INTRODUCTION

Trypanosomes contain a novel form of mitochondrial DNA or kinetoplast DNA (kDNA), which consists of thousands of copies of minicircles and a small number of maxicircles topologically interlocked to form a cup-shaped sheet of DNA termed a DNA network (Simpson, 1987). Maxicircles encode rRNAs and proteins essential for mitochondrial function. Expression of maxicircle genes involves extensive editing of primary transcripts in which U residues are added and/or deleted to create functional mRNAs (Stuart, 1991). The specificity of the editing process is determined by small ‘guide RNAs’ encoded in both maxicircles and minicircles and which serve as templates for the editing process (Sturm and Simpson, 1990).

In trypanosomatids, DNA replication in the nucleus and in the single mitochondrion (or kinetoplast) initiates nearly simultaneously, suggesting that the DNA synthesis (S) phases of the nucleus and the mitochondrion are coordinately regulated. To investigate the basis for the temporal link between nuclear and mitochondrial DNA synthesis phases the expression of the genes encoding DNA ligase I, the 51 and 28 kDa subunits of replication protein A, dihydrofolate reductase and the mitochondrial type II topoisomerase were analyzed during the cell cycle progression of synchronous cultures of Crithidia fasciculata. These DNA replication genes were all expressed periodically, with peak mRNA levels occurring just prior to or at the peak of DNA synthesis in the synchronized cultures. A plasmid clone (pdN-1) in which TOP2, the gene encoding the mitochondrial topoisomerase, was disrupted by the insertion of a NEO drug-resistance cassette was found to express both a truncated TOP2 mRNA and a truncated topoisomerase polypeptide. The truncated mRNA was also expressed periodically with the expression of the endogenous TOP2 mRNA indicating that cis elements necessary for periodic expression are contained within cloned sequences. The expression of both TOP2 and nuclear DNA replication genes at the G1/S boundary suggests that regulated expression of these genes may play a role in coordinating nuclear and mitochondrial S phases in trypanosomatids.

SUMMARY

Periodic expression of nuclear and mitochondrial DNA replication genes during the trypanosomatid cell cycle

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In trypanosomatids, DNA replication in the nucleus and in the single mitochondrion (or kinetoplast) initiates nearly simultaneously, suggesting that the DNA synthesis (S) phases of the nucleus and the mitochondrion are coordinately regulated. To investigate the basis for the temporal link between nuclear and mitochondrial DNA synthesis phases the expression of the genes encoding DNA ligase I, the 51 and 28 kDa subunits of replication protein A, dihydrofolate reductase and the mitochondrial type II topoisomerase were analyzed during the cell cycle progression of synchronous cultures of Crithidia fasciculata. These DNA replication genes were all expressed periodically, with peak mRNA levels occurring just prior to or at the peak of DNA synthesis in the synchronized cultures. A plasmid clone (pdN-1) in which TOP2, the gene encoding the mitochondrial topoisomerase, was disrupted by the insertion of a NEO drug-resistance cassette was found to express both a truncated TOP2 mRNA and a truncated topoisomerase polypeptide. The truncated mRNA was also expressed periodically with the expression of the endogenous TOP2 mRNA indicating that cis elements necessary for periodic expression are contained within cloned sequences. The expression of both TOP2 and nuclear DNA replication genes at the G1/S boundary suggests that regulated expression of these genes may play a role in coordinating nuclear and mitochondrial S phases in trypanosomatids.

Key words: cell cycle, replication, topoisomerase, kinetoplast, mitochondrion
are coordinately regulated through a common cis-acting sequence recognized by a specific transcription factor (Johnston et al., 1991). Since all kinetoplast DNA replication proteins are likely to be nuclear encoded, their expression may be under the same controls as those of genes encoding nuclear replication proteins. To investigate this possibility, we have examined the cell cycle expression of genes encoding both nuclear and kinetoplast replication proteins in the trypanosomatid Crithidia fasciculata.

**MATERIALS AND METHODS**

**Cell synchronization**

*C. fasciculata* was synchronized using a modification of the method described for synchronizing *Leishmania tarentolae* (Simpson and Braly, 1970). Cells were grown to late log phase (~10^8 cells/ml) at 28°C in Difco brain heart infusion medium (BHI) supplemented with 10 µg hemin/ml and 100 µg streptomycin sulfate/ml prior to the addition of 200 µg hydroxyurea/ml for 6 hours. Growth was continued at 28°C in fresh medium lacking hydroxyurea, and samples were removed every 30 minutes.

