Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5962–5966, June 1994
Biochemistry

Mitochondrial minicircle DNA supports plasmid replication and maintenance in nuclei of *Trypanosoma brucei*

STAN METZENBERG AND NINA AGABIAN*

Intercampus Program in Molecular Parasitology, University of California - San Francisco, San Francisco, CA 94143-1204

Communicated by Anthony Cerami, February 25, 1994 (received for review December 14, 1993)

ABSTRACT **In a search for trypanosome DNA sequences that permit replication and stable maintenance of extrachromosomal elements, a 1-kilobase-pair (kbp) fragment from a mitochondrial kinetoplast DNA (kDNA) minicircle of *Trypanosoma brucei* was isolated and characterized. The plasmid pTbo-1, carrying the kDNA element, is maintained in *T. brucei* as a supercoiled concatemer containing approximately seven to nine pTbo-1 monomer units (5.6 kbp each) in a head-to-tail orientation. The concatemer is found in approximately one copy per cell when procyclic trypanosomes are cultured in the presence of 100 μg of hygromycin per ml; however, in the absence of continuous hygromycin selection, the plasmid is lost from the population with a $t_{1/2}$ of approximately 8.7 days (17 cell generations). A second unrelated kDNA minicircle was also able to serve as an autonomously replicating sequence (*ARS*) element in *T. brucei*, suggesting that this is a general property of kDNA minicircles. Replication of mitochondrial DNA in the nucleus may be due to either a specific consensus sequence (such as in yeast *ARS* elements) or nonspecific sequence characteristics (such as the degree of A+T-richness or bent DNA).**

The order Kinetoplastidae contains several genera of parasitic protozoa of agricultural and public health importance. They are also representative of an ancient evolutionary lineage and as such process their genetic information through mechanisms that are unusual in eukaryotes. Transcription generates polycistronic pre-mRNA molecules, from which monocistronic mRNAs are created by the processes of 5′-end trans-splicing and 3′-end cleavage/polyadenylylation (1). The mitochondrial genome, called the kinetoplast DNA (kDNA), is composed of a linked network of approximately 50 identical maxicircle elements, and 5000 heterogeneous minicircle elements (2). These minicircles have not been found in the mitochondria of other orders and appear to be important in RNA editing, an unusual process first described in kinetoplast mitochondria (3, 4).

During the multistage process of DNA replication, the minicircles are unlinked from the network, replicated, and then reattached (5). The growing kDNA then splits into two networks (6), which subsequently are segregated into daughter mitochondria during cell division (7). Although kDNA circles are covalently closed, they are not supercoiled (8). The replication of minicircles in the mitochondrion may depend on conserved sequences, such as the universal minicircle sequence (UMS or CSB-3: 5′-GGGGTTGGTGTA-3′) and conserved sequence characteristics such as purine/pyrimidine strand biases. A specific UMS-binding protein has been identified in kinetoplastids (9); however, it is not known whether it has a role in kDNA replication.

It has recently become possible to transfect *Trypanosoma brucei* with either transient expression vectors or through homologous recombination (10); however, we wished to increase the genetic capabilities of the organism by developing a shuttle vector that would allow the maintenance of multiple extrachromosomal copies of a transfected sequence. By introducing random fragments of total *T. brucei* DNA into a vector carrying a hygromycin-resistance gene, a mitochondrial DNA element was identified† that permits plasmid replication and maintenance in the nuclei of *T. brucei*. The plasmid is maintained stably under continuous drug selection as a supercoiled head-to-tail concatemer composed of approximately eight monomer units. A second kDNA minicircle element, chosen at random and similarly tested, also permits autonomous replication. These findings suggest that minicircles may have the interesting capability of engendering DNA replication in both the mitochondrion and nucleus.

