Nuclear Extracts of Crithidia fasciculata Contain a Factor(s) That Binds to the 5'-Untranslated Regions of TOP2 and RPA1 mRNAs Containing Sequences Required for Their Cell Cycle Regulation*

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The Crithidia fasciculata replication protein A gene, RPA1, and topoisomerase II gene, TOP2, encode proteins involved in the replication of nuclear and mitochondrial DNA, respectively. Transcripts of both genes accumulate periodically during the cell cycle and attain their maximum levels just before S phase. Octamer consensus sequences within the 5'-untranslated region (UTR) of both genes have been shown to be necessary for cycling of these transcripts. Using a gel retardation assay, we show here that nuclear extracts of C. fasciculata contain a protein factor(s) that binds specifically to RNA from 5'-UTRs of TOP2 and RPA1 genes. In addition, mutations in the consensus octamer sequence abolish binding to the RNA in both cases. Ultraviolet cross-linking using a radiolabeled TOP2 5'-UTR probe identified proteins with apparent molecular masses of 74 and 37 kDa in the RNA-protein complex. Nuclear extracts prepared from synchronized cells show that the binding activity varies during the cell cycle in parallel with TOP2 and RPA1 mRNA levels. These results suggest that the cell cycle regulation of the mRNA levels of trypanosomatid DNA replication genes may be mediated by binding of specific proteins to conserved sequences in the 5'-UTR of their transcripts.

The trypanosomatid Crithidia fasciculata is a protozoan parasite containing a single mitochondrion with an unusual form of DNA called kinetoplast DNA (kDNA)1(1, 2). The kDNA consists of a single network of catenated minicircles and maxicircles. During replication of kDNA, the minicircles are released from the network and are reattached to the network periphery after replication, whereas maxicircles replicate while still attached to the network (3). An unusual feature of DNA replication in trypanosomes is that both kinetoplast and nuclear DNA replicate in approximate synchrony (4, 5). In other eukaryotes, mitochondrial DNA replication occurs throughout the cell cycle (6, 7). Since both the kinetoplast and nuclear DNA replication genes are encoded in the nucleus, their coordinated expression may play a role in synchronizing nuclear and kDNA replication.

Expression of protein-coding genes in trypanosomatids involves mechanisms that are different from those in most other eukaryotes. All mRNAs in trypanosomatids contain two exons, a 39-nucleotide minixon at the 5' end of the mRNA and the main coding exon (8, 9). Genes are usually grouped in polycistrionic transcription units, and the polycistrionic pre-mRNAs are processed by 5' trans-splicing and 3' polyadenylation to yield monocistrionic mRNAs. The differential expression of steady state mRNAs from a single polycistrionic transcript involves post-transcriptional controls by mechanisms that are still unclear. Pre-mRNA turnover in combination with differential rates of trans-splicing and polyadenylation and of mRNA turnover may all play a role in regulating mRNA levels. So far, only two RNA polymerase II promoters for protein-coding genes have been described in trypanosomes, the Trypanosoma congolense glutamic acid/alanine-rich protein promoter and the Trypanosoma brucei hsp70 promoter, and neither promoter appears to be transcriptionally regulated (10, 11). The regulated expression of T. brucei hsp70 upon heat shock appears to be controlled entirely at a post-transcriptional level, since heat shock does not induce increased transcription of the hsp70 genes (11).

To determine the mechanism of coordinate expression of nuclear and kinetoplast DNA replication genes, we have studied the cell cycle-regulated expression of RPA1 and TOP2 genes. The TOP2 gene encodes a kinetoplast-associated type II topoisomerase (12), and the RPA1 gene encodes the large subunit of replication protein A, the single-stranded DNA-binding protein that has been immunolocalized to the nucleus (13, 14). The steady state levels of the mRNAs for these two genes and that of dihydrofolate reductase-thymidylate synthase gene are cell cycle-regulated in a similar manner (4). Sequence elements required for periodic accumulation of RPA1 and TOP2 mRNAs are present in the 5'-untranslated region (15, 16). Examination of the 5'-UTR of these genes reveals the presence of a consensus octamer sequence with a conserved hexameric core. Mutation analysis showed that these octamer sequences are necessary for periodic accumulation of TOP2 and RPA1 transcripts. We show here that specific proteins present in nuclear extracts bind to the 5'-UTR RNA of TOP2 and RPA1 genes and that the octamer sequences are necessary for binding to occur.

