Identification of *cis* and *trans* Elements Involved in the Cell Cycle Regulation of Multiple Genes in *Crithidia fasciculata*

RIAZ MAHMOOD, JANE C. HINES, AND DAN S. RAY*

*Molecular Biology Institute and Department of Microbiology, Immunology and Molecular Genetics,
University of California, Los Angeles, Los Angeles, California 90095-1570*

Received 31 March 1999/Returned for modification 9 June 1999/Accepted 15 June 1999

Transcripts of several DNA replication genes, including the *RPA1* and *TOP2* genes, encoding the large subunit of nuclear replication protein A and the kinetoplast topoisomerase II, accumulate periodically during the cell cycle in the trypanosomatid *Crithidia fasciculata*. An octamer consensus sequence, CAUAGAAG, present in the 5' untranslated regions (UTR) of these mRNAs is required for periodic accumulation of the *TOP2* and *RPA1* transcripts and also for binding of a nuclear factor(s) to the 5' UTR RNAs of these genes. We show here that insertion of multiple (six) copies of this octamer sequence (6× octamer) into the 5' UTR of a reporter gene confers periodic accumulation on its transcript. Competition experiments and UV cross-linking studies show that the 6× octamer RNA and *TOP2* 5' UTR RNA bind to the same nuclear factor(s). Single-nucleotide substitutions in the 6× octamer that abolish the RNA gel shift also prevent cyclic accumulation of the reporter gene transcript. A protein termed cycling element binding protein, purified by affinity chromatography using 6× octamer RNA as a ligand, binds to RNAs containing wild-type octamers and not to those with mutant octamers. These results define a small sequence element in *C. fasciculata* mRNAs required for their cell cycle regulation and report the identification and purification of a putative regulatory protein that binds specifically to these elements.

Trypanosomes contain a single mitochondrion with a unique form of DNA called kinetoplast DNA (kDNA) that consists of two types of circular molecules: interlocked minicircles and maxicircles (23, 25, 26). In the trypanosomatid *Crithidia fasciculata* there are approximately 20 to 30 maxicircles of 37 kbp and about 5,000 minicircles of 2.5 kbp each. Replication of the minicircles occurs by a mechanism in which minicircles are released from the network prior to their duplication (8). After replication the daughter minicircles are rejoined to the network periphery while they still contain nicks and gaps (24). Maxicircles appear to replicate while still attached to the network (11).

An unusual feature of kDNA replication is its coordination with nuclear DNA synthesis (7, 19). In most eukaryotes, mitochondrial DNA replication occurs continuously throughout the cell cycle (3, 10, 32). Recent studies with *C. fasciculata* have shown that mRNA levels of both nuclear DNA and kDNA replication genes are regulated in a cell cycle-specific manner (19, 20). The steady-state transcript levels of the genes encoding the kinetoplast topoisomerase II (*TOP2*), dihydrofolate reductase-thymidylate synthetase (*DHFR-TS*), and the large and middle subunits (*RPA1* and *RPA2*) of the nuclear single-strand DNA binding protein replication protein A (RPA) all begin to accumulate prior to S phase and then decline rapidly (19). Synthesis of the kinetoplast topoisomerase II and the large subunit of RPA occurs in parallel with the accumulation of *TOP2* and *RPA1* mRNAs, resulting in a doubling of the levels of these proteins during each S phase (4, 12). A consensus octamer sequence, CATAGAAG, with a conserved central hexamer core, is present in the 5' untranslated regions (UTRs) of these genes (20). Deletion and linker insertion analysis has

shown that the octamer sequence is required for cycling of *TOP2* and *RPA1* gene transcripts (4, 20).

In trypanosomes, most genes transcribed by RNA polymerase II are regulated posttranscriptionally (31). Protein-coding genes are often organized in polycistronic units transcribed from the same promoter but yield very different steady-state mRNA levels or are differentially expressed during the life cycle of trypanosomes (21). Their regulation is exerted largely at a posttranscriptional level and is thought to involve pre-mRNA turnover in combination with differential rates of trans-splicing of a 39-nucleotide mini-exon to the 5' ends of all mRNAs and polyadenylation. At present, there is no evidence for any transcriptional regulation of genes transcribed by RNA polymerase II in trypanosomatids.

The mechanisms involved in regulating the expression of trypanosomatid genes during the cell cycle are unknown. We have initiated studies to identify the *trans*-acting factors involved in conferring periodic expression on kDNA and nuclear DNA replication genes. Using gel retardation assays, we have previously shown that *C. fasciculata* nuclear extracts contain a protein factor(s) that binds to the 5' UTR RNAs of the *TOP2* and *RPA1* genes (17). Mutation of the octamer sequences in the 5' UTRs of these genes abolished binding of the nuclear factor(s). Competition experiments showed that the same factor(s) binds to both *TOP2* and *RPA1* RNAs. These results suggest the interesting possibility that nuclear and mitochondrial DNA replication genes may be coordinately regulated at a posttranscriptional level by a common *trans*-acting RNA binding protein(s).

These studies define at the nucleotide level the sequence element required for periodic accumulation of the *TOP2* and *RPA1* mRNAs during the cell cycle. A protein (cycling element binding protein [CEBP]) purified on the basis of its specific binding to this sequence element is postulated to be a regulator of the levels of multiple mRNAs during the cell cycle.

* Corresponding author. Mailing address: Molecular Biology Institute, 611 Charles E. Young Dr. East, UCLA, Los Angeles, CA 90095-1570. Phone: (310) 825-4178. Fax: (310) 206-7286. E-mail: danray@mbi.ucla.edu.

