Molecular Biology, Vol. 35, No. 1, 2001, pp. 1–10. Translated from Molekulyarnaya Biologiya, Vol. 35, No. 1, 2001, pp. 3–13.

REVIEW

UDC 576.893.161.13

Minicircular Kinetoplast DNA of Trypanosomatidae

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Received April 19, 2000

Abstract—Analysis of primary structure and organization of mitochondrial (kinetoplast) DNA of flagellates occupies a prominent place in the studies of eukaryote mitochondrial genomes, owing to its unusual organization and functioning as well as to the epidemiological role of the Trypanosomatidae family. According to contemporary notions, living zooflagellates are direct descendants of the ancestral forms that gave rise to all eukaryotic kingdoms. Hence, comparative mtDNA studies of recent Trypanosomatidae open broad prospects for phylogenetic reconstructions and analysis of presumable routes of eukaryote evolution. The structure, characteristics, and functions of Trypanosomatidae minicircular kinetoplast DNA are discussed here.

Key words: kinetoplast DNA, minicircle, Trypanosomatidae

INTRODUCTION

Analysis of organization and structure of kinetoplast mtDNA of flagellates occupies a prominent place in eukaryote mtDNA studies owing to its unusual organization, functioning, and the specific role Trypanosomatidae play in epidemiological processes. It is believed that recent zooflagellates descend directly from the ancestral forms that gave rise to all eukaryotic kingdoms, which substantiates phylogenetic reconstructions and eukaryote phylogeny hypotheses.

Data accumulated over more than thirty years of kinetoplast DNA (kpDNA) studies have made it possible to understand the general principles of kpDNA organization and its functional role, to shed light on some unique biological processes such as replication of multicomponent catenated system of circular molecules, and to discover the RNA editing process. However, some aspects of kpDNA structure and functioning remain obscure. For example, why the quantity of kpDNA in cells is so high? Do they have any additional function(s) other than coding for guide RNA required in the editing process? How polymorphic are the individual components of the kpDNA associate? Which genes or other kpDNA sequences could be employed in evolutionary studies and what is the degree and specificity of divergence of individual genes at the species, family, and order levels? Most of these questions raised 15–20 years ago still remain unanswered.

TRYPANOSOMATIDAE SYSTEM AND PHYLOGENY

The Trypanosomatidae family includes monogenetic genera *Crithidia*, *Leptomonas*, *Blastocrithidia*, *Herpetomonas*, and *Wallaceina*, and digenetic genera *Leishmania*, *Phytomonas*, and *Trypanosoma*. All the known species are parasites. Monogenetic species are parasites of invertebrates belonging to the Diptera and Hemiptera orders and infect one host, whereas digenetic species infect two hosts, i.e., invertebrate and vertebrate animals. These unicellular organisms possess some unique characters distinguishing them from other eukaryotes, including an exoskeleton of microtubules completely covering the cell, 5'-terminal posttranscriptional modification of practically all the cytosolic mRNAs with 39 strictly conserved nucleotides [1], the presence of a glycosome, i.e., of a peroxisome-associated membrane organelle including most of the glycolytic enzymes, and one mitochondrion with DNA composed of catenated circular molecules [2].

Such unique characters could exist because the Kinetoplastidae order branched off at the earliest stages of evolution. Digenetic species have a two-stage life, cycle which is directly related to the observed substantial changes in their morphology and physiology. For example, *Trypanosoma* species have a functional respiratory chain in insects but it is practically completely repressed in the circulation of vertebrates. *Leishmania* species were shown to possess lysosomal activity allowing them to invade mammalian macrophages [3].

