Acta Microbiologica et Immunologica Hungarica, 49 (4), pp. 455–467 (2002)

SPECIFIC RECOGNITION OF THE REPLICATION ORIGINS OF THE KINETOPLAST DNA*

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(Received: 30 September 2001; accepted: 5 November 2001)

Origin recognition, an early event in the initiation of chromosome replication

The understanding of the process of chromosome replication initiation and its control in cells and subcellular organelle requires the identification and characterization of origins of replication and other specific control elements in the DNA template, as well as the counterpart proteins that interact with these regulatory sequences. An early event that had been described during replication initiation of bacterial chromosomes, plasmids, bacterial and animal viruses and lower eukaryotes, is the recognition of a unique sequence at the replication origin, known as the origin recognition element (ORE), by a special origin binding protein and the binding of the protein onto this site. This protein–DNA interaction is a key event in chromosome replication initiation. It results in the local, non-catalytic melting of the nearby DNA unwinding element (DUE), a sequence with a relatively lower melting profile, enabling the loading of DNA helicases and the catalytic unwinding of the origin site. Furthermore, binding of the origin binding protein directs, through specific protein–protein interactions, other replication initiation proteins onto the origin site to be assembled into a functional replication initiation complex (reviewed in [1–8]).

We are studying these early events in the replication of DNA in eukaryotes, using the trypanosomatid protozoan *Crithidia fasciculata* as an experimental model. We focus our attention on the replication of a unique extra-chromosomal DNA

*Lecture presented at the International Course for Young Scientists (August 23–27, 2000, Keszthely, Hungary) organized by the Hungarian Society for Microbiology and the UNESCO–Hebrew University of Jerusalem International School for Molecular Biology, Microbiology and Science for Peace.

1217-8950/2002/$ 5.00

structure found in the single mitochondrion of this microorganism, known as the kinetoplast DNA (kDNA). Here we review our observations on the recognition of the kDNA origin and its specific binding by a unique origin binding protein and the recognition of the separate initiation sites for the kDNA minicircle leading and lagging strands synthesis.

Kinetoplast DNA structure and replication

Kinetoplast DNA (kDNA) is a unique DNA structure found in the single mitochondrion of flagellated protozoa of the order Kinetoplastida. It comprises a two-dimensional, disc-shaped DNA network, which has remarkable organization and genetic functions. The network consists of 5,000–10,000 DNA minicircles and 25–50 maxicircles, interlocked in a giant topological catenane. Maxicircles encode mitochondrial ribosomal RNAs and proteins. Their transcripts undergo extensive editing, in which uridine residues are either added or deleted at many specific sites in the mRNA, to create translatable open reading frames. Minicircles function has been a puzzle for many years. However, the discovery that kDNA minicircles encode small guide RNA (gRNA) transcripts, which are complementary to edited maxicircle transcripts, shed light on a physiological function of minicircles, in specifying edited maxicircle mRNA sequences (reviewed in [9–12]). Minicircles within a given network are virtually identical in size (at the range 0.5–2.5 kbp) and sequence complexity among the different species. Cells of most species contain more than one class of minicircle sequences that share a small, strongly conserved region of approximately 100–200 bp, and a large variable region. The overall similarity among conserved sequences of different trypanosomatid species is generally low, however, there are two strictly conserved short sequence motifs (present in most trypanosomatid species examined), that are located at the presumed origins of replication of the two minicircle; DNA strands. These are the dodecamer GGGGTTGGTGTA (known as the universal minicircle sequence, UMS), located in the discontinuously synthesized strand, and the hexamer ACGCCC located in the continuously synthesized one. Minicircles in most trypanosomatid species also contain a single region of bent helix. The local bending of the DNA double helix is directed by stretches of 4–6 adenine residues, which are spaced periodically in phase with the helical repeat. The biological function of the minicircle local bend is yet unknown.

Inside the cell the kDNA network is condensed into a disc, located within the mitochondrial matrix. Electron micrographs revealed that inside the disc the DNA fibers are aligned parallel to its axis. Although the dimensions of the disc vary among the different trypanosomatid species, the thickness of the disc is about half the

circumference of the minicircle. Reconstruction of serial sections of stationary phase *C. fasciculata* cells, studied by confocal microscopy, confirmed that the kinetoplast has a disc structure of about 1 μm in diameter and 0.4 μm thickness (reviewed in [13–19]).