MATERIALS AND METHODS

Transfection of Cells. *T. brucei* (subspecies *brucei*, strain IsTat 1.1) were cultured in Cunningham's medium (11) supplemented with 10% (vol/vol) fetal bovine serum. Electroporation and transfection of *T. brucei* were performed by the method of Kapler (12) with 10–50 μg of plasmid DNA isolated from *Escherichia coli* (SURE strain, Stratagene) by Qiagen (Chatsworth, CA) column chromatography. After electroporation, cells were cultured for 24–48 hr without selection before the addition of 100 μg of hygromycin B (Calbiochem) per ml.

Purification of DNA from *T. brucei*. Total DNA was prepared from *T. brucei* by the method of Milhausen *et al.* (13) or White *et al.* (14) and dialyzed against TE (10 mM Tris chloride/1 mM EDTA, pH 7.5). Supercoiled DNA molecules were fractionated by equilibrium centrifugation (15) in CsCl/ethidium bromide for 24 hr at 53,000 rpm (70 Ti rotor, Beckman). Densities of fractions were determined by refractometry. Supercoiled DNA from pTbo-1 plasmid-carrying *T. brucei* was prepared for EM (performed by Mei Lie Wong) by the method of Koller *et al.* (16). For partial digestion with *Hin*dIII, the enzymatic reaction was performed with 2 units of *Hin*dIII per μg of DNA for 1 hr at 37°C in the presence of 1.4 μg of ethidium bromide per ml (17). Southern analysis was performed with either Nytran membranes (Schleicher & Schuell) for detection with ^{32}P-labeled probes or with Magnagraph membranes (Micron Separations, Westboro, MA) for the Genius detection method (Boehringer Mannheim). ^{32}P-labeled and digoxigenin-labeled probes were prepared by extension of random hexamer primers (15) on Qiagen-purified plasmids or gel-purified DNA fragments. Hybridization was detected and quantified by autoradiography or PhosphorImager analysis (Molecular Dynamics). The minicircle TbmiB10 was donated by K. Stuart (Seattle Biomedical Research Institute). The plasmid pAS12, provided by A. Schneider (University of Basel), is identical to pTbo-1 except

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: kDNA, kinetoplast DNA; *ARS*, autonomously replicating sequence; UMS, universal minicircle sequence.

*To whom reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. UO3908).

that it lacks the 1-kbp *Xba* I fragment (TbmiSM1; indicated by the thick black line in Fig. 1). The DNA sequence for the minicircle TbmiSM1 was determined by using an ABI-370A DNA sequencer (Applied Biosystems).

RESULTS

Selection of an Autonomously Replicating Sequence (*ARS*) Element That Confers Stable Plasmid Maintenance. A plasmid pAS12, containing a procyclin/PARP (38) promoter and hygromycin-resistance gene, was used as a cloning vector for random *Xba* I fragments of total *T. brucei* DNA as described above. The plasmid library was amplified in *Escherichia coli*, and 300 μg of the mixture of supercoiled plasmids was introduced into 4×10^7 *T. brucei* cells by electroporation. After 2 days, 100 μg of hygromycin B per ml was added to initiate drug selection in culture, which continued for an additional 12 days. Hygromycin-resistant cells (2×10^9) could then be obtained for plasmid extraction by the method of White *et al.* (14). Supercoiled DNA from transfected cells was enriched by CsCl/ethidium bromide gradient, and plasmids (containing putative *ARS* elements) were rescued from *T. brucei* by transformation into *E. coli*. In a transformation experiment using 700 ng of fractionated DNA from *T. brucei*, 140 ampicillin-resistant colonies were obtained. Of three colonies initially tested, one contained a plasmid (pTbo-1, see Fig. 1) that, when reintroduced into *T. brucei*, gave rise to hygromycin resistance. The remaining two plasmids and the original plasmid pAS12 failed to confer stable hygromycin resistance following transfection into *T. brucei*.

