steps in the purification of CSBP II activity were performed at 4°C or below. For further purification of the CSBP II activity, the cell extracts were first subjected to 0–40% (of saturation at 0°C) ammonium sulfate precipitation. The precipitated protein was dissolved in buffer E (20 mM Tris-HCl, pH 7.9, 5 mM MgCl₂, 10% glycerol, 1 mM DTT) and centrifuged in a Beckman Ti45 rotor for 30 min at 40,000 rpm at 4°C. The supernatant was collected and dialyzed against buffer E lacking glycerol for 1 h at 4°C. Following dialysis, glycerol was added to the dialyze to 20% final concentration and the dialysate was centrifuged at 12,000 rpm for 15 min in a Sorvall SS34 rotor. The cleared supernatant was loaded onto a 25-ml DE52 column pre-equilibrated with buffer E containing 50 mM KCl. The column was first washed with 5 column volumes of equilibration buffer, and the CSBP II activity was eluted with buffer E containing 125 mM KCl. The active DE52 fractions were pooled and further concentrated with a 0–40% ammonium sulfate cut. The precipitated proteins were dissolved in buffer F (20 mM Tris-HCl, pH 7.9, 20% glycerol, 1 mM EDTA, 1 mM DTT) and divided into three aliquots. Each aliquot was filtered through 0.22 µm SpinX filters (Costar) and subsequently loaded on a UNO Q6 anion exchange column (Bio-Rad) pre-equilibrated with buffer F. The column was washed with 5 column volumes of equilibration buffer, and the bound proteins were eluted with a gradient of 0–35 mM KCl in buffer F. The UNO Q-purified active fractions were diluted with an equal volume of buffer F and then purified further on a 6% octamer RNA affinity column. The column was prepared as described earlier (14) by attaching a RNA with six copies of the wild-type octamer cycling sequence (6× UAUGAAGA) followed by a (A)25 tail on to an oligo(dT) matrix. The column was successively eluted with buffer F containing 0.05, 0.15, 0.3, 0.6, and 1 M salt. The CSBP II activity eluted with buffer containing 1 M salt. The purified CSBP II protein fraction was analyzed by electrophoresis in a 10% polyacrylamide gel containing 0.1% SDS, and the protein bands were visualized by Sypro Ruby staining (Molecular Probes).

**Glycerol Gradient and Gel Filtration Analyses**—UNO Q-purified CSBP II activity was diluted by adding an equal volume of buffer containing 20 mM Tris-HCl, pH 7.9, and 1 mM DTT to reduce the glycerol and salt concentrations. BSA was added to the diluted protein for stabilization followed by concentration on a Centricon-10 column. The final buffer contained 20 mM Tris-HCl, pH 7.9, 100 mM KCl, 10% glycerol, and 1 mM DTT. Fifty microliters of this concentrated sample were analyzed by 10–30% glycerol gradient gel sedimentation as described previously (20). Another 50-µl aliquot of CSBP II was subjected to gel-focusing chromatography on a Superose 12 column (Amersham Biosciences) as detailed earlier (20).

**RESULTS**

**CSBP Gene Knock-out Results in the Loss of Both CSBPA and CSBPB Proteins**—The CSBP gene was disrupted by targeted gene replacement through homologous recombination. Constructs b and c (Fig 1A) were used to sequentially replace the two alleles of the CSBPA gene (construct a), thereby generating clones heterozygous for CSBPA gene (A+/−) and CSBPB null mutants (A−/−), respectively. Southern blots of the wild-type, CSBPA(A+/−), and CSBPB(A−/−) cells confirmed replacement of both alleles of the gene by drug cassettes (data not shown). Western blot analyses were performed with cell lysates from wild-type and mutant cells to confirm the absence of CSBPA protein in the mutant cells (Fig. 1B). The blot was probed with rabbit anti-CSBPA and anti-CSBPB antisera. Levels of both CSBPA and CSBPB were decreased in the heterozygote as compared with the level detected in wild-type cells. CSBPA was totally absent in extracts from CSBPA null mutant cells, but surprisingly, the CSBPB protein was also observed to be absent, even upon prolonged exposure of the blot. Thus, the knock-out of the CSBPA gene eventually depleted the cells of both CSBPA and CSBPB proteins. Northern blot analyses of total RNA from CSBPA null mutant cells (Fig. 2A) show that the CSBPB mRNA level in the mutant cells is similar to that of wild-type cells. Also, episomal expression of CSBPA protein in the CSBPA mutant cells restored wild-type levels of both CSBPA and CSBPB proteins (Fig. 2B). These results indicate that the loss of expression of CSBPA affects CSBPB expression at a post-transcriptional level, possibly at the level of protein stability or synthesis. Although the mechanism of the apparent co-regulation of these proteins is unknown, the mutant cell line has allowed us to search for and identify a second protein complex that specifically binds the octamer consensus sequence.

