Sequences within the 5' Untranslated Region Regulate the Levels of a Kinetoplast DNA Topoisomerase mRNA during the Cell Cycle

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Gene expression in trypanosomatids appears to be regulated largely at the posttranscriptional level and involves maturation of mRNA precursors by *trans* splicing of a 39-nucleotide miniexon sequence to the 5' end of the mRNA and cleavage and polyadenylation at the 3' end of the mRNA. To initiate the identification of sequences involved in the periodic expression of DNA replication genes in trypanosomatids, we have mapped splice acceptor sites in the 5' flanking region of the *TOP2* gene, which encodes the kinetoplast DNA topoisomerase, and have carried out deletion analysis of this region on a plasmid-encoded *TOP2* gene. Block deletions within the 5' untranslated region (UTR) identified two regions (–608 to –388 and –387 to –186) responsible for periodic accumulation of the mRNA. Deletion of one or the other of these sequences had no effect on periodic expression of the mRNA, while deletion of both regions resulted in constitutive expression of the mRNA throughout the cell cycle. Subcloning of these sequences into the 5' UTR of a construct lacking both regions of the *TOP2* 5' UTR has shown that an octamer consensus sequence present in the 5' UTR of the *TOP2*, *RPA1*, and *DHFR-TS* mRNAs is required for normal cycling of the *TOP2* mRNA. Mutation of the consensus octamer sequence in the *TOP2* 5' UTR in a plasmid construct containing only a single consensus octamer and that shows normal cycling of the plasmid-encoded *TOP2* mRNA resulted in substantial reduction of the cycling of the mRNA level. These results imply a negative regulation of *TOP2* mRNA during the cell cycle by a mechanism involving redundant elements containing one or more copies of a conserved octamer sequence within the 5' UTR of *TOP2* mRNA.

The mitochondrial DNA of trypanosomes exhibits several novel features that distinguish it from that of higher eukaryotes (27, 28, 31, 32, 34). This unusual DNA, termed kinetoplast DNA, consists of a single enormous network of catenated minicircles and maxicircles. In the trypanosomatid *Crithidia fasciculata*, there are some 5,000 minicircles and 20 to 30 maxicircles in an unduplicated network. The minicircles in *C. fasciculata* are nearly homogeneous in sequence, whereas minicircles in *Trypanosoma brucei* consist of over 300 sequence classes. Duplication of the kinetoplast network involves the release of individual minicircles from the interior of the network followed by their replication free of the network and rejoining of the newly replicated minicircles to two antipodal sites on the periphery of the network (6). Two kinetoplast replication proteins, a type II DNA topoisomerase (23) and a DNA polymerase (6), have been shown previously to have a similar antipodal localization and may represent components of multiprotein replication complexes.

Maxicircles encode mitochondrial proteins and mitochondrial rRNAs and are analogous to the mitochondrial DNA of higher eukaryotes. Expression of maxicircle genes has been shown recently to involve a novel RNA editing (5, 30) in which small guide RNAs appear to serve as templates for the insertion and/or deletion of uridine residues to produce the mature mRNA (2). The guide RNAs are encoded in both minicircles and maxicircles (35).

Another unusual feature of kinetoplast DNA is its replication in approximate synchrony with that of nuclear DNA (4). In other eukaryotes, mitochondrial replication occurs through-

out the cell cycle (3, 10). Recent studies suggest a possible basis for the temporal link between nuclear and kinetoplast DNA replication in trypanosomes (24). Both kinetoplast and nuclear DNA replication genes are encoded in the nucleus and transcripts of *TOP2*, the kinetoplast DNA topoisomerase gene, those of the nuclear replication genes *RPA1*, *RPA2*, and *LIG1* and that of *DHFR-TS* (dihydrofolate reductase-thymidylate synthase) all accumulate periodically, with maximum levels occurring just prior to S phase in synchronized cultures of *C. fasciculata*. To explore the possibility that coordinated expression of kinetoplast and nuclear DNA replication genes plays a role in coordinating kinetoplast and nuclear S phases, we have initiated studies of the mechanisms involved in regulating the expression of DNA replication genes in trypanosomatids.

DNA replication genes in budding yeast are expressed under cell cycle control by coordinate regulation at the transcriptional level. Transcriptional activation depends on a short sequence (the *MluI* cell cycle box) within the promoters of these genes (20). Expression occurs near the G₁/S phase boundary and involves activation of transcription by a protein complex called DSC1 (or MBF) (14, 17). The periodic activation of replication genes appears to be a consequence of periodic activity of the DSC1 transcription factor (20).

A similar mechanism of regulation of trypanosomatid DNA replication genes is unlikely since regulation of gene expression in trypanosomatids appears to occur largely posttranscriptionally by regulatory loops that involve pre-mRNA turnover in combination with differential rates of *trans* splicing and polyadenylation and of mRNA turnover (26). Nuclear genes in trypanosomatids are often expressed as polycistronic pre-mRNAs which are rapidly processed by coordinated or coupled *trans* splicing and 3'-end cleavage/polyadenylation reactions to yield monocistronic mRNAs (19, 21). *trans* splicing involves the splicing of two unlinked RNA molecules, a spliced

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leader RNA and the pre-mRNA, and results in the addition of a 39-nucleotide spliced leader (or minixen) sequence to every mRNA (for a review, see reference 1). *trans* splicing in trypanosomatids is similar mechanistically to *cis* splicing in other eukaryotes. The 3' splice acceptor site consists of an invariant AG residue and an upstream pyrimidine tract (12). The polypyrimidine tract is a bifunctional element that serves as a determinant both for *trans* splicing and for polyadenylation of the transcript of the upstream gene (19, 21).

The most extensively studied polycistronic transcription units in trypanosomatids are those expressing the *T. brucei* variant surface glycoprotein (VSG) genes and the procyclic acidic repetitive protein (PARP) genes (8). The VSG genes are expressed only in the bloodstream phase of the life cycle of *T. brucei*. In the procyclic form of *T. brucei*, VSG is not expressed because transcription is aborted near the 5' end of the transcription unit. Expression of individual genes within a VSG polycistronic transcription unit can differ as much as 100-fold due to post-transcriptional processes. Unlike VSG genes, PARP genes are regulated at least partly at a transcriptional level during the life cycle. PARP promoters are down regulated to approximately 15% of the level in procyclic trypanosomes. So far, the only example of trypanosome gene regulation exclusively at the transcriptional level is that of the *T. brucei* metacyclic VSG genes, which are expressed as short monocistronic transcripts (9). Both PARP genes and VSG genes are transcribed by α -amanitin-insensitive RNA polymerases, and it is not known whether polymerase II-transcribed genes in polycistronic transcription units display a similar range of regulatory mechanisms.

The mechanisms regulating expression of trypanosomatid genes during the cell cycle represent an unexplored aspect of gene regulation in trypanosomatids. As a first step in determining the mechanism of coordination of the expression of nuclear and kinetoplast DNA replication genes, we have initiated studies of the cell cycle-regulated expression of *TOP2*, the gene encoding the kinetoplast-associated type II DNA topoisomerase of *C. fasciculata* (25). A disrupted form of *TOP2* has been shown to be expressed periodically from a recombinant plasmid during the cell cycle in synchronized *C. fasciculata* cells (24). We show here that redundant sequence elements downstream of the major splice acceptor site within the 5' untranslated region (UTR) of *TOP2* are required for periodic accumulation of the *TOP2* mRNA.

