Interactions of a Replication Initiator with Histone H1-like Proteins Remodel the Condensed Mitochondrial Genome*

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Background: The mechanism of kinetoplast DNA (kDNA) remodeling and its function during replication have been unknown.

Results: Interactions of the replication initiator protein UMSBP with kDNA-condensing proteins result in kDNA decondensation, promoting its accessibility to topoisomerase II.

Conclusion: UMSBP-mediated remodeling may precede and promote the initiation of kDNA replication.

Significance: The function of a replication initiator in remodeling a mitochondrial genome is demonstrated.

Kinetoplast DNA (kDNA), the mitochondrial genome of trypanosomatids, consists of several thousand topologically interlocked DNA circles. Mitochondrial histone H1-like proteins were implicated in the condensation of kDNA into a nucleoid structure in the mitochondrial matrix. However, the mechanism that remodels kDNA, promoting its accessibility to the replication machinery, has not yet been described. Analyses, using yeast two hybrid system, co-immunoprecipitation, and protein-protein cross-linking, revealed specific protein-protein interactions between the kDNA replication initiator protein universal minicircle sequence-binding protein (UMSBP) and two mitochondrial histone H1-like proteins. Fluorescence and electron microscopy, as well as biochemical analyses, demonstrated that these protein-protein interactions result in the decondensation of kDNA. UMSBP-mediated decondensation rendered the kDNA network accessible to topological decatenation by topoisomerase II, yielding free kDNA minicircle monomers. Hence, UMSBP has the potential capacity to function in vivo in the activation of the prereplication release of minicircles from the network, a key step in kDNA replication, which precedes and enables its replication initiation. These observations demonstrate the prereplication remodeling of a condensed mitochondrial DNA, which is mediated via specific interactions of histone-like proteins with a replication initiator, rather than through their posttranslational covalent modifications.

Kinetoplast DNA (kDNA) is a remarkable DNA network found in the single mitochondrion of trypanosomatids. In the species Crithidia fasciculata it consists of ∼5,000 duplex DNA minicircles (of 2.5 kbp) and ∼50 maxicircles (of 37 kbp), which are interlocked topologically into a DNA catenane. According to the current model for kDNA replication (for review, see Refs. 1–3), minicircles are released from the network prior to their replication, by a topoisomerase II, and replicate as free individual circles. Minicircle replication initiates at conserved origin sequences, which are bound by a mitochondrial single-stranded sequence-specific DNA-binding protein, named the universal minicircle sequence-binding protein (UMSBP) (4–9). UMSBP is a CCHC-type zinc finger protein whose binding to origin sequences is regulated by the cell cycle control of the protein redox state (10, 11). Cross-linking analysis has shown that UMSBP binds the kDNA network in vivo (8). Knockdown of the UMSBP genes in Trypanosoma brucei by RNA interference (RNAi) inhibits minicircle replication initiation, blocks the segregation of the kDNA network and the flagellar basal bodies, and impedes the division of the flagella (12). Hines and Ray have recently described two mitochondrial DNA primases (PRI1 and PRI2) and suggested their involvement in kDNA replication initiation (13, 14). Replicating minicircles migrate to two antipodal sites, at the edge of the kDNA disc, where primer excision, gap filling, and sealing of the nicks generated during the synthesis of Okazaki fragments occur. A topoisomerase II then reattaches the minicircles molecules onto the network, where final gap filling and sealing occur, prior to the network scission and segregation (1–3).

The kDNA network is condensed in the mitochondrial matrix into a disc-shape nucleoid. Four kinetoplast-associated proteins (KAP1–4, also known as proteins p21, p18, p17, and p16, respectively) were implicated in the condensation and compaction of the kDNA disc. They are small basic proteins that are rich in lysine and alanine residues. They resemble the nuclear histone H1 protein in their lysine- and alanine-rich domains as well as in their preferential and highly cooperative binding to DNA containing oligo(dA)-oligo(dT) tracts (15–17). KAP proteins were shown to condense kDNA networks in vitro into a tight compact structure (16). Their expression in an Escherichia coli mutant deficient in HU protein rescued a defect of chromosomal condensation and segregation, suggesting that...
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kDNA histone-like proteins could play a role in the condensation of kDNA networks. Immunolocalization analyses revealed that in *C. fasciculata* KAP proteins are localized to the kDNA network (16, 18). Targeted disruption of the two alleles of the KAP1 gene resulted in substantial structural rearrangement of the kDNA disc (19). Double knock-out of both KAP3 and KAP2 slowed cell growth, reduced respiration, and generated abnormal cell morphologies (20).

