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A single-stranded DNA-binding protein from *Crithidia fasciculata* recognizes the nucleotide sequence at the origin of replication of kinetoplast DNA minicircles

(replication/initiation/universal minicircle sequence/telomeres)

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ABSTRACT **A sequence-specific single-stranded DNA-binding protein from the trypanosomatid protozoan *Crithidia fasciculata* binds to a sequence of 12 nucleotides located at the origin of replication of kinetoplast DNA minicircles. This sequence, termed the universal minicircle sequence (UMS), is conserved in the kinetoplast DNA minicircles among species of the family Trypanosomatidae. The purified protein binds specifically to the heavy strand of the DNA at this site, which consists of the sequence 5′-GGGGTTGGTGTA-3′. Binding analyses using mutated UMS dodecamers have revealed the significant contribution of each of the individual residues at the binding site, with the exception of the 3′-terminal adenine residue, to the generation of specific protein–DNA complexes. The possible role of this sequence-specific single-stranded DNA-binding protein in replication of kinetoplast DNA minicircles and the relation of the UMS to chromosomal telomeric sequences are discussed.**

Kinetoplast DNA (kDNA) is a unique extrachromosomal DNA network found in the single mitochondrion of parasitic flagellate protozoa of the family Trypanosomatidae. In *Crithidia fasciculata*, kDNA consists of 5000 duplex DNA minicircles [2.5 kilobase pairs (kb) each] and about 50 maxicircles (37 kb each) interlocked topologically to form a DNA network (reviewed in refs. 1–4). The replication of kDNA occurs during the S phase of the cell cycle (5). Based on *in vivo* observations, Englund (6, 7) has described the replication of kDNA minicircles as a process in which individual minicircles are released from the network, replicate through a Cairns-type mechanism, and reattach to the network. The network increases in size until it doubles and then splits and segregates into two daughter networks.

Extensive studies have been carried out on free minicircle replication intermediates of the trypanosomatids *Trypanosoma equiperdum* (8–12), *Crithidia fasciculata* (7, 13–17), and *Leishmania tarentolae* (18). Minicircles containing nascent heavy (H) strand were highly gapped and nicked (13, 15). One of these gaps was located opposite a conserved hexameric sequence, which was suggested to be the replication-origin site of the H strand (8, 14). Minicircles with a newly synthesized light (L) strand had a single gap of 6–10 nucleotides located at the nascent L strand. This gap was mapped to the site of the suggested origin of L-strand replication and overlapped the 12-nucleotide universal minicircle sequence (UMS) 5′-GGGGTTGGTGTA-3′. To date, this sequence has been found in all sequenced kDNA minicircles (8, 14), except for the sequence AGGGTTGGTGTT found in *Crithidia oncopelti* (19). The presence of ribonucleotides at the 5′ terminus within this gap suggests that L-strand synthesis initiates at this site through an RNA priming mechanism and that the conserved UMS may play a role in the initiation of L-strand DNA replication (9, 14). The present model suggests that L-strand synthesis initiates at the UMS and proceeds continuously. H-strand synthesis is discontinuous and starts when its origin is reached by the proceeding replication fork, through the formation of D-loop intermediates (4).

Each kDNA minicircle in *C. fasciculata* contains two copies of the UMS (20). Similar gaps had been found opposite to either of the two UMSs in the newly synthesized L strand of *C. fasciculata* kDNA minicircles. Since only one gap is found per molecule, replication apparently initiates from either of these sites, but not from both UMSs of the same minicircle (13, 14). The precise role of the UMS elements in the initiation of the minicircle L-strand synthesis is yet to be determined. Unique nucleotide sequences located at origins of replication of bacterial and viral chromosomes serve as specific binding sites for proteins, which function at the initiation of DNA replication, conferring origin specificity upon the priming reaction (reviewed in ref. 21).

We describe here the specific recognition of the UMS element, conserved at the origin of replication of kDNA minicircles, by a protein purified from *C. fasciculata* cell extracts. We discuss its possible role in origin recognition during the initiation of kDNA minicircle synthesis. We will describe elsewhere the purification of the UMS-binding protein and its characterization, as well as the isolation and analysis of the gene encoding this protein in *C. fasciculata*.