**Target Message Levels Continue to Cycle in the Absence of CSBPA and CSBPB Proteins**—We further investigated the role of CSBPA and CSBPB proteins in cycling of the putative target messages using CSBPA null mutant cells depleted of both CSBPA and CSBPB proteins. Northern blot analyses were performed with total RNA isolated at 30-min intervals from hydroxyurea-synchronized cells. The relative levels of TOP2, RPA1, and DHFR-TS mRNAs, which have been shown previously to cycle during the cell cycle, were determined (Fig. 3). The levels of these messages were found to still cycle even in the absence of CSBPA and CSBPB. The message levels were lowest between 90 and 150 min, the period in which the percentage of dividing cells is highest. The message levels then increased during the 180–240-min time period corresponding to the highest levels of DNA synthesis (15). The level of CaBP message detected as the loading control was constant through-

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**Fig. 1.** CSBPA gene knock-out leads to loss of both CSBPA and CSBPB proteins. A targeted disruption of the CSBPA genes a, wild-type CSBPA genomic locus. b and c, targeting constructs for disruption of individual alleles of the CSBPA gene. B, Western blot of whole cell extracts from wild-type (WT), CSBPA heterozygous (A+/−), and CSBPB null mutant cells (A−/−) probed with CSBPA and CSBPB polyclonal antisera.
out the cell cycle. These data indicate that the cycling sequence-binding proteins CSBPA and CSBP are not essential for cycling of the RPA1, TOP2, and DHFR-TS mRNA levels.

Presence of a Cycling Sequence Binding Activity in CSBPA Null Mutant Cells—Evidence of cycling of putative target messages in the CSBPA null mutant cells necessitated further search for any alternate cycling sequence binding activity other than CSBP. Gel-shifts assays were performed with whole-cell extracts of CSBPA null mutant cells using a −291 to −209 5′-UTR transcript of the TOP2 gene as probe (Fig. 4). This 83-nucleotide RNA includes two octamer cycling sequences, and binding to this RNA probe by CSBP was shown previously to depend on these cycling elements (19). The RNA probe (lane 1) containing two wild-type octameric sequences (represented by open squares) is efficiently bound by factor(s) in the CSBPA null mutant cell extract (lane 2). However, when RNA probes with mutations in either one or both of the octamer sequences (represented by solid squares) were used in assays, binding was reduced (lanes 4 and 6) or completely abolished (lane 8). Mutation of the 3′-octamer (lanes 5 and 6) had a greater effect on binding than a mutation in the 5′-octamer (lanes 3 and 4). A similar observation was made previously with wild-type Crithidia nuclear extracts (19). No binding was observed when both the octamer sequences were mutated (lanes 7 and 8). A small shift of each of the radioactive probes relative to the bulk of the free probe was seen even in the absence of extract and is somewhat greater in the presence of extract. The nature of these species has not been investigated further. These results indicate the presence of additional cycling sequence binding protein(s) in CSBPA mutant cells other than CSBPA or CSBPB.

Cycling Sequence Binding Activity in CSBPA Null Mutant Cells Is Sequence-specific—To determine the specificity of binding of the cycling sequence binding protein(s) in CSBPA null mutant cells, hereafter referred to as CSBP II, radiolabeled RNA containing six copies of the wild-type octamer sequence (separated by 2-nucleotide spacers) or its mutant forms with single nucleotide changes in each copy of the octamer were used as probes in gel-shift assays. CSBP II binding activity, which was partially purified on a DE52 column, bound to the wild-type sequence CAUAAGC but not to mutant probes containing single nucleotide substitutions in each copy of the octamer were used as probes in gel-shift assays. CSBP II binding activity, which was partially purified on a DE52 column, bound to the wild-type sequence CAUAAGC but not to mutant probes containing single nucleotide substitutions within the central hexamer (Fig. 5A). A minor and faster migrating species observed with the probe CAUAgGAC was not investigated further.