The Sequence Conferring Autonomous Replication and Maintenance Is Derived from kDNA. The complete DNA sequence of the *Xba* I fragment contained in pTbo-1 was determined (Fig. 2). A comparison of this sequence with the GenBank data base revealed that the inserted element was derived from a kDNA minicircle, as noted by three kinetoplastid-specific conserved sequence blocks (18) (CSB-1, -2, and UMS CSB-3; see boldface nucleotides in Fig. 2) and

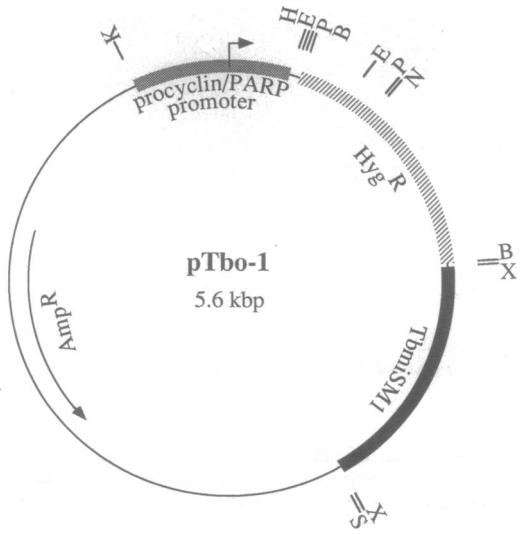

FIG. 1. Structure of 5.6-kilobase-pair (kbp) plasmid pTbo-1. The 1014-nucleotide minicircle element TbmiSM1 is indicated by the thick black line, the hygromycin resistance gene (Hyg^R) is indicated by the hatched line, and the procyclin/PARP (38) promoter and splice acceptor are indicated by the stippled line and clockwise arrow. Vector sequences from bacterial plasmid pBSII$^+$(KS), including the ampicillin resistance gene (Amp^R), are indicated by thin line. Restriction endonuclease cleavage sites for *Kpn* I, *Sac* I, *Hin*dIII, *Nco* I, *Xba* I, *Bam*HI, *Pst* I, and *Eco*RI are indicated outside the circular map by the symbols K, S, H, N, X, B, P, and E, respectively.

```
   1 AGAAAGGTAA AATAATTTAA TAGATAAGTA GTAGTAATAT ATTAAGTTAA
  51 ATATGATATA TATAATGACT AACTAAACTG ATAAAGCAGT AGAAGAGACG
 101 ATGTAATATA TATAATTATT AATTACTGTT AATATATCTA TTATTTTATT
 151 TTATTACTAA GGGAAAGAGA AGATATTAAT AGATAGAACA ATGAATAGAT
 201 AATAAAGTGA AGAATTATAT AAATCCAATA AAAGTGAAAT AGAATCTGAG
 251 AGACTGTGAT TTATACCTGT AAAATGAGAT TTATATTTAT ACTGTATCTA
 301 TTATTTTATT TATGATTGGA GTGGTGAGAT AATGGAGGGA ATCAGAAGTG
 351 AAGCACAGAG TTATTAATTG AAAGAGATTA GTAGTGGATG TAAGAAAATT
 401 ATGGAAAATC GGGTAAAAAT CGAAGAAAAA TGGCTTGAAA AAACCCAAAA
 451 TCTTATGGGC GTGCAAAAAT ACACATACAC AAATCCCGTG CTATTTTGGG
 501 CTGTTTTTTA GGTCCGAGGT ACTTCGAAAG GGGTTGGTGT AATACACACA
 551 TGGTTTTTCC TCGAGATTTC AGGGTTTTGG GGTGATATCT AGTGTAATTA
 601 ATATTGTGTT TTTATAGTCT ACTTAAGGAA TAAAATATAG TAATAGATAA
 651 ATATATAAGT TAGATATATA GCAATTATAA TTAAACTGAA GAGGTTATAA
 701 TACCTCGAAA CTCGCGGTGA TGATTTTATA TTTATTTCTT ATATTACTAT
 751 TTATTAATTT ATTCTCATTC TCGGGATTAC CTAGTGGGAA AGAAATGAGA
 801 TAATAGATAT GTATTGTAGT ATTATAATGA TATATATAGA TATAAGATCA
 851 ACAAAACTGC CATTTTCTAT AGTGAGTGAT GATATATTAT AATTAAATGA
 901 TATTATTATT AAAATCTATT TATTATTTTA TTTATTTATG GAGGATGAAA
 951 TTAATGGGAT TATTCGGTGG TAGAGTGGGA TTAATGTGAT AAATACTGCT
1001 TCATATCTGC GTCT
```