EXPERIMENTAL PROCEDURES

Preparation of Nuclear and Cytosolic Extracts—C. fasciculata Cf-C1 cells were grown in brain heart infusion medium (Difco) supplemented with 20 μg/ml hemin and 100 μg/ml streptomycin sulfate at 28 °C with shaking. The cells were grown to a density of 6–7 × 10^7 cells/ml and then harvested by centrifugation (Sorvall GS-3 rotor, 15 min, 5000 rpm at 4 °C). All procedures for extract preparation were performed at 4 °C.

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1 The abbreviations used are: kDNA, kinetoplast DNA; TOP2, topoisomerase II gene; RPA1, replication protein A gene; TOP2, topoisomerase II gene; UTR, untranslated region; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
were C46 (AGCAGTGCGGCCGCGTGCGAGGATATTGCGGGTAACA-mermers and plasmid pt2–1 as template and then cloning the various PCR
1
the TOP2
wild type octamers (pRM12). These plasmids were constructed by am-
2
III fragment containing the pGEM7Zf(

various RNA probes are given in Table I.

served as a template for run off promoter upstream of the sequence of interest. The PCR product then
1
Amplification of the target DNA yields a PCR product with the T7
2
m

mM HEPES-KOH, pH 7.9, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA,

were incubated with32P-labeled RNA, and the protein-RNA

The plasmids pRM9-pRM12 containing the −291 to −209 region of the
2
gene were constructed (the A residue of the initiating ATG
codon is +1). These plasmids had both mutant octamers (pRM9), left
1
octamer mutant only (pRM10), right octamer mutant (pRM11), or both
2
wild type octamers (pRM12). These plasmids were constructed by am-
plifying the −291 to −209 region of the TOP2 gene by PCR with Vent
DNA polymerase using primers containing wild type or mutant octa-
mers and plasmid pt2–1 as template and then cloning the various PCR
products into the Smal site of pUC19. Oligonucleotides used in PCR
were C46 (AGCAGTGCGGCCGCGTGCGAGGATATTGCGGGTAACA-mermers and plasmid pt2–1 as template and then cloning the various PCR
products into the Smal site of pUC19. Oligonucleotides used in PCR
were C46 (AGCAGTGCGGCCGCGTGCGAGGATATTGCGGGTAACA-CG)
and C47 (GGGGAGTCGGCCGATTCCTCGCAGGCTTTCG-ACTAGAAG
AACTATGCGGTCGGAACGCGGATC

RESULTS

The TOP2 5′-UTR RNA Binding to a Factor(s) in Nuclear Extracts—An 83-base pair DNA fragment from −291 to −209 of the TOP2 5′ UTR has been shown to confer periodic expression to a reporter gene (15). Since gene expression in trypano-
somes is regulated mainly at the post-transcriptional level, we wanted to determine whether C. fascicu-
lata cell extracts contained a factor(s) that would bind to RNA from this region. An electrophoretic method for detecting RNA-protein interactions
was used for this purpose (20). Nuclear and cytosolic extracts were incubated with 32P-labeled RNA, and the protein-RNA
complexes were resolved by electrophoresis on nondenaturing polyacrylamide gels. Heparin, a polyanion, was used to displace proteins bound nonspecifically to the labeled RNA. Gel shift results show that C. fasciculata nuclear extracts contain a factor(s) that binds to the −291 to −209 sense RNA of the TOP2 gene (Fig. 1, lanes 3 and 4). A smaller amount of binding activity was present in cytosolic extract (lane 2) that could be due to some breakage of nuclei during cell lysis. Two gel-shifted bands were seen on the gel with nuclear extracts. At present the basis for the different mobilities of these complexes is unknown. The relative intensity of these two bands varies in different preparations of nuclear extracts.