MATERIALS AND METHODS

Nucleic Acids, Nucleotides, Resins, and Enzymes. kDNA was prepared from *C. fasciculata* as described (22). Synthetic oligonucleotides were prepared using an Applied Biosystems oligonucleotide synthesizer at the interdepartmental unit of the Hebrew University Medical School and poly(dI-dC)·poly(dI-dC) was purchased from Boehringer Mannheim. Phenyl-Sepharose was purchased from Sigma and streptavidin-coated magnetic beads from Dynal (Great Neck, NY). Biotin-16-dUTP and terminal deoxynucleotidyltransferase were purchased from Boehringer Mannheim, T4 polynucleotide kinase from New England Biolabs, and radioactive nucleotides from New England Nuclear.

Purification of UMS-Binding Protein from *C. fasciculata*. Purification of UMS-binding protein was carried out by using its binding to the H strand of the UMS as an assay. A crude cell lysate (fraction I) was prepared from 7 g of *C. fasciculata* cell paste and further fractionated by ammonium sulfate precipitation (23). The protein fraction precipitated at 40–60% (of saturation at 0°C) ammonium sulfate (fraction II) was

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Abbreviations: kDNA, kinetoplast DNA; H, heavy; L, light; UMS, universal minicircle sequence.

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loaded onto a 5.3-ml phenyl-Sepharose column equilibrated with 50 mM Tris Cl, pH 7.5/2% (vol/vol) glycerol/1.5 M ammonium sulfate/1 mM EDTA/2 mM 2-mercaptoethanol. The column was washed with two bed volumes of this buffer and bound proteins were eluted with a linear gradient of 1.5 M ammonium sulfate/2% (vol/vol) glycerol to 50% (vol/vol) glycerol. UMS-binding activity was recovered at the range of 1.3–1.0 M ammonium sulfate and 8.4–18% glycerol, to yield fraction III. This fraction was further purified by DNA affinity chromatography, using biotinylated single-stranded UMS DNA attached to streptavidin-coated beads. Binding was carried out in 25 mM Tris Cl, pH 7.5/40 mM ammonium sulfate/1 mM $MgCl_2$/0.1 mM EDTA/1 mM dithiothreitol. The beads were washed extensively with the binding buffer and the protein was eluted stepwise in this buffer, at the range of 0.5–1.0 M KCl, to obtain fraction IV.

Electrophoretic Mobility-Shift Analysis. The 20-μl standard reaction mixture contained 25 mM Tris Cl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA, 20% glycerol, 20 μg of bovine serum albumin, 0.5 μg of poly(dI-dC)·poly(dI-dC), and 0.2 ng 5′-^{32}P-labeled UMS dodecamer. Reactions were started by the addition of the protein and were incubated at either 8°C or 30°C, as indicated, for 15 min. Reaction products were loaded onto a nondenaturing 8% (wt/vol) polyacrylamide gel (1:30 *N,N′*-methylenebisacrylamide/acrylamide weight ratio) in TAE buffer (6.7 mM Tris acetate/3.3 mM sodium acetate/1 mM EDTA, pH 7.5). Electrophoresis was conducted at 8°C and 8 V/cm for 2.5 hr. Gels were dried and exposed to x-ray films (Agfa Curix RP2 or Kodak X-Omat AR). Quantitation of protein–DNA complexes was by densitometry of the autoradiograms with a G300 scanning densitometer (Hoefer Scientific Instruments). One unit of UMS-binding protein is defined as the amount of protein required for the binding of 1 fmol of UMS DNA H-strand dodecamer, under the standard mobility-shift assay conditions. When competing with M13 DNA, molar ratios are in respect to nucleotides.

RESULTS

A Protein from *C. fasciculata* Binds Specifically to the UMS. A protein from *C. fasciculata* cell extracts bound to the UMS element conserved at the origin of kDNA minicircles. As shown in Fig. 1, binding of the protein at this site was specific only to the H strand of the duplex, which consisted of the nucleotide sequence GGGGTTGGTGTA, whereas the complementary L strand could not form protein–DNA complexes. The double-stranded (HL) form of UMS was unable to support complex formation. A relatively low protein binding (<10% of that observed with the H strand) was measured at 30°C, as a result of partial melting of the duplex, or at 8°C when heat-denatured UMS duplex was used. UMS-binding protein was extensively purified from *C. fasciculata* cell extracts by using its specific binding to the H strand of UMS DNA as an assay. The purified protein had an apparent native mass of 27 kDa. The protein purification, including ammonium sulfate fractionation of crude cell lysates, followed by hydrophobic chromatography on phenyl-Sepharose and a subsequent UMS DNA affinity chromatography, as well as the protein characterization, will be described elsewhere (Y.T., I.K., and J.S., unpublished work).