The replication of kDNA in trypanosomatids (reviewed in [16, 17, 20, 21]), unlike mitochondrial DNA replication of other eukaryotes, is restricted to the S phase of the cell cycle. Replication of kDNA includes the duplication of minicircles and maxi circles and the generation of two progeny kDNA networks that segregate upon cell division. Englund [22] has proposed a model for the replication of the kDNA network that relied on the characterization of intermediates and final products of the replication process. According to this model, G1 phase-networks contain only covalently closed minicircles. During S phase, minicircles are released from the central zone of the network and replicate as free DNA circles and the resulting progeny DNA minicircles reattach to the periphery of the network. At the end of the S phase, the double-size network comprises newly replicated circles, whose nascent DNA strands are nicked and gapped. This pattern of minicircle re-attachment has been confirmed by electron microscopy examination of networks from different stages of replication and by in situ hybridization studies, using minicircle-specific probes detected via fluorescence microscopy [22–25]. Immunolocalization of *C. fasciculata* topoisomerase II, by Dan Ray's laboratory at UCLA [26] has suggested the possible association of this enzyme with replisome-like structures located at two antipodal sites at the perimeter of the kinetoplast disc. Further studies from Paul Englund's laboratory at Johns Hopkins Medical School [27] have shown, by in situ hybridization with minicircle probes and immunolocalization using antibodies against mitochondrial f3-like DNA polymerase, the co-localization of both free replicating minicircles and replication proteins at the two antipodal sites of the kinetoplast disc. Fluorescence in situ hybridization experiments have recently revealed that before their replication, minicircles are released vectorially from the network face nearest the flagellum. Replication initiates in the zone between the flagellar face of the disc and the mitochondrial membrane. The replicating minicircles then move to two antipodal sites that flank the disc-shaped network. In later stages of replication, the number of free minicircles increases, accumulating transiently in this region. The final replication events, including primer removal, repair of many of the gaps, and reattachment of the progeny minicircles to the network periphery, are thought to take place within the antipodal sites [28].

Studies on the replication of minicircles have indicated that synthesis of the minicircle leading strand is initiated by RNA priming at a unique site and proceeds continuously, while synthesis of the lagging strand is discontinuous. The daughter molecules containing the discontinuously synthesized nascent strand have a single gap of 6–10 nucleotides. The gap and remnants of a putative RNA primer were mapped to

the conserved 12 nucleotide UMS. Minicircles containing the discontinuously synthesized nascent strand are highly gapped and nicked. It has been suggested that those discontinuities in the lagging strand, which are located opposite the conserved ACGCCC hexamers, mark the specific lagging strand initiation sites. A proposed scheme, derived from these studies, suggests that minicircle replication begins by initiation of the leading strand synthesis at the dodecameric UMS site. Elongation of the nascent DNA strand displaces the parental DNA strand, forming a D-loop intermediate. Exposure of the lagging strand origin of replication by the proceeding replication fork triggers the initiation of discontinuous synthesis. This occurs at about 100 nucleotides downstream from the site of the leading strand initiation (presumably at, or near, a conserved ACGCCC hexamer). The displaced parental strand serves as a template for multiple initiations of lagging strand synthesis. The final repair of discontinuities in both nascent DNA strands occurs only after the completion of minicircles reattachment to the network [14, 15, 25, 29–33].

UMSBP, the proposed kDNA origin binding protein

Our assumption was that the high conservation of the dodecameric sequence, the UMS, at the replication origin of the minicircle L-strand (*oriL*) and of the hexameric sequence at the H-strand origin (*oriH*) in the various species of trypanosomatids, may reflect the high conservation of specific protein–DNA interactions that take place at these sites. Considering the association of these sequences with the process of replication initiation, the presumption was strong that putative proteins, which may be present in the cell and interact with these sequences, are likely candidates for the role of origin binding proteins that act during kDNA minicircles replication initiation. Following this rationale, we have used the electrophoretic mobility shift analysis (EMSA) and conserved origin sequences probes to detect unique sequence-specific DNA-binding proteins that interact with these conserved elements. As a result, we have detected and purified to apparent homogeneity, a specific UMS-binding protein (UMSBP) from *C. fasciculata* cell extracts [34]. The protein apparent native mass as determined by sedimentation and gel filtration analyses is 27.4 kDa. Under denaturing and reducing conditions it yields a polypeptide of 13.7 kDa. It is suggested that the protein is a homodimer of 27.4 kDa with 13.7-kDa protomer. The calculated molecular axial ratio of approximately 1/7–1/8 implies a non-globular structure, and 12,000 copies of the homodimer are estimated per cell [35]. UMSBP is a sequence-specific single-stranded nucleic acids binding protein. It binds both DNA and RNA at the same sequence specificity. It binds neither the duplex, nor the quadruplex forms of these sequences. Mutational saturation analysis of

the binding sequence revealed the contribution of each residue to the specific binding reaction, emphasizing the importance of 4 out of the 7 G-residues in the sequence for the binding interactions [34, 35].