To investigate the specificity of RNA binding activity in nuclear extracts, we examined binding to an antisense transcript of the −291 to −209 region and to a lacZ transcript. No
binding was observed when the antisense RNA was incubated with cytosolic or nuclear extracts (Fig. 1, lanes 6 and 7). The lacZ sense RNA also showed no binding with cytosolic or nuclear extracts (lanes 9 and 10). The specificity of binding is also indicated by gel retardation assays carried out in the presence of unlabeled competitor RNA (Fig. 2B). A 10-fold molar excess of unlabeled TOP2 291 to -209 sense RNA greatly reduced binding to the labeled RNA (Fig. 2B, lanes 2 and 3). However unlabeled lacZ RNA did not compete for binding at a similar concentration (lane 4). Further experiments were done to characterize the nature and specificity of binding seen to -291 to -209 TOP2 RNA. No complex formation took place when the nuclear extract was pretreated with proteinase K, confirming the involvement of a protein factor(s) in the binding reaction (Fig. 2A, lane 3). However, pretreatment of the nuclear extract with DNase I or inclusion of poly(dI-dC) had no effect on binding (Fig. 2A, lanes 4 and 5), ruling out a role for DNA in the binding reaction. These results show that C. fasciculata nuclear extracts contain a protein factor(s) that binds specifically to an 83-nucleotide RNA from the TOP2 5′-UTR.

Octamer Sequences Present in the TOP2 5′-UTR Are Involved in Binding—The -291 to -209 region of TOP2 gene contains two copies of the consensus octamer sequence found in the 5′ or 3′-UTRs of several C. fasciculata mRNAs that vary as cells progress through the cell cycle (Table I). Deletion of either of the two octamer sequences resulted in reduced cycling of the mRNA of a reporter gene, whereas deletion of both octamers completely abolished cycling of the mRNA (15). To investigate the requirement for the consensus octamer sequence in binding to the RNA, we performed gel retardation assays using labeled RNAs in which one or both octamers were mutated to a different sequence (CAUAGAAC to UGCGAGGC). Mutation of either the left or right octamer sequence greatly reduced binding as compared with wild type RNA (Fig. 3). Interestingly, mutation of the right octamer had a greater effect on binding activity than mutation of the left octamer. We do not know the significance of this observation, although deletion of either octamer sequence reduced reporter mRNA cycling to the same extent.
No binding was seen to an RNA in which both octamers had been mutated. These results suggest that the consensus octamer sequence is required for binding of RNA to a factor(s) in nuclear extracts. Moreover, the results from these binding assays complement those measuring cycling of mRNA levels in synchronized cells. Mutations that reduce or abolish mRNA accumulation of the mRNA-UTR RNA on binding.

Octamer Sequences Are Required for Binding of a Factor(s) to 5′-UTR RNA of the RPA1 Gene—A 349-base pair fragment, from −523 to −174, of the RPA1 5′-UTR is required for periodic accumulation of the RPA1 transcript (16). This region contains two copies of the consensus octamer sequence. Subcloning into a reporter plasmid has shown that a 113-base pair DNA fragment from −343 to −231 containing the two octamer sequences can confer periodic accumulation on a heterologous transcript. Mutation of both octamer sequences abolished cycling of the reporter gene transcript, suggesting that these sequences function in a manner similar to that seen with the TOP2 gene.

We therefore wanted to determine whether C. fasciculata nuclear extracts contained a factor(s) that would also bind to the −343 to −231 RPA1 RNA. Fig. 4 shows that the wild type RPA1 RNA binds to a factor(s) in the nuclear extracts. Two retarded bands are present, as seen earlier with TOP2 RNA. No binding activity is present in the cytosolic extracts. Again, as seen with TOP2 RNA, no binding was observed when an RNA in which both octamers had been mutated to a different sequence was used in the gel retardation assay. These results show that nuclear extracts have a factor(s) that binds to RNA from the 5′-UTR of RPA1, and that octamer sequences present in the RNA are required for binding to occur.