0026-8933/01/3501-0001$25.00

MtDNA OF KINETOPLASTIDS

Subcellular Localization and Organization

The Kinetoplastidae mtDNA constitutes 5–25% of cell DNA [4]. Usually, kpDNA is a complex associate composed of two classes of molecules: mini- and maxicircular [5]. Each associate includes 20–50 maxicircular and $5 \cdot 10^3$ to $5 \cdot 10^4$ minicircular molecules. Maxicircular molecules are transcribed and are functionally similar to mtDNA of other organisms [6]. The high content of compact kpDNA in the cells had allowed it to be revealed by classical cytochemical methods as early as at the end of the XIX century (cited in [2]). The cytological description concerned only the kinetoplast, a specific, as it was then believed, part of the mitochondrion. Many names were suggested for this structure, but only that underlining the relatedness of this structure to the flagellum was finally firmly accepted. Kinetoplast always occupies the place near the flagellum basal body, and this relatedness is never disturbed during the cell life, although the basal body undergoes substantial morphological changes in the Kinetoplastidae life cycle [7]. The point is that the flagellum basal body provides for segregation of the kpDNA associate during cell division, i.e., it functions like centrioles during the cell nucleus division [8].

In the free state, isolated classical kpDNA associate has a highly ordered structure; its size is approximately $15 \times 10 \times 0.3$ μm [9]. KpDNA electron microscopy demonstrated that the associate is a highly ordered, spherical or oval, basket-like or network-like structure, including all intermediate forms except flat ones [10]. Probably, it depends on specific organization, functions and replication of kpDNA (individual DNA molecules are intermeshed like catenanes, producing a three-dimensional network). The contour length of minicircular molecules in some associates was measured. It makes 0.31 μm in *Leishmania gymnodactyli*, 0.43 μm in *Leptomonas pessoai*, 0.60 μm in *Trypanosoma carassii*, and 0.74 μm in *Crithidia fasciculata*. The size of the minicircular molecule is directly related to the associate and kinetoplast dimensions: the larger the minicircles, the larger the associate. The largest associates were found in fish trypanosomes and in species of the *Crithidia* genus, and the smallest ones in mammalian trypanosomes and in leishmanias [3]. If one analyzes the correlation between the dimensions of minicircles and kinetoplasts, the final conclusion is that usually the kinetoplast diameter is linearly related to the minicircular molecule radius. This observation could be explained by specific organization and replication of kinetoplast minicircles: they are folded and twisted in such a way that an 8-like structure is formed.

Individual minicircular molecules are entrapped in associate structure predominantly by catenane-like engagement. Maxicircular molecules do not participate in the formation of associate macrostructure (associates without maxicircles have the same morphology as native ones [11]). Usually, maxicircular molecules are easily observed during segregation when they concentrate in the zone of separation of filial associates. If the three-dimensional structure of associates is projected onto a plane surface, it becomes evident that minicircular molecules produce a pseudoflat, “chain armor” structure, each molecule of which is coupled with three adjacent ones, and maxicircles tie up the periphery of the structure to its center. According to available data, DNA molecules are kept in associates with the aid of proteins. It was demonstrated *in vitro* that p1 and p2 proteins (22 and 21 kDa, respectively) facilitated formation of DNA compact structure [12].

Summing up, the basic principles of organization of the kpDNA associate in the cell are as follows: (i) minicircular molecules form a regular network by multiple engagement with one another; (ii) the three-dimensional structure of the associate is formed with participation of proteins and by interaction of complementary sequences fixed by protein links; (iii) maxicircular molecules also participate in fixation of the three-dimensional structure; (iv) the structure is made compact with the aid of basic proteins.

Some General Characteristics of Minicircular kpDNAs

In contrast to maxicircular molecules (<5% of total DNA) which are functionally analogous and probably homologous to mtDNA of other organisms [13], the minicircular kpDNA is unique in its size (0.47–9.2 kbp) and content (five to fifty thousand per cell) as well as in its unusual three-dimensional organization and functional role [14].

The size of minicircular kpDNA is species-specific and variable (Table). The molecules can be subdivided into three groups: 800–1000, 1600, and 2300–2500 bp. This discreteness might be determined by some specific stereochemical properties of molecular rings, because minimal calculated free energies are characteristic of molecules of such size. Probably, this governs the size of molecules while kinetoplast diameter could be the second factor. These dimensions are probably related to associate replication and are strictly determined for every species.