MATERIALS AND METHODS

Plasmids. Plasmid pΔ10Not has been described previously (20). Plasmids pRM18 and pRM18R each contain six copies of the octamer sequence, separated by 2 bp each, cloned into the *NotI* site of pΔ10Not in opposite orientations (Fig. 1). They were constructed by annealing oligonucleotides C89 (GGCCGACATAGAAGGGCATAGAAGAACATAGAAGATCATAGAAGCGCATAGAAGTTCATAGAAGGC) and C90 (GGCCGCTTCTATGAACCTTCTATGCGCTTCTATGATCTTCTATGTTCTTCTATGCCCTTCTATGTG) and inserting them into the *NotI* site of pΔ10Not (the octamer sequences in C89 and C90 are in boldface). We term this synthetic DNA sequence the 6× octamer. Plasmids pRM25 and pRM27 are similar to pRM18 but have mutant 6× octamers (CATcGAAG and CATAGAcG, respectively) cloned into the *NotI* site of pΔ10Not. (Nucleotide substitutions in the octamer sequence are indicated in lowercase here and elsewhere in the text.) Plasmids pRM16 and pRM16R contain wild-type 6× octamer sequences cloned into the *NotI* site of pGEM13Zi(+) (Promega) in opposite orientations. Plasmids containing mutant 6× octamers cloned into the *NotI* site of pGEM13Zi(+) were constructed as described above by annealing oligonucleotides related to C89 and C90 that differed only in the octamer sequence. These plasmids are pRM19 (CAGAGAAG), pRM20 (CATcGAAG), pRM21 (CtTAGAAG), pRM22M (CATAtAAG), pRM23M (CATAGAcG), and pRM24 (CATAGAcG). Plasmid pRM12 contains the wild-type -291-to-209 region of the *TOP2* gene cloned into the *SmaI* site of pUC19 (17). All plasmids were electroporated separately into *C. fasciculata* as described elsewhere (19).

PCR to generate templates for in vitro transcription. Plasmid pRM12, linearized with *HindIII*, was used as a template in PCR to generate products that were in turn used as templates for in vitro synthesis of -291-to-209 *TOP2* RNA in which both octamers present were either wild type or mutant. The template for making wild-type *TOP2* RNA was prepared by PCR as described previously (17). The PCR product used as a template to generate -291-to-209 *TOP2* RNA in which the two octamers had been changed to CATcGAAG was made by using oligonucleotides D37 (GGATCCTAATACGACTCATATAGGGAGGAGCA TcGAAGTATTGCGGGT) and D38 (CGGGAGTCGCGCGATCTTCTGATGATGGGCTTTCGACACCTCTCT) as 5' and 3' primers, respectively. (The mutant octamer sequences are in boldface). Mutant *TOP2* RNA with CATAGAcG octamers was made from the PCR product by using oligonucleotides D39 (GGATCCTAATACGACTCATATAGGGAGGCGCATAGAcGATTGCGGG) and D40 (CGGGAGTCGCGCGATCTTCTGATGGGCTTTCGACACCTCTCT) as 5' and 3' primers, respectively.

In vitro transcription and gel retardation assay. Wild-type and mutant 6× octamer RNA probes were synthesized by using plasmids linearized with *NotI* (except for pRM16R and pRM22M, which were cut with *HindIII*) as templates for in vitro transcription by T7 RNA polymerase. Wild-type and mutant *TOP2* -291-to-209 RNAs were generated by using PCR products containing a T7 promoter as templates for transcription by T7 RNA polymerase. The in vitro transcription reactions and gel retardation assays were performed by using the Maxiscript kit (Ambion) with [α - 32 P]ATP as described previously (17). Gel retardation assays were performed with 32 P-labeled RNA probes in the presence of 6.7 mg of heparin/ml as a nonspecific competitor. After 15 min at 28°C, the reaction mixtures were electrophoresed at 200 V on nondenaturing 6% polyacrylamide gels (17).

Purification of CEBP. Nuclear extracts were prepared from Nonidet P-40-lysed *C. fasciculata* Cf-C1 cells essentially as described previously (17) and were stored at -70°C. Nuclear extracts were thawed, and solid ammonium sulfate was added (to 40% saturation) with stirring on ice over 15 min. The solution was stirred for 45 min more and centrifuged at 12,000 rpm in a Sorvall GSA rotor for 25 min at 4°C. The pellet was resuspended in buffer E (20 mM Tris-HCl [pH 8.0], 1 mM dithiothreitol [DTT], 5 mM MgCl₂, 20% glycerol) and centrifuged at 40,000 rpm for 30 min in a Beckman 45 Ti rotor. The supernatant was carefully removed and dialyzed against 20 mM Tris-HCl (pH 8.0)-1 mM DTT-5 mM MgCl₂ buffer for 60 min at 4°C. Glycerol was added to a final concentration of 20% (vol/vol), and the solution was centrifuged in a Sorvall SS-34 rotor at 15,000 rpm for 10 min to remove insoluble material. The supernatant was applied on a 40-ml DEAE-cellulose column equilibrated in buffer E containing 50 mM KCl. The column was washed with 150 to 200 ml of buffer E plus 50 mM KCl at a flow rate of 1 ml/min to remove unbound proteins. The *TOP2* RNA binding activity was eluted by washing the column with buffer E plus 100 mM KCl. The proteins, in fractions containing the binding activity, were precipitated by ammonium sulfate (0 to 40% cut) as described above. The pellet was redissolved in buffer F (20 mM Tris-HCl [pH 8.0]-1 mM DTT-1 mM EDTA-20% glycerol) and divided into four aliquots. Each aliquot was run separately on a 1-ml UNO-Q (Bio-Rad) fast protein liquid chromatography (FPLC) column equilibrated in buffer F. The binding activity was eluted with a linear 0 to 300 mM KCl gradient in buffer F.

A 1-ml column of oligo(dT) cellulose was prepared and washed with 10 to 15 ml of 0.1 N NaOH and 15 ml of buffer G (20 mM HEPES [pH 7.9]-1 mM EDTA-0.25 M KCl). A 6× octamer RNA with a 25-base polyadenylate tail at the 3' terminus (200 to 300 μg) was ethanol precipitated and resuspended in buffer G. The RNA was heated to 70°C for 15 min, slowly cooled to room temperature, and applied on the oligo(dT) cellulose column and recycled three to four times. The column was then equilibrated in buffer H (20 mM HEPES [pH 7.9]-1 mM DTT-5 mM MgCl₂-20% glycerol) containing 50 mM KCl. Subsequent opera-

tions were carried out at 4°C. The active fractions from the UNO-Q column were diluted 1:1 with buffer H and loaded on the 6× octamer RNA affinity column. The flowthrough material was reappplied on the column. The column was then washed (flow rate, 0.5 ml/min) successively with 15 to 20 ml of buffer H containing KCl at the following molar concentrations: 0.05, 0.15, 0.3 (alone or with 4 mg of heparin/ml), 0.6, and 1. Fractions were collected (0.5 to 1.0 ml), and 5 μl was assayed for binding activity. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16), and gels were stained with silver (18) or Coomassie blue R-250.