Similar gel-shift experiments with extracts from wild-type Crithidia showed that the cycling sequence binding activity from wild-type Crithidia bound to the wild-type probe and also to a lesser extent to the mutant probes RM23 and RM24 that contain six copies of the mutated sequence CAUAAGG and CAUAAGG, respectively (Fig. 5B). These results indicate that CSBP II has a higher specificity for binding to the cycling sequence than does CSBPA.

Presence of Multiple Cycling Sequence Binding Activities in Crithidia—Identification of cycling sequence binding activity in CSBPA null mutant cells indicates the presence of multiple cycling sequence-binding proteins in Crithidia. CSBP II activity identified in CSBPA null mutant cells could be present in addition to the previously identified CSBP complex in wild-type cells or alternatively may be induced in CSBPA mutant cells. Wild-type RM16 probe and the mutant RM23 probe were used to address this question. Binding activity from wild-type cells bound to both RM16 and RM23 probes (Fig. 6A). Addi-
polypeptides in the wild-type bound to the wild-type and mutant probes (Fig. 6). Five labeled RM16 and RM23 RNA probes to identify polypeptides were cross-linked to the wild-type probe (lane 1), and only the 50- and 52-kDa polypeptides were cross-linked to the mutant probe (lane 2). The CSBPA-immunodepleted extract contained three polypeptides, 68, 52, and 35 kDa in size, that were cross-linked to the wild-type probe (lane 3). No radiolabeled bands were observed with the mutant RNA probe in the CSBPA-immunodepleted wild-type extract (lane 4). These results suggest that the 38- and 50-kDa polypeptides that are missing in CSBPA-immunodepleted extracts are the previously identified CSBPA and CSBPB proteins, which were estimated from SDS gels earlier (18) to have molecular masses of 38 and 48 kDa, respectively. The difference in the estimate of the molecular mass of CSBPB is within the experimental error of such measurements. The 68-, 52-, and 35-kDa polypeptides appear to derive from the CSBP II protein. Since the CSBP complex is probably attributed to CSBP II. UV-cross-linking experiments were performed using radiolabeled RM16 and RM23 RNA probes to identify polypeptides bound to the wild-type and mutant probes (Fig. 6C). Five polypeptides in the wild-type Crithidia extract corresponding to approximate molecular masses of 68, 52, 50, 38, and 35 kDa were cross-linked to the wild-type probe (lane 1), and only the 38- and 50-kDa polypeptides were cross-linked to the mutant probe (lane 2). The CSBPA-immunodepleted extract contained three polypeptides, 68, 52, and 35 kDa in size, that were cross-linked to the wild-type probe (lane 3). No radiolabeled bands were observed with the mutant RNA probe in the CSBPA-immunodepleted wild-type extract (lane 4). These results suggest that the 38- and 50-kDa polypeptides that are missing in CSBPA-immunodepleted extracts are the previously identified CSBPA and CSBPB proteins, which were estimated from SDS gels earlier (18) to have molecular masses of 38 and 48 kDa, respectively. The difference in the estimate of the molecular mass of CSBPB is within the experimental error of such measurements. The 68-, 52-, and 35-kDa polypeptides appear to derive from the CSBP II protein. Since the CSBP complex is probably attributed to CSBP II. UV-cross-linking experiments were performed using radiolabeled RM16 and RM23 RNA probes to identify polypeptides bound to the wild-type and mutant probes (Fig. 6C). Five polypeptides in the wild-type Crithidia extract corresponding to approximate molecular masses of 68, 52, 50, 38, and 35 kDa were cross-linked to the wild-type probe (lane 1), and only the 38- and 50-kDa polypeptides were cross-linked to the mutant probe (lane 2). The CSBPA-immunodepleted extract contained three polypeptides, 68, 52, and 35 kDa in size, that were cross-linked to the wild-type probe (lane 3). No radiolabeled bands were observed with the mutant RNA probe in the CSBPA-immunodepleted wild-type extract (lane 4). These results suggest that the 38- and 50-kDa polypeptides that are missing in CSBPA-immunodepleted extracts are the previously identified CSBPA and CSBPB proteins, which were estimated from SDS gels earlier (18) to have molecular masses of 38 and 48 kDa, respectively. The difference in the estimate of the molecular mass of CSBPB is within the experimental error of such measurements. The 68-, 52-, and 35-kDa polypeptides appear to derive from the CSBP II protein. Since the CSBP complex has been shown to bind to the mutant RM23 probe in gel-shift experiments, UV-cross-linking experiments were performed in wild-type extracts with the addition of 30-fold excess of unlabeled RM23 RNA as competitor to the radiolabeled RM16 probe (lane 5). The cold mutant RNA efficiently competed out binding of the 38- and 50-kDa polypeptides to the wild-type probe, consistent with their identification as CSBPA and CSBPB.

