Binding of the Universal Minicircle Sequence Binding Protein at the Kinetoplast DNA Replication Origin*‡§

Itay Onn†‡, Irit Kapeller‡, Kawther Abu-Elneel‡∥, and Joseph Shlomai†‡∥

From the †Department of Parasitology, The Kuvir Center for the Study of Infectious and Tropical Diseases and the ‡Department of Molecular Genetics and Biotechnology, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

Kinetoplast DNA (kDNA)4 is a unique extrachromosomal DNA found in the single mitochondrion of trypanosomatids. In the species Crithidia fasciculata, 5000 topologically linked duplex DNA minicircles. Their replication initiates at two conserved sequences, a dodecamer, known as the universal minicircle sequence (UMS), and a hexamer, which are located at the replication origins of the minicircle L and H strands, respectively. A UMS-binding protein (UMSBP) binds specifically the 12-mer UMS sequence and a 14-mer sequence that contains the conserved hexamer in their single-stranded DNA conformation. In vivo cross-linking analyses reveal the binding of UMSBP to kinetoplast DNA networks in the cell. Furthermore, UMSBP binds in vitro to native minicircle origin fragments, carrying the UMSBP recognition sequences. UMSBP binding at the replication origin induces conformational changes in the bound DNA through its folding, aggregation and condensation.

Kinetoplast DNA (kDNA)4 is a unique extrachromosomal DNA found in the single mitochondrion of trypanosomatids. In the species Crithidia fasciculata, the kDNA network consists of ~5000 duplex DNA minicircles of 2.5 kbp and 50 maxicircles of 37 kbp that are interlocked topologically to form a DNA network (1–5). Maxicircles contain mitochondrial genes, encoding mitochondrial proteins and rRNA. Minicircles encode for guide RNAs that function in the process of mitochondrial mRNA editing (6–8). Minicircles in most trypanosomatid species are heterogeneous in sequence. However, a few structural and sequence motifs are conserved in all the minicircles within networks of a given species as well as in minicircles of different trypanosomatid species. These include two short sequences that are associated with the process of replication initiation, which are located 70–100 nucleotides apart in the minicircle molecule; they are the dodecameric sequence GGGGT-TGGTGTA, designated the universal minicircle sequence (UMS) and the hexameric sequence ACGCCC. These sequences have been mapped to the sites of the replication origins of the minicircle light (L) and heavy (H) strands, respectively (for review, see Refs. 1–5). Comparison of the complete minicircle sequences of several species of trypanosomatids reveals that minicircles from different trypanosomatid species contain either one (Trypanosoma brucei GenBank™ accession number M15323; Leishmania major, GenBank™ accession number Z32845), two (C. fasciculata, GenBank™ accession number M19266), or three (Trypanosoma cruzi, GenBank™ accession number X56188) copies of the conserved origin region. The two conserved origin regions in C. fasciculata kDNA minicircles are designated OriA and OriB. Each of these origin regions includes the two conserved origin sequences. According to the currently accepted model for kDNA replication, only one of the origins is active during minicircle replication, and the active origin is randomly selected in each replication cycle (9).

Unlike the replication of mitochondrial DNA in other eukaryotic cells, which takes place throughout the cell cycle, kDNA replicates only once, during S phase of the cell cycle, approximately in parallel with the replication of the nuclear DNA (10). According to the current model for kDNA replication, minicircles are released during S-phase from the center of the network by the decatenation activity of a type II DNA topoisomerase and are translocated to the kineto-flagellar zone, located between the kDNA network and the flagellar basal body (11). Each minicircle is an individual replicon that replicates unidirectionally in a semi-discontinuous mechanism, forming two gapped and nicked progeny molecules (12). Minicircles are then transferred onto two antipodal sites, flanking the kDNA disk, in which primer removal, repair of the Okazaki fragment gaps, and reattachment of the progeny minicircles to the network occurs. The final gap-filling and sealing of the topologically linked minicircles take place before the network division (13).