MATERIALS AND METHODS

Determination of 5' splice sites of *TOP2* transcripts. The 5' splice sites of the endogenous *TOP2* transcript and those of plasmids pdN-1 and pdN-1Δ5 were mapped by PCR amplification following reverse transcription of total RNA to generate cDNA primed with *TOP2*-specific primer oligonucleotide 63 (5'GGT CATGCGAGCCCCGCGCACGC3'), complementary to nucleotides +240 to +218 of the *TOP2* coding sequence. The cDNA generated was used as template for 30 cycles of PCR amplification with the 5' primer oligonucleotide 29 (5'AA CGCTATATAAGTATCAGTTTCTG3'), which contains nucleotides 5 to 29 of the *C. fasciculata* minixen and the nested 3' primer oligonucleotide 61 (5'TC TCGATGCTGCCCCACGTACATCTC3'), complementary to nucleotides +61 to +85 of the *TOP2* coding sequence. Total PCR products were analyzed on 2% agarose gels in 45 mM Tris (pH 8.3)–45 mM boric acid–1.25 mM EDTA. The PCR products were cloned and sequenced to determine the minixen splice site junctions.

The 5' splice sites of transcripts from plasmids pdN-1Δ6.4, -Δ3, -Δ7, and -Δ10 were determined by primer extension analysis as described previously (33), using 5'-³²P-labeled oligonucleotide 66 (5'GGGCGCGGTGTTACCCGCAAT3'; -260 to -280) as the primer. Transcripts from plasmids pdN-1Δ6.1, -Δ8, and -Δ9 were determined in the same way, using oligonucleotide 61 as the primer. The same primers were used to generate DNA sequencing ladders from the corresponding plasmid templates. Primer extension products and sequencing reactions were separated by electrophoresis through 6% polyacrylamide-urea sequencing gels, which were then dried and autoradiographed. The positions of splice sites were determined from comparison with the sequencing ladders and

by subtracting 35 nucleotides to correct for primer extension through 35 nucleotides of the 39-nucleotide minixen sequence. Comparison of PCR and primer extension mapping of splice acceptor sites (not shown) indicated that the reverse transcriptase does not efficiently copy the four modified nucleotides at the 5' end of the minixen, consistent with the documented pausing or termination of reverse transcriptase at or before modified bases (13).

Construction of deletion plasmids of pdN-1. The 5' flanking genomic sequence upstream of the *TOP2* gene in pt2-1 had been deleted to various extents previously for DNA sequence analysis (25). The pdN-1 plasmids were constructed by replacing a 1.4-kb *SacII* fragment within the coding region of the *TOP2* gene of each plasmid with the NEO cassette of pX.2-KO (24) as a *SacII* fragment to generate the deletion plasmids pdN-1Δ5.1, pdN-1Δ6.4, pdN-1Δ3, pdN-1Δ5, pdN-1Δ6, pdN-1Δ6.1, and pdN-1Δ6.3. This set of deletion plasmids was used in the construction of a second set of plasmids (pdN-1Δ7, -Δ8, -Δ9, and -Δ10) having internal deletions in the *TOP2* 5' UTR. Plasmid pdN-1Δ7 was constructed by cleavage of pdN-1 with *NarI*, filling in of the ends with Klenow fragment, digestion with *BstXI*, and ligation of the 3,293-bp *NarI/BstXI* fragment to a 7,495-bp *EcoRV/BstXI* fragment of pdN-1. Plasmid pdN-1Δ8 was constructed by ligation of three fragments: a 1,395-bp *EcoRI* (end-filled)/*KpnI* fragment of pdN-1Δ6.1, a 5,904-bp *KpnI/BstXI* fragment of pdN-1, and the 3,516-bp *BstXI/EcoRV* fragment of pdN-1. Plasmid pdN-1Δ8.1 is identical to pdN-1Δ8 except that the central hexamer ATAGAA present in the consensus octamer sequence CATAGAAG was mutated to GCGAGG by the PCR overlap extension technique (11). The base substitutions in pdN-1Δ8.1 were confirmed by DNA sequence analysis. The pair of overlapping mutagenic oligonucleotides used in constructing pdN-1Δ8.1 were oligonucleotides C45 (5'TGGAGTGTTCCTTCGCGGCGTATAAGTA3') and C26 (5'TACTTATACGCCGCGAGGGCAAACACTCCA3'). Outside primers used in this construction were the T7 promoter primer (5'TAATACGACTCACTATAGGG3') within pGEM7 and oligonucleotide A12 (5'CGCAAGCACGCGCTGGCGCA3') within the *Leishmania major* 5' UTR. An internal *KpnI* fragment within the final full-length PCR product was cloned into pdN-1Δ8 to replace the corresponding *KpnI* fragment, yielding pdN-1Δ8.1. The base substitutions in pdN-1Δ8.1 were confirmed by DNA sequence analysis. Plasmid pdN-1Δ9 was constructed by ligation of three fragments: the 1,395-bp *EcoRI* (end-filled)/*KpnI* fragment of pdN-1Δ6.1, the 5,904-bp *KpnI/BstXI* fragment of pdN-1, and the 3,293-bp *NarI* (end-filled)/*BstXI* fragment of pdN-1. Plasmid pdN-1Δ10 was constructed by ligation of three fragments: a 1,250-bp *EcoRI* (end-filled)/*KpnI* fragment of pdN-1Δ6.3, the 5,904-bp *KpnI/BstXI* fragment of pdN-1, and the 3,293-bp *NarI* (end-filled)/*BstXI* fragment of pdN-1. Endpoints of each construct are shown in Fig. 2 and were confirmed by DNA sequence analysis.

Construction of chimeric 5'*TOP2*/CaBP plasmids. The N-terminal *TOP2* sequence in plasmids pdN-1Δ7 and pdN-1Δ10 was precisely replaced with 189 bp of the N-terminal coding sequence of the *C. fasciculata* CaBP (calcium-binding protein) gene by the PCR overlap extension technique (11) to generate plasmids pΔ7C and pΔ10C. Oligonucleotides used in these constructions were B59 (5'ATCGCCAAAAAATGGGGTGCATTCTTCCAAAGTCTACTCAG3'), which contains -15 to -1 of the 5' untranslated region of *TOP2* and +1 to +30 of the CaBP gene, B60 (5'GATCCGCGGTTACTCCATGCTGAGCTTGCCG3'), which contains sequence complementary to +170 to +189 of the CaBP gene and supplies a termination codon and a *SacII* site downstream of the termination codon, and B61 (5'AGAAATGCACCCCATTTTTTTTGGCGATATGTGTCTGTTGGGG3'), which contains sequence complementary to +1 to +15 of CaBP and -1 to -30 of the 5' UTR of *TOP2*, and the T7 promoter primer (5'TAATACGACTCACTATAGGG3'), which primes extension from the T7 promoter in the pGEM-7Zf(+) vector sequence. 5'Δ7*TOP2*/CaBP and 5'Δ10*TOP2*/CaBP PCR products were digested with *AatII* and *SacII* and subcloned into a pGEM-7Zf(+) vector containing the NEO cassette to generate pΔ7C and pΔ10C, respectively. The *TOP2* 5' flanking sequences in both constructs were sequenced to confirm that no changes were introduced by PCR amplification. Plasmid pΔ10Not was derived from pΔ10C by PCR overlap extension mutagenesis (11) to introduce a *NotI* site in place of an *EcoRI* site at the deletion junction in pΔ10C. The mutagenic oligonucleotides were N1 (5'AACGCTCAGAGCGCGCGCTTCGAGTTGC3') and B76 (5'GCAACTCGAAGCGGCCGCTCTGAGCGTT3'), and the outside primers were the T7 promoter primer and oligonucleotide A12. The resulting full-length PCR product was digested with *HindIII* and *SacII* and cloned into *HindIII*- and *SacII*-digested pΔ10C to yield pΔ10Not. Plasmids were introduced into *C. fasciculata* by electroporation as described previously (24).

