Cell Cycle-Dependent Localization and Properties of a Second Mitochondrial DNA Ligase in *Crithidia fasciculata*

Krishna Murari Sinha, Jane C. Hines, and Dan S. Ray*

Molecular Biology Institute and Department of Microbiology, Immunology and Molecular Genetics, University of California at Los Angeles, Los Angeles, California 90095

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The mitochondrial DNA in kinetoplastid protozoa is contained in a single highly condensed structure consisting of thousands of minicircles and approximately 25 maxicircles. The disk-shaped structure is termed kinetoplast DNA (kDNA) and is located in the mitochondrial matrix near the basal body. We have previously identified a mitochondrial DNA ligase (LIG kβ) in the trypanosomatid *Crithidia fasciculata* that localizes to antipodal sites flanking the kDNA disk where several other replication proteins are localized. We describe here a second mitochondrial DNA ligase (LIG kα). LIG kα localizes to the kinetoplast primarily in cells that have completed mitosis and contain either a dividing kinetoplast or two newly divided kinetoplasts. Essentially all dividing or newly divided kinetoplasts show localization of LIG kα. The ligase is present on both faces of the kDNA disk and at a high level in the kinetoflagellar zone of the mitochondrial matrix. Cells containing a single nucleus show localization of the LIG kα to the kDNA but at a much lower frequency. The mRNA level of LIG kα varies during the cell cycle out of phase with that of LIG kβ. LIG kα transcript levels are maximal during the phase when cells contain two nuclei, whereas LIG kβ transcript levels are maximal during S phase. The LIG kα protein decays with a half-life of 100 min in the absence of protein synthesis. The periodic expression of the LIG kα transcript and the instability of the LIG kα protein suggest a possible role of the ligase in regulating minicircle replication.

The mitochondrial DNA of trypanosomatid protozoa, termed kinetoplast DNA (kDNA), is a network of catenated circular DNAs consisting of interlocked minicircles and maxicircles (19, 40). The kDNA of *Crithidia fasciculata* contains approximately 5,000 minicircles and 25 maxicircles. The minicircles are 2.5 kb in size and encode small guide RNAs, which are involved in editing of maxicircle transcripts (44). Maxicircles range in size from 23 kb for *Trypanosoma brucei* to approximately 37 kb for *C. fasciculata* and encode rRNAs and mitochondrial proteins involved in oxidative metabolism (43). In vivo, *C. fasciculata* kDNA is condensed into a disk-shaped structure, 1 µm in diameter and ~0.4 µm thick located at the base of the flagellum, with the flagellum perpendicular to the face of the disk (13, 26). Minicircles in the disk are relaxed, unlike most other circular DNAs in nature, which are negatively supercoiled (36). Electron microscopic studies of sections through the kDNA disk suggest that minicircles are stretched taut and aligned parallel to the axis of the disk, whereas maxicircles appear to be present as a catenated network within the overall network (26, 38, 40).

Replication of kDNA takes place in approximate synchrony with nuclear DNA during S phase (41, 49) unlike in higher eukaryotes, where mitochondrial DNA replicates throughout the cell cycle (6, 14, 48). Each minicircle replicates only once in every cycle, leading to a doubling of the size of the kDNA network, which then segregates into two daughter networks with each cell receiving one progeny network. The unusual kDNA structure and its replication mechanism have been the focus of several recent reviews (19, 20, 25, 40). These studies have shown that different steps of kDNA replication are carried out at discrete sites in and around the kDNA by protein complexes localized at those sites (19). Minicircles are released from the kDNA vectorially as covalently closed circles prior to initiation of replication in a specialized zone called the kinetoflagellar zone (KFZ) at the flagellar face of the kDNA disk (9). Universal minicircle sequence-binding protein, which binds to the two minicircle origins (1) and localizes to two neighboring sites on the flagellar face of the kDNA disk (2), likely plays a role in the initiation of replication. Multiple mitochondrial DNA polymerases have been identified in *T. brucei*, a related kinetoplastid, and RNA interference studies have identified some as being involved in kDNA replication (21). Minicircle replication is RNA primed (5, 31), and the RNA primers are likely synthesized by a DNA primase that localizes to the two minicircle origins (1) and localizes to two neighboring sites on the flagellar face of the kDNA disk (2), likely plays a role in the initiation of replication. Multiple mitochondrial DNA polymerases have been identified in *T. brucei*, a related kinetoplastid, and RNA interference studies have identified some as being involved in kDNA replication (21). 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β (13), structure-specific endonuclease (SSE1) (10), and the mitochondrial DNA ligase, LIG kβ (42). SSE1 has RNase H activity and has been implicated in the removal of RNA primers (15), whereas DNA Pol β, a nonprocessive DNA polymerase, is proposed to fill discontinuities between Okazaki fragments (45). The coimmunoprecipitation of LIG kβ and DNA Pol β suggests that they function together to repair gaps between Okazaki fragments (42). Minicircle replication intermediates are only partially repaired prior to their reattachment to the kDNA network by Topo II (47), with nicks and gaps remaining at the replication origins, which are not closed until all minicircles have replicated. The final sealing of all discontinuities occurs only after all minicircles have been replicated and precedes kinetoplast division (13).