FIG. 2. kDNA minicircle TbmiSM1. The DNA sequence of TbmiSM1 is shown, with nucleotides conserved in *T. brucei* indicated by underlining and sequence blocks conserved in kinetoplastids indicated by boldface font (CSB-1, positions 455–464; CSB-2, 584–591; CSB-3 or UMS, 530–551). The sequence shown is the fragment contained in the *Xba* I site of pTbo-1 (see Fig. 1), with nucleotide 1 distal and nucleotide 1014 proximal to the 3′ end of the hygromycin-resistance gene.

sequences characteristic of *T. brucei* minicircles (underlined nucleotides in Fig. 2). The oligo(dA) stretches and strand asymmetry of purine and pyrimidine bases (nucleotides 395–450) are common features of minicircle sequences, as is the high A+T content of 72% in the 1-kbp element. The 18-bp repeats (nucleotides 9–36, 135–152, 632–649, 748–765, 792–809, and 917–934) and putative guide RNA sequences (nucleotides 60–109 and 840–886) are also typical of a complete kDNA minicircle (K. Stuart, personal communication), and this element has been named TbmiSM1. This sequence is not related to either the *ARS*-like element of tenAsbroek *et al.* (19), which did not function as a shuttle vector, or the single-copy plasmid of Patnaik *et al.* (20) whose autonomous replication originated from a nuclear DNA fragment.

An Average of Five Monomer Copies of pTbo-1 Are Maintained Per Trypanosome. Samples of purified plasmid pTbo-1 grown in *E. coli* and samples of total DNA isolated from *T. brucei* cells transfected with the pTbo-1 were analyzed by agarose gel electrophoresis and Southern blotting. In *E. coli*, the plasmid is 5.6 kbp, although multimeric forms are evident in an undigested sample (Fig. 3*A*, lane 1). The plasmid was grown in a *dam*$^+$ strain of *E. coli*, resulting in the methylation of adenosine residues found in GATC sequences; the plasmid is consequently sensitive to *Dpn* I (lane 2) and *Sau*3AI (lane 4) but not to *Mbo* I (lane 3). In *T. brucei*, the introduced plasmid displays different characteristics. Rather than a monomer, the plasmid is a higher order structure with low electrophoretic mobility (Fig. 3*A*, lane 8); it is resistant to *Dpn* I (lane 9) and sensitive to *Mbo* I (lane 10) and *Sau*3AI (lane 11). Acquisition of *Dpn* I resistance and *Mbo-1* sensitivity upon introduction of pTbo-1 into *T. brucei* (and maintenance under selection with hygromycin for 4 weeks) is consistent with plasmid replication in *T. brucei*, as evidenced by the loss of adenosine methylation pattern.

Digestion of DNA from *T. brucei* transfected with pTbo-1 with enzymes having unique restriction sites in the pTbo-1 monomer (see Fig. 1 and Fig. 3, lanes 12–15) produces restriction fragments comigrating with linearized pTbo-1 derived from *E. coli* (Fig. 3, lanes 5–7). This pattern of digestion could be due to either tandem integration of pTbo-1 copies into the genome or the maintenance of pTbo-1 in a concatemeric plasmid form. The lack of evident junction fragments (in lanes 12–15), which would be expected from a genomic integration event, is consistent with the maintenance of pTbo-1 as a plasmid in *T. brucei*. Digestion with enzymes that cut more than once in the plasmid (Fig. 3, lanes