Various sequences between -387 and -164 of the *TOP2* 5' UTR were cloned into plasmid pΔ10Not by PCR amplification of specific regions, using PCR primers containing *NotI* or *EagI* sites near the 5' end of each primer followed by digestion with *NotI* or *EagI*, ligation into *NotI*-digested pΔ10Not, and transformation into *Escherichia coli*. Oligonucleotides used in these constructions were B70 (5'ATAAGAATGCGGCGCATCGTAGAGCCCTACGCA3') and B73 (5'TATCGACTGCGGCGCGCTCGCTCTCTCTCTTTA3') for pRM4, B92 (5'AGCAGTGGCGGCGCGCATAGAAGTATTGCGGG3') and B93 (5'CGG GAGTCGGCCGATCTTCTATGGGCTTTTCGAC3') for pRM1 and pRM1R, B94 (5'TGTGCGGCGCGCTATTGCGGGTAACACGCC3') and B93 for pX04, B92 and B95 (GTGCTCGGCGGCTGGGCTTTTCGACACTCT3') for pX05; B94 and B95 for pX03, B92 and B71 (5'TATCAAGTCGGCGCGCTGTGTATGGCGCGCGTG3') for pX06, and B72 (5'ATAAGAATGCGGCGCG

ACCCATACACAAACACAG3') and B93 for pX07. Inserts in all plasmids were sequenced to confirm the cloned sequence prior to introduction into *C. fasciculata* by electroporation (24).

Western blot (immunoblot) analysis. Cell lysates were prepared from *C. fasciculata* strains and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described previously (24, 25). The primary antibody used to analyze Western filters was polyclonal rabbit antibody raised against mitochondrial type II topoisomerase (topoIImt) (22), and the secondary antibody used was goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase.

Isolation and analysis of RNA. Hydroxyurea synchronization of *C. fasciculata* cells and isolation of total RNA were performed as described previously (24, 25). The isolation of poly(A)⁺ RNA was performed with Dynabeads oligo(dT) Mag-nabeads as suggested by the manufacturer. mRNA was isolated from total RNA prepared from cells positioned at the 180-min time point of each synchronization for the strains analyzed [with the exception of *C. fasciculata*(pdN-1) and *C. fasciculata*(pdN-1Δ10), for which mRNA was isolated from total RNA prepared from cells at the 150- and 210-min time points, respectively]. Total RNA and poly(A)⁺ RNA (from 75 μg of total RNA) for each sample analyzed were precipitated, electrophoresed on a 2.2 M formaldehyde–1.2% agarose gel, and analyzed by Northern (RNA) blotting (25). The 5' *TOP2* probe used to analyze the Northern blots was a 2.1-kb *Hind*III-*Sac*II fragment of pdN-1 which spans nucleotides –883 to +1186 of the *TOP2* gene and was labeled by random priming (29). The *CaBP* probe was a partial cDNA of the putative *C. fasciculata* homolog of the *Trypanosoma cruzi* 1F8 protein (7). Early blots were analyzed by autoradiography, and later ones (Fig. 11) were analyzed and quantitated by using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Nucleotide sequence accession number. The accession number for the partial cDNA of the putative *C. fasciculata* *CaBP* gene in the GenBank database is U21305.

RESULTS

***TOP2* splice acceptor sites.** The periodic expression during the cell cycle of the 5' 40% of the *TOP2* mRNA from a disrupted form of the *TOP2* gene expressed from a plasmid (pdN-1) suggests that 5' flanking sequences may be important in the cell cycle regulation of the mRNA level (24). We have therefore performed systematic deletions of the *TOP2* 5' flanking sequence in plasmid pdN-1 in order to identify sequences required for the periodic accumulation of *TOP2* mRNA. However, since trypanosomatid genes often have multiple alternative splice acceptor sites for *trans* splicing of the minixon onto the precursor RNA to generate the 5' end of the mature mRNA, the extent of the 5' UTR sequence depends on which of the possible splice acceptor sites is utilized. Deletions that remove a major splice acceptor site can force the utilization of an alternative site and thereby affect the length and sequence of the 5' UTR. To interpret the effect of specific deletions of the *TOP2* 5' flanking sequence, it was therefore necessary to first precisely map the *TOP2* alternative splice sites.

We initially examined the sites of *trans* splicing of transcripts of the endogenous *TOP2* gene. Total RNA was used to map the 5' termini of *TOP2* mRNA by primer extension using a primer complementary to a sequence within the *TOP2* coding region. The primer extension products were amplified by PCR using an additional primer homologous to nucleotides 5 to 29 of the *C. fasciculata* minixon sequence. The major products resulting from the PCR amplification (Fig. 1) were cloned and sequenced. These results allowed precise mapping of splice acceptor sites at –668 (AG_I), –89 (AG_{II}), –75 (AG_{III}), and –65 (AG_{IV}) relative to the initiation codon (Fig. 2A). (The PCR products corresponding to splicing at AG_{III} and AG_{IV} apparently comigrated on the gel in Fig. 1.) AG_{II}, AG_{III}, and AG_{IV} appear to be minor splice acceptor sites and the major splice acceptor site appears to be AG_I, since a single abundant *TOP2* mRNA migrating at 4.8 kb is observed on Northern blots of poly(A)⁺ RNA when hybridized with either a probe spanning the *TOP2* gene (25) or a probe spanning –883 to –235 of the 5' flanking sequence (data not shown). Analysis of the 5' flanking *TOP2* sequence reveals the presence of two polypyrimidine-rich tracts upstream of AG_I, designated PyI and PyII

FIG. 1. Determination of splice acceptor sites of the *TOP2* gene by PCR. The splice acceptor sites within the 5' flanking sequence of the *TOP2* gene were determined by performing PCR with a 5' oligonucleotide identical to residues 5 through 29 of the *C. fasciculata* minixon and a 3' oligonucleotide complementary to nucleotides +61 to +85 of the *TOP2* coding region. The template for the reaction was cDNA derived by primer extension of total RNA with oligonucleotide 63, complementary to nucleotides +240 to +218 of the *TOP2* coding region. PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide and shown in lane 1. Lane 2 contains 500 ng of 1-kb molecular weight ladder from Bethesda Research Laboratories.