We report here the identification of a second mitochondrial DNA ligase from C. fasciculata. We have named it LIG kα based on its kinetoplast localization and its location upstream of LIG kβ. Localization of LIG kα specifically to dividing kinetoplasts during kinetoplast division and segregation suggests that LIG kα may play a role in the final sealing of discontinuities at the replication origins.

**MATERIALS AND METHODS**

Cloning of the LIG kα gene of C. fasciculata. A search of Leishmania major and Trypanosoma brucei genome databases revealed the presence of two putative DNA ligase genes. We have termed these genes LIG kα and LIG kβ (8). A phage isolate obtained by screening a C. fasciculata genomic library in λgEM-11 (Promega) to identify the C. fasciculata LIG kβ gene was subsequently used for identification of the upstream LIG kα gene. A Southern blot of the phage DNA probed with a MuI-Sall 540-bp restriction fragment containing the 5′-flanking region of the LIG kβ gene identified an approximately 7.0-kb Sall fragment, which was cloned into similarly digested pGEM-SZI (+) (Promega) to create the plasmid pLigA2. Sequence analysis of pLigA2 showed that it contained both the LIG kβ and the LIG kα coding sequences (accession number AY380335).

**Episomal expression of epitope-tagged LIG kα.** pX.2-KO (32) was used to construct an expression vector. pLigA2 was used as a template to amplify a 1561-bp fragment containing 900 bp of the 5′-flanking region of LIG kα, along with 661 bp of coding sequence by using primers M36 (GGTACCATTAGCT TGCCAGCAGCA) and M11 (TACAGGCGACAGCAGCAG), and then subcloned into pCRII-TOPO (Invitrogen), yielding plasmid pLigA9. pLigA2 was also engineered to remove one Bspl120I site and introduce an in-frame Bspl120I site just before the termination codon of LIG kα, yielding plasmid pLigA.8. A KpnI- to NcoI digest of pLigA9 released a 1.143-bp fragment providing upstream portions of LIG kα, whereas a NcoI-to-Sall digest of LigA.8 released the remainder of the gene with three copies of the HA epitope tag on a 2.914-bp fragment. These fragments were joined with KpnI-Sall-digested pX.2-KO to produce the expression plasmid pLigA.10. Wild-type C. fasciculata cells were transfected with pLigA.10 and selected on agar plates containing Difco brain heart infusion medium and 50 μg of G418/ml as described previously (32).

**Immunolocalization of LIG kα.** Immunofluorescent localization of HA epitope-tagged LIG kα was performed essentially as described previously (26). In brief, 2 × 10⁵ cells expressing HA-tagged LIG kα were harvested from either an asynchronous culture at 20°C overnight and rehydrated by three washes in PBS, passed through a 18-gauge needle five times and centrifuged at 15,000 × g for 15 min at 4°C. The supernatant was filtered through a 0.45-μm pore-size filter and purified by using His-Bind resin (Novagen). The protein was eluted in 20 mM Tris-HCl (pH 8.0)–0.5 M NaCl with 0.3 M imidazole. A 100-μl aliquot of the peak fraction was loaded onto a 1-mL G25 column in 20 mM Tris-HCl (pH 8.0)–0.1 mM EDTA and fractions of 100 μl were collected. An equal volume of glycerol was added to the eluted fractions, and the protein was stored at −20°C.