Synthesis of 6× octamer-A₂₅ RNA. Plasmid pRM16, containing six copies of wild-type octamer DNA, was linearized with *HindIII*. The 6× octamer DNA was amplified by PCR using *HindIII*-cut pRM16 as a template with a 5' primer that has a T7 promoter sequence (oligo E3, TGAATTGTAATACGACTCACTA TA) and a 3' primer with 25 T residues at the 5' end (oligo E4; T₂₅GAGCGG CCGCCTTCTATGAA). This gives a PCR product with a T7 promoter sequence at the 5' end and 25 T residues at the 3' end. In vitro transcription by T7 RNA polymerase using this PCR product as a template yields 6× octamer RNA with 25 A residues at the 3' end. Unlabeled 6× octamer-A₂₅ RNA (200 to 300 μg) was synthesized by in vitro transcription using the above PCR product as a template (160 to 200 ng) with the T7 MEGA shortscript kit from Ambion. After 3 h of incubation at 37°C, the template DNA was removed by treatment with 2 U of DNase I for 15 min at 37°C. The 6× octamer-A₂₅ RNA was phenol-chloroform extracted and ethanol precipitated. A 6× CAUcGAAG-A₂₅ RNA was prepared in the same manner.

Northwestern blotting. CEBP (50 ng), purified by affinity column chromatography, was electrophoresed on duplicate 10% polyacrylamide gels containing 0.1% SDS (16). Northwestern blotting was performed as described previously (6, 30), and each nitrocellulose transfer membrane was then incubated for 60 min at room temperature with 5 ml of binding buffer (20 mM HEPES [pH 7.9]-50 mM KCl-5 mM MgCl₂-0.5 mM DTT-20% glycerol-2 mg of heparin/ml) containing 1 × 10⁷ to 2 × 10⁷ cpm of 32 P-labeled 6× octamer RNA (wild type) or 6× antisense octamer RNA as a probe. After two washes of 10 min each with washing buffer (20 mM HEPES [pH 7.9]-20 mM KCl-5 mM MgCl₂-0.5 mM DTT-20% glycerol) at room temperature, the filters were air dried and exposed to X-ray film.

Glycerol gradient sedimentation. Affinity-purified CEBP was sedimented for 19 h in a Beckman SW 50.1 rotor through a 4.9-ml 10 to 30% glycerol gradient containing 50 mM HEPES (pH 7.9), 150 mM KCl, 1 mM DTT, and 133 μg of bovine serum albumin/ml at 2°C. T7 RNA polymerase and the Klenow fragment of DNA polymerase I were sedimented in a parallel gradient under the same conditions as markers.

Other methods. Proteins bound to 32 P-labeled 6× octamer RNA were identified by UV cross-linking of gel-purified complexes and analyzed as described previously (17). Synchronization of *C. fasciculata* cultures by hydroxyurea and Northern blot analysis were performed as described previously (19, 20). Quantitation of Northern blots was performed with a Molecular Dynamics Phosphor-Imager and Image Quant software.

RESULTS

The 6× octamer confers cyclic accumulation on a reporter mRNA. Previous experiments have shown that octamer sequences present in the 5' UTRs of the *RPA1* and *TOP2* genes are required for conferring periodic accumulation on the mRNAs (4, 20). We next wanted to determine whether the octamer sequence alone is sufficient to confer periodic accumulation on a heterologous gene transcript. The pΔ10Not reporter plasmid used in these studies contains a partial cDNA sequence of the *C. fasciculata* homolog of the *Trypanosoma cruzi* flagellar calcium binding protein (CaBP) as a reporter (8). The endogenous *CaBP* transcript is expressed at a constant level during the cell cycle in synchronized *C. fasciculata* cells (19). The plasmid also contains the *TOP2* 5' UTR sequences from -883 to -609 and from -40 to +1 (Fig. 1, upper panel). These sequences include the major *TOP2* splice acceptor site at -668 and an upstream polypyrimidine tract. However, it lacks the sequences necessary for periodic accumulation of plasmid-encoded *TOP2* transcript (20). A *NotI* site was introduced at the junction between -609 and -40, allowing insertion of DNA fragments at this site.

A DNA fragment containing six copies of the octamer sequence, separated by 2 bp each, was cloned into pΔ10Not. Plasmids carrying the 6× octamers in the sense (RM18) and the antisense (pRM18R) orientation were introduced into *C. fasciculata*, and the cell cultures were synchronized by treat-

FIG. 1. The 6× octamer confers cyclic accumulation on a reporter transcript. Total RNA was isolated from synchronized cells carrying pRM18 (A and B) or pRM18R (C and D) at the time of release (0 min) or at 30-min intervals after release from hydroxyurea arrest. The RNAs were subjected to Northern blot analysis (A and C) by hybridizing to probes that detect the 6× octamer–CaBP chimeric transcripts and the endogenous CaBP transcripts. The transcript levels in the Northern blots were quantitated with a PhosphorImager (Molecular Dynamics) (B and D). Chimeric RNA transcript levels have been normalized at each time point relative to that of CaBP transcript, which is expressed at a constant level during the cell cycle and serves as a loading control. A diagram of the relevant sequences (not to scale) contained in each plasmid is shown at the top of the figure. NEO is the neomycin phosphotransferase gene with 5′ and 3′ flanking sequences from the *Leishmania major* DHFR-TS gene for expression of G418 resistance in *Crithidia*. AG₁ is the major TOP2 splice acceptor site.

ment with hydroxyurea. Figure 1 shows Northern blot and PhosphorImager quantitation analysis of RNA isolated from synchronized cells carrying these plasmids. The insertion of 6× octamers in the sense orientation in pΔ10Not results in plasmid-encoded chimeric transcripts that accumulate periodically during the cell cycle. The chimeric transcript is present at high levels immediately after release from hydroxyurea arrest and again at 210 min after release. The maximum level at 210 min is 10-fold higher than the minimum at 120 min. The maximum and minimum transcript levels occur at approximately the same times as those observed for endogenous TOP2 and RPA1 transcript levels (4, 20). As expected, the endogenous CaBP transcript is present at a constant level as cells progress through the cell cycle. In contrast, similar analysis of RNA from synchronized cells carrying plasmid pRM18R, in which the 6× octamer is in an antisense orientation, shows less than twofold variation in RNA levels during the cell cycle (Fig. 1C and D). However, the relative levels of the reporter transcript in cells containing pRM18R are about the same as, or higher than, the maximum level of the reporter transcript containing the sense orientation of the 6× octamer (Fig. 1B and D). A pΔ10Not plasmid containing only a single copy of the octamer consensus sequence did not confer periodic accumulation on the chimeric RNA (data not shown). These results indicate that multiple copies of the octamer sequence can confer periodic accumulation on a heterologous transcript in an orientation-dependent manner.