Organization of Minicircular kpDNA

As follows from the Table, associates usually include several classes of minicircles heterogeneous by size and primary structure (e.g., in *C. oncopelti*, *T. scleropori*). However, *T. equiperdum* minicircular DNA is an exception, being composed of identical molecules [15]. Heterogeneity of minicircular DNA

could be observed not only within a species or population, but within an organism, which may have associates including minicircles of different nucleotide sequence. Originally, this was demonstrated by DNA reassociation experiments [53] and found added proof in the results of restriction analysis of minicircular kpDNAs. Analysis of associates composed of uniform minicircles with different primary structures showed that the sum of molecular masses of the restriction fragments formed is usually higher than the molecular mass of intact molecules. Minicircle molecules are usually present in natural associates in the form of minicircle oligomers [54], mostly dimers and trimers; higher oligomers are very rare. Such oligomerization is more abundant among the species of *Crithidia* and *Trypanosoma* and is less frequent among *Leishmania* species.

Proteins capable of selective binding to minicircular motifs recently became the object of profound studies. First, all the proteins participating in processing of guide RNAs should belong to this protein class and, second, some conserved minicircular motifs resemble telomere repeats (GGGGTTGGTGTA and GGGGTTGG); hence such proteins must have much in common with relatively well-characterized telomerases [55].

Size of Minicircles

The size of minicircles is variable, from 465 bp (*T. vivax*) [52] up to 9480 bp (*T. avium*) [44]. As follows from the Table, lower trypanosomatids usually have larger minicircles than higher ones. However, several exceptions were detected. Very large minicircles were found in *T. boissoni* and *T. avium* belonging to higher trypanosomatids. Both species are not mammalian parasites and are thus closer to lower trypanosomatids. An unusual organization of *Herpetomonas ingenoplastis* (a lower trypanosomatid) minicircles was detected. This species has an abbreviated respiratory chain and is actually unable to form functional mitochondria. Probably, this fact explains the presence of two classes of large circular DNA molecules in kinetoplasts which are not homologous to maxicircles or minicircles of other species [56]. Another type of organization of mitochondrial genome was found in *Trypanoplasma borreli*, a free-living trypanosomatid from the Bodonidae which generally have no associates of catenated circular DNA molecules. Mitochondrial genomes of these species include at least two classes of DNA molecules, 170–200 kbp and 80–90 kbp long. The former one includes repetitive 1-kbp sequences making it similar to trypanosomatid minicircles. The latter one includes structural mitochondrial genes. Hence it has much in common with maxicircles [57]. Large DNA molecules of other Bodonidae species: *Bodo caudatus*, *B. saltans*, *Cruzella marina*, and *Cryptobia helicis* were also studied (for details, see [58]).

The size of minicircles may be different not only among species of trypanosomatids, but also among isolates of one and the same species (Table) and even within an organism. It was demonstrated that kpDNAs of many species, such as *C. oncopelti* [18], *Blastocrithidia culicis*, *Herpetomonas roitmani* [59], *T. scelopori* [43], always contain a certain set of minicircles of various classes. The presence of several classes of minicircles could facilitate adaptation of organisms to varying environment.