TOP2 and RPA1 RNAs Compete for Binding to a Factor(s) in Nuclear Extracts—The above results from gel retardation assays show that octamer sequences are required for binding of TOP2 or RPA1 5′-UTR RNA to a factor(s) present in nuclear extracts. To determine whether the same factor(s) is involved in binding to both TOP2 and RPA1 RNA probes, a competition experiment was done in which nuclear extracts were incubated with wild type RPA1-labeled RNA in the presence of unlabeled TOP2 RNA. The presence of unlabeled TOP2 RNA prevents binding of a factor(s) in nuclear extracts to the RPA1 RNA probe (Fig. 5). This suggests that both RNAs bind to the same nuclear factor(s). The elution profile of the TOP2 and RPA1 RNA binding activity was also determined after column chromatography. The binding activity was partially purified by ammonium sulfate precipitation of proteins in the nuclear extract followed by successive chromatography over DEAE-cellulose, phosphocellulose, and heparin-Sepharose columns. A similar elution profile was observed when the heparin-Sepharose column fractions were assayed with either TOP2 or RPA1 RNA probes (data not shown). Results of the competition experiment and the column chromatographic profiles of the factor(s) that binds to RPA1 and TOP2 RNA strongly suggest that the same factor(s) is involved in binding to both RNA probes.

UV Cross-linking of Proteins in Nuclear Extracts to TOP2 5′-UTR RNA—We sought to identify proteins bound to the 5′-UTR of TOP2 RNA in gel-shifted complexes by cross-linking to a radiolabeled probe. The binding activity was partially purified and then incubated with the TOP2 RNA (from −291 to −209 RNA probe). The RNA-protein complexes were separated from other proteins and free probe by nondenaturing polyacrylamide gel electrophoresis. Gel slices containing the shifted bands were irradiated with UV light, and the complexes were eluted from the slices and treated with RNases A and T1. SDS-PAGE analysis of the RNase-treated complexes showed two bands with apparent molecular masses of 74 and 37 kDa (Fig. 6A). These bands were absent when the eluted complexes were treated with protease K (data not shown). At present we do not know if these polypeptides represent two different subunits of the binding protein or if the 74-kDa polypeptide might represent a dimer of the 37-kDa protein held together by a short oligonucleotide resistant to nuclease digestion. The partially purified proteins used in the RNA gel shift were analyzed by
SDS-PAGE. The Coomassie-stained gel showed that the 74- and 37-kDa polypeptides were not major proteins in the partially purified nuclear extract (Fig. 6B). This result indicates that the RNA probe was not simply cross-linked to two abundant nuclear proteins.

The Binding Activity Varies during the Cell Cycle—We have examined the relative level of binding activity in cells synchronized by the hydroxyurea arrest. Northern blot analysis of RNA isolated at various time intervals after release from hydroxyurea arrest has shown that the steady state transcript levels of the genes encoding RPA1, RPA2 (the gene encoding the RPA middle subunit), TOP2, and dihydrofolate reductase-thymidylate synthase gene accumulate periodically during the cell cycle (4), with maximum transcript levels present at G1/S phase. To determine whether the TOP2 and RPA1 5'-UTR RNA binding activities also vary during the cell cycle, cultures of C. fasciculata were synchronized by treatment with hydroxyurea, and nuclear extracts were prepared from cells 90 and 180 min after release from hydroxyurea block. These times represent minimum and maximum levels of the TOP2 and RPA1 mRNAs in the synchronously growing cells (4, 16).

Nuclear extracts were assayed for binding activity using the wild type -291 to -209 TOP2 RNA as probe. Fig. 7 shows that nuclear extracts prepared from cells 180 min after release from hydroxyurea arrest had severalfold higher binding activity than extracts from cells 90 min after hydroxyurea arrest. Similar results were obtained when nuclear extracts were assayed with wild type RPA1 RNA as probe (data not shown). These results show that the binding activity varies during the cell cycle and parallels the mRNA levels of at least four DNA replication genes.
DISCUSSION

We have used a reporter plasmid previously to analyze deletion mutants of 5'-UTRs of TOP2 and RPA1 genes to identify cis elements involved in periodic expression of these genes (15, 16). Both TOP2 and RPA1 genes have 5'-UTR elements that can confer periodic expression on the mRNA of a reporter gene. Comparison of the TOP2, RPA1, RPA2, and dihydrofolate reductase-thymidylate synthase gene 5'-UTR sequences showed the presence of a consensus octamer sequence CATAGAAG that was also shown to be required for cycling of the reporter gene transcript. Furthermore, deletion analysis involving TOP2 5'-UTR showed that the essential sequence elements had to be present on the mature mRNA and not just within the flanking DNA sequence. These results indicated that the regulation of gene expression is primarily at the post-transcriptional level and involves sequences within the 5'-UTR of the TOP2 mRNA. This is in agreement with the general observation that in trypanosomes, the expression of protein-coding genes transcribed by RNA polymerase II is regulated mainly at the post-transcriptional level (21, 22).

