

Intramitochondrial Localization of Universal Minicircle Sequence-binding Protein, a Trypanosomatid Protein that Binds Kinetoplast Minicircle Replication Origins

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Abstract. Kinetoplast DNA (kDNA), the mitochondrial DNA of the trypanosomatid *Crithidia fasciculata*, is a unique structure containing 5,000 DNA minicircles topologically linked into a massive network. In vivo, the network is condensed into a disk-shaped structure. Replication of minicircles initiates at unique origins that are bound by universal minicircle sequence (UMS)-binding protein (UMSBP), a sequence-specific DNA-binding protein. This protein, encoded by a nuclear gene, localizes within the cell's single mitochondrion. Using immunofluorescence, we found that UMSBP localizes exclusively to two neighboring sites adjacent to the face of the kDNA disk nearest the cell's flagellum. This site is distinct from the two antipodal positions at

the perimeter of the disk that is occupied by DNA polymerase β , topoisomerase II, and a structure-specific endonuclease. Although we found constant steady-state levels of *UMSBP* mRNA and protein and a constant rate of UMSBP synthesis throughout the cell cycle, immunofluorescence indicated that UMSBP localization within the kinetoplast is not static. The intramitochondrial localization of UMSBP and other kDNA replication enzymes significantly clarifies our understanding of the process of kDNA replication.

Key words: kinetoplast DNA • kDNA replication • universal minicircle sequence • UMS-binding protein

Introduction

Kinetoplast DNA (kDNA)¹ is the mitochondrial DNA in flagellated protozoa related to the trypanosomes. These parasites have only a single mitochondrion. In the species *Crithidia fasciculata*, this unusual DNA contains ~25 maxicircles (38 kb each) and 5,000 minicircles (2.5 kb each), all of which are catenated into a single giant network. Maxicircles have a genetic function similar to that of mitochondrial DNAs in other eukaryotes, and minicircles encode guide RNAs that control the specificity of editing of maxicircle transcripts. The kDNA network is condensed into a disk-shaped structure within the matrix of the cell's mitochondrion, and it is always positioned near

the basal body of the flagellum (for reviews on kDNA see Ray, 1987; Shlomai, 1994; Shapiro and Englund, 1995).

This paper concerns the unusual replication mechanism of the kDNA network. The replication process takes place during a discrete phase of the cell cycle, approximately concurrent with the nuclear S phase (Cosgrove and Skeen, 1970). Minicircle replication occurs after release of individual molecules from the network. Each free minicircle replicates as a θ structure, forming two progeny that contain gaps. Although many gaps in free minicircles are repaired quickly, some persist even after the minicircle progeny are reattached to the network. Since minicircle reattachment occurs exclusively at the network periphery, the replicating network develops a peripheral zone of gapped minicircles surrounding a central zone of covalently closed minicircles that have not yet undergone replication. As replication progresses, the central zone shrinks and the peripheral zone enlarges. Finally, when network replication is completed the minicircle copy number has doubled, and they all are gapped. Then the gaps are repaired, and finally the network splits in two, a process probably mediated by a topoisomerase II. The result is two progeny networks,

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¹Abbreviations used in this paper: CaBP, calcium-binding protein; kDNA, kinetoplast DNA; pol, DNA polymerase; SSE1, structure-specific endonuclease I; TdT, terminal deoxynucleotidyl transferase; UMS, universal minicircle sequence; UMSBP, UMS-binding protein.

each identical to the parent, and these segregate into the daughter cells during cytokinesis (for reviews on kDNA replication see Ray, 1987; Shlomai, 1994; Shapiro and Englund, 1995).

kDNA replication enzymes are positioned in discrete sites around the kinetoplast disk. Three histone-like proteins (p16, p17, and p18; Xu et al., 1996) localize throughout the kDNA. A DNA topoisomerase II (Melendy et al., 1988), a DNA polymerase (β) (Ferguson et al., 1992), and a structure-specific endonuclease I (SSE1) (Engel and Ray, 1999) are positioned together in antipodal sites, which flank the kinetoplast disk. These sites also contain free minicircle replication intermediates (Ferguson et al., 1992; Johnson and Englund, 1998). In contrast, the mitochondrial DNA primase and p21 localize in two sites adjacent to the faces of the kDNA disk, forming a sandwich around the kDNA (Li and Englund, 1997; Hines and Ray, 1998; Johnson and Englund, 1999).

In this paper, we describe the intramitochondrial localization of the universal minicircle sequence (UMS)-binding protein (UMSBP). This protein, a homodimer containing 13.7-kD subunits, binds to the minicircle-leading strand replication origin, a conserved sequence known as the UMS (Tzfati et al., 1992, 1995; Abeliovich et al., 1993; Avrahami et al., 1995). Recent studies have found that USBP also binds a second site on the minicircle sequence, the proposed initiation site for the first Okazaki fragment of the minicircle discontinuously synthesized strand (Abu-Elneel et al., 1999). USBP may function as a major control protein, whose role is to recognize and bind the replication origin during minicircle replication. We now report that USBP localizes to unique positions within the cell mitochondrion near the kDNA disk. It is positioned in two zones adjacent to the face of the kDNA disk nearest the flagellum. We also report on changes in localization and abundance of USBP that occur during the cell cycle and describe the significant implications of this localization to the kDNA replication mechanism.

Materials and Methods

Cells

C. fasciculata cultures were maintained at 28°C with agitation (150–200 rpm) in brain–heart infusion (Difco) containing 10 μ g/ml hemin. Cultures were synchronized by hydroxyurea arrest as described previously (Pasion et al., 1994) except that the synchronization was conducted at 26°C.

Cell Lysates

Cleared cell lysates (fraction I) were prepared by the gentle disruption of the *C. fasciculata* cell membrane using a nonionic detergent in hypotonic solution, as described previously (Shlomai and Linial, 1986), except that 0.2% (wt/vol) Brij-25 was used (Tzfati et al., 1995).

Antibodies

Anti-UMSBP sera were prepared by immunizing female BALB/c mice by intraperitoneal injections with purified recombinant protein (50 μ g per injection). The initial inoculation was in Freund's complete adjuvant, and six subsequent boosts were at 3-wk intervals using Freund's incomplete adjuvant. The serum (at 1:2,000 dilution) was screened by Western blot analysis for specific recognition of USBP. Affinity purified (1:1,000) rabbit anti-pol β antibodies (prepared against recombinant protein) were described previously (Johnson and Englund, 1998).