As predicted from the UMSBP gene sequence (see below), the majority of the protein consists of two identical protein domains. Each segment includes two CCHC-type zinc finger elements. The majority of the protein has a loop structure. Two short regions form an ordered a-helix structure. There are many potential phosphorylation sites predicted in the protein. This includes two phosphorylation sites for protein kinase C (PKC), one in each segment (between zinc fingers 2, 3 and 4, 5), and a casein kinase II (CK2) site, located between the two protein segments. We have recently shown that the protein is actually phosphorylated. The potential functional role of this modification in the protein, regarding the regulation of its action, is yet to be determined.

Analyses of the UMSBP gene and genomic locus

We have cloned the gene encoding UMSBP in *Crithidia*, from a cDNA expression library, using the 12-mer UMS oligonucleotide as a probe, by a South-Western procedure [36]. The UMSBP gene open reading frame contains 348 base pairs, encoding a potential polypeptide chain of 116 amino acid residues. A striking structural characteristic of this ORF is its potential to form five symmetrically arranged CCHC-type zinc-finger structures. Cloning and sequencing of the entire UMSBP genomic locus in the *C. fasciculata* genome has revealed [37] that it consists of two tandemly arranged UMSBP genes. The ORFs of the two copies are identical, with 5 differences at the nucleotides level, but differ considerably in their 31 and 51 UTRs. Transcription of these two copies of the UMSBP gene yields two distinct processed mRNA transcripts of 2,750 and 3,300 nucleotides long. The knockout of the UMSBP genes, conducted using the double-targeted gene replacement yielded, as yet, the knockout of one of the two UMSBP alleles. Measurements of the level of specific UMSBP mRNAs and protein in the UMSBP mutant revealed no changes, as compared to the wild type cells. Experiments aimed towards the knockout of the second allele have failed, raising the possibility that null UMSBP mutations are lethal. This has been supported recently by an RNA interference approach [38].

Specific recognition of the minicircle replication origins

OriH, the replication origin of the minicircle discontinuously synthesized DNA strand, contains a conserved hexameric sequence, located about 80 base pairs apart

from the *oriL* UMS element in the minicircle molecule. We have detected in trypanosomatid cell extracts a protein that bind specifically to a 14-mer sequence (designated H14) that contains the conserved hexamer and 8 nucleotides flanking it at the *oriH* H-strand. Purification of the *oriH*-binding protein from *C. fasciculata* revealed that the *oriH*-binding activity co-purifies precisely throughout the various purification steps, with the *oriL*-binding protein UMSBP. Several experimental evidence support the notion that UMSBP is not only the *oriL*, but also the *oriH*-binding protein. However, the ultimate evidence that binding of both origin sequences is an intrinsic property of UMSBP came from binding experiments showing that pure recombinant UMSBP that was expressed in *E. coli* forms specific nucleoprotein complexes with both the conserved *oriL* and *oriH* sequences [39]. Binding-competition analyses, as well as direct measurements of equilibrium binding constants, indicate similar binding affinities for the two protein DNA interactions [35, 39].

We have observed that the conserved H-strand hexamer per se does not constitute a binding site for UMSBP. To further define the sequence directing the specific binding of UMSBP onto the *oriH* site, a series of *oriH* H-strand oligonucleotides (ranging from 6 to 26 nucleotides long), each containing the conserved hexamer core and sequences flanking its 3' and/or 5' termini (0–10 residues long), were used as radioactive ligands in electrophoretic mobility-shift analyses. It was found that the minimal UMSBP binding sequence at the *oriH* site is a 14-mer that consists of the conserved hexamer and 8 flanking residues [39].

We were intrigued by the observations that UMSBP, which was defined as a sequence-specific DNA-binding protein, binds two different nucleotide sequences. However, analyzing the two sequences it became apparent that the conserved sequences at the two origin sites share several major common characteristics, that are relevant to the binding of UMSBP [34, 39, Onn and Slomai, unpublished observations].