**Adenylation and deadenylation of recombinant LIG kα.** Adenylation reactions were performed by using recombinant LIG kα protein in reactions containing 10 μl of 20 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.1 mM dithiothreitol, 8% glycerol, 0.02% Triton X-100, 0.2 μCi of [α-32P]ATP, and 20 ng of the recombinant protein. Reactions were incubated at 25°C for 15 min. For deadenylation of the ligase-AMP, the reactions were further incubated with 2 μl of DNAse I-treated calf thymus DNA (2.5 μg/μl), 10 μl of poly[d(A)·d(T)]20 (250 μg/μl), and 10 μl of poly[d(C)·d(G)]·oligo(dT)₅₂ (250 μg/μl) at 25°C for 15 min. Both the adenylation and deadenylation reactions were stopped by boiling with sodium dodecyl sulfate sample buffer for 5 min, and the products were separated by polyacrylamide gel electrophoresis. The gel was stained with 0.3% methanol and 10% acetic acid for 30 min, dried, and autoradiographed (42).

**DNA ligase activity of recombinant LIG kα.** The DNA ligase activity of recombinant LIG kα was assayed by using 5′-end-labeled oligo(dT)₅₂ annealed to poly(dA). The substrate was incubated with various amounts of recombinant
LIG kα at 27°C for 3 h in ligase buffer (Gibco-BRL). The ligation reaction was stopped by heating it at 85°C for 3 min, and the ligation products were analyzed on a 12% polyacrylamide gel containing 6 M urea.

RESULTS

Identification of a second kinetoplast DNA ligase gene in C. fasciculata. We have previously described the identification and characterization of a kinetoplast DNA ligase (LIG kβ) in C. fasciculata. This identification was based on the presence of a putative mitochondrial DNA ligase gene in the L. major genome database (42). Subsequent examination of both the T. brucei and L. major genome databases revealed the presence of a second putative mitochondrial DNA ligase gene (LIG kα) upstream of LIG kβ. We have cloned the C. fasciculata LIG kα gene and describe here the characterization of the encoded ligase. The open reading frame of the C. fasciculata LIG kα consists of 663 amino acid residues and contains six colinear motifs characteristic of ATP-dependent DNA ligases (46), including the conserved active site motif–K-DG– (amino acid residues 283 to 286), which is essential for ligase activity. The predicted LIG kα protein does not contain a mitochondrial leader sequence at its N terminus similar to the 9-amino-acid cleavable presequences present in several C. fasciculata kinetoplast proteins (45, 50). However, the MitoProt II computational method for predicting mitochondrial imported proteins predicts mitochondrial import with a probability score of 0.9895. Like LIG kβ, LIG kα does not have significant amino acid sequence homology outside of the conserved motifs with any other known ligases, including human mitochondrial DNA ligase, LIG III (22). The C. fasciculata LIG kα has 37% identity with the T. brucei LIG kα and 58% identity with the L. major LIG kα. The C. fasciculata LIG kα and LIG kβ are only 24% identical (8).

Localization of LIG kα is cell cycle dependent. Immunostaining of an asynchronous C. fasciculata culture expressing HA-tagged LIG kα from a plasmid construct showed that localization of LIG kα was not detected in all cells but was primarily observed in cells undergoing division and having a dividing kinetoplast or recently divided kinetoplast and two nuclei (Fig. 1A, a to c; B, a to h; and D, a to b; and Table 1). Some cells containing a single nucleus that also show LIG kα localization possibly represent newly divided cells in which the localization is still observable (Fig. 1A, d to f, and C, a and b). In cells from a synchronized culture harvested at a time when ca. 30% of the cells contain either two nuclei and a dividing kinetoplast or two nuclei and two newly divided kinetoplasts, essentially all of the dividing cells show kinetoplast localization of LIG kα. The lack of detection of localization of LIG kα in a few of the dividing cells may be a consequence of the signal being below the level of detection in those cells.