Immunofluorescence

Unless otherwise stated, all steps were performed at room temperature and a humid chamber was used for incubations. Cells (2×10^6) in PBS were spread on poly-L-lysine-coated slides and allowed to adhere for 20 min. The cells were then fixed in 4% (wt/vol) paraformaldehyde for 5 min. Fixation was stopped by two consecutive washes in PBS containing 0.1 M glycine, pH 8.6, for 5 min. Slides were washed once in PBS containing 0.025% (vol/vol) Triton X-100 for 5 min and once in PBS for 3 min, and then immersed in methanol at -20°C for 1 h. After fixation, slides were rehydrated with three 5-min washes in PBS before a 30-min incubation in blocking buffer containing 1% (wt/vol) BSA in PBS containing 0.1% (vol/vol) Tween-20 (PBST). The slides were incubated for 90 min with primary antibody diluted in blocking buffer, 1:20 for anti-UMSBP and 1:50 for anti-pol β (when double immunolocalization was performed). Slides were washed three times for 5 min in PBST, and then were incubated for 45 min with the secondary antibody as follows: for USBP, a FITC-rat anti-mouse monoclonal antibody (Sigma-Aldrich) diluted 1:20 and for pol β , a Texas red goat anti-rabbit (Molecular Probes) diluted 1:250. Slides were washed three times for 5 min in PBST, stained with 0.1 μ g/ml DAPI (Sigma-Aldrich) in PBS, followed by one wash with PBS. Finally, slides were mounted in Vectashield (Vector Laboratories) as an antifade agent.

In Situ Labeling of kDNA Networks by Alexa-dUTP and Terminal Deoxynucleotidyl Transferase

The method based on the procedure of Gavrieli et al. (1992), which specifically labels the gaps in kDNA minicircles either free or in networks, was conducted as described previously (Guilbride and Englund, 1998; Johnson and Englund, 1998) except that the fluorescent nucleotide used was Chromatide Alexa Fluor 568-5-dUTP (Molecular Probes).

Microscopy

Slides were examined using a ZEISS Axioscope microscope and images captured by a Photometrics SenSys CCD camera (Photometrics, Ltd.) using IPLab Spectrum software. All of the cells were photographed using the same exposure time of 1 s for the DAPI staining, 5 s for the Alexa-dUTP and Texas red, 10 s for FITC, and 2 s for phase microscopy.

Northern Blot Hybridization Analysis

Total cell RNA was isolated from aliquots (15 ml) withdrawn from synchronized *C. fasciculata* cell cultures using RNAZOL™ (Biotex Laboratories) according to the manufacturer's instructions. Total RNA (15 μ g) from each time point was denatured and electrophoresed in a 1% denaturing agarose gel that was washed, blotted onto a Hybond-N membrane, and UV cross-linked. Hybridization was carried out using a random-primed radiolabeled 350-bp fragment of the *C. fasciculata* USBP gene ORF (Abeliovich et al., 1993) and a 260-bp fragment of the *C. fasciculata* calcium-binding protein (*CaBP*) gene ORF (a gift from Dr. Dan S. Ray, University of California, Los Angeles, CA) as probes. Northern blots were quantified by PhosphorImager using a Bio Imaging Analyzer (BAS1000; Fuji).

Metabolic Labeling Using [³⁵S]Methionine/Cysteine

Metabolic labeling of *C. fasciculata* cultures was conducted following the procedure of Hines and Ray (1997) using Trans[³⁵S]Label™ (Amersham Pharmacia Biotech) (>1,000 Ci/mmol methionine [70%] and cysteine [30%]). Aliquots (10 ml) of synchronized cells were harvested at 30-min intervals, washed twice with PBS, and resuspended in 2.5 ml of fresh growth media (DME; Biological Industries) lacking methionine to which 250 μ Ci of Trans[³⁵S]Label™ was added. The cells were labeled for 15 min at 28°C. Labeling was stopped by the addition of NaN_3 to 0.04%. Cells were washed twice with PBS and then lysed in a solution containing 10 mM sodium pyrophosphate, 1% (vol/vol) NP-40, 0.5 M NaCl, 0.02% (wt/vol) NaN_3 , 2 mM EDTA, and protease inhibitors (1 mM PMSF, 5 μ g/ml aprotinin, 1 μ g/ml leupeptin, 3 mM benzamide, 1 μ g/ml antipain). Protein (0.8 mg) in the cleared cell lysates from each time point was immunoprecipitated with 20 μ l of anti-UMSBP antibody and 70 μ l of protein A-Sepharose (Amersham Pharmacia Biotech) as described previously (Harlow and Lane, 1988). The precipitates were then analyzed by electrophoresis in a 16.5% Tris-tricine SDS-polyacrylamide gel. The proteins were transferred onto nitrocellulose membranes, and radioactivity was

measured by a PhosphorImager. The blots were then subjected to a Western blot analysis as described below.

Western Blot Analysis

Aliquots (10 ml) were withdrawn from a synchronized *C. fasciculata* culture at 30-min intervals after the release from hydroxyurea arrest. Cells were harvested by centrifugation at 2,500 g for 7 min and washed with PBS. Samples (20- μ g protein) of cleared cell lysates (fraction I; Tzfati et al., 1995) were mixed with the loading buffer to final concentrations of 50 mM Tris-HCl, pH 6.85, 4% (wt/vol) SDS, 3.5% (vol/vol) β -mercaptoethanol, 10% (vol/vol) glycerol, and 10 mM EDTA. Samples were boiled for 10 min and loaded onto a 16.5% Tris-tricine SDS-polyacrylamide gel. Upper electrophoresis buffer was 0.1 M Tris-tricine, pH 8.25, containing 0.1% SDS; lower buffer was 0.2 M Tris-HCl, pH 8.9. Protein bands were transferred onto Immobilon P membranes. Membranes were blocked by incubation for 30 min in 5% skim dry milk (Difco) in PBST and probed for 90 min with 1:1,000 dilution of either anti-UMSBP or anti-pol β antibodies. Membranes were probed for 45 min with a 1:10,000 dilution of HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies, followed by ECL detection as recommended by the manufacturer (Amersham Pharmacia Biotech). Quantification was conducted using densitometry.