(Fig. 2A), which may play a role in the *trans* splicing of the *TOP2* transcript.

Unidirectional deletions of *TOP2* 5' flanking sequences affect *trans* splicing and cell cycle regulation. We have carried out a deletion analysis of *TOP2* 5' flanking sequences in pdN-1 to investigate the possible role of 5' flanking sequence in modulating the level of the mRNA during the cell cycle. The *TOP2* gene in plasmid pdN-1 (Fig. 2B) is disrupted by the insertion of a 3.3-kb NEO cassette in place of 1.4 kb of *TOP2* coding sequence (24). The NEO cassette contains the neomycin phosphotransferase (*NPT*) gene and 5' and 3' flanking sequences from the *L. major* *DHFR-TS* gene and confers resistance to the drug G418 (18). *C. fasciculata* cells transformed with plasmid pdN-1 express p45, a truncated amino-terminal fragment of topoIImt, and show periodic accumulation of a truncated *TOP2* transcript (2.0 kb) in synchronized cells in parallel with the periodic accumulation of the 4.8-kb endogenous *TOP2* transcript (24). Both the size of the p45 polypeptide and that of the 2.0-kb mRNA are consistent with those predicted from the nucleotide sequence of the pdN-1 disruption construct. A stop codon within the 5' *DHFR-TS* flanking sequence of the NEO cassette would generate a protein of the observed size, and the utilization of the polyadenylation site within the *L. major* 5' *DHFR-TS* sequence of the NEO cassette (15, 18) would yield an mRNA of 2.0 kb for a transcript spliced at AG_I and about 1.4 kb for transcripts spliced at AG_{II}, AG_{III}, or AG_{IV}. A minor *TOP2* transcript of 1.35 to 1.4 kb is observed in addition to the 2.0-kb transcript in *C. fasciculata*(pdN-1) cells (reference 24 and this work).

To demonstrate the functionality of the truncated *TOP2* mRNAs, a series of plasmids (Fig. 2C) containing deletions upstream of the *TOP2* gene were introduced into *C. fasciculata* cells by electroporation, and G418-resistant clones were analyzed by Western blotting. Figure 3A shows the results of Western blot analysis of cell lysates prepared from *C. fasciculata* strains carrying pdN-1 or the deletion plasmids. All plasmids except pdN-1Δ6.3 induced expression of the p45 protein.

FIG. 2. (A) Organization of the 5' flanking sequence of the *C. fasciculata* *TOP2* gene. Nucleotides -883 to +17 of the *TOP2* gene are shown, with the initiation codon ATG underlined (A residue of the initiation codon is +1). Splice acceptor sites mapped by PCR and primer extension are indicated by the circled AG and labeled AG_I, AG_{II}, AG_{III}, AG_{IV}, and AG_V. The 3' deletion endpoints of nested deletions of pN-1 are designated by arrows and labeled Δn, where n refers to the construct name pN-1Δn. The nucleotides which are boxed identify polypyrimidine-rich sequences and are labeled PyI or PyII. Octamer sequence elements are indicated by overlines. The horizontal arrow underlines the sequence complementary to oligonucleotide 66. (B) Circular map of pN-1 (not to scale). *TOP2* 5' seq. is -883 to -1 (relative to the coding sequence) of the *TOP2* 5' flanking sequence; *TOP2* 3' seq. is +1 to +1186 of the *TOP2* coding sequence; *C-term TOP2* is +2652 to +3717 of the *TOP2* coding sequence; *Lm DHFR* 5' seq. and *Lm DHFR* 3' seq. are the 5' and 3' flanking sequences of the *L. major DHFR-TS* gene contained in plasmid pX.2-KO (24); *NEO* is the coding sequence of the *NTP* gene from pX.2-KO; *TOP2* 3' seq. is 1.5 kb of *TOP2* 3' flanking sequence; pGEM7 is plasmid pGEM-7Zf(+) cleaved at the *HindIII* site. (C) Map (not to scale) of deletions of plasmid pN-1. The splice acceptor sites AG_I through AG_V are indicated with labeled vertical arrows within the 5' flanking *TOP2* sequence. The three horizontal arrows above the schematic indicate the three different transcripts generated by usage of the AG_I, AG_V, or AG_{II-IV} cluster splice acceptor sites. Below the schematic, the 3' endpoint of the

The lack of expression of p45 in cells carrying pN-1Δ6.3 is apparently the result of deleting all known splice acceptor sites upstream of the initiation ATG. Consequently, this strain was not analyzed further.

To examine the effects of these upstream deletions on the cyclic accumulation of the plasmid-derived *TOP2* transcript, *C. fasciculata* strains carrying the deletion plasmids were synchronized with hydroxyurea and analyzed by Northern blotting. Total RNA was isolated at 30-min intervals from the synchronized cells after release from hydroxyurea arrest [at 45-min intervals for *C. fasciculata*(pN-1Δ5.1)] and subjected to Northern blot analysis. As shown in Fig. 4, several transcripts were detected when these blots were hybridized with the 5' *TOP2* probe, which spans -883 to +1186 of the *TOP2* gene. The 5' *TOP2* probe detected the 4.8-kb *TOP2* transcript encoded by the endogenous chromosomal *TOP2* gene in strains carrying pN-1 or the deletion plasmids. As observed previously (24), the steady-state level of the endogenous *TOP2* transcript exhibited periodicity with peaks occurring at 30, 180, and approximately 360 min after release from hydroxyurea arrest. The fluctuation of the transcript level was not due to differences in the amount of RNA in each lane, as demonstrated by the panel labeled pN-1/*CaBP* in Fig. 4. The blot labeled pN-1/5' *TOP2* was stripped and hybridized with a probe for the *C. fasciculata* *CaBP* gene, which has a constant transcript level throughout the cell cycle. Similar results were obtained after each of the Northern blots analyzing RNA from strains carrying the deletion plasmids was stripped and hybridized with the *CaBP* probe (data not shown).

The 5' *TOP2* probe also detected three transcripts smaller than the endogenous 4.8-kb transcript in strains carrying pN-1 or the deletion plasmids. Transcripts larger than the 4.8-kb endogenous transcript are not observed when the disrupted form of *TOP2* in pN-1 is present on the chromosome (data not shown) and apparently result from polyadenylation of plasmid transcripts at sites further downstream within plasmid sequences and consequently have not been investigated further. Cells carrying pN-1Δ5.1 or pN-1Δ6.4 show periodic accumulation of the 2.0-kb transcript similar to that observed in cells carrying pN-1. Since the 3' deletion endpoint of pN-1Δ6.4 lies between the pyrimidine-rich sequences PyI and PyII, PyII is not required for splicing at the AG_I splice acceptor site or for periodic accumulation of the 2.0-kb transcript. Deletion past PyI to six nucleotides upstream of AG_I in pN-1Δ3 or deletion past AG_I to -603 in pN-1Δ5 unmasked a cryptic splice acceptor site AG_V and resulted in the appearance of a smaller 1.85-kb transcript in cells carrying pN-1Δ3 or pN-1Δ5. The 3' deletion endpoints of pN-1Δ6.4 and pN-1Δ3 define a sequence of 43 nucleotides spanning PyI that is required for splicing at the AG_I splice acceptor site. Although AG_I is present on pN-1Δ3, it is no longer a functional splice acceptor site and the cryptic splice acceptor site AG_V is utilized. The 3' deletion endpoint of pN-1Δ5 extends 65 nucleotides past AG_I, deleting this splice acceptor site and resulting in splicing at the cryptic splice acceptor site (AG_V) to produce a 1.85-kb transcript. This cryptic splice acceptor site was mapped to -548 relative to the initiation codon by PCR amplification of cDNA, using oligonucleotides homologous to coding sequence and to the minixon followed by cloning and

nested deletions and the 5' and 3' boundaries of the internal deletions of the pN-1 deletion plasmids are indicated and labeled to the right with the deletion plasmid name and the major splice site utilized by each construct. +, transcripts that cycle. -, those that do not cycle.