In the present work we have attempted to identify trans-acting factor(s) that interact with RNA encoded by the 5'-UTR elements shown to be required for periodic expression of TOP2 and RPA1 genes. Our results provide evidence that proteins present in nuclear extracts of C. fasciculata interact specifically with 5'-UTR of TOP2 and RPA1 mRNA. The interaction was shown to be specific by three independent criteria. First, excess unlabeled 5'-UTR RNA of the TOP2 gene prevents formation of complexes, whereas no reduction in binding is seen with an unlabeled nonspecific RNA as competitor. Second, mutation of both octamer sequences present in either TOP2 or RPA1 RNA probes abolished binding to the probes. Since deletion of these octamer sequences or mutation to a different sequence abolishes cycling of the reporter gene transcript, it appears that these elements function by virtue of being recognized by nuclear proteins at the RNA level. Third, ultraviolet cross-linking of the gel-shifted complexes identified two specific proteins cross-linked to the probe RNA.

The proteins that bind to the 5'-UTR RNA of TOP2 and RPA1 genes appear to be the same for several reasons. First, binding activities to both RNAs are present in the nuclear extracts and not in cytosolic extracts. Second, the same sequence elements are required for binding of both RNAs; mutations in octamer sequences abolish binding to both RNA molecules. Third, both activities vary during the cell cycle in a similar manner. Fourth, the activities that bind to both RNA molecules show identical chromatographic elution profiles. Finally, the addition of unlabeled TOP2 RNA eliminates binding to labeled RPA1 RNA probe. Thus we suggest that the cell cycle regulation of genes involved in replication of nuclear DNA and kDNA is mediated at a post-transcriptional level by a common protein factor(s). It remains to be determined whether the coordinate regulation of DNA replication genes plays an important role in synchronizing nuclear DNA and kDNA replication in C. fasciculata.

There are several examples of 5'- and 3'-UTR elements affecting gene expression in trypanosomes. In Leishmania the intergenic region of the tubulin gene was shown to be essential when placed on the 5'-side of the reporter chloramphenicol acetyltransferase gene. The region contained signals that did not affect transcription rate and probably modulated RNA stability (23). The developmentally regulated Leishmania donovani A2 genes are regulated by differential RNA stability that involves the 3'-UTR of A2 mRNA (24). In T. brucei, the expression of procyclin mRNA is regulated at the post-transcriptional level by cis elements in the 3'-UTR (25). In this case regulation occurs at the level of both mRNA stability and translation. Our results also indicate that the 5'-UTR of TOP2 and RPA1 regulates gene expression at a post-transcriptional level.

An interesting observation in these studies is that the level of binding activity varies in nuclear extracts prepared from synchronized cells at different stages of the cell cycle. The binding activity is severalfold higher in nuclear extracts prepared from cells 180 min after release from a hydroxyurea block, the time at which DNA replication gene transcript levels are maximal, as compared with extracts from cells 90 min after release from hydroxyurea, the time at which these transcript levels are at a minimum (4, 15). As a working model we propose that the presence of the conserved hexamer sequence in an mRNA targets the RNA for destruction and that a binding activity expressed periodically during the cell cycle protects the RNA from destruction when bound to the RNA. The target mRNAs would therefore vary during the cell cycle in parallel with the level of the binding activity. Another feature of this model is the protection from degradation of mRNAs in which the hexamer sequence is mutated. In this case, the mRNA level would no longer cycle regardless of the variation of the level of the binding factor. Further studies are aimed at testing this model and elucidating the biochemical mechanism of the cell cycle regulation of DNA replication genes in trypanosomatids.

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