Electrophoretic Mobility Shift Analysis

Analyses were carried out as described previously (Tzfati et al., 1992, 1995; Abu-Elneel et al., 1999). Samples of 10-ng protein of *C. fasciculata*-cleared cell lysate (fraction I; Tzfati et al., 1995), prepared as described above, were withdrawn at the time intervals indicated and added to the 10- μ l standard-binding reaction mixture containing 25 mM Tris-Cl, pH 7.5, 2 mM MgCl₂, 1 mM dithiothreitol, 20% (vol/vol) glycerol, 2 μ g bovine serum albumin, 0.5 μ g poly(dI-dC)·poly(dI-dC), and 12 fmol of 5'-³²P-labeled UMS (5'-GGGGTTGGTGTGA-3') DNA. Reactions were incubated at 30°C for 30 min and electrophoresed in an 8% native polyacrylamide gel (1:32, bisacrylamide/acrylamide) in TAE buffer (6.7 mM Tris-acetate, 3.3 mM sodium acetate, 1 mM EDTA, pH 7.5). Electrophoresis was conducted at 2–4°C and 16 V/cm for 1.5 h. Protein–DNA complexes were quantified by exposing the dried gels to an imaging plate and analyzing by PhosphorImager. 1 U of USBP is defined (Tzfati et al., 1992) as the amount of protein required for binding of 1 fmol of UMS DNA.

Results

The Intraorganelle Localization of USBP

We first localized USBP relative to a previously studied kDNA replication protein, pol β , by immunofluorescence using antibodies raised against the two proteins. Fig. 1 A shows images of cells from an asynchronous logarithmic culture of *C. fasciculata*. In all of the examples, DAPI staining reveals the brightly stained kinetoplast disk (K) and a weakly stained nucleus (N). In Fig. 1 A, a, b, and d, the disk is viewed edgewise, whereas in c the disk has partially tipped so that the flat surface is visible (Johnson and Englund, 1998). In all of these examples, pol β immunofluorescence is detected in the two well-characterized antipodal sites that flank the kinetoplast disk (Ferguson et al., 1992). In contrast, USBP has a novel location in two discrete zones adjacent to the face of the disk closest to the cell flagellum (see below; Fig. 2). In this location, USBP resides in the same region in which DNA primase has been previously localized (Li and Englund, 1997; Johnson and Englund, 1999). The image in d shows a slightly different localization of USBP, in which it is distributed in a single elongated zone adjacent to the kDNA disk. It is likely that this example also contains two zones of USBP that are not resolved, possibly because the cell has rotated slightly so that the space between the two zones is obscured.

We have also studied the localization of USBP relative to the location of the replicating free minicircles that are associated with the replication proteins in the two antipodal sites on the perimeter of the kDNA disk. These experiments, in which gapped kDNA is labeled in situ with Alexa-dUTP using terminal deoxynucleotidyl transferase (TdT) (Johnson and Englund, 1998), are based upon the well-characterized distribution of covalently closed and gapped minicircles in prereplication, replicating, and post-replicated kDNA in isolated networks (Guilbride and Englund, 1998) and in vivo (Ferguson et al., 1992). Whereas unreplicated minicircles are covalently closed, minicircles that have completed replication are gapped and thus are selectively labeled by the fluorescent dUTP. The relatively weak labeling of the kDNA disk in all panels of Fig. 1 B is due to the fact that replicated network minicircles have only one or a small number of gaps. In contrast, the strong labeling of the antipodal sites is due to the presence of abundant free minicircle replication intermediates that are multiply gapped (Kitchin et al., 1984; Johnson and Englund, 1998). Visualization of USBP in these cells confirms that it is in a unique location and not colocalized with the two antipodal sites.

In Fig. 2, A and B, we present overlays of the images from Fig. 1, A and B (a–c). They confirm that USBP is located in two sites adjacent to the flagellar face of the kDNA disk. Fig. 2, A and B, c, both show images of cells in which the kDNA disk appears to be at least partially tipped over (roughly 10% of the cells analyzed had their kDNA in a tipped orientation). In these cases, a face of the disk is visualized. In both cases, the antipodal sites visualized by either pol β immunofluorescence (Fig. 2 A) or Alexa-dUTP incorporation (Fig. 2 B) still flank the disk, implying that these sites remain associated with the disk even when it changes orientation (see Ferguson et al., 1992, for more images supporting this contention). In contrast, USBP seems to dissociate from the tipped disk, remaining in its original position, suggesting that it may be bound not to the disk but to other components of the mitochondrial matrix.

Visualization of 289 cells in an asynchronous logarithmic population of *C. fasciculata* revealed that all of the cells undergoing replication (65% of the total population, as judged by labeling of kDNA with Alexa-dUTP) contained USBP. The remaining cells (35%) did not label with Alexa-dUTP and therefore were not undergoing replication. Of these, some had USBP in the location shown in the examples in Figs. 1 and 2 (16% of the total cells), some did not contain detectable USBP (9% of the total), and the remainder (~10% of the population) had other organizations of USBP that will be discussed below.

Localization of USBP throughout the Cell Cycle

To determine whether the changes in USBP localization are coordinated with the cell cycle, we examined a synchronized culture of *C. fasciculata*. Cells synchronized by hydroxyurea arrest (Pasion et al., 1994) were sampled at 30-min intervals. We performed cell counts by hemocytometry, detected dividing cells containing two nuclei by fluorescence and phase microscopy, and determined whether kDNA was undergoing replication in individual cells by observing Alexa-dUTP fluorescence (using the