Several of the proteins involved in the replication of the kDNA network have been identified, including the origin-binding protein, designated UMS-binding protein (UMSBP). UMSBP has been purified from C. fasciculata, and its encoding
gene and genomic locus were cloned and analyzed (14–17). Genes encoding for homologous proteins have been identified in other trypanosomatid species (18) (supplemental Table 1). The protein binds specifically to the two conserved sequences located at the minicircle replication origins, the UMS dodecamer and a 14-mer sequence (H14) containing the conserved hexamer core and 8 flanking nucleotides, in their single-stranded conformation (14, 15, 19). The 13.7-kDa protein contains five tandemly arranged CCHC-type zinc-knuckle motifs. This motif forms a compact zinc finger that has been associated with the binding of single-stranded nucleic acids (20–22). We have previously reported on the effect of redox on the binding of UMSBP to the origin sequence as well as on the protein oligomerization and suggested that redox potential may play a major role in the regulation of UMSBP action at the replication origin (23). Immunolocalization of UMSBP within the kinetoplast has revealed two distinct protein foci at the kinetoflagellar zone, near the suggested minicircle replication site (24). As a single-stranded DNA-binding protein, an intriguing question is, How does UMSBP bind the native origin in the duplex kDNA minicircle? This question had been addressed in earlier studies, demonstrating that the minicircle origin region is distorted and adopts a single-strand conformation (25). Yet no direct evidence has been provided in these studies for the binding of the full-length native origin, including both the L-strand and H-strand initiation sites, by UMSBP, and biochemical and structural analyses of this intriguing nucleoprotein complex have not yet been described.

In this study we present evidence for the interaction of UMSBP with kDNA networks in vivo. We further describe the interactions of UMSBP with the sequences conserved at the native minicircle replication origin and show that UMSBP binding induces a significant conformational change in the DNA structure at the native minicircle replication origin.

**EXPERIMENTAL PROCEDURES**

**Preparation of UMSBP—Cloning of the C. fasciculata UMSBP**

Gene open reading frame (15) and the preparation of pure recombinant UMSBP were conducted as we have previously described (23).

**In Vivo Cross-linking and Purification of Kinetoplast Nucleoprotein Complexes—** 700 ml of C. fasciculata cell culture were grown to logarithmic phase (4.4 × 10^7 cells/ml) as described elsewhere (14, 19). Cross-linking of kinetoplasts and their partial purification were carried out following the procedure of Xu and Ray (26) with modifications as follows. Formaldehyde (Sigma) was added to a final concentration of 0.75%, and the cells were incubated for 10 min at 37 °C with shaking followed by chilling in ice water. Cells were harvested in a Sorvall GS-3 rotor at 5000 rpm for 10 min at 4 °C and washed once with phosphate-buffered saline and once in NET buffer (10 mM Tris-Cl pH 8.0, 100 mM NaCl, 100 mM EDTA). Cells were then resuspended to final density of 1.25 × 10^6 cells/ml in NET buffer containing 3% sarkosyl and heated for 10 min at 65 °C. The cell lysate was fractionated through a series of three step gradients as described by Xu and Ray (26) as follows. Each 12.0-ml lysate was loaded on the top of a step gradient containing a layer of 23 ml of 20% sucrose in TNES buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Sarkosyl) and a bottom layer of 3 ml of 20% sucrose in TNES, containing NaI to yield a density of 1.36 g/ml, and was centrifuged for 5 min at 10,000 rpm in a Beckman SW28 rotor at 25 °C. The bottom 7.6 ml was discarded, and the step-gradient was refilled from the bottom with 5 ml of 20% sucrose in TNES, 3 ml of 20% sucrose, NaI solution (1.36 g/ml), and 3 ml of saturated NaI in TNE (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA) and was centrifuged at 20,000 rpm for 10 min in a Beckman SW28 rotor at 25 °C. The bottom 10 ml of the gradient was fractionated into 10 1.0-ml fractions. To monitor their content of kDNA, samples withdrawn from the gradient fractions were electrophoresed in a 1% agarose gel and stained with ethidium bromide. The DNA-containing fractions (5–8) were pooled, diluted with equal volume of TNES, and centrifuged in an Eppendorf 5417R at 14,000 rpm and 4 °C for 1 h. The pellets, containing kDNA networks, were resuspended in 18 ml of TNES. Each 9.0-ml suspension of DNA was re-centrifuged as described above through a step gradient containing (from top to bottom) 23 ml of 20% sucrose, 3 ml of 20% sucrose, NaI (1.36 g/ml) in TNES, and 3 ml of saturated NaI solution in TNE. The bottom 10 ml of the gradient was fractionated, and the fractions containing kDNA were diluted and centrifuged as described above. The nucleoprotein pellets were resuspended in 200 μl of TNES and loaded onto a 5.0 ml of sucrose/NaI linear gradient at the range of 10–30% sucrose, saturated NaI in 50 mM Tris-Cl, pH 8.0, 100 mM NaCl. The gradients were centrifuged at 20,000 rpm for 20 min in a Beckman SW55.5 rotor at 25 °C. 20 fractions of 250 μl were collected from the bottom of the tubes. Samples withdrawn from each fraction were analyzed for their kDNA content by electrophoresis in ethidium bromide-stained agarose gel followed by Southern blot hybridization analysis using a radioactively labeled minicircle probe following the published procedures (27). Two of the DNA-containing fractions were randomly selected for further analysis. Each fraction was divided into halves. One-half was incubated overnight at 65 °C to reverse the formaldehyde cross-linking of the nucleoprotein complex, and the other was incubated overnight at 4 °C. The fractions were then added to loading buffer and incubated at 37 °C for 30 min and then electrophoresed in 16.5% SDS–PAGE as described below along with protein markers and recombinant UMSBP. Gels were analyzed by Western blot analysis using anti-UMSBP antibodies and Envision+ System HRP (DakoCytonation) secondary antibodies by ECL as described below.