Primary Structure of Minicircular kpDNA and Methods of Analysis of Minicircles

As mentioned earlier, the primary structure of minicircular DNA is heterogeneous, and differences in nucleotide sequence could be revealed among species of a genus (species-specific differences), within a species (differences between populations and isolates), and even within individual kinetoplasts [3]. To evaluate the number of individual classes, a criterion of kinetic complexity of mitochondrial genome is employed, i.e., for trypanosomatids, the temporal characteristics of kpDNA reassociation. Homogeneous DNA reassociates completely in a time t which is taken as a unit time. It could be calculated from the molecular weight of reassociated DNA and its approximate GC content calculated from DNA melting experiments. KpDNA usually reassociates in a time nt, where $n > 1$ and corresponds to the number of different minicircle classes. However, such calculations are based on the assumption that the maxicircular component of kpDNA of a species is nearly homogeneous and does not substantially influence the reassociation kinetics because of its low relative concentration [60]. The kinetic complexity of kpDNA of trypanosomatids determined by this method varies from 1 (*T. equiperdum*) to 300 (*T. brucei*). In other words, *T. equiperdum* minicircles are practically homogeneous, while approximately 300 classes of minicircular molecules constitute the kpDNA associate of *T. brucei*. Kinetic complexity is determined by not only the heterogeneity of the primary structure of minicircles, but by the genome size as well.

Another approach to the problem of minicircular kpDNA heterogeneity is based on restriction analysis allowing one to obtain preliminary estimates of the structure of a population of minicircles and to reveal classes of molecules of different size [61]. In practice, enzymes recognizing four-nucleotide sequences such as *Msp*I, *Taq*I, etc. were widely used. As the number of such short recognition sites in kpDNA molecules is high, the reaction products are of low molecular weight. If a minicircular kpDNA associate is structurally homogeneous, the sum of molecular weights of

Size of trypanosomatid minicircular molecules

Species	Size, in kbp	Reference
	Monogenetic species	
Crithidia fasciculata	2515	[15]
C. luciliae	2500	[16]
C. acanthocephali	2500	[17]
C. oncopelti	1300, 1650, 1848, 2350	[18]
C. guilhermei	2500	Kolesnikov, unpubl.
C. deanei	2500	Kolesnikov, unpubl.
C. roitmani	>2500	Kolesnikov, unpubl.
Blastocrithidia culicis	1800–2000	Brack, unpubl.
B. gerricola	1900	[19]
Wallaceina brevicula	1477	[20]
W. inconstans	1519, 1479	[20]
Leptomonas sp.	1700	[19]
L. (*Herpetomonas*) *pessoai*	1350	[21]
Herpetomonas muscarum	1100	[22]
Phytomonas davidi	1065	[23]
P. serpens	1457, 1476	[24]
P. sp	1457	[25]
	Digenetic species	
Leishmania		
Subgenus *Sauroleishmania*		
Leishmania (*S.*) *tarentolae*	826	[26]
L. (*S.*) *gymnodactyli*	920	[27]
L. (*S.*) *guliki*	920	[28]
L.(*S.*) *adleri*	890	[27]
Subgenus *Leishmania*		
L. (*L.*) *gerbilli*	900	[27]
L.(*L.*) *arabica*	860	[27]
L.(*L.*) *turanica 3720*	970	Kolesnikov, unpubl.
L.(*L.*) *turanica 3ı66*	860	Kolesnikov, unpubl.
L.(*L.*) *turanica 9567*	920	Kolesnikov, unpubl.
L.(*L.*) *aethiopica*	900	[29]
L.(*L.*) *sp 48*	900	[29]
L.(*L.*) *major*	683	[27, 30]
L.(*L.*) *tropica*	920	[27, 31]
L.(*L.*) *tropica*	756	Aluen, unpubl.
L.(*L.*) *sp LDJ(L. tropica?)*	920	[28]
L.(*L.*) *sp UR6*	825	[32]
L.(*L.*) *donovani*	719, 750, 792, 819, 829	Bassellin, unpubl.
L.(*L.*) *donovani*	805	[33]
L.(*L.*) *infantum sp.*	617, 778	Aransay, unpubl.
L.(*L.*) *infantum AJS-IPTPS*	805, 814	[34]
L.(*L.*) *infantum AJS-D2PST*	803	[33]
L.(*L.*) *sp. ZMA*	780	[27]
L.(*L.*) *mexicana*	859	[35]

Table. (Contd.)