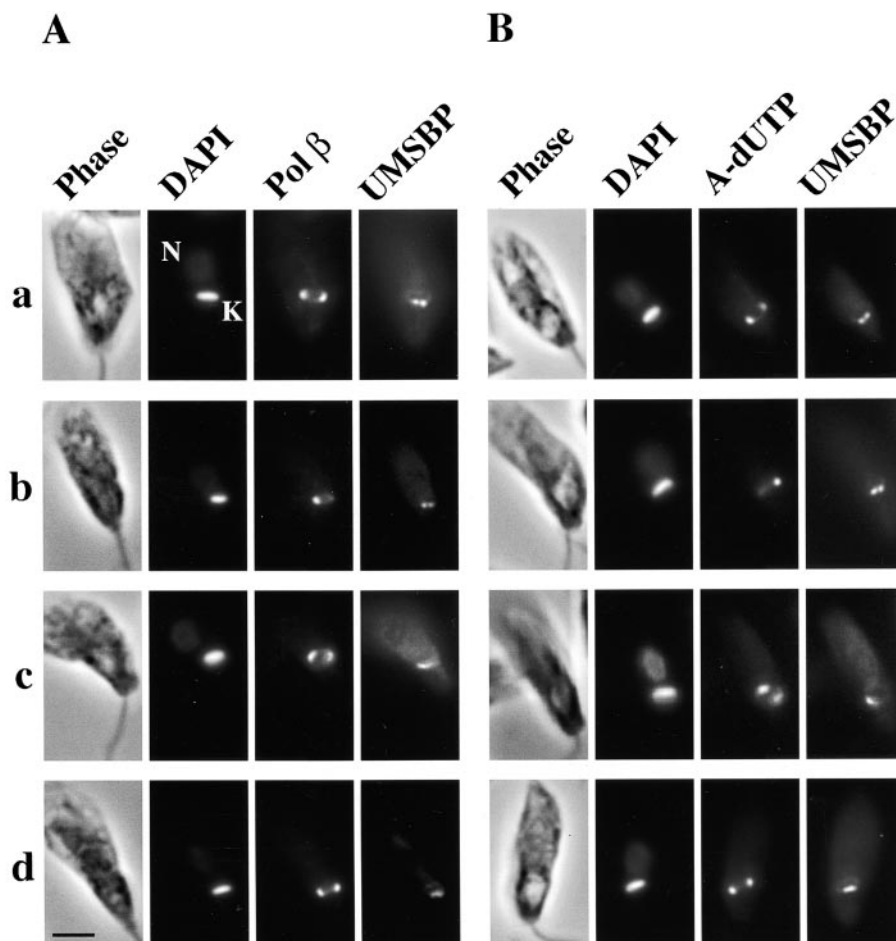


Figure 1. Immunolocalization of UMSBP in an asynchronous *C. fasciculata* cell culture. Phase and fluorescence microscopy are described in Materials and Methods. With DAPI staining, the kDNA disk displays bright fluorescence, and the nucleus fluoresces weakly. (A) Immunofluorescence of pol β and UMSBP. For pol β , the secondary antibody was goat anti-rabbit conjugated to Texas red, and for UMSBP it was rat anti-mouse conjugated to FITC. (B) Immunofluorescence of UMSBP in cells labeled in situ with Alexa-dUTP (using TdT as described in Materials and Methods). a, b, and d present edgewise views of the kDNA disk, whereas c shows a disk that is partially tipped over. K, kDNA; N, nucleus; A-dUTP, Alexa-dUTP. Bar, 3 μ m.

method shown in Fig. 1 B). We also used immunofluorescence to determine the status of UMSBP. Fig. 3 quantifies these data as a function of time over the entire cell cycle (~ 210 min). Virtually all of the cells in the culture are undergoing kDNA replication upon hydroxyurea release, but only $\sim 5\%$ have replicating kDNA at the time of maximal cell division (150 min; Fig. 3). UMSBP is present in virtually all of the cells during replication but is found in only 70% of the cells at the time of cell division.

Fig. 4 A shows examples of cell images captured at the various time intervals after the release from hydroxyurea arrest, revealing different patterns of UMSBP and Alexa-dUTP staining during progression through the cell cycle. The relative abundance of cells with these patterns, as a function of time, is shown in Fig. 4 B. The timing of appearance and disappearance of each of these patterns reveals the changes in behavior of UMSBP throughout the cell cycle.

Upon release from hydroxyurea arrest, most cells as expected are in an “early replication” stage, containing both Alexa-dUTP labeling and UMSBP labeling (Fig. 4 B, filled circles). An example of this pattern is shown in Fig. 4 A, a. The next stage, “late replication,” is characterized by a kinetoplast disk with a larger diameter (Fig. 4 B, filled triangles). These cells peak at 90 min and are labeled by both Alexa-dUTP and UMSBP. An example is shown in Fig. 4 A, b. In this example, Alexa-dUTP labels not only the antipodal sites flanking the kDNA disk but also a re-

gion adjacent to the face of the kDNA disk nearest the flagellum. The accompanying paper (Drew and Englund, 2001) shows that this labeling is due to free minicircle replication intermediates. This is the likely site of initiation of minicircle replication, but the free minicircles accumulate here only in the late stage of replication. In cells of this type, the UMSBP staining is typically more diffuse. The subsequent stage, “postreplication,” has cells beginning to undergo division (Fig. 4 B, open triangles). Since these cells have no gapped minicircles, they do not label with Alexa-dUTP, but they do contain UMSBP. In the example shown in Fig. 4 A, c, the diffuse pattern of UMSBP seems to be sub-dividing so that there are four zones of UMSBP per dividing cell.

The next stage, appearing at 120 min, is “prereplication, without UMSBP” (Fig. 4 B, open boxes). These cells have no detectable UMSBP, do not label with Alexa-dUTP, and contain kDNA disks of relatively small diameter (Fig. 4 A, d). The final stage, “prereplication with UMSBP,” appears just before the initiation of kDNA synthesis at 150 min. These cells do not label with Alexa-dUTP, but they do contain UMSBP (Fig. 4 B, filled boxes). An example is presented in Fig. 4 A, e.

Expression of UMSBP Protein in Synchronized Cell Cultures

We next explored whether the changes in the abundance and localization of UMSBP during the cell cycle could be a