**Immunoprecipitation of Nucleoprotein Complexes in Vitro—OriA region of the minicircle was amplified by PCR, using as template either a C. fasciculata kDNA minicircle or a plasmid containing a full-length minicircle (linearized by cleavage at its XhoI site). All the sets of primers (A–I) used for preparation of wild-type (WT) and mutant origin region in this work are described in supplemental Table 2. The DNA fragments were biotinylated using 5′-biotinylated primers.

PCR amplification was carried out using Failsafe PCR kit (Epícenter Technologies). PCR products were purified using PCR cleaning column (Qiagen). PCR using primers set A (supplemental Table 2) yielded a 313-bp fragment (positions 319–631 (28)) that includes the OriA. Mutated OriA sequences were
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generated in two steps by PCR amplification using in the first step a 313-bp fragment of kDNA minicircle as the DNA template and two sets of mutated primers for each mutant, primers set B and C (supplemental Table 2), yielding two fragments with an overlap (underlined) at the H14 site. In the second step the fragments described above were used as templates with primers set A, yielding a 313-bp fragment that includes a mutated H14 (positions 396–409) and WT UMS sequence. In the first step primers set D and E yielded two fragments with an overlap (underlined, supplemental Table 2) at the UMS site. In the second step the fragments described above were used as template with the primers set A (supplemental Table 2), yielding a 313-bp fragment that included a WT H14 and mutated UMS sequences (positions 489–500). The template for the generation of a double mutant, mutated in both H14 and UMS was the PCR product of the 313-bp fragment that includes a WT H14 and mutated UMS. The two steps of PCR amplification were conducted as in the production of the mutated H14, as described above. PCR products produced in the first and second steps of the preparation of all mutants and the WT were purified by electrophoresis in agarose gel followed by their extraction using a gel extraction preparation (QIAquick, Qiagen).

Binding of UMSBP to the double-stranded origin fragment was assayed in a 10-μl reaction mixture containing 150 ng (0.75 pmol) of the origin-containing PCR-amplified DNA fragments, 25 mM Tris-Cl, pH 7.5, 5 mM dithiothreitol, 2 mM MgCl₂, and 1 mg/ml bovine serum albumin. Reactions started by the addition of IP (60–500 RU) binding reaction mixture containing 25 mM Tris-Cl, pH 7.5, 2 mM MgCl₂, 5 mM dithiothreitol, 10% glycerol, 1 μg/ml bovine serum albumin, 0.25 μg of poly(dl-dC)-poly(dl-dC), and 25 fmol of [γ-32P]ATP and T4 polynucleotide kinase (MBI Fermentas).

EMSA—Analyses were carried out as described previously (14, 16). Samples of UMSBP as indicated were incubated in the 10-μl binding reaction mixture containing 25 mM Tris-Cl, pH 7.5, 2 mM MgCl₂, 5 mM dithiothreitol, 10% glycerol, 1 μg/ml bovine serum albumin, 0.25 μg of poly(dl-dC)-poly(dl-dC), and 25 fmol of [γ-32P]ATP and T4 polynucleotide kinase (MBI Fermentas).