**Measurement of DNA synthesis**

Total DNA synthesis was measured by labeling 65 µl of culture with 10 µl of [^3H]thymidine (1 mCi/ml; 79 Ci/mmol) at 28°C for 10 minutes. Cells were lysed and incorporation of [^3H]thymidine into trichloroacetic acid-precipitable form was measured. Mitochondrial DNA synthesis was measured by scintillation counting of [^3H]thymidine label in minicircle DNA bands on Southern blots following digestion with *Xhol* and aagarose gel electrophoresis.

**Northern blots**

RNA extracted from 10^9 cells by guanidinium isothiocyanate lysis was used for northern blotting and hybridization as described (Pasion et al., 1992). Either 50 µg (for *CIRPA1*) or 25 µg (all others) of RNA was fractionated. Probes for analysis of *C. fasciculata* RNA were a 980 bp *EcoRI-Mul* fragment of the CfARPA1 gene (EMBL accession number Z23163), a 606 bp *HaeII* fragment of the CfARPA2 gene (EMBL accession number Z23164), a 630 bp *EcoRI-Aval* fragment of the CfALIG1 gene (EMBL accession number Z23078), a 2000 bp *Xhol* fragment of the CfATOP2 gene encompassing amino acid residues 311 to 1007 (Pasion et al., 1992) and a 260 bp fragment of a *C. fasciculata* gene designated *CaBP* due to its sequence similarity to trypanosome calcium-binding proteins (Lee et al., 1990). The probe for the analysis of Cf (pdN-1) RNA was a 2.1 kb *HindIII-SacII* fragment of pdN-1 designated 5′ TOP2 US and CfaTOP2 (1-1186) in Fig. 2. Northern blots were quantitated using a Molecular Dynamics PhosphorImager.

**Transformation of Crithidia fasciculata**

*C. fasciculata* cells were cultured in BHI media supplemented with 10 µg hemin/ml and 100 µg streptomycin sulfate/ml to a density of 1×10^8 cells/ml. Cells were harvested in a clinical centrifuge at 4°C for 10 minutes at 1,500 rpm. The supernatant was decanted and the cells were resuspended in the residual media and plated onto 1% agar plates containing BHI plus 10 µg hemin/ml, 100 µg streptomycin sulfate/ml and 80 µg G418/ml. The plates were wrapped in parafilm and incubated at 28°C. G418-resistant colonies appeared within 4 to 5 days.

**Construction of the pdN-1 plasmid**

The vector pX.2 was derived from pX (LeBowitz et al., 1990), which contains a mutant neomycin phosphotransferase (NPT) gene by replacing the 350 bp *Ball-Ncol* fragment contained within the NPT gene by the corresponding 350 bp *Ball-Ncol* fragment from the wild-type NPT gene from pSV2neo (Yenofsky et al., 1990). Two oligonucleotides (Kpn1 1: 5′-CGCGGATCCCTCTAGAG-GTAC-3′ and Kpn 2: 5′-CTCTAGAGGATCCGGGTAC-3′) were synthesized, annealed, and subcloned into the unique *KpnI* site of pX.2 introducing *BanHI*, *SacII* and *Xhol* sites flanked by *KpnI* sites and generating the vector pX.2-KO. This construct allows the movement of the 3.3 kb NEO cassette (the wild-type NPT gene with flanking *Leishmania major* DHFR-TS sequences) as a *BanHI*, *SacII* or *Xhol* fragment.

To create a disruption of the plasmid-encoded CfATOP2 gene, the NEO cassette was subcloned as a SacII fragment from pX.2-KO into the p21 plasmid (Pasion et al., 1992), which contains the CfATOP2 coding region and 883 bp 5′ flanking and 1.5 kb 3′ flanking sequences. The p21 plasmid was constructed by inserting a 6.1 kb genomic fragment into the bacterial cloning vector pGEM-TZI(+), which contains no other trypanosomatid sequences. The inserted NEO cassette replaces a 1.4 kb SacII fragment within the CfATOP2 coding sequence to generate pdN-1 in which the CfATOP2 gene and the NPT gene are in the same orientation.