As indicated by the lack of protein binding to both the UMS duplex and the L strand (Fig. 1), the UMS-binding protein is a sequence-specific single-stranded DNA-binding protein. A >2 orders of magnitude excess of an unrelated oligomer could not compete with the H strand of the UMS element in the formation of protein–DNA complex (data not shown). This was further demonstrated in binding competition experiments (Fig. 2) using bacteriophage M13 single-stranded DNA, which is of a higher sequence complexity. More than 500-fold molar excess of M13 DNA, but only 5-fold excess of the competing unlabeled UMS H strand, was required to displace >90% of the radioactively labeled H strand from the protein–DNA complex. Further, a point-mutated UMS H-strand dodecamer (in which the guanine residue at position 10 was replaced by an adenine residue), supported a considerably less efficient binding of the protein (Fig. 2).

Generation of Specific Protein–UMS DNA Complexes Is Tightly Dependent Upon the Conserved UMS Sequence. Does the high conservation of the UMS elements at the minicircle origin of replication in trypanosomatids reflect a highly specific recognition of this site by specific binding proteins? To address this question we assessed the contribution of each of the individual nucleotide residues at the UMS binding site to the specific protein–DNA interactions. A series of point-mutated dodecamers of the H strand were used in competition analyses against the radioactively labeled wild-type UMS. These binding-competition studies, using 5-, 10-, and 15-fold molar excess of mutated to wild-type UMS (10-fold excess is presented in Fig. 3) revealed several significant features of the UMS binding site. A transition mutation introduced at the 3′-terminal adenine residue of the sequence did not affect the binding capacity of UMS (Fig. 3). This mutated oligonucleotide competed as efficiently as the unla-

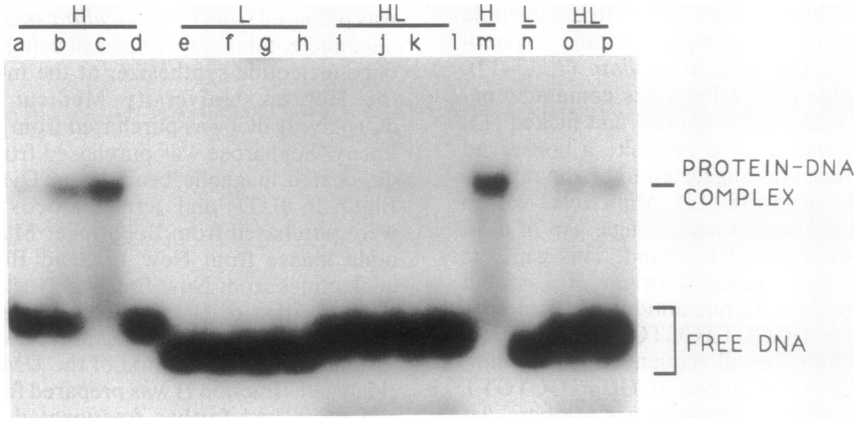

FIG. 1. Binding of UMS-binding protein to the H strand of UMS. Samples of 8.6, 25.8 and 77.5 units of UMS-binding protein (fraction IV) were incubated under the standard assay conditions (at 8°C) with 46 fmol of 5′-^{32}P-labeled UMS dodecanucleotides: lanes a, b, and c, H strand of UMS; lanes e, f, and g, L strand of UMS; lanes i, j, and k, double-stranded (HL) form of UMS. Lanes m, n, and o, 77.5 units of UMS-binding protein were incubated, at 30°C, with the UMS H, L, and HL forms, respectively. Lane p, the double-stranded (HL) UMS DNA was heat-denatured at 95°C for 10 min prior to its addition into the binding reaction, which was conducted at 8°C. Lanes d, h, and l, free single-stranded H and L, and double stranded (HL) UMS DNAs. Mobility-shift analysis was as described under *Materials and Methods*.