Species	Size, in kbp	Reference
L.(*L.*) *amazonensis*	677, 699	[36]
L.(*L.*) *aethiopica*	864	[37]
L.(*L.*) *chagasi*	800	[34]
Subgenus *Viannia*		
L. (*V.*) *peruviana*	751	[38]
L. (*V.*) *braziliensis*	749	[39]
Trypanosoma		
Trypanosoma boissoni	5200	[40]
T. carassii	1599	[41]
T. mega	2300	[42]
T. scelopori	1700, 2050, 2300	[43]
T. avium A493	5900	[44]
T. avium A1412	9480	[44]
T. cruziY	1423	[45]
T. rangeli H9	1587, 1764	[46]
T. congolense	958, 964	[47]
T. lewisi	1018	[48]
T. brucei	1014	[49]
T. brucei	983	[50]
T. equiperdum	1012	[46]
T. evansi	999	[51]
T. vivax	465	[52]

the restriction fragments will exactly coincide with that of the native minicircle. Otherwise, the sum of the former will be greater than the latter. Such an approach allows one to evaluate unequivocally the heterogeneity of minicircular populations [44].

With the advent and further broad application of genetic engineering, methods of cloning and sequencing of minicircles [3], direct cross-hybridization [61], or express PCR methods [62] have occupied leading positions in kpDNA analysis.

Common Structural and Functional Features of kpDNA

1. Structure. Conserved and variable sequences (CS and VS, respectively) were found in all the minicircles studied to date. High interspecies homology is characteristic of CSs of many species of trypanosomatids. Three regions of very high (90–100%) homology were identified within CSs: CSB1 (GGGCGT), CSB2 (CCCCGTAC), and CSB3 (GGGGTTGGTGTA) (CSB is Conserved Sequence Block [63]). Sequences of CSB1 and CSB3 are practically identical among higher as well as lower trypanosomatids, while CSB2 may differ from the canonical one.

It is agreed that CSB3 is structurally and functionally related to initiation of replication of the DNA L-strand, and CSB1, of the H-strand. The size of CS is variable, but usually it falls within 100–200 bp. It is of interest that its structure is practically identical even in minicircles of different classes from individual associates. Adjacent to the 5' terminus of CSB1 is a region of relative or species-specific conservatism. It is conserved in different clones, strains, and isolates of a species. Regions including CSs and such species-specific, conserved sequences are usually employed to reconstruct the evolutionary relatedness of species.

The variable region of minicircles carries information on the structure of the so-called “guide RNAs” (gRNA) directly participating in posttranscriptional modification of mRNA known as “uridylate editing” [64].

2. Copy number. The number of minicircular DNA molecules per cell is up to 50,000 [2], i.e., hundreds and thousands of completely identical molecules joined together and performing one and the same functional role are present even in the most heterogeneous associates (more than 300 classes of molecules). The meaning of this excess remains obscure.

However, if one remembers that minicircles serve as templates for gRNA synthesis, this excess does not seem unusual; other explanations could be suggested.

3. The presence of regular oligo(A) sequences inducing formation of "bent helix" DNA structures due to the presence of $(dA)_{5–6}$ repeats corresponding to the helix pitch and causing formation of the so-called "twisted helix". Such regions were found in many minicircular DNAs [65] and appear to be related to their replication. The presence or absence of twisted helix in CS is species-specific and might be employed as an additional character in comparative mitochondrial genotype analysis.

4. Trypanosomatid minicircle DNAs practically do not include modified bases. However, an unusual base, β-D-glucosyl-hydroxime-ethyluracil (J) was found in the nuclear genome of trypanosomatids. Hypothetically, this base might take part in regulation of expression of some genes at certain stages of development, e.g., it might inhibit expression of the variable surface antigen.