**Western blot analysis**

Cell lysates prepared from *C. fasciculata* strains were analyzed on 7% acrylamide-SDS gels as described previously (Pasion et al., 1992). Each lane contained lysate from 2.5×10^9 cells. Blots were probed with polyclonal antisera (1:5000) raised against purified topolImt (Melendy and Ray, 1989) and detected with alkaline phosphatase conjugated to goat anti-rabbit IgG.

**RESULTS**

**Expression of DNA replication genes in synchronized cells**

*C. fasciculata* cultures undergoing synchronous progress through the cell cycle were prepared by a hydroxyurea arrest method (Simpson and Braly, 1970). Cells were released from the hydroxyurea block and sampled every 30 minutes to determine the cell number, the percentage of dividing cells and the incorporation of pulse label into DNA. Cultures released from hydroxyurea treatment entered S phase immediately, as indicated by the high level of DNA synthesis at 0 and 30 minutes (Fig. 1A). Following this peak of DNA synthesis, the percent of cells undergoing division (cells with two nuclei) increased from <1% to 30% at 120 minutes post-release followed by an approximate doubling of the cell number by 150 minutes post-release. The culture then entered another round of DNA synthesis, with peak incorporation at 210 minutes, followed by another round of division at about 300 minutes. Incorporation of [^3H]thymidine into mitochondrial minicircle DNA was also measured by quantitation of radioactivity in linear minicircle DNA following digestion of a sample of the pulse-labeled DNA with *Xhol*, an enzyme that cuts all minicircles once, and gel electrophoresis and blotting to a
nitrocellulose membrane. Minicircle DNA synthesis occurred in synchrony with total DNA synthesis during the second S phase, with peak synthesis at 180 minutes, but was delayed relative to total DNA synthesis immediately following release from the hydroxyurea block in the experiment shown (Fig. 1c). This delay was an anomaly and was not reproducible in subsequent synchronizations. The culture gradually became increasingly asynchronous during the course of the experiment, as evidenced by a broadening of the peaks of DNA synthesis and percentage dividing cells in the second cell cycle. The gradual loss of synchrony can be attributed, at least in part, to natural variation in individual cell cycle times. In Trypanosoma brucei, for example, cell cycle kinetic studies on asynchronous populations of cells indicate that individual cells may have a G2 phase ranging from 1.44 hours to 2.5 hours (Woodward and Gull, 1990). In the first division cycle the cell number does not exactly double, probably due to the presence of inviable cells, as hydroxyurea treatment is selectively lethal to cells in S phase (Simpson and Braly, 1970).

Cell cycle expression of genes encoding topoIImt (the kinetoplast DNA topoisomerase) (Pasion et al., 1992) and those of DNA ligase I (Brown and Ray, 1992), the 51 and 28 kDa subunits of replication protein A (Brown et al., 1994) and dihydrofolate reductase (Hughes et al., 1989) were examined on northern blots of RNA prepared from synchronized cultures (Fig. 1B). The Crithidia RP-A protein is the trypanosome homolog of the human RP-A protein, which has been shown to be required for SV40 DNA replication in vitro. *Crithidia* RP-A substitutes for human RP-A in the T-antigen-dependent unwinding of the SV40 origin of replication and stimulates both DNA synthesis and DNA priming by human DNA polymerase α/primase (Brown et al., 1992). CfaRPA1 and CfaRPA2 encode the 51 kDa and 28 kDa subunits of the heterotrimeric *C. fasciculata* RP-A protein (Brown et al., 1994). CfaLIG1 encodes a DNA ligase with properties similar to other type I nuclear DNA ligases (Brown and Ray, 1992) and the *C. fasciculata* DHFR-TS gene encodes dihydrofolate reductase-thymidylate synthetase (Hughes et al., 1989), which is required for the synthesis of DNA precursors. The analysis of the CfaBP transcript, which was found to be expressed at a constant level during cell cycle progression, is included as an internal loading control.

The steady state levels of the CfaRPA1, CfaRPA2, and DHFR-TS transcripts varied during cell cycle progression, with peak mRNA levels at 0-30 minutes and 150-210 minutes, both during late G1 and S phases, with little expression between these peaks, during G2 and M phases. All three transcripts were most abundant at 180 minutes, just before the peak of DNA synthesis, indicating that maximal expression is at the transition from G1 phase to S phase. The three transcripts were induced 5- to 7-fold between 90 and 180 minutes following release from hydroxyurea. The CfaLIG1 transcript is periodically expressed, but reached maximum levels at 210 minutes, at the peak of S phase. The approximately 3-fold induction of CfaLIG1 expression as cells progress into S phase is not as great as that seen for the CfaRPA1 and CfaRPA2 genes and the DHFR-TS gene. These results suggest that the mechanism regulating expression of CfaLIG1 may be distinct from that regulating expression of the DHFR-TS and CfaRPA1 and CfaRPA2 genes.