Micrococcal Nuclease Assay—Samples of UMSBP as indicated were added to a reaction mixture containing 0.25 pmol of 5′-32P-labeled 313 bp minicircle DNA fragment (positions 319–631, GenBank™ accession number M19266), 25 mM Tris-Cl, pH 7.5, 2 mM MgCl₂, 5 mM CaCl₂, and 20 mM dithiothreitol. The reaction was incubated for 60 min at 30 °C followed by the addition of 1 unit of micrococcal nuclease (MBI Fermentas) for 10 min at 37 °C. The reaction was stopped by the addition of 25 mM EGTA, and the reaction products were loaded onto a 6% native polyacrylamide gel (1:29, bisacrylamide/acrylamide) along with a sequencing reaction of kDNA (see above) was PCR-amplified using Insafe PCR kit (Epicenter Technologies). PCR primer set F (supplemental Table 2) was used for the preparation of the WT OriA-containing fragment yielding a 142-bp fragment (positions 377–518). Mutated OriA sequences were generated by PCR amplification using primers that include mutated origins. The 142-bp fragment containing OriA served as template. Primers sets (supplementary Table 2) were as follows. Set G yielded a 144-bp fragment (positions 377–520) that included a mutated H14 and WT UMS sequences; set H yielded a 144-bp fragment (positions 377–520) that included a WT H14 and a mutated UMS sequence, and set I yielded a 144-bp fragment (positions 377–520) that included a double mutant (a mutated H14 and a mutated UMS sequence). The WT and mutated PCR products were purified by electrophoresis in agarose gel followed by their extraction from the gel using a gel extraction preparation (QIAquick). The primers used for the preparation of ligands for micrococcal nuclease digestion of nucleoprotein complexes were 5′-32P-labeled using [γ-32P]ATP and T4 polynucleotide kinase (MBI Fermentas).
**Thermodynamic Analysis**—Structural properties of the *C. fasciculata* minicircle sequence (GenBank™ accession number M19266) were analyzed for DNA curvature according to Bolshoy et al. (29) and propeller-twist, as a measure of helix rigidity, as described by El Hassan and Calladine (30). Window size was 100 bp, and slide was 10 bp.

**Analysis of Proteins by SDS-PAGE**—Protein samples in loading buffer containing 50 mM Tris-Cl, pH 6.85, 4% SDS, 3.5% β-mercaptoethanol, 10% (v/v) glycerol, and 10 mM EDTA were incubated at 37 °C for 30 min and loaded onto a 16.5% Tris-Tricine SDS-polyacrylamide gel (31) along with protein size markers (Rainbow pre-stained low molecular weight, Amersham Biosciences). Upper electrophoresis buffer was 0.1 M Tris-Tricine, pH 8.25, containing 0.1% SDS; lower buffer was 0.2 M Tris-Cl, pH 8.9.

**Western Blot Analysis**—Protein samples were analyzed by SDS-PAGE electrophoresis, as described above. Protein bands were transferred onto a Protran BA85 cellulose nitrate membrane (Schleicher & Schuell). Membranes were blocked by incubation for 30 min in 5% skim dry milk (Difco) in phosphate-buffered saline containing 0.1% (v/v) Tween 20 and probed for 90 min with anti-UMSBP antibodies that were raised in rabbit. Membranes were probed for 45 min with a 1:13,000 dilution horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (DacoCytonation) followed by ECL detection as recommended by the manufacturer (Amersham Biosciences).

**RESULTS**

**UMSBP Interacts in Vivo with kDNA Networks**—UMSBP was immunolocalized within the kinetoplast to two discrete foci located at the kineto-flagellar zone (24) at the site implicated with kDNA minicircle replication initiation (11). Moreover, immunofluorescence analysis of synchronized *C. fasciculata* culture revealed a higher abundance of UMSBP at this site during S-phase, in correlation with the progress of kDNA replication. However, whereas binding of UMSBP to single-stranded origin sequences has been previously demonstrated in DNA binding assays *in vitro*, its interaction with native kDNA networks *in vivo* has not yet been challenged. This question was addressed here using an *in vivo* protein-DNA cross-linking approach following a procedure modified from Xu and Ray (26). Lysates prepared from cross-linked cells were fractionated by centrifugation through a series of sucrose/NaI stepwise gradients (26), and pooled kDNA-containing fractions obtained were fractionated by a linear sucrose/NaI gradient. Two kDNA-containing fractions (Fig. 1A) from the final linear sucrose/NaI gradient were monitored by SDS-PAGE analysis followed by Western blot analysis using anti-UMSBP antibodies either with or without heat reversal of the nucleoprotein cross-linking (Fig. 1B). In both kDNA-containing fractions, reversal of the formaldehyde cross-linking by heating at 65 °C overnight (26) resulted in the release of UMSBP as indicated by the subsequent SDS-PAGE and Western blot analysis, whereas only traces of protein were released from the untreated complexes (Fig. 1B). Under the denaturing and reducing conditions used in this analysis the majority of released protein migrated in the gel as monomers. The minor slower migrating bands observed (arrowheads, Fig. 1B) may represent traces of higher oligomeric forms of UMSBP, generated under the cross-linking conditions, that resisted the denaturing and reducing electrophoresis conditions. These results indicate that UMSBP interacts *in vivo* with kDNA networks. Interactions of UMSBP with free kDNA minicircles are undetectable by the method employed here, since the purification of the large kDNA-protein complexes in the sucrose/NaI gradients apparently excludes unlinked free minicircles.