In Fig. 1A (a and d) and B (a) localization of LIG kα appears to localize to the two faces of kDNA disk in dividing cells and in a cell containing a single nucleus and a nondividing kinetoplast. An unstained central region is evident in these kinetoplasts, indicating an absence of the ligase in the interior of the disk. Although the epitope-tagged protein appears to localize on both faces of the kinetoplast disk, the localization observed in Fig. 1B (d and h) and D (a) is more pronounced on the flagellar face of the disk in the KFZ, which is proposed to be the site for initiation of minicircle replication (9). Figure 1C shows a pair of cells each having a single nucleus but with the upper cell having greater localization of LIG kα in the KFZ, while the lower cell has greater localization of LIG kβ on the opposite face of the kDNA disk. The significance of this latter localization is unknown. LIG kβ colocalizes with DNA Pol β, Topo II, and SSE1 at the two antipodal sites (42) but to a lesser degree also to the two faces of the kDNA disk similar to that of DNA primase (24).

Adenylation and DNA ligase activity of the recombinant LIG kα. To demonstrate the enzymatic activity of the ligase protein, a His-tagged form of the recombinant LIG kα was expressed and purified from E. coli (Fig. 2). Although a high level of expression of the recombinant protein was obtained (Fig. 2A, lane 2), the protein was mostly insoluble and was removed by centrifugation. However, a sufficient amount of soluble protein remained in the supernatant (Fig. 2A, lane 3) for further purification by metal chelate affinity chromatography and gel filtration (Fig. 2B). The purified recombinant LIG kα was assayed for its adenylation, deadenylation, and DNA ligase activities. ATP-dependent DNA ligases form a ligase-AMP complex as an intermediate in the ligation reaction. Recombinant LIG kα was adenylated in the presence of [α-32P]ATP as shown in Fig. 3A, lane 1. The 32P-labeled ligase-AMP intermediate was subsequently discharged upon incubation with DNA ligase substrates (lanes 3 to 5). Nicked calf thymus DNA and oligo(dT)20 annealed to poly(dA) or to poly(rA) were all effective in deadenylation of the ligase-AMP. DNA joining activity of the recombinant protein was also demonstrated in reactions using 5′-end-labeled oligo(dT)20 annealed to poly(dA) as a substrate. Analysis of the reaction products on a denaturing polyacrylamide gel showed that the oligonucleotides were ligated into higher oligomers (Fig. 3B), confirming the identification of the protein as a DNA ligase.

Transcripts of LIG kα and LIG kβ vary out of phase with each other during the cell cycle. In light of the cell cycle-dependent localization of LIG kα protein, we have investigated the variation in transcript levels of the LIG kα and LIG kβ genes during the cell cycle. A Northern blot analysis of the transcript levels of LIG kα, LIG kβ, and DHFR-TS in a synchronized wild-type C. fasciculata cell culture showed that the variation in the transcript levels of both LIG kα and LIG kβ during the cell cycle are out of phase with one another (Fig. 4). The variation in the transcript level of LIG kβ is identical to that of DHFR-TS, which was shown previously to be maximal during S phase and minimal during cell division (32). The transcript levels of both LIG kβ and DHFR-TS peak immediately after the release from a hydroxyurea block when the cells are in S phase and then increase again during 180 to 210 min after hydroxyurea release during the S phase of the next cycle. In contrast, the transcript levels of LIG kα are maximal during 60 to 120 and 210 to 270 min after hydroxyurea release. These periods correspond to the passage of the cells through mitosis and subsequent cell division.

Cycling sequences flanking the LIG kα and LIG kβ genes. Previous studies identified an octameric consensus sequence CAUAGAAA(A/G) present in the 5′ and/or 3′ untranslated regions of mRNAs encoding several DNA replication proteins in C. fasciculata (3, 7, 27, 30, 32, 34). The central hexamer
AUAGAA is highly conserved in these transcripts and has been used, along with additional constraints, to screen the genome database of Leishmania major, a closely related parasite (51). This screen was highly successful in identifying genes expressed during S phase in L. major. We have examined the DNA sequences flanking the C. fasciculata LIG kα/LIG kβ genes for the presence of the conserved hexamer sequence. Although the LIG kβ transcript cycles similarly to those of TOP2, KAP3, RPA1, and DHFR-TS, the hexamer sequence is not present within sequences flanking the LIG kβ gene (Fig.