The CfaTOP2 gene, which is also encoded in the nucleus, is also under cell cycle control. The periodic expression pattern of the CfaTOP2 transcript is similar to that of the CfaRPA1 and CfaRPA2 transcripts and the DHFR-TS transcript, with the peak of expression occurring just prior to the peak of total DNA synthesis (Fig. 1A) and mitochondrial minicircle DNA synthesis (Fig. 1C). A 5-fold difference in CfaTOP2 transcript levels occurred between the 90 and 180 minute time points. The CfaTOP2 gene is the first example of a mitochondrial

![Fig. 1. Expression of RP-A p51 and p28, DHFR-TS, DNA ligase I, topoIImt, and calcium-binding protein genes during progression through the cell cycle in *C. fasciculata* synchronized by hydroxyurea treatment. (A) Cell number (squares), percent of cells with two nuclei (triangles), and incorporation of [3H]thymidine pulse label (circles), measured every 30 minutes following release from the hydroxyurea block to monitor synchrony and cell cycle position. (B) Northern blot analysis of RNA carried out on synchronized *C. fasciculata* cells. The transcript levels of CfaRPA1, CfaRPA2, DHFR-TS, CfaLIG1 and CfaTOP2 are shown together with the RNA control and percentage dividing cells in the second cell cycle. (C) Incorporation of [3H]thymidine into mitochondrial minicircle DNA in synchronized cells.](image-url)
Expression of a disrupted TOP2 gene in plasmid pdN-1

A 6.1 kb genomic fragment containing the TOP2 gene and 5’ and 3’ flanking sequences cloned in plasmid pt2-1 (Pasion et al., 1992) was disrupted by insertion of a neomycin phosphotransferase drug-resistance cassette (NEO) between two SacII sites within the TOP2 coding sequence to yield the plasmid pdN-1 (Fig. 2). The inserted NEO cassette replaces approximately 1.4 kb of TOP2 coding sequence and confers resistance to G418 in C. fasciculata. A clone expressing drug resistance from extrachromosomal pdN-1 plasmid was examined for possible expression of the amino-terminal portion of topoIImt. In addition to full-length topoIImt, western blots of C. fasciculata (pdN-1) show the presence of a 45 kDa protein recognized by rabbit polyclonal antibodies against topoIImt consistent with the molecular mass predicted based on the location of an in-frame stop codon within the NEO cassette (Fig. 3). Some less intense bands present in both lanes of Fig. 3 appear to be non-specific proteins recognized by this polyclonal antiserum.

In addition to the 4.8 kb TOP2 mRNA expressed from chromosomal copies of TOP2, cells carrying the pdN-1 plasmid also express a truncated TOP2 mRNA of approximately 2.0 kb, which corresponds to the size predicted based on the utilization of the most frequently used TOP2 splice acceptor site located at −670 relative to the ATG initiation codon (unpublished results) and the polyadenylation site contained in the Leishmania major DHFR-TS 5’ flanking sequence of the NEO cassette (Kapler et al., 1990).

We have investigated the possibility that sequences required in cis for the periodic expression of TOP2 might be present in the pdN-1 plasmid. C. fasciculata (pdN-1) cells were synchronized in the same manner as the wild-type C. fasciculata and analyzed by northern blotting at 30 minute intervals. As shown in Fig. 4, both the plasmid-expressed 2.0 kb TOP2 mRNA and the endogenous 4.8 kb TOP2 message are expressed with the same periodicity as observed for the wild-type TOP2 mRNA in Fig. 1. Both the 4.8 and 2.0 kb transcripts are expressed at a high level at the time of release from the hydroxyurea block and then again at 180 minutes after release. Both transcript levels go through minima at 90-120 minutes and then again at 300 minutes. A minor RNA species migrating at approximately 1.35 kb possibly represents an mRNA resulting from the utilization of alternate splice acceptor sites...
in the TOP2 5′ flanking sequence located at −77 and −67 relative to the TOP2 initiation codon (unpublished results). The higher molecular mass species migrating at 5.85, 7.7 and 10 kb appear to represent RNAs expressed from the extrachromosomal plasmid, which are polyadenylated at sites within plasmid sequences. These latter species are not observed in cells lacking the plasmid or in cells in which the disrupted TOP2 gene is integrated into one of the chromosomal copies of TOP2 (S. G. Pasion and O. S. Ray, unpublished results).