Factors in nuclear extracts bind to 6× octamer RNA. Nuclear extracts of *C. fasciculata* contain a protein factor(s) that binds to RNA encoded by 5′ UTR elements required for pe-

riodic accumulation of TOP2 and RPA1 mRNAs (17). Mutations in the 5′ UTR that abolished cycling of the reporter gene transcript also eliminated binding of the nuclear factor(s) to the corresponding mutant RNA probes. Since six copies of the octamer sequence were able to confer periodic accumulation on a heterologous gene transcript, it was of interest to determine whether the nuclear factor(s) would bind to the 6× octamer RNA. Labeled 6× octamer RNA was incubated with *C. fasciculata* nuclear extracts and then electrophoresed on a non-denaturing polyacrylamide gel to separate free and bound RNA. Two gel-shifted bands are seen, indicating the presence of a nuclear factor(s) that binds to the 6× octamer RNA (Fig. 2). No binding to an antisense 6× octamer RNA probe was observed. When nuclear extracts were incubated with TOP2 –291-to–209 RNA, two shifted bands were observed as before (17). Interestingly, severalfold-higher binding of the nuclear factor(s) to the 6× octamer RNA is observed compared to that of the TOP2 RNA. These results show that nuclear extracts of *C. fasciculata* contain a factor(s) that exhibits specific and strong binding to the 6× octamer RNA.

Effect of mutations on cycling of reporter gene mRNA and binding of a nuclear factor(s) to 6× octamer RNA. The role of individual nucleotides in the binding of RNA to a nuclear factor(s) was investigated by using mutant 6× octamer RNA probes in the gel retardation assay. Single-nucleotide substitutions were introduced into the central hexamer core of the octamer, since the hexamer is completely conserved in several *C. fasciculata* gene transcripts that show periodic accumulation during the cell cycle (20). RNA probes in which all six octamers contained the same single-nucleotide substitution were pre-

FIG. 2. 6× octamer RNA binds factors in nuclear extracts. RNA probes were incubated with nuclear extracts (NE) for 15 min at 28°C and analyzed by non-denaturing PAGE. Probes used were 6× octamer sense RNA, 6× octamer antisense RNA, and -291-to-209 *TOP2* RNA. The retarded complexes are indicated by a bracket.

pared. The wild-type and mutant 6× octamer RNA probes were incubated with nuclear extracts and analyzed for binding by gel retardation assay. Mutation of any of the first 5 nucleotides of the hexamer abolished the formation of specific RNA-protein complexes (Fig. 3). More rapidly migrating minor bands observed with each of these five mutant RNAs were not characterized further. Binding of nuclear factors to 6× CAUAGAcG RNA was observed but was significantly reduced compared to their binding to wild-type 6× octamer RNA. Therefore, the first 5 nucleotides of the hexamer core appear to be critical for the binding of a factor(s) to the 6× octamer RNA.

FIG. 3. Single-nucleotide substitutions in the 6× octamer RNA prevent binding by nuclear factors. Wild-type or mutant 6× octamer RNA probes were incubated in the absence (-) or presence (+) of 17 μg of nuclear extracts (NE) and analyzed by nondenaturing PAGE.

FIG. 4. Northern blot analysis of total RNA isolated from hydroxyurea-synchronized *C. fasciculata* strains carrying plasmid pRM25 or pRM27. RNA was isolated from synchronized cells carrying pRM27 (A and B) or pRM25 (C and D) and analyzed by Northern blotting as in Fig. 1.

We next determined the effect of these substitutions on the ability of mutant 6× octamers to confer periodic accumulation on the reporter gene transcript. Two mutations were selected based on results of the gel retardation assay: CAUcGAAG, which abolished specific complex formation, and CAUAGA cG, which reduced the binding of a factor(s) to 6× octamer RNA. DNA fragments containing 6× CATcGAAG or 6× CATAGAcG were cloned into pΔ10Not in the sense orientation to give plasmids pRM25 and pRM27, respectively. These plasmids were then electroporated separately into *C. fasciculata*. RNA was isolated from hydroxyurea-synchronized cells carrying pRM25 or pRM27 and subjected to Northern blot analysis (Fig. 4). Cloning of 6× CATAGAcG into pΔ10Not (plasmid pRM27) conferred periodic accumulation on the chimeric transcript. The maximum transcript level at 210 min after release from hydroxyurea block is 10.6-fold higher than the minimum transcript level at 120 min (Fig. 4B). The chimeric transcript levels in synchronized *C. fasciculata* cells carrying pRM25 or pRM27 are similar to those from cells carrying pRM18 (compare Fig. 1B and 4B). In contrast, plasmid pRM25 produced a chimeric transcript that did not accumulate in the manner seen for pRM18 and pRM27 (Fig. 4D). In this case the trough was shifted to 150 min, and the transcript levels increased by only approximately twofold thereafter, although, as for pRM18R, the reporter transcript levels expressed from pRM25 were generally at or above the maximum level of the transcript from the construct that cycles (pRM27). Since the mutant 6× CATAGAcG could confer periodic accumulation on the chimeric transcript, the reduced level of binding by the corresponding mutant 6× octamer RNA is apparently sufficient to confer periodic accumulation on the transcript.