FIG. 3. Western blot analysis of wild-type and transformed *C. fasciculata* strains carrying pdN-1 deletion plasmids. Cell lysates were prepared from wild-type or transformed *C. fasciculata* cells and analyzed by Western blotting. The strains examined are labeled above the lanes, and positions of the molecular weight markers are indicated to the left. The blots were probed with rabbit antibody raised against topoIImt. Cell lysates analyzed were from wild-type *C. fasciculata* cells (Cf) or cells transformed with pdN-1 or pdN-1 nested deletion plasmids (A) and from wild-type *C. fasciculata* cells or cells transformed with pdN-1 or pdN-1 internal deletions plasmids (B).

sequence analysis of the PCR products (data not shown). Utilization of AG_V by pdN-1Δ3 also was confirmed by primer extension analysis (Fig. 5). One of the primer extension products observed in this case corresponds to splicing at AG_V. A second primer extension product five nucleotides shorter is unexplained. The periodic accumulation of the 1.85-kb transcript resulting from splicing at AG_V indicates that splicing at the major splice acceptor AG_I is not specifically required for the periodic accumulation of the mRNA.

Further deletion of the upstream sequence results in the stable expression of a transcript of approximately 1.35 kb in cells carrying pdN-1Δ6 or pdN-1Δ6.1. Primer extension analysis of poly(A)⁺ RNA from cells carrying pdN-1Δ6.1 indicates that this transcript is generated by splicing of the minixon at splice acceptor sites AG_{II} and AG_{III} (Fig. 5). Comparison of the expression pattern of transcripts from this family of plasmids in the synchronized cells shows that the levels of the 2.0- and 1.85-kb transcripts, derived by utilizing AG_I or AG_V, fluctuate as cells progress through the cell cycle in a pattern mimicking the cycling of the endogenous *TOP2* transcript, but the level of the 1.35-kb transcript, derived by utilizing AG_{II} or AG_{III}, does not vary significantly during the cell cycle.

While cells harboring plasmid pdN-1Δ6 or pdN-1Δ6.1 express the 1.35-kb transcript at a constant steady-state level throughout the cell cycle, strains carrying pdN-1 or the deletion plasmid pdN-1Δ5.1, pdN-1Δ6.4, pdN-1Δ3, or pdN-1Δ5 do not express the 1.35-kb transcript. To resolve these different transcripts without distortions caused by the abundant rRNAs, strains carrying pdN-1 or the other deletion plasmids were analyzed by Northern blotting of polyadenylated mRNAs (Fig. 6). The polyadenylated mRNAs show the 4.8-kb endogenous transcript and the smaller plasmid-derived transcripts encoding the p45 polypeptide. Although the 1.35-kb transcript is readily detected in mRNA from cells carrying pdN-1Δ6 and pdN-1Δ6.1, it is absent in mRNA from cells carrying pdN-1, pdN-1Δ5.1, pdN-1Δ6.4, pdN-1Δ3, or pdN-1Δ5. As deletions of the 5' flanking *TOP2* sequence extend further into the flanking sequence, the splice acceptor site utilized switches from AG_I to AG_V and finally to AG_{II}, AG_{III}, or AG_{IV}. Consequently, the major plasmid-derived transcript encoding the p45 polypeptide

is detected as a 2.0-kb transcript when AG_I is utilized, a 1.85-kb transcript when the cryptic splice acceptor site AG_V is utilized, and finally as a 1.35-kb transcript when AG_{II}, AG_{III}, or AG_{IV} is utilized (Fig. 2C).

Block deletions of *TOP2* 5' flanking sequences. Analysis of the results of the synchronization experiments with *C. fasciculata* strains harboring the nested deletion plasmids derived from pdN-1 suggests that an element downstream of the 3' deletion endpoint of pdN-1Δ5 at position -604 of the *TOP2* gene is required for the periodic fluctuation of the truncated *TOP2* transcript in synchronized cells. To further delineate this element, plasmids derived from pdN-1 and containing internal deletions within the 5' flanking sequence of the *TOP2* gene were generated. Plasmids pdN-1Δ7 (deletion of -608 to -388), pdN-1Δ8 (deletion of -387 to -186), pdN-1Δ9 (deletion of -608 to -186), and pdN-1Δ10 (deletion of -608 to -41) were constructed (Fig. 2C) and introduced into *C. fasciculata* cells by electroporation. Cell lysates were prepared from strains harboring these internal deletion plasmids and analyzed by SDS-PAGE and Western blotting to determine whether the strains express the p45 polypeptide. As shown in Fig. 3B, *C. fasciculata* strains carrying the internal deletion plasmids pdN-1Δ7, pdN-1Δ8, pdN-1Δ9, and pdN-1Δ10 all express the p45 protein.

To examine the effects of these block deletions on the expression of the transcript encoding the p45 protein, total RNA was isolated from synchronized cells at the time of release and at 30-min intervals after release from hydroxyurea arrest. The results of the Northern blot analysis of these samples are shown in Fig. 7. As seen previously, the steady-state level of the 4.8-kb endogenous *TOP2* transcript fluctuates as cells progress through the cell cycle. (The RNA samples loaded in lane 8 of the pdN-1Δ8 blot and lane 13 of the pdN-1Δ10 blot were partially degraded.) Deletion of 5' flanking *TOP2* sequence resulted in the loss of the cryptic splice acceptor site, AG_V, in plasmids pdN-1Δ7, pdN-1Δ9, and pdN-1Δ10. The AG_{II-IV} splice acceptor site cluster was retained in plasmids pdN-1Δ7, pdN-1Δ8, and pdN-1Δ9. The major splice acceptor site utilized for each internal deletion plasmid appears to be splice acceptor site AG_I, since utilization of the AG_{II-IV} splice acceptor site