Based on its encoding cDNA, the native UMSBP homodimer isolated *from C. fasciculata* contains the total of 10 CCHC-type zinc finger structures. The potential functional roles of each of these 10 zinc fingers in the binding of the ligand, in dimerization of the protein and its interactions with other replication proteins are currently being studied. Stoichiometry analyses revealed that one UMSBP molecule binds only one site of either of the two origin sequences, but not both sites simultaneously [35, 39]. Structural-functional studies of UMSBP, using site-directed mutagenesis and genetic analysis in a yeast two-hybrid system have indicated the role of the various UMSBP domain in either the binding of the nucleic acid ligand or in the protein dimerization [Onn *et al*. unpublished observations].

The intracellular and intra-organelle localization of UMSBP

UMSBP is encoded by a nuclear gene and the protein product does not have a characteristic N-terrninal mitochondrial targeting sequence [36]. The apparent lack of N-terrninal mitochondrial targeting sequences is also the case with the kinetoplast SSE1 and topoisomerase II enzymes [40, 41]. However, other kDNA associated proteins, such as the kinetoplast histone-like proteins (p16, p17 and p18), p21 and pol β3 have more typical cleaved N-terminal targeting signals [42–44]. Despite the lack of an obvious targeting sequence in UMSBP, the specificity and high binding affinity to two conserved sequences at the minicircle replication origin led to our proposal that this protein functions in the mitochondrion [34]. An interesting question, which is pertinent to UMSBP function during kDNA replication initiation, was the precise intra-organelle localization of the protein within the kinetoplast. It has been previously found that kDNA replication enzymes are positioned in discrete sites around the kinetoplast disc. Three histone-like proteins (p16, p17 and p18 [44] localize throughout the kDNA disc. A DNA topoisomerase II [26], a DNA polymerase β3 (pol β3) [27] and a structure-specific endonuclease (SSE1) [40] are positioned together in antipodal sites, which flank the kinetoplast disc. These sites also contain free minicircle replication intermediates [27, 45]. In contrast, the mitochondrial DNA primase and p21 localize in two sites adjacent to the faces of the kDNA disc, forming a sandwich around the kDNA [42, 46, 47]. We have recently localized UMSBP relative to a previously studied kDNA replication protein, pol β3, by immunofluorescence using antibodies raised against the two proteins [48]. In cells from an asynchronous logarithmic culture of *C. fasciculata*, pol β3 immunofluorescence is detected in the two well-characterized antipodal sites that flank the kinetoplast disc [27]. In contrast, these analyses have revealed that UMSBP has a novel location, in two discrete zones, adjacent to the face of the disc closest to the cell flagellum. In this location UMSBP resides in the same region in which DNA primase has been previously localized [46, 47]. We have also studied the localization of UMSBP relative to the location of the replicating free minicircles that are associated with the replication proteins in the two antipodal sites on the perimeter of the kDNA disc. These experiments, in which gapped kDNA is labeled in situ with fluorescently-labeled Alexa-dUTP, using terminal deoxynucleotidyl transferase (TdT) [45], are based upon the well-characterized distribution of covalently closed and gapped minicircles in pre-replication, replicating, and post-replicated kDNA in isolated networks [49] and in vivo [27]. Whereas unreplicated minicircles are covalently closed, minicircles that have completed replication are gapped and thus are selectively labeled by the fluorescent dUTP. The antipodal sites are strongly labeled due to the presence of abundant free minicircle replication intermediates that are

multiply gapped [45, 50]. Visualization of UMSBP in these cells confirms that it is in a unique location, and not co-localized with the two antipodal sites. This location is consistent with UMSBP's proposed function as the kDNA minicircle origin binding protein that controls the initiation of kDNA replication. Recent studies have indicated [28] that minicircles are released vectorially from the kDNA disc, towards the face of the disc nearest to the flagellum. Replication initiates in this zone and ultimately the progeny minicircles accumulate in the antipodal sites [28]. Within the antipodal sites, primer removal and filling of some of the gaps are thought to occur prior to minicircle reattachment to the network. In the framework of this replication model, UMSBP is ideally positioned to recognize free minicircles once they are released from the network. It is likely that UMSBP facilitates the assembly of the primase and other proteins (such as the replicative polymerase) to initiate replication at the origin sequence. Moreover, UMSBP could provide the specific origin recognition required for the origin-specific priming of kDNA replication. As UMSBP recognizes specifically the replication origins of both the L-strand [34, 35] and the H-strand [39] of the minicircle, such an interaction could facilitate the coordinated initiation of leading and lagging strand synthesis. Given these considerations, our currently proposed model is that minicircles are released vectorially from the central region of the network. Then the free minicircles associate with UMSBP at the location of UMSBP foci or outside these sites, which allows them to assemble with primase and other proteins and begin replication. Replicating minicircles accumulate in the central region adjacent to the flagellar face of the kinetoplast disc and then move to the antipodal sites to finish their replication and preparere for attachment to the network.