![Image](image_url)

**TABLE 1. Localization of LIG kα in 1N and 2N cells**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Total no. of cells</th>
<th>No. of ligases localized</th>
<th>% Localized</th>
</tr>
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<tbody>
<tr>
<td>Exponential culture&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1N</td>
<td>375</td>
<td>41</td>
<td>11</td>
</tr>
<tr>
<td>2N</td>
<td>11</td>
<td>8</td>
<td>73</td>
</tr>
<tr>
<td>Synchronous culture&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1N</td>
<td>114</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>2N</td>
<td>42</td>
<td>40</td>
<td>95</td>
</tr>
</tbody>
</table>

<sup>a</sup> Exponential culture at $2 \times 10^7$ cells per ml.

<sup>b</sup> Synchronized culture harvested 120 min after hydroxyurea release.

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**FIG. 1. Immunolocalization of HA-tagged LIG kα.** (A) A dividing cell stained with mouse HA.11 antibodies conjugated with Alexa Fluor 488 (a) and DAPI (b) and a phase-contrast image (c) are shown. A cell with a single nucleus stained overnight with mouse HA.11 antibodies conjugated with Alexa Fluor 488 (d) and with DAPI (e) and a phase-contrast image (f) are also shown. (B) Dividing cells stained with mouse 12CA5 antibodies and goat anti-mouse IgG conjugated with Alexa Fluor 594 and with rabbit anti-RPA1 and goat anti-rabbit IgG conjugated with Alexa Fluor 488 to stain the nucleus (a and c) and DAPI (b), a DAPI and AlexaFluor488 merged image (f), phase images (c and g), and merged images (d and h) are shown. (C) Cells with a single nucleus stained with mouse 12CA5 antibodies and goat anti-mouse IgG conjugated with AlexaFluor 594 and with DAPI (a) and a phase image (b). (D) Confocal image of a dividing cell stained with mouse HA.11 antibodies conjugated with Alexa Fluor 488 and with DAPI shown in false color (red) (a); a phase image is also shown (b).
However, there are five copies of the sequence ATAGA in 5’ and 3’ flanking sequences and one copy of the sequence CATAGAGG. DNA LIG kα on the other hand, has two copies of the conserved hexamer ATAGAA, one of which is within the sequence CATAGAA (Fig. 5). Transcripts of LIG kα would have been predicted to cycle in the same manner as those of TOP2, KAP3, RPA1, and DHFR-TS. Instead, the LIG kα mRNA cycles out of phase with the other cycling transcripts.

**LIG kα is unstable.** Since ligation of remaining discontinuities at the replication origins of newly synthesized minicircles appears to be inhibited until all minicircles have been replicated, periodic expression of LIG kα mRNA would be particularly relevant if the LIG kα protein had a short half-life. We have therefore investigated the possibility that LIG kα might be an unstable protein. We examined the relative levels of LIG kα by Western blotting of cell extracts from cells in which protein synthesis was inhibited by cycloheximide (Fig. 6A). LIG kα was observed to decay with a half-life of approximately 100 min under conditions where the cell doubling time was 3 to 3.5 h, whereas the C. fasciculata CSBPA protein was stable. No turnover of LIG kβ was observed under identical conditions (Fig. 6B). The striking difference in the signals of CSBPA in Fig. 6A and B is due to the much longer exposure of the blot in Fig. 6A required to detect the LIG kα signal and reflects a lower abundance of LIG kα compared to that of LIG kβ.

**DISCUSSION**

The repair of discontinuities in newly replicated minicircles is a highly regulated process and may play an important role in regulating kDNA replication (25, 40). Many, but not all, discontinuities in the newly synthesized minicircle strands are filled and sealed before their attachment to the kDNA network. Pol β and the SSE1 nuclease, present at the antipodal sites, have been shown to be capable of primer removal and gap filling (15). LIG kβ, also present at the antipodal sites is proposed to be involved in joining Okazaki fragments at these sites. However, discontinuities at the replication origins consist of a nick or gap of a few nucleotides and are particularly resistant to closure (5, 18). These remaining discontinuities have been suggested to distinguish the replicated from unreplicated minicircles (12). Once all of the minicircles are replicated there is a final sealing of the discontinuities before the segregation of progeny kDNA networks to the daughter cells (35).