**UMSBP Binds in Vitro Double-stranded DNA Fragments Carrying the Minicircle Replication Origin**—We have previously shown that UMSBP is a single-stranded DNA-binding protein that binds specifically the 12-mer UMS and the 14-mer minicircle sequence containing the core hexamer (H14) in their single-stranded conformation (14, 16, 19). In binding competition analyses duplex native kDNA minicircles, either free or topologically linked, competed efficiently with a radioactively labeled single-stranded UMS on the binding of UMSBP (25). However, no direct evidence has yet been reported, demonstrating the binding of UMSBP to the native minicircle origin, which includes both its UMS and H14 sequences in a duplex kDNA conformation. To examine the binding of UMSBP at the origin and its dependence upon the presence of its UMS and/or H14 binding sites, we have studied the interactions of UMSBP with an OriA sequence, which was mutated at either its UMS,
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FIGURE 2. Binding of UMSBP to the native OriA region is mediated through the recognition of the UMS and H14 sequences. A, schematic representation of the wild type and mutant 313 bp PCR-generated fragments containing the OriA region (positions 319–631 (28)). B, 0.75 pmol of a 313-bp minicircle DNA fragment containing either a WT OriA site (lanes a–b) or this site mutated at either its H14 (lanes c–d), UMS (lanes e–f), or at both sites (lanes g–h) were incubated with (lanes b, d, f, and h) or without (lanes a, c, e, and g) 7.5 pmol of pure recombinant UMSBP. The nucleoprotein complexes were UV cross-linked and immunoprecipitated with anti-UMSBP antibodies, and the extracted DNA was analyzed by Southern blot hybridization using a minicircle DNA probe as described under “Experimental Procedures.” C, a quantitative representation of the hybridization data described in panel B; values above the columns indicate percentage of DNA precipitation relative to the value measured for the WT sequence.

H14, or both sequences. Mutated OriA sequences were prepared by PCR amplification using mutated oligonucleotides primers in which purines were substituted for purines and pyrimidines for pyrimidines and the 313-bp OriA (positions 319–631 (28)) template (Fig. 2A) as described under “Experimental Procedures.” DNA fragments were incubated with UMSBP at DNA:protein molar ratio of 1:10 and were UV-cross-linked and then immunoprecipitated using anti-UMSBP antibodies. The precipitated protein-DNA complexes were then treated with proteinase K, and the remaining DNA was analyzed by agarose gel electrophoresis followed by Southern blot hybridization analysis using a radiolabeled minicircle probe. The hybridization analysis (Fig. 2B) demonstrated clearly that immunoprecipitation of OriA DNA fragment mutated in either their H14 or UMS sites (lanes d and f) was significantly less efficient (36.7 and 24.3%, respectively) than that of the WT OriA sequence (lane b) and was further significantly reduced (6.5% immunoprecipitation, respectively, compared with that of the WT OriA) when both sites were mutated (compare lanes h and b).