The same nuclear factor(s) binds to wild-type 6× octamer RNA and *TOP2* 5' UTR RNA. UV cross-linking of the 6× octamer RNA to bound proteins was performed to identify the proteins in gel-shifted complexes. The binding activity was partially purified by ammonium sulfate precipitation of proteins in nuclear extracts followed by chromatography on a DEAE-cellulose column (17). The partially purified protein was used in the gel retardation assay with wild-type 6× octamer RNA as a probe. The gel slice containing the RNA-protein complexes was irradiated with UV light for 1 min, and the cross-linked complex was eluted from the gel. After digestion with RNases A and T₁, the labeled proteins were electrophoresed on SDS-polyacrylamide gels and visualized by auto-

FIG. 5. UV cross-linking of proteins to 6 $\times$ octamer RNA. Proteins cross-linked to 32 P-labeled 6 $\times$ octamer RNA were analyzed by SDS-PAGE and autoradiography after digestion of gel-isolated complexes with RNases A and T_1 .

radiography. Two polypeptides of approximately 74 and 38 kDa were labeled by the 6 $\times$ octamer RNA (Fig. 5). Similar-sized proteins have been shown to be cross-linked to *TOP2* -291-to--209 RNA (17). Thus, the 6 $\times$ octamer and *TOP2* RNA probes bind to proteins of the same molecular mass.

To confirm that the 6 $\times$ octamer RNA and *TOP2* RNA probes bind to the same protein(s), nuclear extracts were incubated with a labeled -291-to--209 *TOP2* RNA probe in the absence or presence of unlabeled 6 $\times$ CAUAGAAG (wild type) or 6 $\times$ CAUCGAAG (mutant) RNA. The 6 $\times$ CAUCGAAG RNA was used as a control, since it does not bind to a nuclear factor(s) (see Fig. 3). The presence of unlabeled 6 $\times$ CAUAGAAG RNA reduced the binding of the nuclear factor(s) to the *TOP2* RNA probe (Fig. 6, lanes 3 and 4), but unlabeled 6 $\times$ CAUCGAAG RNA did not have a significant effect (lanes 5 and 6), indicating that the *TOP2* RNA and 6 $\times$ octamer RNA bind to the same factor(s).

Effects of point mutations on binding to *TOP2* 5' UTR RNA. The *TOP2* -291-to--209 5' UTR RNA contains two octamers, one at either end of the RNA. We have previously shown that mutation of both octamers to a different sequence eliminated binding of a factor(s) to the mutant RNA (17). Since the same nuclear factor(s) binds to the *TOP2* 5' UTR and the 6 $\times$ octamer RNA, it was important to determine whether single-nucleotide substitutions would have a similar effect on binding to mutant *TOP2* RNA as they did on binding to mutant 6 $\times$ octamer RNA. Both octamers in *TOP2* RNA were changed either to CAUCGAAG or to CAUAGAcG. These two mutations were selected because the 6 $\times$ CAUCGAAG RNA shows no binding, while 6 $\times$ CAUAGAcG shows reduced binding, to a nuclear factor(s) (see Fig. 3). The *TOP2* RNA in which both octamers were CAUCGAAG does not bind to a nuclear factor(s) (Fig. 7, lanes 3 and 4). In contrast, the *TOP2* RNA with both CAUAGAcG octamers shows binding that is significantly reduced compared to that seen with wild-type *TOP2* RNA (Fig. 7; compare lanes 2 and 6). Therefore, mutations that eliminate or reduce binding to 6 $\times$ octamer RNA have similar

FIG. 6. Competition between *TOP2* 5' UTR and 6 $\times$ octamer RNAs for binding to a factor(s) in nuclear extracts. The 32 P-labeled -291-to--209 *TOP2* RNA probe was incubated with 17 μ g of nuclear extract in the absence (lane 2) or presence of a three- or sixfold molar excess of unlabeled wild-type (lanes 3 and 4) or mutant (CAUCGAAG) (lanes 5 and 6) 6 $\times$ octamer RNAs. The complexes formed were analyzed by nondenaturing PAGE. Lane 1, *TOP2* RNA probe in the absence of nuclear extract.

effects on binding to *TOP2* RNA containing the corresponding mutant octamers.

The binding activity varies during the cell cycle. Since the mRNA levels of several DNA replication genes vary during the cell cycle, with maximum levels present at G₁/S phase (19), we wanted to determine whether the specific RNA binding activity observed here also varied during the cell cycle. Nuclear extracts were prepared from synchronous cultures at 30-min intervals after release from hydroxyurea block and were assayed for binding activity by using the -291-to--209 *TOP2* RNA probe. Total RNA was also isolated from the synchronized cells and subjected to Northern blot analysis to detect *TOP2* RNA. As shown in Fig. 8, the *TOP2* RNA binding activity varies during the cell cycle (Fig. 8A) and follows the same pattern as *TOP2* mRNA levels (Fig. 8B).

Purification of CEBP. The *TOP2* RNA binding activity was purified from crude nuclear extracts of *C. fasciculata* cultures by successive chromatography on a DEAE-cellulose column, a UNO-Q FPLC anion-exchange column, and an RNA affinity column by using 6 $\times$ octamer RNA as a ligand. SDS-PAGE analysis and silver staining of protein fractions containing the binding activity from the affinity column demonstrated the presence of two polypeptides with molecular masses of approximately 38 and 48 kDa (Fig. 9B) that were eluted with 0.6 M KCl. During this purification scheme, it was important to partially purify the binding protein before applying it to the affinity

FIG. 7. Gel retardation assay with wild-type or mutant *TOP2* 5' UTR RNA. The -291-to-209 *TOP2* RNA probes containing both wild-type (CAUAGAAG) or mutant (CAUcGAAG or CAUAGAcG) octamer sequences were incubated in the absence (-) or presence (+) of 17 μ g of nuclear extracts (NE) and analyzed by nondenaturing PAGE.

column; otherwise, several other polypeptides were present in fractions containing the binding activity. Control experiments were also done to determine the specificity of binding. A 6 $\times$ CAUcGAAG RNA affinity column was prepared as described above, and the partially purified proteins (FPLC fractions) were applied to it. A 6 $\times$ CAUcGAAG RNA column was prepared, since no binding is seen to the 6 $\times$ CAUcGAAG RNA (Fig. 3). When the 6 $\times$ CAUcGAAG column was washed with KCl, most of the specific RNA binding activity eluted in the 0.15 M wash and some eluted in 0.3 M KCl fractions. No binding activity was observed in 0.6 M KCl fractions. SDS-PAGE analysis of 0.6 M KCl fractions showed the absence of the 38- and 48-kDa polypeptides (data not shown). When the 0.15 and 0.3 M KCl fractions were pooled and applied on a 6 $\times$

FIG. 8. (A) *TOP2* 5' UTR RNA binding activity in nuclear extracts prepared from hydroxyurea-synchronized cells. Cultures of *C. fasciculata* were synchronized with hydroxyurea, and nuclear extracts were prepared at 30-min intervals after release from hydroxyurea arrest. The nuclear extracts (8 μ g) were assayed for binding activity with the *TOP2* -291-to-209 RNA probe. (B) Total RNA was isolated from synchronized cells and subjected to Northern blot analysis by hybridizing with a probe that detects *TOP2* mRNA.