Cycling of CSBP II Activity in CSBPA Mutant Cells—The
nuclear extracts of CSBPA mRNA levels of transcripts containing cycling sequences indicate that the binding activity of CSBP II, similar to previous reports, cycling sequence binding activity in *Crithidia* cells was shown to cycle in parallel with the levels of the putative target mRNAs that accumulate periodically during the cell cycle (18, 20). To determine whether the CSBP II activity also varies during the cell cycle, we prepared whole cell extracts from cell samples removed at 30-min intervals from a hydroxyurea-synchronized culture of *CSBPA* mutant cells. These extracts were assayed for their ability to bind RM16 RNA in gel-shift assays, which were quantitated by phosphorimaging analysis.

The relative levels of CSBP II binding activity and *DHFR-TS* mRNA were quantitated by phosphorimaging analysis of the dried RNA gel-shift assay gel and Northern blot, respectively. Shaded circles indicate the percent of cells having two nuclei (doublets) and represent the cells that have undergone nuclear division but have not yet undergone cytokinesis.

Prior analysis of CSBP II binding activity cycles in parallel with that of a putative target mRNA. *Crithidia* CSBPA null mutant cells were collected at 30-min intervals from a hydroxyurea-synchronized culture for the preparation of whole cell extracts or RNA. A, autoradiograph of RNA gel-shift assays of extracts containing 10 μg of protein and 0.6 fmol of 32P-labeled 6× octamer RNA. B, Northern blot analysis of the total RNA using a probe that detects *DHFR-TS* mRNA. C, graphical representation of the 6× octamer RNA binding activity (open squares) and *DHFR-TS* mRNA levels (solid diamonds) in synchronized cells taken out at 30-min intervals. The relative levels of 6× octamer binding activity and *DHFR-TS* mRNA were quantitated by phosphorimaging analysis of the dried RNA gel-shift assay gel and Northern blot, respectively. Shaded circles indicate the percent of cells having two nuclei (doublets) and represent the cells that have undergone nuclear division but have not yet undergone cytokinesis.

Previously reported cycling sequence binding activity in *Crithidia* cells showed that the CSBP II activity also varies during the cell cycle. We prepared whole cell extracts from cell samples removed at 30-min intervals from a hydroxyurea-synchronized culture of *CSBPA* mutant cells. These extracts were assayed for their ability to bind RM16 RNA in gel-shift assays, which were quantitated by phosphorimaging analysis. The relative levels of CSBP II binding activity (Fig. 7A) and of the putative target mRNA (*DHFR-TS*) in the Northern blot (Fig. 7B) cycled synchronously. Relative levels of CSBP II binding activity are shown graphically in Fig. 7C. The percentage of dividing cells is shown as an indication of synchrony in the culture. Following release from hydroxyurea arrest, the CSBP II binding activity reaches its peak within 30–60 min after cell division, attaining a maximum level around 180 min. The *DHFR-TS* mRNA levels follow the same trend and increase to a maximum at approximately the same time. CSBP II binding activity and *DHFR-TS* message levels decline rapidly between 180 and 300 min as the cell enters the G2/M phase. The CSBP II activity and *DHFR-TS* mRNA levels increase once more following another cell division. These results indicate that the binding activity of CSBP II, similar to that of CSBP, varies during the cell cycle in parallel with the mRNA levels of transcripts containing cycling sequence elements.