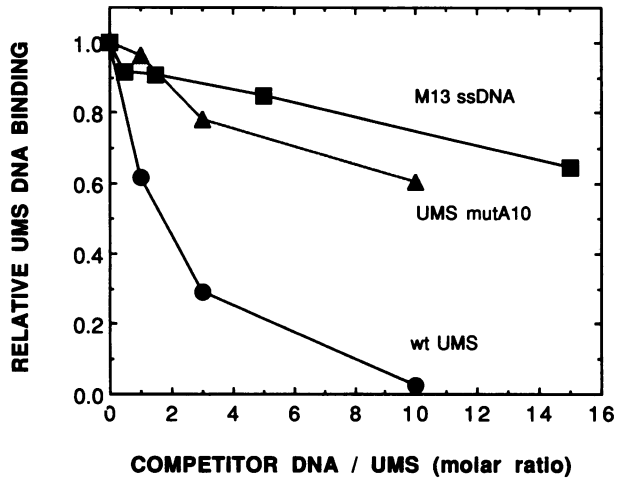

FIG. 2. Sequence specificity of UMS-binding protein. UMS-binding protein (fraction IV, 1 ng) was incubated under the standard assay conditions with 46 fmol of 5′-^{32}P-labeled H-strand UMS in the presence of various concentrations of unlabeled H strand [wild-type (wt) UMS], single-stranded M13mp8 DNA (M13 ssDNA) or mutated H strand in which a guanine residue at position 10 (numbering from the 5′-terminal residue) was replaced by an adenine residue (UMS mutA10). Electrophoretic mobility-shift analysis and quantitation of protein–DNA complexes were as described under *Materials and Methods*.

beled wild-type sequence in the generation of the protein–DNA complex. In contrast, mutations introduced at all the other positions of the UMS resulted in decreased binding affinities, as compared to the wild-type sequence. Of these, mutations introduced at five out of the seven guanine residues of this sequence, located at positions 3, 4, 7, 8, and 10, had the most significant effect on the binding of the protein. At 10-fold molar excess, these mutated dodecamers competed poorly with the radioactively labeled wild-type UMS. Competition with these mutated oligomers reduced the generation of labeled protein–DNA complexes by only 68%, 35%, 32%, 56%, and 45% of the control, respectively, while a reduction of 99% was observed with the wild-type UMS competitor. Dodecamers mutated at the other positions of the

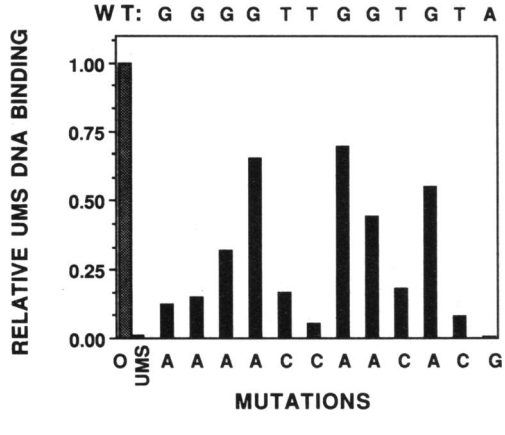

FIG. 3. Dependence of the binding interactions upon a conserved UMS element. Forty-six femtomoles of 5′-^{32}P-labeled H-strand UMS was incubated under standard binding-assay conditions with 1 ng of UMS-binding protein (fraction IV), in the absence of a competing DNA (0), in the presence of 10-fold molar excess of unlabeled H-strand UMS (UMS), or in the presence of a 10-fold molar excess of each of the 12 point-mutated H-strand UMSs. Point-mutated dodecamers were prepared by substituting a purine for a purine and a pyrimidine for a pyrimidine. Wild-type (WT) sequence is shown at the top. Mobility-shift analysis and quantitation of protein–DNA complexes were as described.

binding site competed more efficiently with the wild-type UMS, reducing complex formation by 82–85% for the guanine residue at position 2 and the thymine residues at positions 5 and 9, and by as much as 88–95% for the guanine residue at position 1 and the thymine residues at positions 6 and 11.