Although KAP proteins were implicated in the condensation and compaction of kDNA into a mitochondrial nucleoloid (16, 18), the mechanism of kDNA decondensation has not yet been described. We presumed that similar to other genomes, kDNA replication and transcription would require the decondensation or “opening” of the condensed kDNA nucleoloid to enhance its accessibility to the replication and transcription machineries. In this study we challenged a hypothesis that decondensation of the kDNA network is required for its replication initiation and searched for a potential mechanism that mediates kDNA remodeling. Here, we report on a potential novel function of the origin-binding protein UMSBP in the remodeling of the kDNA nucleoloid. We describe the interactions of UMSBP with two kinetoplast-associated histone H1-like proteins, KAP3 and KAP4 (16, 18) and demonstrate that these interactions result in the decondensation of the kDNA network. We further show that the UMSBP-mediated decondensation of kDNA is the result of specific protein-protein interactions between KAP proteins and UMSBP rather than through competition on the binding of the DNA. Moreover, we show that decondensation of the kDNA network by UMSBP enhances the network accessibility to DNA-interacting proteins, enabling the topological decatenation of kDNA by a DNA topoisomerase II, to yield free minicircle monomers. Based on these observations, we suggest that UMSBP interactions with histone-like proteins may enable *in vivo* the prereplication release of minicircles from the network, activating a key preinitiation step in minicircle replication.

**EXPERIMENTAL PROCEDURES**

**Co-immunoprecipitation**—Anti-KAP1–4 antibodies were the generous gift of Dr. Dan S. Ray, from the Molecular Biology Institute, UCLA. Log phase *C. fasciculata* cells were harvested and resuspended to the concentration of 5 × 10^7 cells/ml in Nonidet P-40 lysis buffer (10 mM sodium phosphate, pH 7.2, 1% Nonidet P-40, 2 mM EDTA, 150 mM NaCl, 5 mM DTT) supplemented with protease inhibitors. The cells were incubated for 30 min on ice and then precipitated, and the supernatant incubated overnight, at 4 °C, with protein A-Sepharose beads (Amersham Biosciences) that were preincubated with each of the following rabbit sera: preimmune, anti-CfUMSBP, anti-CfKAP1, anti-CfKAP2, anti-CfKAP3, and anti-CfKAP4. The precipitates were washed three times with PBS containing 0.1% Nonidet P-40 and twice with PBS. The bound proteins were eluted from the protein A beads using 100 mM glycine, pH 2.5, neutralized using 100 mM Tris-Cl, pH 8.0, and assayed for UMSBP activity. When nuclelease-treated cell lysates were used, lysates were supplemented with 5 mM CaCl_2_, and 5 units of micrococcal nuclease (Sigma) were added, followed by a 15-min incubation, at 30 °C, prior to the immunoprecipitation reaction.

**Preparation of Kinetoplast and Cell Extracts**—*C. fasciculata* cells were grown to logarithmic phase and were disrupted using a Stansted Homogenizer–Air operated model 0612W-195 AFC, and kinetoplasts were isolated by differential centrifugation. Frozen kinetoplasts or cells pellets were thawed on ice, and then 4 volumes of ice-cold water and protease inhibitors (1 mM EDTA, pH 8.0, 1 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 3 mM benzamidine, 1 μg/ml antipain), and 5 mM DTT were added. The suspension was incubated on ice, for 5 min, and then Brij-58 was added to the final concentration of 0.2%. Incubation of the suspension on ice, for 10 min, was followed by the addition of 1/9 volume of lysis buffer (0.5 M Tris-Cl, pH 8.0, 40 mM EDTA, pH 8.0, 200 mM spermidine-Cl_2_, 50% (of saturation, at 0 °C) ammonium sulfate, and 20 mM β-mercaptoethanol). The lysate was centrifuged at 38,720 × g, at 4 °C, for 2 h. The supernatant was dialyzed against dialysis buffer (20 mM Tris-Cl, pH 7.5, 2 mM β-mercaptoethanol, 1 mM MgCl_2_) for 2 h.