To monitor directly and quantify the interaction of UMSBP with the native minicircle replication origin, we used the SPR BIAcore approach. A 5′-biotinylated oligonucleotide primer was used to synthesize a 313-bp DNA fragment containing the minicircle OriA region, which was immobilized to a streptavidin-coated BIAcore SA sensor chip. In the mobile phase we have used increasing concentrations of UMSBP to measure the biochemical parameters of the reaction. Because the DNA fragment contained two UMSBP recognition sequences (the 12-mer UMS and the 14-mer H14), the sensograms obtained were analyzed according to a heterogeneous ligand binding model (Fig. 3). An equilibrium binding constant value \(K_D\) of \(3.97 \times 10^{-9}\) M was measured for the interaction of UMSBP with one binding site (Fig. 3, Site 2). This value is similar to the values obtained in our previous analyses using a single-stranded UMS (16, 23) or H14 (19) oligonucleotides. The \(K_D\) value measured for the interaction of UMSBP at the second site (Fig. 3, Site 1) was considerably higher, \(3.11 \times 10^{-8}\) M, indicating a significantly lower affinity of the interaction of UMSBP with this binding site. The kinetics data (Fig. 3) revealed that whereas similar association rates were measured for the interactions of UMSBP with its two binding sequences in OriA, a difference of an order of magnitude was measured in the corresponding dissociation rates, indicating the different random GT stretches in the fragment. These results demonstrate that binding of UMSBP to the native minicircle origin site is mediated through the recognition of its UMS and H14 binding sequences and that the full capacity of UMSBP binding at the OriA occurs only in the presence of both UMSBP binding sites.
stability of the nucleoprotein complexes generated at the two UMSBP binding sites. These BIAcore data could not per se match the UMSBP interacting sites 1 and 2 with either the UMS or the H14 binding sites. However, the complementary analyses of the interactions of UMSBP with OriA sequences, mutated in either their UMS or H14 sites (Fig. 2), revealed, reproducibly, a more efficient binding of UMSBP to the WT UMS sequence, which may implicate the UMS and H14 sequences with the higher and lower affinity binding sites, respectively.

The Two UMSBP Binding Sites at the Minicircle Origin Differ in Their Predicted Structural Properties—Next we have studied the structural properties of the two UMSBP binding sequences at the minicircle replication origin. Structural properties of the C. fasciculata minicircle sequence were analyzed for DNA curvature according to Bolshoy et al. (29) and propeller-twist, as a measure of helix rigidity, following El Hassan and Calladine (30). Analysis of the minicircles sequence revealed two significant differences between the UMS and the hexamer regions. The sharp curvature (29) observed at the UMS site (Fig. 4) may induce the local unwinding of the DNA double helix at this region. These results are in accord with our previously reported experimental observations, indicating that the UMS sequence resides within locally unwound or otherwise sharply distorted DNA structure (25). On the other hand, the H14 region resides within a duplex DNA region, whose low propeller-twist angle measured indicates the low rigidity of the DNA helix (30) and thereby its capacity to adopt a stem-loop structure. Indeed, an Mfold single-stranded DNA secondary structure analysis (32–34) of the H14 sequence predicts the potential formation of a stem-loop structure (data not shown) at this site. Comparison of the thermodynamic data for the OriA and OriB regions does not reveal any significant difference in their intrinsic curvature. The local peak of propeller-twist, however, is wider in OriA than in OriB. The implications of these different thermodynamic characteristics of the binding of UMSBP to the two replication origins has yet to be explored.

UMSBP Binding Induces Conformational Changes in the Bound DNA—Previous EMSA studies using short 32P-labeled single-stranded UMS or H14 oligonucleotides revealed, as
expected, the formation of stable protein-DNA complexes with electrophoretic mobility lower than that of the free DNA ligands (14, 16, 19). To further study the interaction of UMSBP with the native minicircle origin region, we used a radiolabeled 142-bp duplex DNA fragment containing either a WT OriA sequence or an OriA sequence (144 bp) that was mutated partially in its UMS, H14, or both sequences as a ligand in an EMSA analysis. As described in Fig. 5, UMSBP interacts with the duplex DNA fragment to yield a protein-DNA complex. However, unexpectedly, unlike the observations with single-stranded oligonucleotide ligands, the electrophoretic mobility of the nucleoprotein complex generated with the native WT origin fragment was higher than that of the free, unbound DNA ligand (Fig. 5A, lanes b–d). Extraction of the DNA from the nucleoprotein complex followed by agarose gel electrophoresis analysis along with DNA size markers excluded the possibility that the higher electrophoretic mobility of the complex observed has resulted from a nucleolytic cleavage of the bound DNA (not shown). These observations indicated that the enhanced electrophoretic mobility of the nucleoprotein complex could have resulted from a conformational change induced in the complexed DNA molecule through its interaction with the bound UMSBP. Involvement of UMSBP in the generation of the high mobility nucleoprotein complexes was strongly supported by requirement for the presence of UMSBP binding sequences UMS and H14 in the DNA ligand. Generation of the nucleoprotein complexes was significantly less efficient with origin sequences mutated in either their UMS or H14 sites (compare Fig. 5A, lanes b–d to lanes f–h and j–l), whereas mutation of both sites resulted in the complete inhibition of complex formation (compare lanes b–d and n–p) (Fig. 5, A and B).