Some Special Features of Organization of *Trypanosoma, Leishmania,* and *Crithidia Minicircles*

Trypanosoma brucei, T. evansi,* and *T. equiperdum. Probably, the simplest minicircles containing only one CS [50] approximately 1000 bp long were found in these species (Table). However, kpDNA of *T. brucei* causing cattle disease "surra" transferred by tsetse flies has up to 300 classes of minicircles, whereas populations of minicircles of *T. evansi* and *T. equiperdum* which are morphologically similar to *T. brucei* and could be recognized only by using special clinical or biochemical tests are structurally homogeneous and predominantly (if not all of them) belong to one and the same primary structure class. It is worth noting that these African trypanosomes (Stercoraria), which are characterized by the same invasion type and area of distribution as *T. brucei*, have lost the part of their life cycle in insects and are transferred "from blood to blood" during contacts of mammals [67]. They have a reduced respiratory chain, and their maxicircles have undergone substantial changes, e.g., very large deletions were found in the corresponding coding sequences of *T. equiperdum* that are not detected in AMB3 and ILRAD B-32 *T. evansi* isolates [68].

Minicircles of *T. equiperdum* are so homogeneous that direct sequencing of one of them omitting intermediate cloning procedure appeared to be possible after multistep purification of associate in CsCl gradient and by other methods [46]. Minicircle DNA is AT-rich (72.8%), has six direct repeats 12 bp long, and a region 130 bp long highly homologous to CS of *T. brucei*, but lacks long open reading frames (ORF). Complete nucleotide sequence of a *T. equiperdum* minicircle was determined also in some other laboratories [22]. It appeared that two independently sequenced minicircles are homologous only within 130 bp sequence of CS. In alternative experiments, minicircular molecules were initially cloned in pUC18 and then sequenced. A potential ORF localized 165 bp apart from the "twisted helix" region was detected. Gel retardation experiments demonstrated anomalous mobility of minicircular fragments. These contradictory results could be explained by structural heterogeneity of associates and by the presence of two classes of molecules with different primary structures. As direct sequencing of minicircles was performed, one might suppose that the relative content of "minor type" molecules which were obviously cloned in [22] is very low (<1%).

In sequenced minicircle DNA of *T. evansi* the length of CS is also 130 bp. Its 5' terminus is flanked with a palindrome repeating a part of conserved region. If compared with the canonical structure, GGGCGT, sequence of the origin of replication (*ori*) (GGGGCGT) of the H-strand has one additional G. Within a species, the distance between *ori*L and *ori*H is rather conservative and is similar (73 bp) in *T. evansi* and in *T. brucei*. Close relatedness of *T. evansi* and *T. brucei* following from the results of analysis of primary structures of minicircular kpDNAs finds added proof in the results of hybridization and restriction analysis of nuclear genomes [68].

Among trypanosomatids, the structure of *T. brucei* minicircles was most often studied, probably because this organism does not infect humans and is of substantial economic importance. Within certain limits, the data obtained in these studies could be extrapolated to other trypanosomes. The kinetic complexity of the minicircular associate is approximately 300 kbp, i.e., it might be composed of 300 classes of molecules 1000 bp long each. However, the homology of the first two minicircular molecules of *T. brucei* following from the results of sequencing appeared to be only 27%, determined mostly by 120 bp of CS which is practically identical in the analyzed species. When the primary structures of additional four molecules isolated from two other lines of *T. brucei* were determined, it was found that the structure of CS in them does not differ from that of the CSs studied earlier [23]. Compared with other parts of minicircles, CSs are enriched in GC pairs (23 and 50%, respectively). Short (20 bp) imperfect inverted repeats are dispersed in the minicircle variable region.

Analysis of hybrid *T. brucei* lines demonstrated that minicircles are inherited from both parents, contrary to maxicircles, which are inherited from one of the parents only [69]. *In vivo* experiments with tsetse flies allow one to suppose that, at least in certain conditions and at certain stages of the parasite life cycle, crossingover occurs between mitochondrial genomes.