**Protein Cross-linking Analyses**—Purified recombinant UMSBP (400 pmol) (21) and KAP3 (100 pmol) (16) were incubated at 25 °C for 5 min in a 10-μl reaction mixture containing 50 mM sodium phosphate buffer, pH 8.0, 20 mM NaCl, and 0.1% Triton X-100. Formaldehyde was added to a final concentration of 1%, and the reaction mixture was incubated for additional 5 min at 25 °C, followed by its 5-fold dilution in triple-distilled water. Then, loading buffer was added, and the protein samples were electrophoresed in 16.5% polyacrylamide gel, as described previously (10).

**Isolation of kDNA Networks**—*C. fasciculata* cell cultures were grown to a density of ~10^8 cells/ml, as described previously (5). Purification of kDNA was carried out following Sauvée et al. (22), as modified for purification of *C. fasciculata* kDNA (23), except that 0.7 mg/ml protease K was used, ultracentrifugation was conducted for 15 min, and ethidium bromide was extracted using 0.1× SSC-saturated isooamyl alcohol. When further purification was required, kDNA was re-treated with protease K as above, followed by sequential phenol and chloroform extractions.

**Analysis of kDNA Network Condensation by Fluorescence Microscopy**—200 ng of purified *C. fasciculata* kDNA networks were incubated in a 25-μl reaction mixture containing 50 mM Tris-Cl, pH 8.0, and 20 mM NaCl, with the *C. fasciculata* KAP3, KAP4, or human histone H1 (Sigma) and/or UMSBP, as indicated, for 5 min at 25 °C. The reaction mixture was adhered to poly-L-lysine-coated slides (Electron Microscopy Sciences) for 30 min in a humidity chamber and then stained with 25 μl of 2 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma), for 2 min, and washed twice with 25 μl of PBS for 5 min. Slides were examined in a Zeiss Axioscope 2 imaging E fluorescence microscope and images captured by a RETIGA Exi fast 1394 camera (Q imaging) using Image Pro Plus version 5.1.2 (Media Cybernetics). Counting of stained networks was performed using the above software. Counting included the average of 600 kDNA networks per each reaction.

**Sucrose Gradient Sedimentation Analysis**—200 ng kDNA was incubated in a 10-μl reaction mixture containing 50 mM Tris-Cl, pH 8.0, 20 mM NaCl, 0.1% Triton X-100, 2 mM DTT,
and KAP4 and UMSBP as indicated, at 25 °C for 5 min. Reaction products were loaded on a 5-ml linear (5–20%) sucrose gradient, in the reaction buffer, containing 1 mM EDTA. Centrifugation was for 5 min at 4°C at 20,000 rpm, in a TST 55.5 Kontron rotor. Each gradient was fractionated into 21–23 fractions. The refractive index of each fraction in the gradient was recorded. To follow the profile of kDNA sedimentation, a sample of each fraction was dot-blotted onto a nitrocellulose membrane, which following UV cross-linking was submitted to Southern blot analysis using a 32P-labeled minicircle probe.

Electron Microscopy—Electron microscopy analyses of kDNA networks were performed using an FEI (Hillsboro, OR) Tecnai 12 TEM, with a Gatan (Pleasanton, CA) Ultrascan 400 CCD camera, following Thresher and Griffith (24). Briefly, kDNA networks, products of the condensation-decondensation reactions, were incubated for 10 min at 25 °C with glutaraldehyde (0.6%). Ammonium acetate (250 mM) was added, and the mixture was incubated for 10 min at room temperature. Then, cytochrome c (6.8 μg/ml) was added, and the mixture was placed on Parafilm for 90 s. kDNA networks were loaded onto the grid by its contact for 10 s with the mixture, followed by dehydration, sequentially, using 75 and 90% aqueous ethanol solutions, for 45 and 3 s, respectively. Shading was conducted as described previously (7).