**Purification of CSBP II Activity**—CSBP II was purified from nuclear extracts of *CSBPA* mutant cells based on its ability to bind RM16 RNA. For purifying CSBP II, we followed the basic chromatographic steps used to purify CSBP (18). Initial purification was achieved upon DE52 and UNO Q fast protein liquid chromatography. CSBP II activity eluted from the UNO Q column with 180–210 mM KCl in buffer. CSBP II was finally purified on an RNA affinity column to which RM16 RNA containing an A25 tail was bound to an oligo(dT) matrix. As the CSBP II binding to the octamer sequence was sensitive to the presence of magnesium ion, the UNO Q-purified CSBP II activity was loaded onto the RNA affinity column in the absence of magnesium ion in the buffer, which instead contained 1 mM EDTA. This buffer condition allows most efficient CSBP II binding to the RNA affinity column. Following subsequent washes with buffers containing up to 0.6 M KCl, the binding activity was finally eluted from the column with buffer containing 1 mM EDTA. SDS-PAGE analysis of the active fractions (Fig. 8) eluted with 1 mM KCl shows a prominent polypeptide at ~68 kDa and a doublet of very closely migrating polypeptides ~50–52 kDa in molecular mass. Two additional polypeptides with molecular masses ~35 and ~73 kDa are also present in the affinity-purified fraction.

**Three Polypeptides in RNA Affinity-purified CSBP II Bind 6× Octamer RNA**—UV-crosslinking was performed to identify the octamer-specific RNA-binding polypeptides in the purified CSBP II following binding reactions using the radiolabeled RM16 or RM23 mutant probes and RNA affinity-purified CSBP II complex from *CSBPA* mutant cells. The UV-cross-linked RNA-protein complexes were digested with RNase A and RNase T1, after which the samples were analyzed by SDS-PAGE followed by autoradiography (Fig. 9). Three polypeptides of approximate molecular masses 35, 52, and 68 kDa were labeled when the wild-type RM16 RNA was used as the probe. No labeled protein band was visible in experiments using the mutant probe. These results are in agreement with our observations with CSBPA-depleted wild-type extracts where three polypeptides of 35, 52, and 68 kDa were also cross-linked specifically to the wild-type RM16 probe. Polypeptides of comparable size to the cross-linked ones were also observed in the purified CSBP II fractions upon SDS-PAGE analysis (Fig. 8). The ~73-kDa protein in the affinity-purified CSBP II was not cross-linked to the labeled probe.

**CSBP II Has a High Molecular Mass**—Previous analysis of CSBP indicated that the protein complex has a molecular mass in excess of 200 kDa. Since gel shifts obtained with CSBP and CSBP II are of similar magnitude, CSBP II is likely to have a similar molecular mass. To estimate the molecular mass of CSBP II, we utilized the sedimentation coefficient ($S_{20,w}$) and the Stokes’ radius of the binding activity of CSBP II determined by glycerol gradient sedimentation and gel-sieving chromatography, respectively. UNO Q-purified CSBP II activity was sub-
electrophoresed on a 10% SDS-polyacrylamide gel. Digested with various concentrations of RNase A and T1 before being probes following a binding reaction. The cross-linked samples were digested with various concentrations of RNase A and T1 before being electrophoresed on a 10% SDS-polyacrylamide gel.

To further examine the role of CSBPA and CSBPPB proteins and their possible involvement in mRNA cycling, we performed a knock-out of the CSBPA gene by gene replacement. Knockout to velocity gradient sedimentation on 10–30% glycerol gradient containing 80 μg/ml BSA. The position of CSBP II activity was determined by RNA gel-shift analysis of the collected fractions following centrifugation. From the relative sedimentation of marker proteins of known S20,w, the S20,w for the CSBP II activity was estimated to be −5.2 S (Fig. 10A).

The Stoke’s radius for CSBP II was determined by gel-sieving chromatography on a Superose 12 column (Amersham Biosciences). The CSBP II binding activity eluted from the column consistent with a Stoke’s radius of 61 Å (Fig. 10B). These data predict a native molecular mass of the protein of ~160 kDa with a frictional ratio (f/f0) of 1.41 (24). This predicted molecular mass of CSBP II is consistent with the presence of the three RNA-binding proteins in the complex but appears to be too small to include the 73-kDa protein as well. We cannot exclude the possibility that the 73-kDa protein has a labile association with the other three proteins. Further characterization of the molecular architecture of CSBP II is required to more precisely define its structure.