These data revealed the high significance of the guanine residues at positions 3, 4, 7, 8, and 10, the relatively lower significance of the residues at positions 1, 2, 5, 6, 9, and 11, and the insignificance of the 3′-terminal adenine residue for protein–DNA interactions. Based on these observations we suggest that the high conservation of UMS elements reflects the high specificity of the protein–DNA interactions at the UMS sites. Such interactions may take place at the UMS elements located at the origin of kDNA minicircles and contribute to the origin specificity of L-strand replication initiation.

Interaction of UMS-Binding Protein with Chromosomal Telomeric Sequences. The similarity of the H strand of the kDNA UMS to the single-stranded G-rich 3′-overhanging sequences of 12–16 nucleotides found in telomere termini (24) has raised the possibility that the UMS-binding protein may also function in the cell as a telomere-binding protein. To explore this possibility, we determined the binding of the purified protein to 12-mer sequences, composed of two hexamers, as found in the 3′-overhanging telomeric sequences. We used the hexameric element AGGGTT (found in the chromosomal telomeres of trypanosomatids, including *Crithidia*, and vertebrate cells) and the hexameric element GGGGTT (found in telomeres of *Tetrahymena* and other ciliated protozoa) (25, 26). Binding-competition analyses (Fig. 4) demonstrated the relatively low affinity of the UMS-binding protein for the repetitive sequence found in the chromosomal telomeres of trypanosomatids. A 10-fold molar excess of unlabeled UMS, but a 100-fold excess of the trypanosomal telomeric dodecamer AGGGTTAGGGTT, was required to displace most (>90%) of the labeled DNA from its complex with the UMS-binding protein. However, as predicted from analyses using mutated UMS oligomers (Fig. 2), the 12-mer sequence GGGGTTGGGGTT, representing the repetitive sequence found in telomeres of *Tetrahymena*,

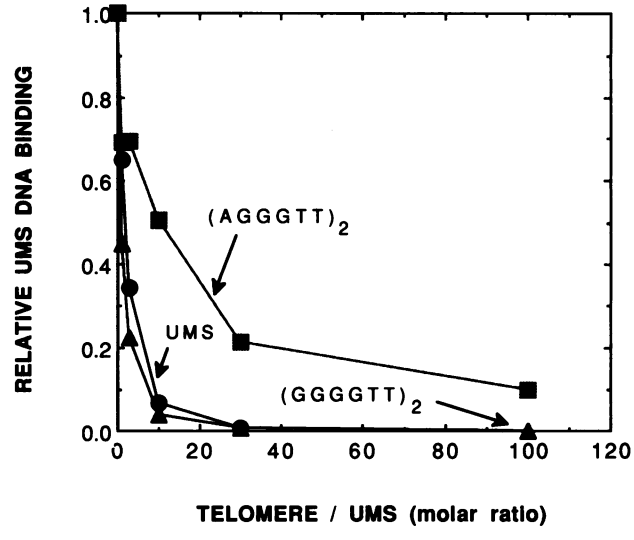

FIG. 4. Binding of UMS-binding protein to telomeric sequences. UMS-binding protein (fraction IV, 1 ng) was incubated under the standard binding-assay conditions with 46 fmol of 5′-^{32}P-labeled H-strand UMS in the presence of various concentrations of unlabeled competitor DNAs: H-strand UMS (●), the 12-mer telomeric sequence $(GGGGTT)_2$ (▲), or the 12-mer telomeric sequence $(AGGGTT)_2$ (■). Mobility-shift analysis and quantitation of protein-DNA complexes were as described.

competed as efficiently as the unlabeled UMS in the binding assay. Although these observations, made using short (dimeric) repeats of the sequence, do not rule out a possible telomere-binding function of the UMS-binding protein in *Crithidia*, its high affinity for the UMS element *in vitro* supports a potential functional role for this protein at the origin of kDNA minicircle replication, through its specific interaction with the UMS.