We have sought to identify the proteins involved in the late steps of kDNA replication. The LIG kα identified here is an ATP-dependent DNA ligase and is only distantly related to...
other mitochondrial DNA ligases and bacterial DNA ligases. Unlike the *E. coli* DNA ligase that uses NAD$^+$ for activation of the enzyme, LIG $\kappa$ utilizes ATP and forms an enzyme-AMP intermediate in the ligase reaction like most other eukaryotic DNA ligases (23).

LIG $\kappa$ showed a cell cycle-dependent localization to the kDNA disk and was also concentrated in the KFZ. Localization of LIG $\kappa$ was observed primarily in cells that had undergone mitosis and contained a dividing kinetoplast or newly divided kinetoplast. In some of these dividing cells LIG $\kappa$ was present on both faces of the kDNA disk in addition to being present in the KFZ. Cells containing only a single nucleus rarely showed kinetoplast localization of LIG $\kappa$ and possibly represent newly divided cells with residual LIG $\kappa$ associated with the kDNA. In these cells LIG $\kappa$ was often present on the opposite faces of the kDNA disk and was absent from the interior of the disk.

Cell cycle-dependent localization of kinetoplast DNA replication proteins is a common feature of several kinetoplast replication proteins and has been observed for DNA Pol $\beta$, Topo II (16), and SSE1 (10) in *C. fasciculata* cells. These

![FIG. 4. Transcript levels of LIG $\kappa$ and LIG $\kappa\beta$ genes during the cell cycle.](image)

**FIG. 4.** Transcript levels of LIG $\kappa$ and LIG $\kappa\beta$ genes during the cell cycle. (A) Transcript levels of LIG $\kappa$, LIG $\kappa\beta$, and DHFR-TS genes in a synchronized *C. fasciculata* culture were analyzed by Northern blot after release from a hydroxyurea block. Numbers at the top indicate the time at which cell aliquots were collected for RNA isolation after hydroxyurea release. (B) PhosphorImager quantitation of the transcript levels in panel A. The relative transcript levels are shown as a function of time after release from the hydroxyurea block.

![FIG. 5. Sequences related to mRNA cycling sequence elements flanking kinetoplast DNA ligase genes.](image)

**FIG. 5.** Sequences related to mRNA cycling sequence elements flanking kinetoplast DNA ligase genes. The highly conserved central hexamer ATAGAA of the consensus octamer cycling sequence (7, 34) and the related sequence ATAGA are shown flanking the coding sequences of LIG $\kappa$ and LIG $\kappa\beta$. Other mitochondrial DNA ligases and bacterial DNA ligases. Unlike the *E. coli* DNA ligase that uses NAD$^+$ for activation of the enzyme, LIG $\kappa$ utilizes ATP and forms an enzyme-AMP intermediate in the ligase reaction like most other eukaryotic DNA ligases (23).

LIG $\kappa$ showed a cell cycle-dependent localization to the kDNA disk and was also concentrated in the KFZ. Localization of LIG $\kappa$ was observed primarily in cells that had undergone mitosis and contained a dividing kinetoplast or newly divided kinetoplast. In some of these dividing cells LIG $\kappa$ was present on both faces of the kDNA disk in addition to being present in the KFZ. Cells containing only a single nucleus rarely showed kinetoplast localization of LIG $\kappa$ and possibly represent newly divided cells with residual LIG $\kappa$ associated with the kDNA. In these cells LIG $\kappa$ was often present on the opposite faces of the kDNA disk and was absent from the interior of the disk.

Cell cycle-dependent localization of kinetoplast DNA replication proteins is a common feature of several kinetoplast replication proteins and has been observed for DNA Pol $\beta$, Topo II (16), and SSE1 (10) in *C. fasciculata* cells. These

![FIG. 6. Turnover of LIG $\kappa$ in the absence of protein synthesis.](image)

**FIG. 6.** Turnover of LIG $\kappa$ in the absence of protein synthesis. *C. fasciculata* cultures expressing HA epitope-tagged LIG $\kappa$ (A) and HA epitope-tagged LIG $\kappa\beta$ (B) were treated with cycloheximide at 100 $\mu$g/ml. Cell aliquots were collected at 1-h intervals and analyzed by Western blotting of total cell extracts by probing with 12CA5 monoclonal antibodies and rabbit polyclonal antibodies to the CSBPA protein used as loading control.
proteins are localized to antipodal sites at the edges of the kDNA disk. Localization of the *C. fasciculata* universal minicircle sequence-binding protein was also observed to vary during the cell cycle (2). However, unlike these examples, the localization of LIG kα appears to be specific for dividing kinetoplasts or newly divided kinetoplasts. Periodic assembly of kinetoplast replication proteins at distinct sites relative to the kDNA disk may dictate the timing and order of individual steps in kDNA replication as cells progress through the cell cycle.