Localization of UMSBP throughout the cell cycle

To determine whether the changes in UMSBP localization are coordinated with the cell cycle, we examined a synchronized culture of *C. fasciculata*. Cells, synchronized by hydroxyurea arrest [51], were sampled at 30 min intervals. We performed cell counts by hemocytometry, detected dividing cells, containing two nuclei, by fluorescence and phase microscopy, and determined whether kDNA was undergoing replication in individual cells by observing Alexa-dUTP fluorescence. We also used immunofluorescence to determine the status of UMSBP. We found that UMSBP localization, in two zones on the flagellar face of the kDNA disc, is dynamic during the *C. fasciculata* cell cycle. Although virtually every cell has UMSBP detectable by immunofluorescense during the time of kDNA replication, over 30% of the cells lose virtually all UMSBP during the time of cell division. In addition, we have found that the localization pattern of UMSBP also changed during the later stages of

kDNA replication, with the UMSBP zones becoming more elongated and diffused, in correlation with the progress in kDNA replication and cell cycle. These changes were not due to alteration in the level of UMSBP in the cell, as we detected no alterations in the level 1 UMSBP specific mRNA, UMSBP synthesis rate, UMSBP protein abundance, or its UMS binding activity, throughout the cell cycle. A possible explanation to the loss of UMSBP fluorescence upon cell division and the changes is that the protein molecules may move from the two zones near the flagellar face during the time of cell division. A similar loss of fluorescence detection during cell division is found for pol β3, topo II, and SSE1 [40, 45]. In contrast, primase immunofluorescence is constant throughout the cell cycle [45].

Structure of the minicircle replication origin

Based on its specific binding to the origin-associated UMS element, we have previously suggested that UMSBP functions as an origin binding protein in the process of kDNA replication initiation [34]. However, considering the strict specificity of UMSBP to single-stranded DNA, it would be difficult to explain such a role for UMSBP in the double-stranded minicircle molecule. It was therefore essential to determine whether UMSBP actually binds to the native kDNA minicircle. This question was addressed [52] in a binding competition analysis in which UMSBP was incubated with the 12-mer UMS H-strand probe, in the presence of increasing concentrations of unlabeled kDNA networks or decatenated, covalently-sealed kDNA minicircles. This analysis demonstrated clearly that kDNA networks competed efficiently with the binding of the protein. Direct binding of UMSBP to the minicircle origin region was shown by mobility shift electrophoresis analysis, revealing that UMSBP binds specifically to a duplex minicircle DNA fragment containing the UMS element.

Since UMSBP was characterized as a single-stranded nucleic acids binding protein [34, 35], its binding to the origin site would require the availability of the UMS as a single-stranded sequence in the overall double-stranded DNA minicircle. The possibility that local melting of the double helix is facilitated by a negative superhelical torsion of the minicircle could be excluded in this case, since kDNA minicircles are topologically relaxed [53]. Electrophoresis analyses [52], using minicircle fragments containing the UMS site indicated that a local change in the geometry of the DNA double helix at the minicircle origin region, may support the distortion and the consequent unwinding of the double-helix at the origin site, providing the binding site for UMSBP. In these studies we have found that several unusual characteristics of the DNA indicate the intrinsic curving of the DNA double helix at the origin region.

Computer analysis [54] also predicted that the UMS element resides within a curved region. Being located within a curved DNA segment, the UMS double helix may be susceptible to distortion through the induction of sequence-dependent kinks at sites, which are potentially "kinkable" [55–57]. We hypothesized that locally unstacked (kinked) base pairs in UMS may provide the nucleation for unwinding at this sequence [52], and that the unwound DNA conformation may be trapped in the protein–DNA complex. This was challenged experimentally using potassium permanganate [58], which attacks preferentially unwound DNA and other sharply distorted DNA structures in the double helix, modifying primarily thymine residues to form thymine glycols. The primer extension reaction, using the modified DNA strand as a template, clearly demonstrated that thymine residues at and around the UMS element, were modified by permanganate, implying their presence within a single stranded or otherwise distorted DNA structure [52].

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