DISCUSSION

Origin-directed initiation of DNA replication in bacterial and viral systems is regulated by unique sequence elements, which are recognized by specific trans-acting cellular and virus-encoded proteins. The detection of ARS (autonomously replicating sequence)-binding proteins in eukaryotic cells (27–32) indicates that similar interactions may occur during the initiation of DNA replication in eukaryotic replicons. We report here that the 12-nucleotide sequence conserved at the origin of replication of kDNA minicircles is recognized by a sequence-specific single-stranded DNA-binding protein from the trypanosomatid *C. fasciculata*. Binding of the purified protein is highly specific to the H strand of the UMS. Neither the double-stranded form of the UMS nor the complementary L strand is recognized as a substrate for the generation of specific protein–DNA complexes (Fig. 1). Since whole-cell extract preparations were used for the purification of the UMS-binding protein, the possibility of its intracellular association with the kinetoplast organelle has yet to be explored. However, its high specificity for the minicircle origin-associated UMS element implies an *in vivo* origin-recognition function for this protein. Since no measurable ATPase activity, either DNA-dependent or DNA-independent, was found to be associated with this protein (data not shown), it is presumed that binding of the protein at the H strand of the UMS should be supported by the local melting of the duplex at the binding site through the action of other replication proteins, such as DNA helicases.

Computer scanning of viral and eukaryotic cell genomes has revealed the high abundance of repetitive, as well as nonrepetitive, sequences of the consensus GGGG(C/T)(C/T)GGGG(C/T)(C/T). Such sequences are particularly abundant among the herpesviruses. For example, the genome of herpes simplex virus 1 was found to contain 17 tandem repeats of the sequence GGGG(CT/TT)GGGG(CT/TT) (44). The kDNA UMS deviates from the above consensus sequence by the two residues at positions 9 and 12. Indeed, the introduction of transitional mutations at these positions of the UMS has little or no effect on binding of the protein. In general, the variation found in the two pyrimidines of the above consensus correlates well with the relatively minor effect caused by mutating the pyrimidines at positions 5, 6, 11, and 12. The major residues affecting the binding interactions are the five guanine residues at positions 3, 4, 7, 8, and 10 ($G^1G^2G^3G^4T^5T^6G^7G^8T^9G^{10}T^{11}A^{12}$) (Fig. 3).

Considering the similarity of the H strand of the UMS and the G-rich 3′-overhanging sequences found in telomeres of eukaryotic chromosomes, we studied the binding of the purified UMS-binding protein to two telomeric sequences abundant in eukaryotic cells. Binding competition showed that a dodecamer consisting of two copies of the *Tetrahymena* telomere repeated sequence GGGGTT, which matches the above consensus, was as efficient a substrate for binding by the protein as the UMS. On the other hand, that of the trypanosomal telomeric sequence AGGGTT, which deviates from the consensus, did not compete efficiently with the wild-type UMS (Fig. 4). This is in agreement with the binding analyses of point-mutated UMS (Fig. 3) and implies that the affinity of UMS-binding protein correlates with the presence of the consensus sequence in the DNA substrate. These results suggest that the kDNA minicircle origin of replication, rather than the chromosome telomere, is the target for specific binding by the protein in the *Crithidia* cell.

UMS-binding protein may belong to a larger family of proteins that specifically bind G-rich single-stranded DNA. Of the few other sequence-specific single-stranded DNA-binding proteins described so far, Msbp-1 binds the G-rich strand of the core sequence of minisatellite DNA (33) and Mf3 interacts with the G-rich, noncoding, strand of muscle gene elements (34). Telomere-binding proteins have been found to bind specifically the G-rich 3′-overhanging telomeric sequences in species of *Oxytricha nova* (35–39) and *Euplotes crassus* (40) and probably in *Physarum polycephalum* (41) and *Stylonychia mytilis* (42). Furthermore, methylation interference analysis carried out with the telomere-binding protein of *Oxytricha nova* (38) has revealed a binding pattern similar to the one reported here for the UMS-binding protein. Finally, a primase activity specific to the 3′-overhanging telomeric sequence was detected in *Oxytricha nova* (43). Whether this finding may indicate a possible functional relation between chromosomal telomeric sequences and sequence elements that function at the origin of replication of kDNA minicircles has yet to be explored. We have recently cloned the gene from *C. fasciculata* encoding the UMS-binding protein. The genetic analysis made possible with the cloning of this gene, as well as further biochemical analysis of minicircle replication, will shed light on the physiological function of the UMS-binding protein and its possible role of conferring origin-specificity on the initiation of kDNA minicircle replication.

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