The increase observed in the electrophoretic mobility of the nucleoprotein complex may reflect the higher-order organization of the bound DNA molecule through its folding, aggregation, or condensation, as a result of UMSBP binding. These observations suggest a role for both UMSBP binding sites in the generation of the high mobility UMSBP-OriA complex. They are in accord with previous reports showing that many site-specific DNA-binding proteins bind several target sites on the DNA and self-associate by protein-protein interactions to generate highly organized nucleoprotein structures (35–40). In this context it is noteworthy that we have previously reported on the high capacity of UMSBP monomers to conduct protein-protein interactions and to oligomerize in solution (23).

To study the nature of the conformational change induced in the bound DNA by UMSBP, we used the 32P-labeled 313-bp DNA fragment as a ligand in a binding reaction in the presence of increasing molar ratios (within 2 orders of magnitude) of UMSBP. The complexes generated were treated with micrococcal nuclease followed by electrophoresis analysis on denaturing polyacrylamide sequencing gel along with a sequencing reaction of the minicircle origin region. Fig. 6 shows the presence of a series of nuclease hypersensitive sites along the DNA ligand that are induced only in the presence of UMSBP. The hypersensitive sites are distributed in the bound DNA at intervals of 10–14 bp, a periodicity that apparently would not support a regular wrapping of the DNA molecule around the protein, but would rather indicate its otherwise distortion through folding, bending, and unwinding of the DNA double helix. Remarkably, the most hypersensitive site in the bound DNA

![FIGURE 6. UMSBP induces local hypersensitivity of the bound DNA to micrococcal endonuclease. UMSBP was incubated at increasing concentrations with 0.25 pmol of a 32P-labeled DNA fragment containing the minicircle origin region (lanes a, and c–e) in the standard binding reaction mixture supplemented with 5 mM CaCl₂ as described under “Experimental Procedures.” The protein-DNA complexes were treated with micrococcal nuclease (MCN) (lanes c–e), and the reaction products were analyzed by denaturing gel electrophoresis alongside a sequencing reaction as described under “Experimental Procedures.” In lane a, no micrococcal nuclease added; b, no UMSBP added; c, 0.25 pmol UMSBP; d, 2.5 pmol UMSBP; e, 25 pmol of UMSBP. The arrow indicates the location of a hypersensitive site in proximity to the 3’ end of the UMS. The vertical lines and numbers indicate the location of the UMS and the H14 sequences on the minicircle molecule (GenBank™ accession number M19266).]
was mapped next to the UMS sequence (Fig. 6). Because micrococcal nuclease has a cleavage preference for single-stranded DNA, these results may suggest that the interaction of UMSBP with the replication origin induces conformational changes that results in the stable unwinding of the replication origin region. Such a model is in agreement with previous observations, indicating that the minicircle origin region is bent and unwound or otherwise highly distorted (25). It is also in accord with previous reports showing that in many of the nucleoprotein structures, including those formed by the binding of origin binding proteins at their corresponding replication origins, the bound DNA assumes special deformations in which the DNA appears to be bent, wrapped, looped, or unwound (35–40).

**DISCUSSION**

Earlier studies have shown that UMSBP binds specifically a 12-mer (UMS) sequence conserved at the L-strand replication origin (OriL) and a 14-mer (H14) sequence, located at the H-strand origin (OriH), in their single-stranded conformation (14, 16, 19). Studies on the protein interactions with native minicircles revealed the bent and distorted nature of the double helix at the native minicircle origin region, suggesting the availability of a single-stranded UMS site for binding by UMSBP (25). The **in vivo** cross-linking experiment reported here indicates the binding of UMSBP to native kDNA minicircles in the cell. Our analysis could neither detect the interaction of UMSBP with free minicircles, which are being excluded during the purification of the kDNA-protein complexes, nor could it distinguish between the interactions of UMSBP with newly replicated **versus** mature minicircles in the network. Under these limitations of the analysis one could speculate that if UMSBP was bound to mature minicircles in the network, then its interaction with kDNA minicircles may have occurred either before their pre-replication release from the network or after their post-replication reattachment to it. In the latter case UMSBP could interact with either newly replicated gapped minicircles in the network or covalently sealed minicircles before the network segregation. Alternatively, UMSBP may be bound **in vivo** to kDNA networks throughout the cell cycle. In the latter case one could speculate that an inactive bound UMSBP may be activated to act at the replication origin during the S phase of the cell cycle at the onset of kDNA replication initiation. This hypothesis is currently under study in synchronized *C. fasciculata* cell cultures.