Electrophoretic Mobility Shift Analyses (EMSA)—The 20-μl standard reaction mixture contained 25 mM Tris-Cl, pH 7.5, 2 mM MgCl2, 1 mM EDTA, 20% glycerol, 20 μg of BSA, 0.5 μg polynucleotide ligase, and 12.5 fmol of 5'-32P-labeled UMS DNA (G6G7TGGTGT). When UMSBP–KAP4–DNA interactions where assayed, glycerol, BSA, and polynucleotide ligase were omitted, and 0.1% Triton X-100 and 20 mM NaCl were included in the reaction mixture. The ligand used was a 117-bp double-stranded DNA fragment. KAP3 and UMSBP were added to the reaction mixture as indicated. Reaction mixtures were incubated at 30 °C for 30 min (or 20 min at 25 °C, for analyses of UMSBP–KAP3–DNA interactions) and electrophoresed in 8% native polyacrylamide gel (1:29 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 4 °C and 16 V/cm, for 1.5 h. Gels were analyzed by a PhosphorImager. A UMSBP unit was defined as described previously (7).

Topoisomerase Assay—Decatenation of kDNA networks was conducted in a 20-μl reaction mixture containing 50 mM Tris-Cl, pH 8.0, 110 mM KCl, 10 mM MgCl2, 0.5 mM DTT, 30 μg/ml BSA, 0.5 mM ATP, 200 ng of kDNA, and KAP4 and UMSBP as indicated. Human topoisomerase II was first examined by the yeast two-hybrid analyses (Fig. 1A and supplemental Fig. S1). For this purpose, CjUMSBP open reading frame was cloned into a “bait” plasmid, yielding a construct that expresses UMSBP fused to the LexA DNA binding domain. The prey plasmids encode for KAP1–4 proteins fused to the transcriptional activation domain B42. Following the transformation of yeast cells with these constructs and their expression (supplemental Fig. S1), protein–protein interactions of UMSBP with KAP3 and KAP4 were detected, as well as the expected UMSBP–KAP3–KAP4 interaction, which was previously shown to yield UMSBP homodimers (11). Under these assay conditions, we could not detect such interactions with KAP1 and KAP2, as judged by the lack of β-galactosidase (β-gal) activity in the cell lysates (Fig. 1A), as well as the lack of leucine prototrophy (see supplemental Fig. S1C). However, these observations do not exclude the possibility that protein–protein interactions of lower affinity, which may occur between UMSBP and KAP1 or KAP2, had not been detected by these analyses.

Next, we examined the occurrence of protein–protein interactions between UMSBP and KAP proteins in the trypanosomatid cell lysate, by co-immunoprecipitation of UMSBP using rabbit sera that were raised against the KAP proteins, followed by elution of the proteins from the immunoprecipitates. Considering the high specificity and sensitivity of the UMSBP EMSA (6, 7), we examined UMSBP DNA binding activity in the eluted fractions by EMSA (Fig. 1B). As expected, anti-UMSBP