***Trypanosoma cruzi*.** Among South- and Pan-American trypanosomatids, *T. cruzi* belonging to Salivaria group [67] is of great importance. It is well characterized by biochemical and immunological methods. The heterogeneity of minicircular DNA in isolates of this species is rather low (approximately 20 classes of molecules), but minicircles obtained from individual isolates could differ substantially. This follows from the results of restriction and hybridization analysis [70], as well as from the data obtained by PCR amplification of variable regions [71]. It should be mentioned that the variable region rapidly changes in evolution. This observation followed from the experiments when mice were infected with two different (but well characterized) isolates of *T. cruzi*. It appeared that nucleotide sequence of minicircles isolated from such a "system" differs from that of the parental trypanosome isolates. Primarily, differences were registered in the variable region of minicircles [72]. Analysis of the primary structure of minicircles of different classes of minicircles of *T. cruzi* revealed that a 120-bp CS is present in four copies per molecule, and distances between them are always similar, i.e., they are localized symmetrically. They have all the regions typical of minicircular CSs (CSB1, CSB2, and CSB3). It is noteworthy that the *T. cruzi* CS has no potential "bent helix" region, and this might be characteristic of other Salivaria species.

***Leishmania* sp.** In all phylogenetic reconstructions, the *Leishmania* genus forms a far more compact clade than the *Trypanosoma* genus or other trypanosomatids [73]. *Leishmania* minicircles are organized by the trypanosomatid general plan and include CSs with typical CSB1–3 and variable region. Structure of CS of *Leishmania* minicircles is nearly identical even in geographically distant isolates of a species and is very similar within the genus. The fact that leishmanias cause dangerous infectious diseases stimulated elaboration of express methods for revealing and identifying *Leishmania* species based on hybridization of specific (primarily, minicircular) sequences or on PCR [74]. Such methods allow quick (3–4 h) identification of parasites.

It was established that the *Leishmania* minicircles encode gRNA, and one class of minicircles encodes only one class of gRNA. Basing on this fact, the number of minicircle classes (approximately 60) involved in the expression of all the gRNAs participating in RNA editing was estimated [73].

***Crithidia* and other monogenetic parasites of insects.** Organization of the minicircular component of lower trypanosomatids has not been studied in detail, and most of the data obtained pertain to the *Crithidia* species. Recently, some other lower trypanosomatids were involved in analysis. The minicircular component of *C. fasciculata* isolate Cf-C1 includes the major class (90% of the total number of minicircles, interclone variability 0.3%) and 3–16 minor classes [16]. The complete nucleotide sequence of minicircles of the major class was determined [15]. Two CS were found in it, with two potential origins of replication. In one of the adjacent regions a "bent helix"-like structure is present, as well as flanking regions of a complex (probably, cloverleaf) secondary structure. Individual minicircular kpDNA molecules of *C. fasciculata* were also described which exist apart from the catenated associate. Kinetics of incorporation of [^{3}H]thymidine into this fraction shows that they replicate according to the Cairns model [75].

Restriction analysis demonstrated that *C. oncopelti* kpDNA contains four major classes of minicircles: 1.3, 1.6, 1.85, and 2.4 kbp. The 1848-bp minicircle was characterized in detail [18]. It has trypanosomatid-universal CSB1 and CSB3 regions, but its CSB3 differs from the canonical GGGGTTGGTGTA: the first G in it is substituted by A, and there is an additional T at the end, i.e., the primary structure of CSB3 of *C. oncopelti* is AGGGTTGGTGTTA. In CSB1 the *ori*H sequence, GGGCGT, remains unaltered. Cross-hybridization did not reveal homologies in kpDNA of *C. oncopelti* and other species of the *Crithidia* genus [76]. Summing up, *C. oncopelti* differs from other members of the *Crithidia* genus in many molecular-biological characters.

Minicircular molecules of *Wallaceina brevicula* and *W. inconstans* of approximately 1500 bp were recently analyzed in our laboratory. By the GC content (approximately 51%) they differ drastically from all other trypanosomatids.