**DISCUSSION**

The coordination of nuclear and mitochondrial DNA replication in trypanosomatids is unique among eukaryotes, where mitochondrial DNA replication is typically not restricted to any one period of the cell cycle (Guttes et al., 1967). Our results show that the steady state levels of the mRNAs of a mitochondrial replication gene as well as those of nuclear replication genes are cell cycle regulated in the trypanosomatid C. fasciculata. These results are similar to those found for nuclear DNA replication genes in S. cerevisiae where at least 18 genes encoding DNA replication proteins are expressed periodically (Johnston and Lowndes, 1992). These include genes encoding subunits of DNA polymerase α, DNA polymerase δ and DNA primase, PCNA (proliferating cell nuclear antigen, a pol δ processivity factor) and DNA ligase. In addition, some genes encoding enzymes involved in the production of dNTPs are also expressed periodically. These include thymidylate kinase, thymidylate synthetase and ribonucleotide reductase. These genes, as well as the B-type cyclin genes, are all activated at or soon after cells undergo START. This set of periodically expressed replication and cell cycle control genes appears to be coordinately regulated by a transcription factor (DSC1) that recognizes an upstream element consisting of one or more copies of the 6 bp sequence ACGCGT, the MutI restriction sequence. Similar ACGCGT elements and near matches are also involved in regulating a DNA synthesis gene in Schizosaccharomyces pombe. In this case, binding sites for the regulatory factor are present at sites located both upstream and downstream of the transcription start site (Lowndes et al., 1992). Although no perfect ACGCGT sequences are present in the TOP2 5′ US sequence contained in pdN-1, two near matches of 5 out of 6 are present. However, even if the DSC1 control mechanism is conserved in trypanosomatids, the recognition sequence may not be.

While the similarity to the periodic expression observed for yeast DNA replication genes suggests that a similar mechanism of regulation may be involved in the regulation of trypanosomatid DNA replication genes, post-transcriptional mechanisms require consideration as well. This alternative is suggested by the generally unsuccessful search for RNA polymerase II promoters in trypanosomatids. The current view of modulation of gene expression in trypanosomatids is that regulation is primarily achieved post-transcriptionally by regulatory loops that involve pre-mRNA turnover in combination with differential rates of trans-splicing and polyadenylation, and mRNA turnover (LeBowitz et al., 1993; Matthews et al., 1994; Huang and Van der Ploeg, 1991). In this alternative view, an unstable trans-acting factor affecting RNA splicing of the DNA replication genes might be expressed periodically during the cell cycle. Such a factor might be similar to the Drosophila tra-2 protein whose binding to dsx pre-mRNA appears to be necessary for sex-specific splicing and polyadenylation (Hedley and Maniatis, 1991).

So far, a promoter responsible for expression of TOP2 has not been identified, however, since many protein-coding genes in trypanosomes are transcribed polycistronically from putative upstream promoters (Johnson et al., 1987), transcription may possibly initiate at some distance. Even in the event that the chromosomal TOP2 might be expressed as a polycistronic transcript, the periodic accumulation of the 2.0 kb TOP2 transcript expressed from the pdN-1 plasmid indicates that cis-acting sequences necessary for regulation are contained within the deletion clone. With the availability of the pdN-1 plasmid, it will now be possible to identify and characterize the cis elements required for the periodic expression of the TOP2 gene.

In light of the early divergence of trypanosomes from the main eukaryotic lineage, relative to the divergence of plants, animals and fungi (Sogin, 1991), the cell cycle regulation of DNA replication gene expression may be a common feature of all eukaryotes. The experiments presented here suggest that coordinate expression of nuclear and mitochondrial DNA replication genes may be responsible, at least in part, for the nearly-coincident initiation of nuclear and mitochondrial DNA replication in trypanosomatids. Further dissection of the regulatory network responsible for the periodic expression of these genes will be required to establish where the factors involved in coordinating nuclear and mitochondrial S phases fit into the hierarchy of regulatory molecules controlling progression through the cell cycle.

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