**FIGURE 1.** Interactions of *C. fasciculata* UMSBP with KAP proteins. A, yeast two-hybrid analysis, following Paroush et al. (47). Yeast cells were co-transformed with pSH2–UMSBP bait plasmid, expressing LexA–UMSBP fusion and pJG4–prey plasmids, each containing one of the genes tested, expressed as B42 fusion protein. Interactions of the KAP1–4 proteins with UMSBP were monitored by assaying for β-gal activity in a liquid assay. Additionally, an assay for leucine prototrophy was performed, by plating serial dilutions of the cells on leucine-deficient medium (supplemental Fig. S1C). Column 1, B42–UMSBP; 2, B42–KAP1; 3, B42–KAP2; 4, B42–KAP3; and 5, B42–KAP4, where examined (see also supplemental Fig. S1). B, co-immunoprecipitation analysis of UMSBP by antibodies directed against KAP1–4 proteins conducted as described under “Experimental Procedures.” *C. fasciculata* cell extracts were used in immunoprecipitation analysis with the sera indicated below. Samples of glycine-elicited immunoprecipitates were subjected to EMSA, using UMS DNA ligand, and UMSBP activity was monitored and quantified by phosphorimaging. The following rabbit sera were used: lane 1, anti-UMSBP; lanes 2 and 3, no serum added; lane 4, serum added; lane 5, anti-KAP1; lane 6, anti-KAP2; lane 7, anti-KAP3; and lane 8, anti-KAP4.
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serum efficiently precipitated UMSBP (the total of 109.7 units was measured in the immunoprecipitate), whereas the preimmune, anti-KAP1 and anti-KAP2 sera did not (<0.1 UMSBP unit). Anti-KAP3 and anti-KAP4 antibodies precipitated UMSBP from the trypanosomatid cell extract. UMSBP activity measured in these co-immunoprecipitates was 3.4 and 5.7 units, respectively (~50- and ~80-fold higher than the activity measured with the preimmune serum, respectively). These results corroborate the observations obtained by the yeast two-hybrid analyses, indicating the interactions of KAP3 and KAP4 with UMSBP. To account for the possibility that these interactions were mediated by nucleic acids in the cell lysates, a control experiment was conducted in which the interactions of UMSBP with the two histone-like proteins KAP3 and KAP4 were monitored in a micrococcal nuclease-pretreated *C. fasciculata* cell lysate. No decrease in the co-immunoprecipitation of UMSBP (the total of 109.7 units measured in these co-immunoprecipitates) was observed in the reaction mixture has not resulted in detectable changes in the structure of uncondensed kDNA networks (data not shown). These observations revealed the capacity of UMSBP to modulate the structure of the KAPs-condensed kDNA networks efficiently.

Electron microscopy analyses of kDNA networks (Fig. 4) demonstrates the condensation of kDNA networks by KAP3 (Fig. 4B) and their decondensation following interaction with UMSBP (Fig. 4C), in accord with the fluorescence microscopy observations. Electron microscopy analysis performed using KAP4 for the condensation of kDNA yielded similar results (data not shown).

Next, we monitored the structural changes in kDNA networks following their interactions with KAPs or following their further interaction with UMSBP, using sucrose gradient sedimentation analysis. Fig. 5 demonstrates that treatment of purified uncondensed networks (Fig. 5A) with UMSBP has no significant effect on their sedimentation profile in sucrose gradient (Fig. 5B), in accord with the lack of detectable binding of UMSBP to DNA under these conditions (see below, Fig. 6). However, incubation of uncondensed networks with KAP4 (Fig. 5C) results in a dramatic increase in the sedimentation velocity of the kDNA networks, reflecting their conversion into more compact, condensed kDNA structures. Remarkably, when these KAP-condensed kDNA networks were interacted with UMSBP (Fig. 5D), a significant change was observed in their sedimentation rate, as most (67%) of the networks restored the sedimentation profile observed prior to their interaction with the KAP protein, indicating the significant decrease in the network compactness as a result of the interaction with UMSBP. A similar analysis, performed using KAP3 for the con-
kDNA Decondensation Is Mediated via Protein-Protein Interactions between KAPs and UMSBP—Is decondensation of kDNA by UMSBP mediated via protein-protein interactions with KAP proteins, or is it rather the result of their competition on the binding of DNA? It is noteworthy that a priori it would be quite unlikely to expect that UMSBP, a sequence-specific single-stranded origin-binding protein, would support the extensive displacement of KAPs from nonspecific duplex kDNA sequences, via protein-DNA interactions. Nevertheless, we addressed this question experimentally by EMSAs, using a double-stranded DNA ligand that does not contain the UMSBP binding sequence. Binding of KAP3 and UMSBP to DNA as well as the effect of UMSBP on KAP3-DNA interactions were examined. The data presented in Fig. 6 describe such an analysis, where KAP3 is used as the DNA-binding protein. It clearly demonstrates that whereas KAP3 tightly binds the duplex DNA fragment, no significant binding of UMSBP to the DNA ligand could be observed under these conditions, even in the presence of high concentrations of UMSBP (compare lanes a and b with lane c). Furthermore, in the presence of UMSBP, binding of KAP3 to kDNA was significantly inhibited (compare lanes c and d), at the same KAPs:UMSBP molar ratios, in which UMSBP-mediated decondensation of kDNA was observed by fluorescence microscopy (Fig. 3). Under these concentrations of UMSBP, no significant binding of this protein to the DNA could be measured. These observations demonstrate that although no significant binding of UMSBP to DNA could be detected, its presence significantly destabilized the KAP3-DNA nucleoprotein complex, indicating that UMSBP decondensation activity is independent of its DNA binding capacity.