REPLICATION OF MINICIRCULAR KPDNA

DNA molecules undergo doubling in the S phase of the cell cycle, and during this process minicircles leave associates and replicate like θ-structures with the aid of ATP-dependent topoisomerase II. Using antibodies to this enzyme, it was demonstrated that in *C. fasciculata* this protein is localized at two opposing parts of the associate [77]. DNA polymerase was also isolated and purified from *C. fasciculata* and *L. mexicana* kinetoplasts [78, 79]. In its properties it differs substantially from mitochondrial DNA polymerase γ and is closer to eukaryotic DNA polymerase β. The kinetoplast enzyme is of modest size (43 kDa), has low processivity and fidelity, and does not exhibit endonuclease activity. However, it repairs underreplicated regions, just as the eukaryotic enzyme is believed to do. Probably, this enzyme is imported from the cytoplasm.

Replication of DNA in the presence of [^{3}H]thymidine followed by autoradiography disclosed two zones in kinetoplasts. The central one includes covalently closed minicircles, and the periphery contains open minicircles with a gap in one strand, which

have undergone replication [80]. The fate of replicated molecules may be different. In *T. brucei* such newly synthesized molecules are localized in opposite positions in two regions of the associate "attachment points" [81]. On the contrary, in *C. fasciculata* newly synthesized molecules are uniformly distributed at the associate periphery. In all other species studied to date (*L. tarentolae*, *L. donovani*, *T. cruzi*, and *P. serpens*) minicircles are distributed after replication in the same manner as in *C. fasciculata* [80]. Mechanisms of such distribution and its functional role remain obscure.

Approximately 1000 minicircles, all of them nicked and replicated, are present per cell by the end of the S phase. Several ribonucleotides were detected at the gap terminal sequence which includes a part of CSB3 and probably serves as a signal preventing repeated replication and which perhaps remain after removal of the replication primer [5]. Such ribonucleotides could serve as markers differentiating signal gaps from accidental ones that are to be repaired. Such gaps are not filled up to the end of associate replication.

At the next step, minicircles are repaired and finally produce two daughter associates [9]. Maxicircles are also replicated according to the Cairns model. Immunofluorescence analysis demonstrated that the flagellum basal body takes part in segregation of mitochondrial genomes. Such type of segregation under strict control might be evolutionarily ancient [8].

DO MINICIRCLE SEQUENCES CODE FOR POLYPEPTIDES?

Initially, participation in protein synthesis was considered to be the main functional load of minicircles [83]. However, as soon as the first minicircles were sequenced, this idea was practically abandoned, because minicircle sequences included too many termination codons to code for long polypeptides. However, practically all the minicircle kpDNA sequences contain ORFs. For example, six ORFs were detected in *C. fasciculata* minicircles which potentially could code for a polypeptide of 100 amino acid residues. The same number of ORFs was found in minicircles of *C. oncopelti*. The largest of them corresponds to a polypeptide of 152 amino acids [18].

However, attempts to find appreciable homology of such polypeptides with any other protein failed, and estimates of such sequences by the Shepherd method proved to be very low.

The functional role of minicircles is still an open question. Periodically, papers are published describing a specific RNA homologous to a minicircle ORF, or even its protein product. For example, an ORF of 284 bp was described [84] coding for a highly hydrophobic protein (10.5 kDa) similar to transport proteins TAP1, TAP2, and NRAMP of *L. donovani*. However, attempts to isolate the protein translated from this template failed. Summing up, one comes to the conclusion that the main genetic function of minicircles is not related to protein synthesis. This idea finds support in the results of experiments demonstrating fast "laboratory evolution" of minicircles, and in the fact that minicircles of one size (class) can substantially differ in nucleotide sequences [44]. One can hardly imagine that such rapidly evolving DNA could code for proteins vitally important for the cell.

ACKNOWLEDGMENTS

The authors are indebted to L.P. Martinkina, Yu.Yu. Vengerov, and E.M. Merzlyak for their help and collaboration in preparing this review.

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