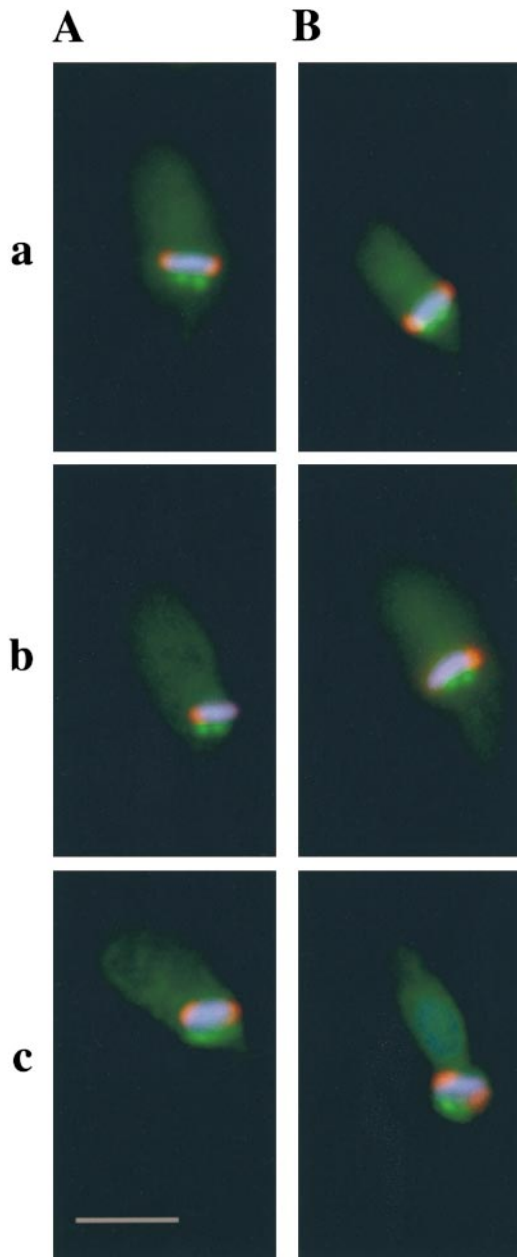


Figure 2. Localization of UMSBP, pol β , and minicircle replication intermediates. Phase and fluorescence microscopy were conducted as described in Materials and Methods. (A) An overlay presentation of DAPI staining (blue), pol β (red), and UMSBP (green) immunostaining of the cell images presented in Fig. 1 A, a–c. (B) An overlay presentation of DAPI staining (blue), Alexa-dUTP fluorescence (red), and UMSBP (green) of the cell images presented in Fig. 1 B, a–c. Bar, 3 μ m.

consequence of a periodic expression of the protein during the cell cycle. With the same synchronization method used in the experiments described in Figs. 3 and 4, we followed the expression of the *UMSBP* gene by hybridization analysis of total cell RNA using the *UMSBP* ORF as a probe. Abundance of specific *UMSBP* mRNA transcripts, when compared with transcripts of the constantly expressed *C. fasciculata* calcium-binding protein (Pasion et al., 1994), revealed a similar pattern of constant steady-state levels of

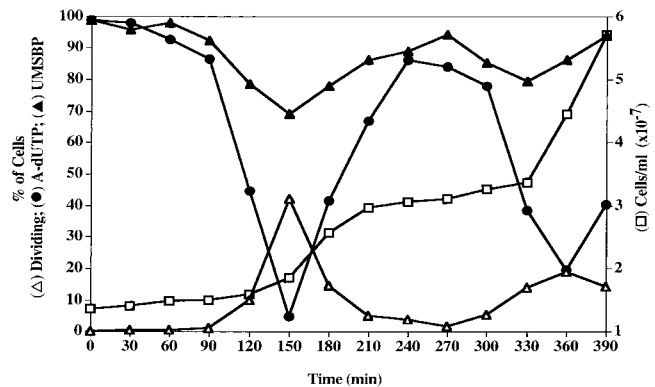


Figure 3. UMSBP in synchronized cell cultures. Cell cultures were synchronized as described previously (Pasion et al., 1994). Cells (10^7 cells/ml) were incubated for 6 h at 26°C in the presence of 200 μ g/ml hydroxyurea, centrifuged (6,000 g, 15 min), resuspended in fresh culture medium, and grown for an additional 6.5 h. Samples were withdrawn at the indicated times and counted (open squares). The percent of cells undergoing division containing two nuclei was determined by fluorescence and phase microscopy (open triangles), and the percent of cells displaying UMSBP (filled triangles) and Alexa-dUTP (A-dUTP) (filled circles) fluorescence was determined by fluorescence microscopy.

UMSBP mRNA throughout the various stages of the cell cycle (Fig. 5 A). The *UMSBP* genomic locus consists of two tandemly arranged copies of the *UMSBP* gene that are transcribed as two distinct mRNA transcripts of 3.3- and 2.75-kb long, at a ratio of \sim 1:3, respectively (Abeliovich et al., 1993; Tzfati and Shlomai, 1998). Quantification of these two *UMSBP* mRNA bands (Fig. 5 A) indicates no significant changes in the ratio between the two *UMSBP* transcripts throughout the cell cycle.

We next followed the rate of UMSBP synthesis during the progress of cell cycle in synchronized cell cultures. We measured the incorporation of [³⁵S]methionine/cysteine into UMSBP during short (15 min) pulse-labeling, at 30-min intervals after release from hydroxyurea arrest. Gel electrophoresis analysis of UMSBP immunoprecipitated from cell lysates by anti-UMSBP antibodies revealed no detectable changes throughout the cell cycle in the rate of incorporation of the ³⁵S-labeled amino acids into newly synthesized UMSBP (Fig. 5 B). Western blot analyses of lysates obtained from synchronized *C. fasciculata* cell cultures using specific anti-UMSBP antibodies also failed to detect any significant changes in the abundance of UMSBP during the different stages of the cell cycle (Fig. 5 C). These results are similar to those obtained using antibodies directed against pol β (Fig. 5 C), a replication protein whose constant abundance throughout the different stages in the cell cycle has been reported previously (Johnson and Englund, 1998).

The constant abundance of UMSBP throughout the cell cycle also yields constant levels of UMS-binding activity at its various stages, as measured in cleared cell lysates by the mobility shift electrophoresis analysis (Fig. 5 D). All of the observations in Fig. 5 indicate that the changes observed in the patterns of UMSBP localization, in synchronized *C. fasciculata* cultures, do not reflect the abundance of

UMSBP-specific mRNA, the rate of USBBP synthesis, or its level in the cell and its UMS DNA-binding activity.

Discussion

A remarkable characteristic of the kDNA replication apparatus is the specific location of the various replication enzymes and DNA-binding proteins in unique sites surrounding the kDNA disk. So far, there are three such distinct sites. Three enzymes, topoisomerase II (Melendy et al., 1988), pol β (Ferguson et al., 1992), and SSE1 (Engel and Ray, 1999), colocalize with free minicircle replication intermediates (Ferguson et al., 1992; Johnson and Englund, 1998) in two antipodal sites on the perimeter of the kinetoplast disk. In contrast, histone-like proteins, p16, p17, and p18 (Xu et al., 1996), localize throughout the kDNA disk. Finally, the mitochondrial DNA primase and p21 localize in two sites close to the two faces of the kDNA disk (Li and Englund, 1997; Hines and Ray, 1998; Johnson and Englund, 1999). In this paper, we have shown that USBBP localizes in a fourth site, distinct from those described previously.