FIGURE 3. Interaction of UMSBP with KAP-condensed networks results in kDNA decondensation. Analyses of DAPI-stained kDNA networks by fluorescence microscopy (A–E) and their quantification (F), were conducted as described under “Experimental Procedures.” 200 ng of untreated purified kDNA networks (A) were interacted with 45 pmol of KAP3 or KAP4 (B and D, respectively) and subsequently with 90 pmol of UMSBP (C and E, respectively). $F$, columns a–e present the quantification of the reactions described in panels A–E, respectively. The average of 600 kDNA networks was counted per each reaction.
UMSBP-mediated Decondensation Shows Preference for Interaction with KAP Proteins—Next, we examined the specificity of the UMSBP-mediated decondensation reaction. For this purpose, we used the fluorescence microscopy assay described above, monitoring the decondensation of kDNA which has been condensed by human histone H1 protein (Fig. 7A), with increasing concentrations of UMSBP (Fig. 7, B–E). The results show that compared with the decondensation of KAP-condensed kDNA (Fig. 3), UMSBP-mediated decondensation of the H1-condensed networks is relatively inefficient. Unlike the case of KAP3- or KAP4-condensed networks, where 2-fold molar excess of UMSBP to KAP3 or KAP4 yielded almost complete decondensation (98.1 and 100%, respectively) of the networks (Fig. 3F), only approximately 2.3% decondensation could be detected under these conditions with H1-condensed kDNA (Fig. 7, B and F). 5-fold molar excess of UMSBP to H1 results in only 5.7% decondensation of the H1-condensed networks (Fig. 7, C and F), and an order of magnitude molar excess of UMSBP over H1 increased the fraction of decondensed (and partially decondensed) networks to 54.4% (Fig. 7, D and F). Almost complete decondensation (of 95.9%) is observed at a UMSBP:H1 ratio of 20 (Fig. 7, E and F). These observations suggest a relatively lower affinity of the protein-protein interactions between UMSBP and human histone H1 compared with KAPS-UMSBP interactions, indicating the specificity of UMSBP in its interactions with the KAP proteins.

Decondensation Renders the kDNA Networks Accessible to Enzymes—In analogy to the remodeling of eukaryotic chromatin, our working hypothesis has been that decondensation of the kinetoplast nucleoid is similarly required to enable the access of the kDNA replication machinery to the mitochondrial genome. Following this rationale, we predicted that decondensation of the kDNA network would facilitate the access of replication enzymes to kDNA. It had been observed previously (25) that the first step in the replication of kDNA minicircles is their release from the network

![Electron microscopy images of UMSBP-mediated decondensation of kDNA](image1)

![Sucrose gradient sedimentation analysis of UMSBP-mediated decondensation of kDNA](image2)
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As covalently closed free DNA circles, which serve as the templates for kDNA replication initiation. Because topological decatenation of the network by topoisomerase II is a key pre-initiation step in kDNA replication, we studied its capacity to decatenate the KAP-condensed network and the effect of UMSBP-mediated decondensation of the network on the topological reaction. For this purpose we used agarose gel electrophoresis analyses of kDNA networks, which were condensed with KAP3 and then served as substrate for topoisomerase II, either directly or following their interaction with UMSBP. Because the trypanosomal DNA topoisomerase II that functions in the prereplication release of minicircles has not yet been identified (26, 27), we used human topoisomerase II in these experiments. In these analyses, the large kDNA networks cannot enter the gel, whereas their decatenated minicircle monomers migrate as covalently closed free DNA circles, which serve as the templates for kDNA replication initiation.