FIG. 9. Purification of CEBP. Samples from each stage of purification were separated on 10% polyacrylamide gels containing 0.1% SDS. (A) Coomassie blue-stained gel showing proteins in nuclear extracts (NE), ammonium sulfate precipitate (A.S.), DEAE-cellulose pooled fractions, and UNO-Q FPLC column fractions. (B) Silver-stained gel showing proteins in the 0.6 M KCl wash of the 6 $\times$ octamer RNA affinity column. The numbers indicate the sizes of the molecular weight markers (in kilodaltons).

CAUAGAAG column, the binding activity eluted in the 0.6 M KCl fraction and contained the 38- and 48-kDa polypeptides (data not shown). These results suggest that the two polypeptides are subunits of the binding protein.

To confirm the association of these two polypeptides with the binding activity, purified CEBP was subjected to glycerol gradient sedimentation in order to estimate the native molecular mass of the binding protein. Purified CEBP was sedimented through a 10 to 30% glycerol gradient containing 133 μ g of bovine serum albumin per ml (Fig. 10). The position of CEBP in the gradient was determined by gel retardation assay of fractions after centrifugation. CEBP sedimented at approximately the same rate as T7 RNA polymerase in a parallel gradient. Within the uncertainty of such measurements, this result is consistent with CEBP being a heterodimer with subunits of approximately 38 and 48 kDa. A small peak of more rapidly sedimenting binding activity may represent a higher oligomeric form of CEBP.

FIG. 10. Glycerol gradient sedimentation of CEBP. Purified CEBP was sedimented through a 10 to 30% glycerol gradient as described in the text. Fractions were collected through a 21-gauge needle and assayed immediately for CEBP binding activity. T7 RNA polymerase (pol) and DNA polymerase I Klenow fragment were sedimented in a parallel gradient as sedimentation markers.

FIG. 11. Interaction of purified CEBP with RNA. RNA probes were incubated in the absence (–) or presence (+) of 10 μ g of CEBP and analyzed by nondenaturing PAGE. Probes used are *TOP2* –291-to–209 RNA with both wild-type (*TOP2* wt) or both mutant (*TOP2* mut) octamers, *RPA1* –341-to–231 RNA with both wild-type (*RPA1* wt) or both mutant (*RPA1* mut) octamers, 6 $\times$ CAUAGAAG RNA (6 $\times$ oct wt), and 6 $\times$ CAUCGAAG RNA (6 $\times$ oct mut).

Binding specificity of purified CEBP. Gel retardation analysis shows that purified CEBP binds to *TOP2* and *RPA1* RNAs containing wild-type octamers (Fig. 11). No binding was seen to corresponding RNAs in which both octamers were mutated to a different sequence. Similarly, binding was seen to the wild-type 6 $\times$ CAUAGAAG RNA but not to the mutant 6 $\times$ CAUCGAAG RNA. These results are consistent with previous observations where no binding was seen when crude nuclear extracts were incubated with mutant *TOP2* and *RPA1* RNAs (17) or 6 $\times$ CAUCGAAG RNA. Purified CEBP binds more strongly to 6 $\times$ CAUAGAAG RNA than to *TOP2* RNA, as seen earlier with nuclear extracts. Interestingly, the binding of CEBP to wild-type *RPA1* RNA is stronger than that to *TOP2* RNA (Fig. 11), and at present we do not know the significance of this observation. At the level of CEBP used in these experiments, only a single gel-shifted band is seen with *TOP2* RNA, but two are seen with 6 $\times$ octamer RNA and three with *RPA1* RNA. The additional bands seen with 6 $\times$ octamer and *RPA1* RNAs appear to represent higher-order complexes due to binding of additional protein molecules. At higher levels of CEBP, a second gel-shifted band is observed with a *TOP2* RNA probe; this likely represents the binding of CEBP to both of the octamer elements on the RNA probe (17).

The 38-kDa protein binds to RNA. Northwestern blotting was performed to identify the protein containing the RNA binding activity. Purified CEBP was subjected to SDS-PAGE to separate the 38- and 48-kDa proteins and then transferred to a nitrocellulose filter. The filter was probed with 32 P-labeled 6 $\times$ octamer RNA and subjected to autoradiography. A band of approximately 38 kDa was observed on the autoradiograph (Fig. 12A, lane 2). This band corresponds to the 38-kDa protein in purified CEBP. The same band was detected by the probe when proteins present in the 0 to 40% ammonium sulfate precipitate were resolved by SDS-PAGE (Fig. 12A, lane 1). Control experiments in which the filters were probed with 6 $\times$ octamer antisense RNA showed no binding to any polypeptide (Fig. 12B). Thus, the 38-kDa protein contains the RNA binding activity and can bind to RNA even in the absence of the 48-kDa protein.

FIG. 12. Northwestern blot analysis. Proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with 32 P-labeled 6 $\times$ octamer RNA (wild type) (A) or with 32 P-labeled 6 $\times$ octamer RNA (antisense) (B). Lane 1, ammonium sulfate cut (20 μ g); lane 2, purified CEBP (50 ng).