FIG. 4. Northern blot analysis of total RNA isolated from synchronized *C. fasciculata* strains carrying the pdN-1 nested deletion plasmids. Total RNA was isolated from synchronized cells carrying pdN-1 or a pdN-1 nested deletion plasmid (pdN-1Δ6.4, pdN-1Δ3, pdN-1Δ5, pdN-1Δ6, or pdN-1Δ6.1) at the time of release (lanes 0) or at 30-min intervals after release from hydroxyurea arrest. The time of isolation of each RNA sample is indicated above alternate lanes. For cells carrying pdN-1Δ5.1, RNA was isolated at 45-min intervals, and the timing of these RNA samples is indicated in parentheses. The RNAs were subjected to Northern blot analysis and hybridized with the 5' *TOP2* probe or with a 260-bp probe derived from a partial cDNA encoding the *C. fasciculata* homolog to the *T. cruzi* flagellar calcium-binding protein. Each blot is labeled to the left or right, with the plasmid construct carried by the strain above the bar and the probe utilized below the bar. The transcripts detected and their sizes are indicated by the bars to the left or right.

cluster would have resulted in truncated transcripts of the same size in strains carrying pdN-1Δ7, pdN-1Δ8, or pdN-1Δ9. However, the transcript encoding p45 was detected as different sizes in the strains due to the deletion of 5' flanking sequence of the *TOP2* gene, consistent with utilization of the splice acceptor site AG₁. The truncated transcripts detected by the 5' *TOP2* probe were 1.8 kb (pdN-1Δ7 or pdN-1Δ8, deletion of 221 or 202 bp, respectively), 1.6 kb (pdN-1Δ9, deletion of 423 bp), and 1.4 kb (pdN-1Δ10, deletion of 568 bp). Each of these

truncated RNAs was shown to be polyadenylated by Northern blot analysis of poly(A)-selected RNAs as in Fig. 6 (data not shown). Primer extension analysis of each of these transcripts confirmed the utilization of the AG₁ splice acceptor site (data not shown). Results for the pdN-1Δ7 transcript are shown in Fig. 5.

The 1.8-kb plasmid-derived truncated transcripts detected in strains carrying either pdN-1Δ7 or pdN-1Δ8 fluctuate in parallel with the endogenous *TOP2* transcript as cells progress

FIG. 5. Mapping of 5' ends of *TOP2* transcripts from plasmids pdN-1Δ3, pdN-1Δ6.1, and pdN-1Δ7. Oligonucleotide 66, with its 5' end at -260, was used for primer extension of RNAs from cells transformed with pdN-1Δ3 and pdN-1Δ7. Oligonucleotide 61, with its 5' end at +85, was used for primer extension of RNA from cells transformed with pdN-1Δ6.1. The 32 P-labeled 5'-end mapping products were separated by electrophoresis through a polyacrylamide-urea sequencing gel along with sequencing reactions using oligonucleotide 66 to prime synthesis on pdN-1 DNA (for pdN-1Δ3 and pdN-1Δ7) and oligonucleotide 61 to prime synthesis on pdN-1Δ6.1 DNA. Splice sites identified for these and other plasmid transcripts are shown in Fig. 2A.

through the cell cycle. The sequences deleted in pdN-1Δ7 and pdN-1Δ8 are essentially reciprocal deletions, since the basic difference between pdN-1Δ7 and pdN-1Δ8 is that each plasmid retains the sequence which has been deleted in the other. Interestingly, it is the cumulative loss of both sequences deleted in pdN-1Δ7 and pdN-1Δ8 that results in the perturbation of the cell cycle regulation. As shown in Fig. 7, the truncated transcripts generated in strains carrying pdN-1Δ9 or pdN-1Δ10 do not fluctuate periodically as cells progress through the cell cycle. In contrast to what had been observed with strains carrying pdN-1Δ7 or pdN-1Δ8, the level of the 1.6-kb pdN-1Δ9 truncated transcript appears to remain constant over the first 240 min and then decreases. The simplest interpretation of this result is that there are two elements residing within -608 to -186 of the *TOP2* 5' flanking sequence which singly provide the signal to allow periodic expression of the transcript carrying the element, but the deletion of both elements releases the transcript from cell cycle regulation. pdN-1Δ10 is identical to pdN-1Δ9 except that the 3' deletion endpoint extends further downstream to -41, resulting in the loss of the splice acceptor site cluster AG_{II}, AG_{III}, and AG_{IV} and the generation of a 1.4-kb transcript defined by the single available splice acceptor site AG_I. Since the same elements required for periodicity deleted in pdN-1Δ9 also have been deleted in pdN-1Δ10, the level of the 1.4-kb pdN-1Δ10 truncated transcript also does not fluctuate periodically in synchronized cells.

The 5' UTR of *TOP2* confers periodic expression on a reporter gene. To determine whether the 5' flanking sequence of the *TOP2* gene is sufficient to confer cell cycle-regulated expression on a heterologous gene, plasmids pΔ7C and pΔ10C

(Fig. 8) were constructed. These plasmid DNAs are identical to the parental plasmids pdN-1Δ7 and pdN-1Δ10 except for the deletion of both the C-terminal third of the *TOP2* gene and the 3' flanking *TOP2* sequence and the precise replacement of the amino-terminal third of the *TOP2* gene (the p45 coding region) with the partial cDNA of the *C. fasciculata* *CaBP* gene. The *CaBP* gene was selected as the heterologous reporter gene since the steady-state expression of the endogenous *CaBP* transcript does not vary during the cell cycle in synchronized wild-type or transformed *C. fasciculata* cells (24). The only *C. fasciculata* sequences present in pΔ7C and pΔ10C outside of the *CaBP* gene sequence are derived from the 5' flanking *TOP2* sequence.

C. fasciculata(pΔ7C) and *C. fasciculata*(pΔ10C) were synchronized with hydroxyurea, and total RNAs were isolated from the synchronized cells. The results of the Northern blot analysis of total RNAs isolated from these strains carrying 5' *TOP2*/*CaBP* chimeric genes are shown in Fig. 9. Analysis of Northern blots hybridized with a *TOP2*-specific probe (Figs. 9A and B) reveals that *C. fasciculata*(pΔ7C) cells express 3.1- and 0.8-kb transcripts periodically in parallel with the endogenous *TOP2* transcript (Fig. 9A), whereas *C. fasciculata*(pΔ10C) cells express a 0.5-kb transcript at a constant level throughout the cell cycle (Fig. 9B). When these Northern blots were analyzed for *CaBP* expression (Fig. 9C and D), hybridization of the 0.8-kb transcript in *C. fasciculata*(pΔ7C) (Fig. 9C) and the 0.5-kb transcript in *C. fasciculata*(pΔ10C) (Fig. 9D) with the *CaBP* probe identified these transcripts as the chimeric Δ7*TOP2*/*CaBP* and Δ10*TOP2*/*CaBP* transcripts, re-

FIG. 6. Northern blot analysis of polyadenylated RNAs isolated from *C. fasciculata* strains carrying the pdN-1 nested deletion plasmids. Polyadenylated RNA was purified from 75 μ g of total RNA isolated from synchronized cells. The lanes are labeled above the blot to indicate the *C. fasciculata* (Cf) strain analyzed. The total RNA used for mRNA purification was isolated from cells at 150 to 210 min after hydroxyurea release. The mRNAs were subjected to Northern blot analysis and hybridized with the 5' *TOP2* probe. The transcripts detected and their sizes are indicated by the bars to the right.