Condensation of the kDNA network in vitro was performed in this study by mitochondrial histone H1-like proteins, which were implicated in vivo in the packaging of kDNA (16). Packaging and condensation of genomes have been observed in cells and DNA-containing organelles, in which genomes are found as intricate nucleoprotein complexes, forming the nucleoid structure in prokaryotic cells and mitochondria and the chromatin structure in the eukaryotic nucleus. It is remarkable that whereas bacterial DNAs are packaged into a nucleoid structure by proteins, which are structurally different from eukaryotic nuclear histones and HMG proteins (28), mitochondrial DNAs are packaged into nucleoid forms by eukaryotic HMG-like proteins and histone H1-like proteins but not by proteins of bacterial ancestry. It has been suggested that the use of these proteins, of eukaryotic origin, results from the severe reduction of mitochondrial genomes during evolution in eukaryotic cells (29) with loss of many of the bacterial endosymbiont original genes, including genes encoding nucleoid-associated proteins. Consequently, the bacterial nucleoid-associated proteins were replaced by functionally similar, but structurally unrelated, eukaryotic proteins (30). Hence, the organization of mitochondrial nucleoids was found to be different from both the nuclear chromatin and the bacterial nucleoid (30–34). The main packaging proteins of mitochondrial DNA, studied in yeast and metazoa systems, are proteins that contain HMG boxes, such as Abf2 in yeasts and mtTFA in mammalian cells. In trypanosomatids, these functions are conducted by the eukaryotic kinetoplast-associated histone H1-like KAP proteins. In the slime mold Physarum polycephalum the histone H1-like protein Glom, also contains two HMG domains (35). Preliminary analyses of C. fasciculata KAP3 and KAP4 sequences also predict potential HMG-like domains in these proteins. Their nature and potential function have yet to be determined.4

Due to the release of kDNA minicircles from the network, enabling the initiation of minicircle replication.

DISCUSSION

Condensation of the kDNA network in vitro was performed in this study by mitochondrial histone H1-like proteins, which were implicated in vivo in the packaging of kDNA (16). Packaging and condensation of genomes have been observed in cells and DNA-containing organelles, in which genomes are found as intricate nucleoprotein complexes, forming the nucleoid structure in prokaryotic cells and mitochondria and the chromatin structure in the eukaryotic nucleus. It is remarkable that whereas bacterial DNAs are packaged into a nucleoid structure by proteins, which are structurally different from eukaryotic nuclear histones and HMG proteins (28), mitochondrial DNAs are packaged into nucleoid forms by eukaryotic HMG-like proteins and histone H1-like proteins but not by proteins of bacterial ancestry. It has been suggested that the use of these proteins, of eukaryotic origin, results from the severe reduction of mitochondrial genomes during evolution in eukaryotic cells (29) with loss of many of the bacterial endosymbiont original genes, including genes encoding nucleoid-associated proteins. Consequently, the bacterial nucleoid-associated proteins were replaced by functionally similar, but structurally unrelated, eukaryotic proteins (30). Hence, the organization of mitochondrial nucleoids was found to be different from both the nuclear chromatin and the bacterial nucleoid (30–34). The main packaging proteins of mitochondrial DNA, studied in yeast and metazoa systems, are proteins that contain HMG boxes, such as Abf2 in yeasts and mtTFA in mammalian cells. In trypanosomatids, these functions are conducted by the eukaryotic kinetoplast-associated histone H1-like KAP proteins. In the slime mold Physarum polycephalum the histone H1-like protein Glom, also contains two HMG domains (35). Preliminary analyses of C. fasciculata KAP3 and KAP4 sequences also predict potential HMG-like domains in these proteins. Their nature and potential function have yet to be determined.4