The variation in the transcript level of LIG kα differs from those of *TOP2, KAP3, RPA1, and DHFR-TS* (32), transcripts that are expressed at their highest levels during S phase. The transcript level of LIG kα is low after the release from a hydroxyurea block when the cells are in S phase and then increases 90 to 120 min after the release when the cells are going through mitosis and cell division. This also corresponds to a phase of the cell cycle when nuclear S phase has been completed but kinetoplast duplication is not yet completed, as evidenced by the presence of cells containing two nuclei but still have a single elongated kDNA that has not yet divided. The possible links between the expression of the LIG kα transcript, the cell cycle-dependent kDNA localization of the LIG kα protein, and the presence of networks having all of the minicircles nicked/gapped at the replication origins remain to be investigated.

There is still much to be learned concerning the mechanisms of cell cycle regulation of mRNA levels in trypanosomatids and analysis of the cycling of the LIG kα and LIG kβ transcripts should provide new opportunities for further defining the factors regulating transcript levels. Even though sequences flanking LIG kβ do not contain the consensus octamer sequence CATAGA(A/G) that has been shown to be necessary for cycling of the *TOP2, KAP3, RPA1, and DHFR-TS* transcripts, the LIG kβ transcripts nonetheless cycle in the same manner. There are, however, five copies of the sequence ATAGA and a single copy of the sequence CATAGAG near the 5′ and 3′ termini of the LIG kβ coding sequence. Earlier studies showed that multiple copies of closely related sequences including a single copy of the sequence CATAGACC were sufficient to confer cycling on a truncated form of the RPA1 gene (7). In addition, binding to the octamer consensus sequence by the cycling sequence binding protein CSBP in *C. fasciculata* extracts is reduced but not eliminated by an A-to-C substitution at nucleotide 7 in the octamer sequence (nucleotide 6 in the conserved hexamer core) (27). In contrast, single nucleotide substitutions in any of the first five nucleotides of the hexamer essentially abolished binding by CSBP. Taken together, these results implicate the sequence ATAGA as the most important element in the cycling of these transcripts.

It is much less clear why the LIG kα transcripts do not cycle in the same manner but are 180 degrees out of phase with transcripts of these other genes. The LIG kα gene has two copies of the conserved hexamer sequence 5′ of the LIG kα coding sequence, one of which is in the sequence CATAGGA. It also contains the sequence ATAGAG just 3′ of the LIG kα coding sequence. This result suggests that whereas these conserved sequence elements may be required for transcript cycling in the manner of *TOP2, KAP3, RPA1, and DHFR-TS*, they are not sufficient. Further examination of the factors involved in the cycling of the LIG kα transcripts may reveal new aspects of the cycling mechanism.

There are previous instances where multiplets of replication proteins have been found in kinetoplastid parasites. *T. brucei* has four mitochondrial Pol I-like DNA polymerases (21) and two DNA Pol β-like polymerases (37). Distinct localizations of these polymerases may be indicative of distinct roles in kDNA replication. The different localization and expression patterns of LIG kα and LIG kβ may also reflect involvement in different stages of kDNA replication. LIG kβ is suggested to be primarily involved in the repair of Okazaki fragments at the two antipodal sites (42), whereas LIG kα may be involved in the final sealing of the discontinuities at the origin of the newly replicated minicircles. LIG kα may have additional roles as well. The higher level of LIG kα in the KFZ where minicircle replication initiates suggests that LIG kα might also participate in joining Okazaki fragments in nascent minicircles prior to their attachment to the antipodal sites. Regulation of LIG kα at the levels of periodic mRNA expression, protein stability, and kinetoplast localization suggests that LIG kα may play a key role in the timing of the final closure of the remaining discontinuities in the minicircle population prior to scission of the double-size networks.

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