FIG. 7. Northern blot analysis of total RNA isolated from synchronized *C. fasciculata* strains carrying the pdN-1 internal deletion plasmids. Total RNA was isolated from synchronized cells carrying the deletion plasmid pdN-1Δ7, pdN-1Δ8, pdN-1Δ9, or pdN-1Δ10 at 30-min intervals after release from hydroxyurea arrest. The RNAs were subjected to Northern blot analysis and hybridized with the 5' *TOP2* probe. The blots are labeled as in Fig. 4.

spectively. As observed in previous synchronization experiments, the endogenous *CaBP* transcript is expressed at a constant level as cells progress through the cell cycle. Upon longer exposure, the 3.1-kb transcript in *C. fasciculata*(pΔ7C) is detected with the *CaBP* probe. The 3.1-kb transcript present in *C. fasciculata*(pΔ7C) was also found to hybridize with a probe specific for the neomycin *NPT* transcript and appears to represent a polycistronic transcript in which the downstream *NPT* transcript had not been *trans* spliced resulting in the fusion of the upstream chimeric *CaBP* transcript with the *NPT* transcript (data not shown). The apparent difference in the expression levels of the 0.8- and 0.5-kb chimeric transcripts detected with the *TOP2* probe likely results from the fact that the Δ7*TOP2*/*CaBP* transcript contains 447 bases of identity with the *TOP2* probe whereas the Δ10*TOP2*/*CaBP* transcript contains only 106 bases of identity with the same *TOP2* probe. Similar differences in hybridization intensity are not observed with the *CaBP* probe since the chimeric transcripts carry the same *CaBP* sequences. These results demonstrate that the 5' flanking sequence of the *TOP2* gene present in pΔ7C (−883 to −609 and −387 to −1 of the *TOP2* 5' flanking sequence) is sufficient to confer periodic expression to a heterologous transcript and that an element present within −387 to −186 of the 5' UTR of the *TOP2* gene is required for periodic expression.

An octamer consensus sequence is required for normal cycling of the mRNA level. Plasmid pΔ10C was modified for use in further identifying sequences necessary for cycling of mRNA levels. A *NotI* site was introduced at the deletion junction in pΔ10C to create plasmid pΔ10Not (Fig. 8). Various sequences between −164 and −387 of the *TOP2* 5' UTR were amplified by PCR with primers containing *NotI* or *EagI* sites at their 5' ends and subsequently cloned into pΔ10Not. Northern

blot analysis of whole cell RNAs from hydroxyurea-synchronized cells was performed, and the RNA levels were quantitated by phosphor image analysis and normalized to the level of the *CaBP* transcript in each lane. The plasmid constructs used in these experiments are shown in Fig. 10A, and the results of the quantitated Northern blots are presented in Fig. 11.

For the endogenous *TOP2* and for plasmid pdN-1, a minimum level of the *TOP2* mRNA occurs at 90 to 120 min after release from the hydroxyurea block and a maximum level occurs at 180 to 210 min after release (24). In both cases, the ratio of the mRNA maximum to minimum levels (defined as

FIG. 8. Deletions in the *TOP2* flanking sequence in the plasmids pΔ7C and pΔ10C illustrated as in Fig. 2C. *CaBP* is 189 bp of the amino-terminal coding sequence of the *C. fasciculata* *CaBP* gene. The 5' and 3' boundaries of the deletions within the *TOP2* 5' flanking sequence are indicated. Plasmid pΔ10Not was derived from pΔ10C by insertion of a *NotI* restriction site at the junction of the deletion in pΔ10C. Other designations are as in Fig. 2B.

FIG. 9. Northern blot analysis of total RNA isolated from synchronized *C. fasciculata* strains carrying the chimeric plasmid pΔ7C or pΔ10C. Total RNA was isolated from synchronized cells carrying plasmid pΔ7C (A and C) or pΔ10C (B and D) at 30-min intervals after release from hydroxyurea arrest. The RNAs were subjected to Northern blot analysis and hybridized with the 5' *TOP2* probe (A and B) or with the *CaBP* probe (C and D). The endogenous *TOP2* and *CaBP* transcripts are labeled. The chimeric 3.1-kb 5'Δ7*TOP2*/*CaBP*/NPT, 0.8-kb 5'Δ7*TOP2*/*CaBP*, and 0.5-kb 5'Δ10*TOP2*/*CaBP* transcripts are indicated by arrows.

the cycling ratio) ranges from seven to ninefold. Insertion of the entire region from -387 to -164 into pΔ10Not to give pRM4 restored a level of cycling of the chimeric RNA to that of the endogenous *TOP2* mRNA (Fig. 10 and 11). Plasmid pRM1, which contains only 83 bp of the functional sequence, was also found to confer cycling at a near-normal level. Insertion of the 83-bp sequence in the opposite orientation in pRM1R resulted in a reduction of the cycling ratio to 2.0, a shift of the minimum mRNA level to 120 to 150 min, and a nearly constant level of the mRNA from 180 min onward.

Examination of this 83-bp insert reveals two interesting features: two copies of an octamer and two overlapping direct repeats (Fig. 10). The octamer CATAGAAG is a perfect match to a consensus octamer sequence found in the 5' UTRs of the *C. fasciculata* *RPA1* and *DHFR-TS* genes as well as in *TOP2* (Table 1). All three genes show similar periodic accumulation of their mRNAs during the cell cycle (24). In contrast, the *CaBP* mRNA level does not vary during the cell cycle and the *CaBP* 5' UTR does not contain the consensus octamer. Plasmid pdN-1Δ8, which lacks the entire region from -164 to -387 , shows cyclic accumulation of the *TOP2* mRNA (Fig. 7) similar to that of the endogenous *TOP2* mRNA and contains only a single copy of the consensus octamer. However, the two overlapping direct repeat sequences present in the 83-bp sequence are not present in the *TOP2* 5' UTR of pdN-1Δ8, indicating that these repeat sequences are not required for mRNA cycling. Deletion of either of the two octamer sequences in the 83-bp sequence (plasmids pXO4 and pXO5 in Fig. 10A) resulted in reduced levels of cycling of the mRNA, and deletion of both of the octamers (plasmid pXO3) essentially abolished cycling of the mRNA (Fig. 10A and 11). Similarly, insertion of the two halves of the 83-bp sequence into pΔ10Not (plasmids pXO6 and pXO7) did not rescue the cycling defect even though each plasmid, like pdN-1Δ8, contained a single copy of the octamer sequence.

To further investigate the requirement for the consensus octamer sequence for periodic accumulation of *TOP2* mRNA during the cell cycle, the single consensus octamer sequence in the *TOP2* 5' UTR of pdN-1Δ8 was mutated. The central hexamer within the consensus sequence was mutated from ATA GAA to GCGAGG (plasmid pdN-1Δ8.1). Northern blot analysis of mRNA expressed from the plasmid-encoded, truncated *TOP2* gene during the cell cycle showed substantial reduction of the cyclic accumulation of the mRNA in cells carrying pdN-

FIG. 10. (A) Map of fragments inserted into plasmid pΔ10Not for analysis of cell cycle regulation shown in Fig. 11. Cycling ratio is the ratio of the maximum reporter RNA level at 180 to 210 min to the minimum level at 90 to 120 min. Open boxes and filled boxes represent the sequences in panel B indicated by boxes and by thick horizontal arrows, respectively. (B) Sequence of the insert in plasmid pRM1. Arrows denote direct repeat sequences.