Condensation and packing of genomes limit their accessibility to the replication and transcription apparatus. This has been studied extensively in the case of nuclear chromatin, revealing that uncoiling of the nucleosomal complex, a process known as “chromatin remodeling,” is mediated by histone posttranslational modifications, especially acetylation of lysine residues, which decreases their affinity to DNA (36). Whether or not reversible condensation of mitochondrial DNA affects its functionality in a similar way is not yet clear. Unlike in nuclear chromatin, the mitochondrial DNA, packed in the nucleoid, is not associated with histones in a nucleosomal structure. Hence, it has been suggested that in mitochondrial systems, where DNA packing is mediated by HMG-like proteins (such as in yeast and mammalian cells), the DNA-bound HMG-like proteins might not pose a strong barrier to the phage homologous mitochondrial RNA polymerase (30, 37–40). Nevertheless, the current study suggests that kDNA decondensation is essential for kDNA replication initiation. This is because association of kDNA with KAP3 protein poses a barrier to the DNA topoi-

4 G. Kapach and J. Shlomai, unpublished data.
somesar II, which catalyzes the first reaction in kDNA replication, the prereplication decatenation of kDNA, providing the monomeric minicircle templates for replication initiation (Fig. 8).

Does UMSBP mediate the decondensation of the kDNA network in vivo? In a previous study we observed that silencing of the two T. brucei UMSBP orthologues by RNAi resulted in the complete inhibition of the kDNA segregation and caused changes in the organization of the kDNA network (12). However, these in vivo analyses have not clarified the effect of UMSBP-mediated decondensation per se on the abnormal organization of the network. This is because it was difficult to distinguish between the distinct contributions of the suppression of the network decondensation versus the inhibition of its segregation.

Intriguingly, the observations presented here demonstrate that UMSBP, which functions as a kDNA replication initiator protein, may also function during a prereplication stage. Previous RNAi analyses have shown (12) that prereplicated minicircles were released from the network in the UMSBP RNAi-induced cells, but failed to start their initiation and be converted into replication intermediates. If the function of UMSBP was required at a preinitiation stage to enable the release of minicircles from the network, then its knockdown should have resulted in the lack of released prereplicated minicircles, rather than in their failure to initiate their replication. One explanation to this apparent discrepancy is based on the observation that the silencing of the UMSBP genes, as indicated in that report (12), was incomplete. In fact, it was this leakiness in silencing of the UMSBP genes that enabled a fraction of the kDNA networks in the RNAi-induced culture to escape the inhibition of minicircle replication initiation and generate unsegregated kDNA networks, which revealed the downstream function of UMSBP in kDNA segregation (12).

Similarly, it is quite plausible that this leakiness in UMSBP knockdown (12) enabled the partial decondensation of kDNA

FIGURE 7. Decondensation of histone H1-condensed kDNA networks, by interaction with UMSBP. Analyses of DAPI-stained kDNA networks by fluorescence microscopy (A–E) and their quantification (F), were conducted as described under "Experimental Procedures." 200 ng of untreated purified kDNA networks were interacted with 20 pmol of histone H1 (A) and subsequently with increasing concentrations of UMSBP at UMSBP:histone H1 molar ratios of 2, 5, 10, and 20 (B–E, respectively). F, columns a–e present the quantification of the reactions described in panels A–E, respectively. The average of 600 kDNA networks was counted per each reaction.
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networks and their consequent decatenation, revealing the function of UMSBP during replication initiation (12).

Finally, the in vitro observations presented here demonstrate an example of a different route for DNA decondensation, which is mediated via protein-protein interactions rather than through the posttranslational covalent modification of the DNA-condensing proteins. It is noteworthy that observations made in several other systems also indicate the occurrence of interactions between replication initiator proteins and DNA-condensing proteins. Interaction of E. coli dnaA with the nucleoid organizers HU and IHF proteins was shown to stimulate unwinding of the oriC (41). Interactions of the simian virus 40 (SV40) T antigen and of human papilloma virus E1 with histone H1 displace histone H1 from the origin region, promoting replication initiation and elongation (42, 43). In eukaryotic cells the replication initiator, origin recognition complex was shown to play a role during DNA replication initiation (44). Together, these observations indicate that interactions between replication initiator proteins and DNA-condensing proteins may play an important role during DNA replication initiation.

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