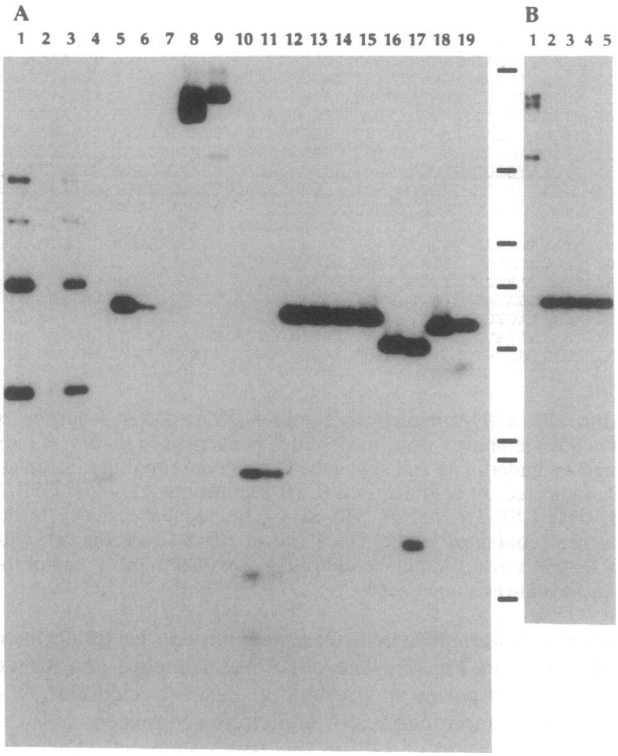

FIG. 3. Southern analysis of pTbo-1 and pTbmi-b10, probed with a hygromycin-resistance gene. (*A*) pTbo-1 extracted from *E. coli* (lanes 1–7) or in genomic DNA from *T. brucei* stably transfected with the plasmid pTbo-1 (lanes 8–19). DNA was either loaded undigested (lanes 1 and 8), or cleaved with enzymes *Dpn* I (lanes 2 and 9), *Mbo* I (lanes 3 and 10), *Sau* 3AI (lanes 4 and 11), *Kpn* I (lanes 5–7 and 12), *Sac* I (lane 13), *Hin*dIII (lane 14), *Nco* I (lane 15), *Xba* I (lane 16), *Bam*HI (lane 17), *Pst* I (lane 18), or *Eco*RI (lane 19). Purified plasmid was loaded into lanes as follows: 150 pg in lanes 1–5, and 15 pg and 5 pg, respectively, in lanes 6 and 7; 450 ng of genomic DNA was loaded in each of lanes 8–19. Lanes 1–4 have a lower hybridization signal than in lanes 8–11, respectively, because less of the *E. coli*-derived plasmid was loaded on the gel. (*B*) Hygromycin-resistance plasmid detected in DNA of *T. brucei* stably transfected with pTbmi-b10. Genomic DNA (1.4 μg) was loaded undigested (lane 1) or cleaved with *Kpn* I (lane 2), *Sac* I (lane 3), *Hin*dIII (lane 4), or *Nco* I (lane 5) and analyzed with a digoxigenin-labeled hygromycin-resistance gene. For *A* and *B*, the positions of the gel origin and molecular weight markers, as indicated by the bars, are derived from a *Hin*dIII digest of phage λ (23.1, 9.4, 6.5, 4.3, 2.3, 2.0, and 0.56 kbp).

16–19) produces patterns that are consistent with the structure of the *E. coli*-derived plasmid (Fig. 1). There is no evidence for rearrangement of the plasmid sequence in *T. brucei*, suggesting that pTbo-1 is maintained as a concatemeric molecule. Comparison of the hybridization signals of linearized *T. brucei*-derived pTbo-1 (Fig. 3*A*, lanes 12–15) with standards of *E. coli*-derived pTbo-1 (lanes 5–7) indicates that each *T. brucei* cell contains an average of approximately five monomer equivalents of the pTbo-1 plasmid sequence. Results of restriction analysis of total DNA from cells grown continuously under hygromycin selection for a period of 10 months (>600 cell generations) were not significantly different than that shown in Fig. 3*A* (data not shown).