The **in vitro** cross-linking experiments reported here indicate the binding of UMSBP to a duplex kDNA fragment of the *C. fasciculata* minicircle containing the proposed minicircle replication origin. These observations revealed the dependence of UMSBP binding to the origin upon the binding of its UMS and H14 binding sites. Moreover, the results also indicated that an efficient binding of UMSBP to the origin required the presence of both origin sequences, implying an apparent cooperativity in the binding of UMSBP to the two origin sequences.

The induction of structural changes in the DNA at replication origins by the binding of their initiator proteins has been shown to play an important role in the initiation of DNA replication. A common feature of the interactions between replication origins and their origin binding proteins is the bending or wrapping of the origin DNA around the origin binding protein in the nucleoprotein complex. Wrapping of the DNA around the protein was first observed for the interactions of bacteriophage λ replication origin with protein O (37, 41, 42) and of *Escherichia coli* dnaA with oriC (35, 43). Interaction of T-antigen with the SV40 origin induces a sharp DNA bending adjacent to the T-antigen recognition site (43, 44). The structural changes induced in herpes simplex virus 1 origin through its interaction with OBP suggest either bending of the central part of the origin sequence, bringing together the two OBP binding sites, or wrapping of the DNA around the protein core (45, 46). Considerable bending of the replicator sequence was also observed in the interaction of Epstein-Barr virus with the interacting EBNA1 protein at the two pairs of binding sequences (47–49). Interaction of the replication origin of bovine papilloma virus with proteins E1 and E2 also yields the sharp bending of the bound DNA as a result of the cooperative binding of the two proteins. The pattern of hypersensitive micrococcal nuclease sites observed with the nucleoprotein complex generated by UMSBP at the minicircle replication origin is in accord with the second type of interaction, which has been shown to induce unwinding of the DNA (50, 51). The results reported in this work indicate that induction of the conformational change in the minicircle replication origin results in the unwinding of this site. Other CCHC-type zinc knuckle proteins have been shown to act as nucleic acid chaperons, such as the HIV-1 nucleocapsid, which is involved in replication initiation, genome dimerization, condensation and the formation of compact G-quartets structure (52–55).

Finally, functioning as an origin binding protein during minicircle replication initiation, UMSBP is expected to be the first replication protein that interacts with the origin site, stabilizing an unwound DNA structure and directing the assembly of other replication proteins for the generation of a replication fork. The results presented here indicate the stabilization of an unwound DNA structure at the replication origin. Preliminary observations suggested that UMSBP interacts with several replication proteins. Recent structure-function analyses of UMSBP have revealed that whereas the UMSBP C-terminal domain is involved in the binding of DNA, its N-terminal domain mediates protein-protein interactions (23). The kinetics rates measured here for UMSBP interactions with the minicircle origin sequences (Fig. 3), are in accord with the dynamic nature of the interaction of an origin binding protein with the replication origin that would enable the fast proceeding of the newly generated replication fork from this site. The results presented here provide a mechanistic rationale for the previously proposed model of minicircles replication initiation (for a recent review, see Ref. 5). Here we speculate that minicircle replication may initiate by the binding of UMSBP onto the partially unwound UMS site, stabilizing its single-stranded conformation. At this stage a concomitant binding of UMSBP to the OriH site is suppressed either by virtue of the double-stranded conformation of this site or, alternatively,

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5 N. Milman-Shtepel and J. Shlomai, unpublished observations.
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through its capacity to form a stem and loop structure at this site. Next, UMSBP may recruit other replication proteins onto this site, and a replication fork is assembled at or near this site. Proceeding at the L-strand, unidirectional replication toward the oriH site induces the unwinding of its double-stranded conformation (or the disruption of the stem-loop structure generated at this site), enabling the binding of UMSBP onto the H14 sequence. Consequently, the OriH is activated to fire and initiates the unidirectional replication of the minicircle H-strand.

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