FIG. 11. Northern blot analysis of total RNA isolated from hydroxyurea-synchronized *C. fasciculata* strains carrying the chimeric plasmids diagrammed in Fig. 10A. Blots were probed with both the 5' *TOP2* probe and the *CaBP* probe. Levels of plasmid-expressed transcripts and of endogenous *CaBP* transcripts were quantitated with a PhosphorImager (Molecular Dynamics). Chimeric RNA levels have been normalized at each time point relative to that of the *CaBP* transcript, which is expressed at a constant level throughout the cell cycle. In some experiments, a zero time point sample was not taken.

1Δ8.1 (Fig. 12). Together, these results suggest that the consensus octamer sequence is necessary but not sufficient for cycling of the mRNA levels.

DISCUSSION

The periodic expression of plasmid-encoded forms of the *TOP2* gene in synchronous cultures has provided a means for identifying possible *cis* elements involved in regulating expression of *TOP2* and potentially other DNA replication genes as well (24). Deletion analysis of the *TOP2* 5' UTR in plasmid pDN-1 identified two regions of the 5' UTR sequence involved in conferring cell cycle regulation of the *TOP2* mRNA level. Deletion of either one of the two approximately 200-bp 5' UTRs had no effect on the periodic accumulation of the mRNA, whereas deletion of both regions resulted in greatly reduced cycling of the mRNA level. At least one of these sequences had to be contained in the mature mRNA for periodic accumulation of the mRNA. In mutant constructs in which the most distal splice acceptor sites (AG_I and AG_V) were deleted, forcing the utilization of the proximal splice acceptor sites (AG_{II} to AG_{IV}), the mRNA level did not cycle. This result indicates a requirement for the presence of an essential sequence element in the mature mRNA and not just in the flanking DNA sequence. Also, deletion of distal 5' sequence up to and including the major splice acceptor (AG_I)

TABLE 1. Conserved 5' UTR sequence elements

Gene ^a	Sequence	Location ^b
<i>TOP2</i>	CATAGAAG	-428 to -421
	CATAGAAG	-289 to -282
	CATAGAAG	-217 to -210
<i>RPA1</i>	CATAGAAA	-315 to -310
	CATAGAAA	-262 to -255
<i>DHFR-TS</i>	AATAGAAG	-397 to -390
Consensus	cATAGAAG	

^a Accession numbers for these genes are X59623, Z23163, and M22852 (*TOP2*, *RPA1*, and *DHFR-TS*, respectively).

^b Locations are based on coding sequence beginning at +1.

had no effect on cyclic accumulation of the mRNA. These observations are consistent with regulation entirely at a post-transcriptional level and involving sequences within the 5' UTR of the mRNA.

Sequences required for normal cycling of the *TOP2* gene were localized further by subcloning of the *TOP2* 5' UTR into a plasmid carrying a gene construct in which both of the 200-bp regions of the *TOP2* 5' UTR were deleted. In addition, *TOP2* coding sequence and 3' UTR sequence were replaced in this plasmid by a fragment of the *C. fasciculata* *CaBP* gene as a reporter. These experiments have shown that an octamer sequence is necessary but not sufficient for normal cycling of the mRNA. Comparison of the *TOP2* 5' UTR sequence with those of the *C. fasciculata* *RPA1* and *DHFR-TS* genes, which also show cyclic expression, revealed the presence of similar sequences in one or more copies in the 5' UTRs of these genes. A central hexamer is perfectly conserved in each sequence, and there is a 7-of-8 identity of each sequence with a consensus sequence of cATAGAAG (Table 1). Mutation of this hexamer sequence from ATAGAA to GCGAGG in plasmid pDN-1Δ8, which contains only a single copy of the consensus octamer in the deleted form of the *TOP2* 5' UTR, substantially reduced

FIG. 12. Northern blot analysis of total RNA isolated from hydroxyurea-synchronized *C. fasciculata* strains carrying plasmid pDN-1Δ8 or pDN-1Δ8.1. Blots were probed and quantitated as in Fig. 11. The 1.8-kb *TOP2* transcript levels were normalized at each time point relative to that of the *CaBP* transcript.

cycling of the *TOP2* transcript. In addition to this sequence element, additional features of the *TOP2* 5' UTR appear to be required for normal cycling of the mRNA. Plasmids pX06 and pX07 each contain a single copy of the consensus octamer, yet neither plasmid showed cyclic accumulation of the chimeric *TOP2* transcript. Further experiments will be required to identify additional features or sequences in the *TOP2* 5' UTR required for normal cycling of the mRNA.

Although it remains to be shown that these same elements function in the genomic context of the chromosomal *TOP2* genes, it is a reasonable extrapolation that the elements perform the same function for the endogenous *TOP2* transcript, since the 5' UTRs of the endogenous 4.8-kb and the truncated 2.0-kb transcripts are identical and their patterns of expression are also identical. This variation in *TOP2* mRNA levels during the cell cycle is reflected in a similar periodic synthesis of topolmt, the protein product of the *TOP2* gene (unpublished results). The observed cycling of the mRNAs of the *RPA1* and *DHFR-TS* genes (24) in synchronized *C. fasciculata* suggests that synthesis of the RPA protein, a nuclear replication protein, and dihydrofolate reductase-thymidylate synthase may also be periodic. An investigation of the mechanisms regulating these genes may reveal common features that could serve as points of coordinate regulation and could lead to the identification of *trans*-acting factors.

The experiments reported here support a negative regulation of the *TOP2* mRNA level, since the loss of a *cis* element in the 5' UTR resulted in expression of the *TOP2* transcript with greatly reduced variation in level during the cell cycle. As one possibility, these results could be explained by the loss of a site required for periodic repression of *trans* splicing of the *TOP2* transcript by a labile factor, leading to the subsequent degradation of the transcript. In this case, the element might repress *trans* splicing during G₂ and M phases, resulting in accumulation of the mature mRNA only during the G₁ and S phases of the cell cycle. Deletion of the element would lead to a stable mRNA level throughout the cell cycle. Although *trans* splicing of transcripts serves a fundamentally different purpose in trypanosomatids compared to *cis* splicing in other eukaryotes, it is worth noting that S-phase regulation of the mouse thymidylate synthase gene occurs by posttranscriptional mechanisms involving a modulation of splicing of thymidylate synthase transcripts during the cell cycle (16).

Alternatively, regulation of the *TOP2* gene could be at the level of mRNA stability. The *cis* element in the 5' UTR might target the mature mRNA for enhanced degradation by cell cycle-dependent nuclease activity. An instability determinant in the 5' UTR could interact with labile nucleases or other *trans*-acting factors that regulate the accessibility of the domain to nucleases. In higher eukaryotes, instability determinants are often found in the 3' UTRs of transcripts. In light of the many differences in gene organization and expression between trypanosomes and higher organisms, the presence of *cis* regulatory sequences in the 5' UTR of the *TOP2* gene does not exclude the possibility that these sequences serve as instability determinants. Identification of *trans*-acting factors involved in modulating the *TOP2* mRNA level will be necessary for distinguishing among these and other possibilities.

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