To determine whether plasmid replication and stable maintenance can be conferred by other minicircles, a 1-kbp *Sau*3AI fragment containing the minicircle element TbmiB10 (provided by K. Stuart) was introduced into the *Xba* I site of pAS12. The resulting plasmid pTbmi-b10 was introduced into *T. brucei* by electroporation, and, as with pTbo-1, readily gave rise to hygromycin-resistant cells. Southern analysis of DNA extracted from pTbmi-b10-transfected cells revealed a higher order structure (Fig. 3*B*, lane 1) in uncut DNA. The three bands in the uncut sample, which are likely to correspond to supercoiled, nicked, and linearized multimers, were reduced to a monomeric size of 5.6 kbp by digestion with restriction enzymes that linearize pTbmi-b10 (Fig. 3*B*, lanes 2–5). The minicircle elements in pTbo-1 and pTbmi-b10 were cloned in opposite orientations to each other; however, the respective distances from the 3′ end of the UMS sequence to the site of linearization were nearly identical (469 and 484 nucleotides, respectively). Therefore, stable plasmid maintenance occurs irrespective of the orientations of the hygromycin gene transcript and the asymmetric minicircle features (e.g., UMS sequence, oligo(dA), and purine/pyrimidine strand bias).

The pTbo-1 Plasmid Is Maintained as a Supercoiled Concatemer in *T. brucei*. Total DNA from *T. brucei* carrying pTbo-1 under drug selection was mixed with a 4.9-kbp supercoiled plasmid standard (pAgglo$^-$) and fractionated on a CsCl/ethidium bromide gradient. Fractions were collected and analyzed by Southern blot with a ^{32}P-labeled probe of either a hygromycin gene fragment for detection of pTbo-1 (Fig. 4*A*) or a chloramphenicol acetyltransferase gene fragment for detection of pAgglo$^-$ (Fig. 4*B*). As evident from Fig. 4*A*, approximately half of the pTbo-1 sequences were located in fractions 17–23 (ρ = 1.62–1.61 g/ml) and migrated with the supercoiled plasmid standard (Fig. 4*B*). A significant portion of the pTbo-1 sequence was located in fractions 41–46 (ρ = 1.58–1.57) and migrated with a trace amount of nicked pAgglo$^-$ standard. This lower density pTbo-1 fraction must either have been nicked during the preparation and purification of the DNA or was not supercoiled when extracted from *T. brucei*.

To confirm that the structure of pTbo-1 in *T. brucei* is a concatemeric circle, EM was performed on DNA that had been enriched from supercoiled DNA fractions of the CsCl/ethidium bromide gradient. As shown in Fig. 5 *A* and *B*, the