UMSBP is encoded by a nuclear gene, and the protein product does not have a characteristic NH₂-terminal mitochondrial-targeting sequence (Abeliovich et al., 1993). The apparent lack of NH₂-terminal mitochondrial-targeting sequences is also the case with the kinetoplast SSE1 and topoisomerase II enzymes (Pasion et al., 1992; Engel and Ray, 1999). However, other kDNA-associated proteins, such as the kinetoplast histone-like proteins (p16, p17, and p18), p21, and pol β , have more typical cleaved NH₂-terminal targeting signals (Torri and Englund, 1995; Xu et al., 1996; Hines and Ray, 1998). Despite the lack of an obvious targeting sequence in USBBP, the specificity and high binding affinity to two conserved sequences at the minicircle replication origin led to our proposal that this protein functions in the mitochondrion (Tzfati et al., 1992). In the study described here, we have conclusively localized USBBP to the parasite's single mitochondrion. Furthermore, we found that USBBP is located at two adjacent sites close to the face of the kDNA disk nearest the cell flagellum (Fig. 1). As discussed in the next paragraph, this location is consistent with USBBP's proposed function as the kDNA minicircle origin-binding protein that controls the initiation of kDNA replication.

Minicircles are released from the kDNA network by a topoisomerase for the purpose of replication (Englund, 1979). Studies in the accompanying paper indicate that minicircles are released vectorially from the kDNA disk towards the face of the disk nearest to the flagellum. Replication initiates in this zone and ultimately the progeny minicircles accumulate in the antipodal sites (Drew and Englund, 2001). Within the antipodal sites, primer removal and filling of some of the gaps are thought to occur in preparation for minicircle reattachment to the network. In the framework of this replication model, USBBP is ideally positioned to recognize free minicircles once they are released from the network. It is likely that USBBP facilitates the assembly of the primase and other proteins (such as the replicative polymerase) to initiate replication at the origin sequence. Moreover, USBBP could provide the specific origin recognition required for the origin-specific

priming of kDNA replication. Since USBBP recognizes specifically the replication origins of both the L-strand (Tzfati et al., 1992, 1995) and the H-strand (Abu-Elneel et al., 1999) of the minicircle, such an interaction could facilitate the coordinated initiation of leading and lagging strand synthesis.

One interesting feature of the USBBP localization, not yet understood, is its presence in two distinct foci (Figs. 1 and 2). This pattern is distinct from that of the primase, which we have found to uniformly cover the entire face of the disk (Johnson and Englund, 1999). We have no information on the distribution of USBBP when viewed towards the face of the disk except that it forms two zones (Figs. 1 and 2). Nevertheless, the two observed sites of

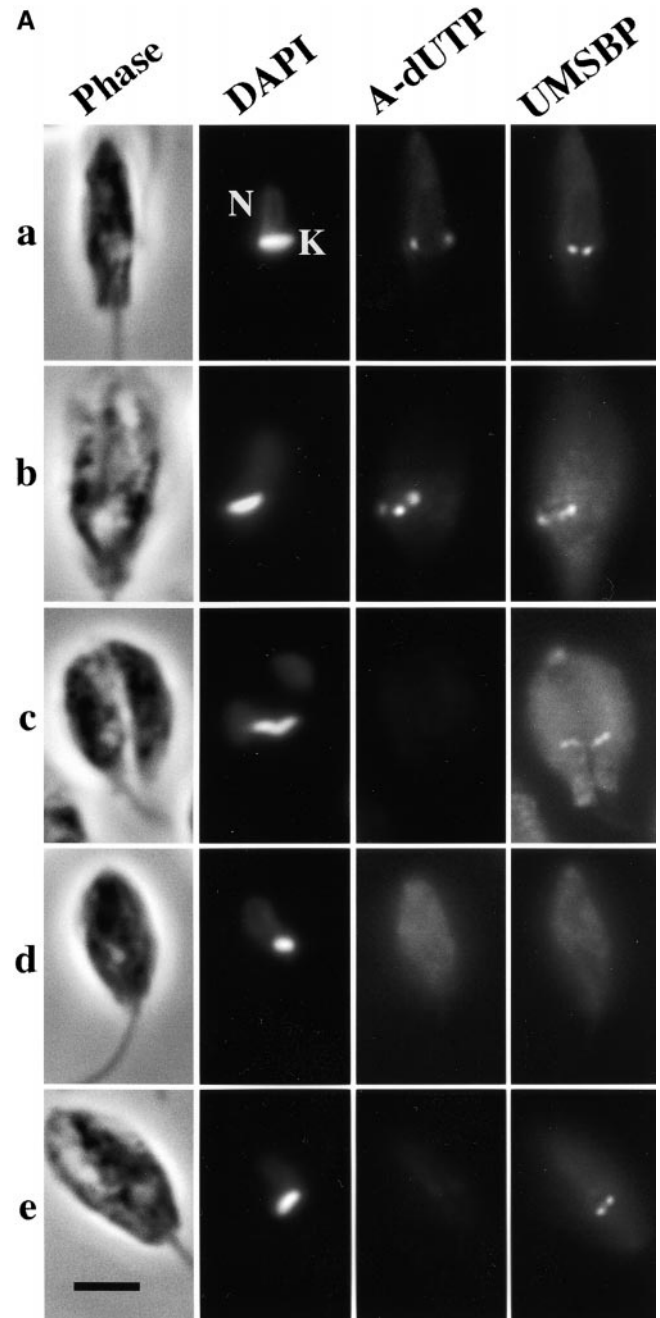


Figure 4 (continues on facing page)

UMSBP localization could represent regions of storage for this protein rather than for its function. This speculation is based on the fact that each cell contains 12,000 dimers of USBP (Tzfati et al., 1995) in vast excess over the number of free minicircles (see Drew and Englund, 2001) and especially over the small number of free minicircle θ structures (Englund, 1979). Given these considerations, our current view (partly based on speculation) is that minicircles are released vectorially from random sites in the central region of the network. Then, the free minicircles associate with USBP at the location of USBP foci or outside these sites, which allows them to assemble with primase and other proteins and begin replication. In later stages of kDNA replication, the minicircle progeny accumulate in the central region adjacent to the flagellar face of the kinetoplast disk (see example in Fig. 4 A, b). Finally, the minicircle progeny move to the antipodal sites to prepare for attachment to the network. If USBP is still associated with these free minicircles, its concentration would be too low for detection by immunofluorescence. For further discussion of this model see the accompanying paper (Drew and Englund, 2001).