DISCUSSION

We have initiated studies to identify the *cis*-acting elements and *trans*-acting factors involved in conferring periodic expression on DNA replication genes in *C. fasciculata*. An investigation into the mechanism regulating the *TOP2* and *RPA1* genes has revealed two common features that could serve to mediate the coordinate regulation of these genes. First, octamer sequences within the 5' UTRs of these genes have been shown to be required for conferring periodic accumulation of their mRNAs (4, 20). Second, the same nuclear factor(s) binds to the 5' UTR RNAs of both these genes (17). To further characterize the *cis* sequences involved in this unusual cell cycle regulatory mechanism, we have inserted multiple copies of the octamer into the 5' UTR of a reporter gene of a plasmid transfected into *C. fasciculata*. We have shown here that six copies of the octamer sequence cloned into the 5' UTR confer strong cycling on a reporter gene transcript only when they are present in the sense orientation. The 6 $\times$ octamer RNA also showed strong and specific binding to a nuclear factor(s). Thus, multiple copies of the octamer sequence can both confer cycling on a reporter gene transcript and binding to a specific nuclear factor(s). In addition, a single-base change in this sequence element is sufficient to abolish both the binding of specific nuclear factors in vitro and cycling of a reporter gene in vivo. Since the 6 $\times$ octamer is a small sequence with just 2 nucleotides separating the octamers, specific RNA secondary structures do not appear to be critical for its function. To our knowledge, this is the first report of the identification of a small (<10-nucleotide) posttranscriptional regulatory element in trypanosomes whose function has been studied at the single-nucleotide level.

We have exploited the strong binding of the nuclear factor to the 6 $\times$ octamer RNA to affinity purify the binding protein. Purified CEBP binds to *TOP2*, *RPA1*, and 6 $\times$ octamer RNAs but not to RNA probes with mutant octamers. Proteins with molecular masses of approximately 38 and 48 kDa copurify with the binding activity, consistent with a multimeric structure for CEBP. The 38-kDa protein contains the RNA binding activity and binds RNA even in the absence of the 48-kDa protein. The high specificity of binding of 6 $\times$ octamer RNA to the 38-kDa protein is illustrated by the specific binding to this protein even in crude nuclear extracts.

In other eukaryotes, specific RNA binding proteins have

been found to regulate gene expression posttranscriptionally by activating splicing enhancer elements. Multiple copies of RNA binding sites have been shown to function as strong splicing enhancers for two different members of the SR family of splicing factors (27, 28). Also, multiple copies of the hexamer TGCATG have been shown to regulate the alternative splicing of fibronectin pre-mRNA and a heterologous prepro-tachykinin pre-mRNA (15). Trypanosome genes do not contain introns, and splicing of primary transcripts is limited to the *trans* splicing of the 39-nucleotide mini-exon sequence at alternate splice acceptor sites in the 5' flanking region of each gene (21). The cell cycle regulation of specific trypanosomatid mRNAs observed here is unlikely to involve a cell cycle regulation of *trans* splicing of primary transcripts for the following reasons. First, consensus octamer sequences are present between distal and proximal splice acceptor sites in the 5' flanking sequences of both the *TOP2* and *RPA1* constructs examined earlier, yet mRNAs spliced at the distal sites contain the octamer sequences and cycle while mRNAs spliced at the proximal splice acceptor sites lack the octamer sequences and do not cycle (4, 20). Thus, the presence of the octamer sequences on the primary transcript is not sufficient to confer cycling; the octamer sequences must be present on the mRNA. Second, recombinant constructs in which the octamer sequences are transposed from the 5' UTR to the 3' UTR still express mRNAs that cycle, indicating that proximity of the octamer sequences to splice acceptor sites is not essential for conferring cycling on the mRNA level (4).

Studies of the developmental regulation of mRNA levels in trypanosomes have shown that regulation is determined posttranscriptionally and that the sequences responsible are usually contained within the 3' UTR of the transcripts under investigation (2, 14, 22). In the case of procyclins, the major surface glycoproteins of the insect form of *Trypanosoma brucei*, both positive and negative regulatory elements are contained in the procyclin 3' UTR (9). Both a conserved 16-mer in the 3' UTR of procyclin transcripts and the first 40 nucleotides of the 3' UTR serve as positive elements in regulating the mRNA level, while a separate element within a 73-nucleotide sequence (LII) negatively regulates the mRNA level. A 26-nucleotide polypyrimidine tract within this negative element acts to accelerate turnover of the procyclin mRNA in bloodstream forms (13). The differential expression of the genes for the glycoproteins gp63 and gp46 in *Leishmania chagasi* is posttranscriptionally regulated by elements in their 3' UTRs (1, 22). The 3' UTR elements also posttranscriptionally regulate expression of the amastin genes, which encode an abundant protein on the surface of *T. cruzi* in the amastigote stage of the parasite (29). Similarly, A2 genes in *Leishmania donovani*, which encode an amastigote-specific protein, are posttranscriptionally regulated by 3' UTR elements (5). However, in none of these cases has a protein factor(s) that binds to sequences performing the regulatory function been identified and purified. To our knowledge, the present studies represent the first example for trypanosomes of the purification of a protein shown to bind to sequences affecting gene expression.

We suggest that the octamer sequences in *TOP2* and *RPA1* 5' UTRs play a negative role in regulating mRNA levels during the cell cycle and that binding of CEBP to these sequences overcomes the negative regulation. Cycling of the binding activity would therefore result in a parallel cycling of target mRNA levels, consistent with results presented here and in earlier studies (17). Also, mutation of the octamer sequences in a target mRNA would be expected to eliminate cycling of the mRNA and to result in mRNA levels similar to the maximum levels achieved by the wild-type mRNA during the cell

cycle. This model is supported by the observations in Fig. 1 and 4 and in previous studies (20) showing that mutation of octamer sequences in reporter constructs reduces the cycling and results in mRNA levels throughout the cell cycle near the maximum level attained by the wild-type construct. The availability of purified CEBP should now facilitate the cloning of the genes encoding the subunits of CEBP and the further analysis of this novel mechanism of gene regulation.

ACKNOWLEDGMENTS

We thank Lisa Brown for performing the experiment with a single octamer cloned in pΔ10Not.

This research was supported by NIH grant GM53254.