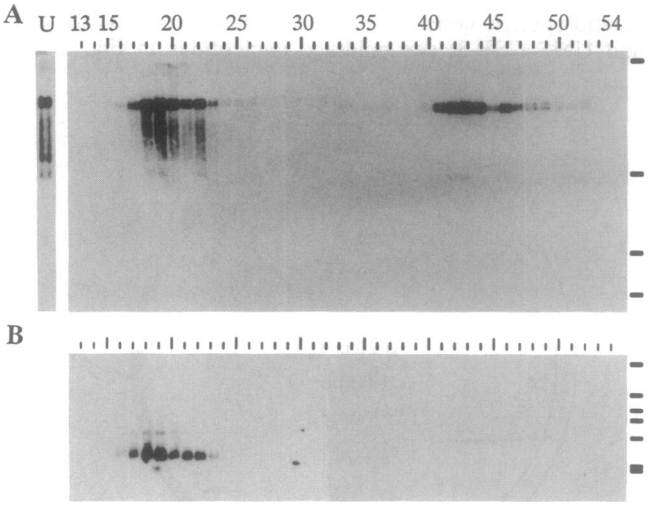

FIG. 4. Detection of supercoiled pTbo-1 isolated from transfected *T. brucei*. Genomic DNA (11 μg) from *T. brucei* stably transfected pTbo-1 was combined with 10 ng of a supercoiled 4.8-kbp plasmid pAgglo$^-$ and fractionated on a CsCl gradient containing ethidium bromide. Of each fraction, 10% was tested by Southern analysis by using a radiolabeled hygromycin gene fragment (for specific detection of pTbo-1 in *A*) or a radiolabeled chloramphenicol acetyltransferase gene fragment (for specific detection of pAgglo$^-$ in *B*). Peak fractions 19 and 43 had densities of 1.616 g/ml and 1.578 g/ml, respectively. The lanes in *A* and *B* are shown aligned by fraction number. Lane U (*A*) was loaded with 66 ng of unfractionated DNA from *T. brucei* stably transfected with pTbo-1. *A* and *B* correspond to separate Southern blots; the gel origin and molecular weight markers, as indicated by the bars to the right, are derived from a *Hin*dIII digest of phage λ (A: 23.1, 9.4, and 6.5 kbp; B: 23.1, 9.4, 6.5, 4.3, 2.3, and 2.0 kbp).

enriched fractions contained large circular DNAs with a contour length 8.1 times (±8% standard deviation) the contour length of nicked pTbo-1 monomers derived from *E. coli* (Fig. 5*C*). The large concatemers appeared relaxed in the electron micrographs, and it is assumed that they were nicked during preparation of the sample for EM. Additional evidence for the proposed concatemeric structure was observed from a partial digestion of the *T. brucei*-derived pTbo-1 plasmid with *Hin*dIII. The resulting ladder contains seven detectable bands (Fig. 5*D*, lane 2), consistent with the interpretation that the concatemeric form of pTbo-1 contains approximately seven to nine monomer units. Given our previous estimate of approximately five monomer units per cell (Fig. 3*A*), it would appear likely that the average number of copies of concatemeric pTbo-1 per cell is close to 1.

In the Absence of Continuous Drug Selection, the Plasmid pTbo-1 Is Lost from the Cell Population. *T. brucei* cells carrying the plasmid pTbo-1 were cultured in the absence of hygromycin selection for periods up to 45 days (90 cell generations) to determine whether the pTbo-1 sequences are stably maintained. Southern analysis of undigested DNAs from cells grown for 0, 17, 30, or 45 days in the absence of selection indicates that the plasmid content decreased significantly in the population during that period (Fig. 6*A*). The hybridization signals were quantified directly by phosphor imaging and are plotted on a semilogarithmic scale (Fig. 6*B*) as a function of days cultured in the absence of selection. Replicate flasks grown without selection gave nearly identical decay curves (filled vs. open squares), from which a loss rate of $t_{1/2}$ = 8.7 days (17 generations) could be calculated. If each *T. brucei* cell contains, on the average, a single copy of the concatemeric pTbo-1, then this rate of loss in the absence of selection is not high, and segregation of replicated copies between the daughter cells at mitosis may be nonrandom.