DISCUSSION

We have reported here the identification, purification, and characterization of a second RNA-binding protein complex in C. fasciculata that specifically binds to the consensus octamer sequence (C/A)AUAGAA(G/A) shown to be required for periodic accumulation during the cell cycle of mRNAs containing these elements. We had previously described another protein complex consisting of two proteins, CSBPA and CSBPPB, that has high binding affinity for the octamer RNA sequence (18, 19). Both recombinant CSBPA and CSBPPB had been shown to bind to the wild-type RNA probe, and incubation with antibodies against the CSBPA protein resulted in supershift of the RNA-protein complex. However, complete supershift of the probe was never achieved even with excess antibodies (20) implying the occurrence of an unrelated binding activity. The octamer sequence binding activity in wild-type cells was shown to vary periodically in parallel with the levels of the putative target messages containing the sequence. We hypothesized that the periodic binding of the CSBP complex to the octamer sequence might mediate the cycling of the target mRNAs.

To further examine the role of CSBPA and CSBPPB proteins and their possible involvement in mRNA cycling, we performed a knock-out of the CSBPA gene by gene replacement. Knockout of CSBPA resulted in the loss of expression of both CSBPA and CSBPPB in CSBPA null mutant cells. However, the cycling of the target mRNA levels remained unaffected even in the absence of both the CSBPA proteins. Thus, although CSBPA and CSBPPB proteins have a high specificity for binding to the octameric cycling sequence, they are not essential for the cell cycle regulation of the levels of mRNAs containing the octamer sequences.

We have identified an alternate cycling sequence-specific binding activity called CSBPB in CSBPA null mutant cells. CSBPB II RNA binding activity is found to be sensitive to point mutations and very specific for the hexameric core of the cycling sequence. Three polypeptides in the RNA affinity-purified CSBPB II activity, 68, 52, 35 kDa in molecular mass, are radioiodelabels specifically when the wild-type 6 × CAUAGAAG RNA was used as probe in “in-solution” UV-cross-linking experiments. Similar cross-linking experiments with the purified wild-type Crithidia extract show five radioiodelabeled polypeptides approximately 68, 52, 50, 38, and 35 kDa in mo-

![Fig. 9. UV cross-linking of purified CSBP II protein to 6× octamer RNA.](http://www.jbc.org/)

![Fig. 10. Glycerol gradient sedimentation and Superose 12 column chromatography analysis of CSBP II.](http://www.jbc.org/)

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where polypeptides of molecular mass. This finding differs from our earlier observation that the polypeptides present in CSBP and CSBP II activities are expressed in the wild-type *Crithidia* cells.

Binding of CSBP II to the wild-type probe in extracts of the mutant cells varies during the cell cycle, reaching maximum levels as the cells transit S phase and declining sharply prior to cell division. Levels of the cycling target mRNAs also follow a similar pattern. These results are similar to those observed in wild-type cells (20). We had earlier hypothesized that the periodic binding of trans-regulatory factor(s) to cycling sequence elements in target mRNAs could stabilize the messages in a cell cycle-dependent manner. Parallel cycling of cycling sequence binding activity and target mRNA levels in the absence of CSBPA and CSBPB proteins in *CSBP* null mutant cells is consistent with but does not prove a role for the CSBP II complex in the cycling of target mRNAs. Identification of proteins directly involved in the degradation of target mRNAs will be essential for further assessing the possible role of CSBP II in mRNA cycling.

It will also be important to determine the molecular composition of the CSBP II complex. Three of the five polypeptides in the RNA affinity-purified activity are specific RNA-binding proteins. The molecular masses of these three proteins taken together add up to ~160 kDa, which is comparable to the molecular mass calculated for the CSBP II complex with data available from size exclusion chromatography and glycerol gradient experiments. Whereas the presence of multiple specific RNA-binding proteins in a single complex is unusual, we note that previous studies of CSBP indicated that both CSBPA and CSBPB subunits bind to the cycling sequence element and also co-immunoprecipitate (15). SDS-PAGE shows the presence of an additional protein of ~73 kDa in the purified fraction apart from the three cycling sequence-binding proteins. Cloning of the genes encoding the proteins in the CSBP II complex will allow us to develop the immunological reagents necessary for addressing these questions. Also, it will enable us to examine the intracellular localization of CSBP II and its association with specific mRNA sequences in the cell. A more complete picture of the proteins that interact with the cycling mRNAs will be needed to understand the mechanism of cycling of these mRNA levels.

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Presence of Multiple mRNA Cycling Sequence Element-binding Proteins in *Crithidia fasciculata*

Bidyottam Mittra, Krishna M. Sinha, Jane C. Hines and Dan S. Ray

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