Given the complex arrangement of all of the replication proteins that surround the kDNA disk, it is of great interest to understand the intramitochondrial-targeting mechanisms. Although almost nothing is known about this subject, our data raise the possibility that USBP and primase-targeting mechanisms may be different even though the two proteins colocalize. Most strikingly, primase covers both faces of the kDNA disk (Li and Englund, 1997; Johnson and Englund, 1999), whereas USBP localizes exclusively to two zones only on the flagellar face of the disk (Figs. 1 and 2). Second, primase

seems to associate directly with the kDNA disk, since we have found that in the few cells in which the disk is tipped (that is, not in its usual orientation perpendicular to the axis of the flagellum) the primase remains associated with the disk (Johnson and Englund, 1999). Similarly, pol β (Fig. 1 A; Johnson and Englund, 1998) and free minicircle replication intermediates in the antipodal sites (Ferguson et al., 1992) remain associated with the kDNA when the disk is tipped. In contrast, when we observed USBP localization relative to a tipped disk (for examples see Fig. 1, A, c, and B, c), we found that it appeared to remain in its original location. This fact suggests that USBP does not interact directly with the kDNA disk but instead is bound to some other component in the mitochondrial matrix. It is already known that there are direct cytoskeletal interactions between the kDNA network and the basal body of the flagellum (Robinson and Gull, 1991). The physical connection between the kinetoplast and the basal body complex has been demonstrated directly by isolation of flagella with attached kinetoplasts. The molecular nature of this link is still unknown. However, a tripartite attachment complex that links the two structures through sets of fibrils has been described recently using EM (Gull, 1999). It may be the unknown cytoskeletal components involved in linkage of the kDNA disk and the basal body that interact with USBP. The restriction of USBP localization to two zones could be due to the distinct organization of these cytoskeletal components.

An interesting question is why primase is localized on both faces of the kDNA disk, whereas USBP is restricted to the flagellar face (the site of minicircle replication). We have speculated elsewhere that the nonflagellar face of the disk is the site of maxicircle replication and that

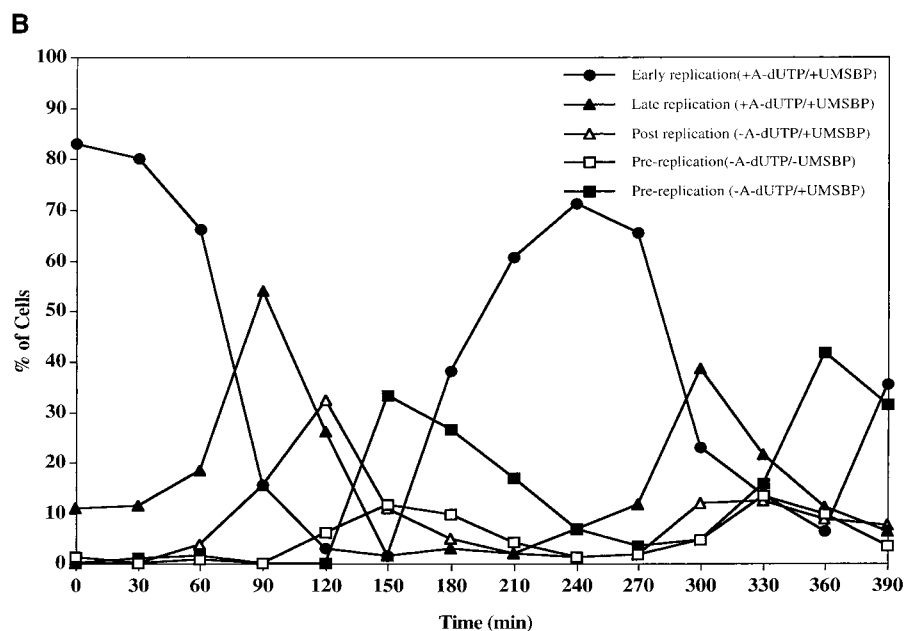


Figure 4. Variations in the intraorganellar localization of USBP during the cell cycle. Cell cultures were as described in the legend to Fig. 3. Phase and fluorescence microscopy were conducted as described in Materials and Methods. Phase microscopy, DAPI fluorescence, and immunofluorescence of USBP in cells labeled in situ with fluorescent Alexa-dUTP are presented. (A) Typical examples of cells sampled at various times after release from hydroxyurea arrest: (a) cell at an early stage of kDNA replication (at 30 min and then at the subsequent cell cycle at 240 min after the release from hydroxyurea arrest); (b) cell at a late stage of kDNA replication (90 and then 300 min); (c) cell at a postreplication stage (120 and then 300–330 min); (d and e) cells in a prereplication stage (150–180 and then 330–360 min). (B) Quantification of cell images throughout two consecutive cell cycles. Cells

displaying both Alexa-dUTP and USBP fluorescence at an early stage of kDNA replication, like that in A (a, filled circles), or a late stage, like that in A (b, filled triangles). Cells displaying USBP fluorescence but little or no Alexa-dUTP fluorescence at a post-kDNA replication stage, like that in A (c, open triangles). Cells at a stage before undergoing kDNA replication (no Alexa-dUTP labeling), either with USBP, like that in A (e, filled squares), or without USBP, like that in A (d, open squares). K, kDNA; N, nucleus; A-dUTP, Alexa-dUTP. Bar, 3 μ m.