REFERENCES

1. Beetham, J. K., K. S. Myung, J. J. McCoy, M. E. Wilson, and J. E. Donelson. 1997. Glycoprotein 46 mRNA abundance is post-transcriptionally regulated during development of *Leishmania chagasi* promastigotes to an infectious form. *J. Biol. Chem.* **272**:17360-17366.
2. Berberof, M., L. Vanhamme, P. Tebabi, A. Pays, D. Jefferies, S. Welburn, and E. Pays. 1995. The 3'-terminal region of the mRNAs for VSG and procyclin can confer stage specificity to gene expression in *Trypanosoma brucei*. *EMBO J.* **14**:2925-2934.
3. Bogenhagen, D., and D. A. Clayton. 1977. Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. *Cell* **11**:719-727.
4. Brown, L. M., and D. S. Ray. 1997. Cell cycle regulation of *RPA1* transcript levels in the trypanosomatid *Crithidia fasciculata*. *Nucleic Acids Res.* **25**:3281-3289.
5. Charest, H., W. W. Zhang, and G. Matlashewski. 1996. The developmental expression of *Leishmania donovani* A2 amastigote-specific genes is post-transcriptionally mediated and involves elements located in the 3'-untranslated region. *J. Biol. Chem.* **271**:17081-17090.
6. Chen, X., J. Sadlock, and E. A. Schon. 1993. RNA-binding patterns in total human tissue proteins: analysis by Northwestern blotting. *Biochem. Biophys. Res. Commun.* **191**:18-25.
7. Cosgrove, W. B., and M. J. Skeen. 1970. The cell cycle in *Crithidia fasciculata*. Temporal relationships between synthesis of deoxyribonucleic acid in the nucleus and in the kinetoplast. *J. Protozool.* **17**:172-177.
8. Englund, P. T. 1979. Free minicircles of kinetoplast DNA networks in *Crithidia fasciculata*. *J. Biol. Chem.* **254**:4895-4900.
9. Furger, A., N. Schurch, U. Kurath, and I. Roditi. 1997. Elements in the 3' untranslated region of procyclin mRNA regulate expression in insect forms of *Trypanosoma brucei* by modulating RNA stability and translation. *Mol. Cell. Biol.* **17**:4372-4380.
10. Guttus, E. W., P. C. Hanawalt, and S. Guttus. 1967. Mitochondrial DNA synthesis and the mitotic cycle in *Physarum polycephalum*. *Biochim. Biophys. Acta* **142**:181-194.
11. Hajduk, S. L., V. A. Klein, and P. T. Englund. 1984. Replication of kinetoplast DNA maxicircles. *Cell* **36**:483-492.
12. Hines, J. C., and D. S. Ray. 1997. Periodic synthesis of kinetoplast DNA topoisomerase II during the cell cycle. *Mol. Biochem. Parasitol.* **88**:249-252.
13. Hotz, H. R., C. Hartmann, K. Huober, M. Hug, and C. Clayton. 1997. Mechanisms of developmental regulation in *Trypanosoma brucei*: a polypyrimidine tract in the 3'-untranslated region of a surface protein mRNA affects RNA abundance and translation. *Nucleic Acids Res.* **25**:3017-3026.
14. Hug, M., V. B. Carruthers, C. Hartmann, D. S. Sherman, G. A. Cross, and C. Clayton. 1993. A possible role for the 3'-untranslated region in developmental regulation in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **61**:87-95.
15. Huh, G. S., and R. O. Hynes. 1994. Regulation of alternative pre-mRNA splicing by a novel repeated hexanucleotide element. *Genes Dev.* **8**:1561-1574.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (London)* **227**:680-685.
17. Mahmood, R., and D. S. Ray. 1998. Nuclear extracts of *Crithidia fasciculata* contain factors that bind to the 5' untranslated regions of *TOP2* and *RPA1* mRNAs containing sequences required for their cell cycle regulation. *J. Biol. Chem.* **273**:23729-23734.
18. Merril, C. R. 1990. Gel-staining techniques. *Methods Enzymol.* **182**:477-488.
19. Pasion, S. G., G. W. Brown, L. M. Brown, and D. S. Ray. 1994. Periodic expression of nuclear and mitochondrial DNA replication genes during the trypanosomatid cell cycle. *J. Cell Sci.* **107**:3515-3520.
20. Pasion, S. G., J. C. Hines, X. Ou, R. Mahmood, and D. S. Ray. 1996. Sequences within the 5' untranslated region regulate the levels of a kinetoplast DNA topoisomerase mRNA during the cell cycle. *Mol. Cell. Biol.* **16**:6724-6735.
21. Pays, E. 1993. Genome organization and control of gene expression in trypanosomatids, p. 127-160. In P. Broda, S. G. Oliver, and P. F. G. Sims

- (ed.), The eukaryotic genome: organization and regulation. Press Syndicate of the University of Cambridge, Cambridge, United Kingdom.
22. **Ramamoorthy, R., K. G. Swihart, J. J. McCoy, M. E. Wilson, and J. E. Donelson.** 1995. Intergenic regions between tandem gp63 genes influence the differential expression of gp63 RNAs in *Leishmania chagasi* promastigotes. *J. Biol. Chem.* **270**:12133–12139.
 23. **Ray, D. S.** 1987. Kinetoplast DNA minicircles: high-copy-number mitochondrial plasmids. *Plasmid* **17**:177–190.
 24. **Shapiro, T. A., and P. T. Englund.** 1995. The structure and replication of kinetoplast DNA. *Annu. Rev. Microbiol.* **49**:117–143.
 25. **Simpson, L.** 1987. The mitochondrial genome of kinetoplastid protozoa: genomic organization, transcription, replication, and evolution. *Annu. Rev. Microbiol.* **41**:363–382.
 26. **Stuart, K.** 1983. Kinetoplast DNA, mitochondrial DNA with a difference. *Mol. Biochem. Parasitol.* **9**:93–104.
 27. **Tacke, R., Y. Chen, and J. L. Manley.** 1997. Sequence-specific RNA binding by an SR protein requires RS domain phosphorylation: creation of an SRp40-specific splicing enhancer. *Proc. Natl. Acad. Sci. USA* **94**:1148–1153.
 28. **Tacke, R., and J. L. Manley.** 1995. The human splicing factors ASF/SF2 and SC35 possess distinct, functionally significant RNA binding specificities. *EMBO J.* **14**:3540–51.
 29. **Teixeira, S. M., L. V. Kirchhoff, and J. E. Donelson.** 1995. Post-transcriptional elements regulating expression of mRNAs from the amastin/tuzin gene cluster of *Trypanosoma cruzi*. *J. Biol. Chem.* **270**:22586–22594.
 30. **Towbin, H., T. Staehlin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
 31. **Vanhamme, L., and E. Pays.** 1995. Control of gene expression in trypanosomes. *Microbiol. Rev.* **59**:223–240.
 32. **Williamson, D. H., and E. Moustacchi.** 1971. The synthesis of mitochondrial DNA during the cell cycle in the yeast *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **42**:195–201.