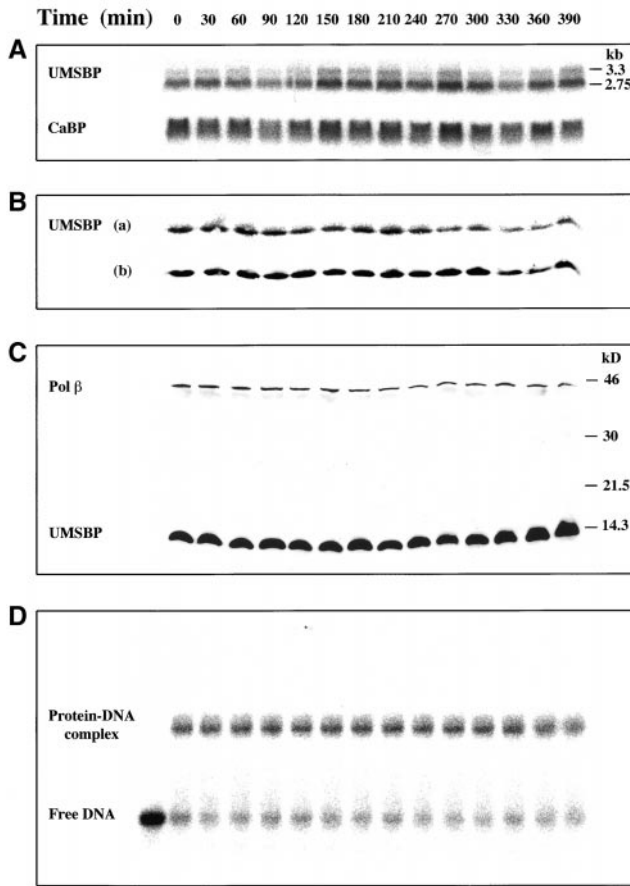


Figure 5. Expression of UMSBP throughout the cell cycle. *C. fasciculata* cell cultures were synchronized as described in the legend to Fig. 3. In A, the steady-state levels of *UMSBP* mRNA in synchronized cell cultures were determined by Northern blot hybridization analyses of total cell RNA (15 μ g/lane) isolated at 30 min intervals after the release from hydroxyurea arrest using *UMSBP* and *CaBP* ORFs probes as described in Materials and Methods. In B, the rate of UMSBP synthesis during the cell cycle was followed in synchronized cell cultures, monitoring the incorporation of [³⁵S]methionine/cysteine during short (15 min) pulse labeling at the indicated time intervals after the release of hydroxyurea arrest. Metabolic labeling of *C. fasciculata* cultures was conducted as described in Materials and Methods. Sampled cells were lysed, and the cleared cell lysates were immunoprecipitated with anti-UMSBP antibody and protein A-Sepharose and analyzed by electrophoresis in 16.5% Tris-tricine SDS-polyacrylamide gels as described in Materials and Methods. Indicated are ³⁵S-labeled immunoprecipitates of UMSBP quantified by phosphorImager (a) relative to their respective Western blots developed by ECL and quantified by densitometry (b). In C, the abundance of UMSBP in synchronized *C. fasciculata* cell cultures was determined by Western blot analyses as described in Materials and Methods. Cells withdrawn at the indicated time intervals after the release from hydroxyurea arrest were lysed, and cleared cell lysates were electrophoresed (20 μ g protein/lane), blotted onto membranes, and probed with anti-UMSBP and anti-pol β antibodies. Detection of immune complexes by ECL was described in Materials and Methods. In D, UMSBP activity in synchronized cell cultures was conducted by the electrophoretic mobility shift analysis as described in Materials and Methods. Lysates (fraction I; Tzfat et al., 1995) of cell aliquots withdrawn at the indicated time intervals were used (10 ng protein/assay), and 12 fmol of the ³²P-labeled UMS DNA ligand were used. Indicated are UMSBP-UMS DNA complexes and free UMS DNA.

primase also functions in this process (Drew and Englund, 2001). It is interesting that the *Trypanosome brucei* maxicircles contain two sequences nearly identical to the *C. fasciculata* minicircle UMS (Myler et al., 1993). However, it was unexpected to find that these sequences are not at the *T. brucei* maxicircle origin of replication (Carpenter and Englund, 1995). Therefore, if the speculation that maxicircles replicate on the nonflagellar face is correct we would predict that a distinct origin-binding protein that recognizes the maxicircle replication origin colocalizes with primase on the nonflagellar face of the kDNA disk.

We found that the UMSBP localization in two zones on the flagellar face of the kDNA disk is dynamic during the *C. fasciculata* cell cycle. Although virtually every cell has UMSBP detectable by immunofluorescence during the time of kDNA replication, >30% of the cells lose virtually all UMSBP during the time of cell division (Fig. 3). This change was not due to alteration in the level of UMSBP concentration, since we detected no alterations in UMSBP mRNA level (by Northern blot), UMSBP synthesis rate (by pulse radiolabeling), UMSBP protein level (by Western blot), or UMS-binding activity (by gel shift assays of cell extracts) (Fig. 5). Therefore, the most likely explanation for the loss of UMSBP fluorescence is that the protein molecules move from the two zones near the flagellar face during the time of cell division. A similar loss of fluorescence detection during cell division is found for pol β , topo II, and SSE1 (Johnson and Englund, 1998; Engel and Ray, 1999). In contrast, primase immunofluorescence is constant throughout the cell cycle (Johnson and Englund, 1998).

Finally, we found that the localization pattern of UMSBP also changed during the later stages of kDNA replication, with the UMSBP zones becoming more elongated and diffused (Fig. 4 A, b). All of the changes, determined from images and kinetics of reorganization of UMSBP during the cell cycle (Fig. 4, A and B), are summarized schematically in Fig. 6. According to this scheme, during the G1 phase the cells contain prereplication kDNA networks with covalently closed minicircles that do not incorporate fluorescent dUTP; UMSBP fluorescence localizes to two distinct sites close to the face of the kDNA disk nearest the flagellum (Fig. 6 A). Entering S phase, with the initiation of kDNA replication, the cells accumulate gapped minicircle replication intermediates (detected by fluorescent dUTP labeling) mainly at the antipodal sites at the perimeter of the disk; UMSBP is localized in these early S phase cells to its typical two sites at the disk face (Fig. 6 B). As kDNA replication progresses, the diameter of the kDNA disk increases, and gapped free minicircles accumulate on the flagellar face of the kDNA disk (detected by fluorescent dUTP); UMSBP fluorescence indicates that the protein is no longer focused in spots but instead is diffuse and seems to be undergoing subdivision (Fig. 6 C). Entering into its post replication stage, the cell contains a single elongated (V-shaped) kinetoplast disk, and UMSBP is still located close to the kDNA disk facing the cell flagellum, but its two fluorescing spots are continuing to subdivide. At this stage, there are no longer any free minicircle replication intermediates detected by fluorescent dUTP (Fig. 6 D). At the final stage of cell division, some cells have lost UMSBP completely (Fig. 6 E). The

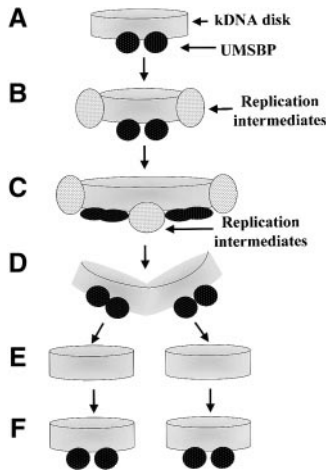


Figure 6. Schematic description of UMSBP localization throughout the cell cycle. The scheme is based upon fluorescence microscopy of UMSBP immunolocalization, DAPI staining of kDNA networks, and incorporation of fluorescently labeled dUTP into gapped kDNA replication intermediates in synchronized cell cultures. See text for details. Note that in these diagrams, the basal body of the flagellum is positioned just below the kDNA disk.

cycle is completed with the appearance of kDNA disks with two spots of UMSBP (Fig. 6 F).

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