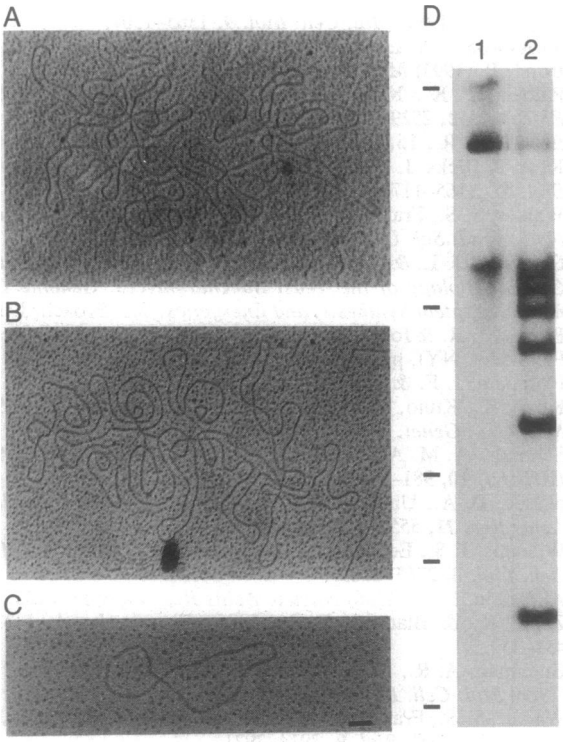

FIG. 5. Concatemeric structure of pTbo-1 isolated from *T. brucei*. (*A–C*) Electron micrographs of pTbo-1 plasmid as a concatemer isolated from *T. brucei* by CsCl/ethidium bromide gradient (*A* and *B*) or as a DNase-nicked monomer isolated from *E. coli* (*C*). Micrographs are shown at identical magnifications. (Bar = 100 nm; see *C*). (*D*) Southern analysis of pTbo-1 concatemers. *T. brucei* genomic DNA (2.5 μg) was loaded on a gel either undigested (lane 1) or partially digested with the enzyme *Hin*dIII (lane 2). The radiolabeled probe is as described in Fig. 3. The bars to the left indicate the position of the gel origin and molecular weight markers from a *Hin*dIII digest of phage λ (23.1, 9.4, 6.5, and 4.3 kbp).

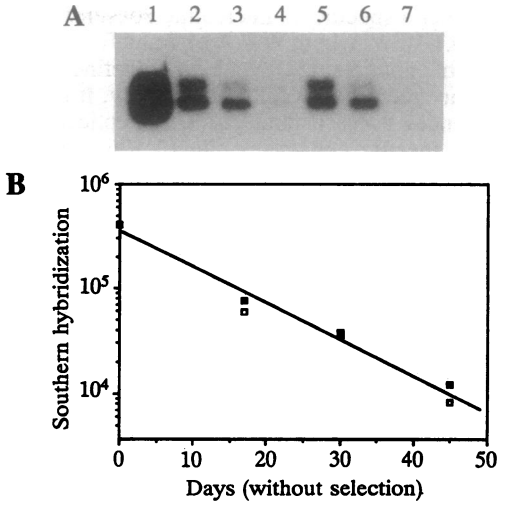

FIG. 6. Loss of pTbo-1 in *T. brucei* grown in the absence of hygromycin selection. (*A*) Undigested genomic DNA (200 ng) from *T. brucei* stably transfected with pTbo-1 was analyzed by Southern blot (an exposure on x-ray film is shown) following growth in the absence of hygromycin selection for 0 days (lane 1), 17 days (lanes 2 and 5), 30 days (lanes 3 and 6), or 45 days (lanes 4 and 7). Samples in lanes 2–4 and 5–7 were taken from independent cultures, respectively. The radiolabeled probe was the same as described in Fig. 3. (*B*) Hybridization of the probe was quantified by phosphor imaging, and plotted as a function of the days of growth in the absence of hygromycin selection. ■, Data from lanes 1–4 of *A*; □, data from lanes 5–7. The best-fit line was determined by linear regression analysis of the logarithm of hybridization signal (arbitrary units), plotted as a function of time.

DISCUSSION

The isolation of an autonomously replicating plasmid, allowing the introduction of multiple copies of a DNA sequence in *T. brucei*, represents a major stride forward in our ability to apply molecular genetic technologies to the study of this important class of protozoan pathogens. The fact that the sequences conferring this capability originated from mitochondrial minicircles is both practically and experimentally significant; it allows us to confer autonomous replication on any plasmid containing trypanosome gene sequences. This work is also relevant to DNA replication because it allows direct comparison between sequences that are replicons in the mitochondrion and nucleus and the interaction of the DNA replication machinery in those compartments.

In this paper, it is shown that kDNA minicircle sequences behave as *ARS*-like elements in *T. brucei* in that they confer stable maintenance under drug selection to recombinant plasmids. Although we think it likely that minicircle sequences act as origins of replication, these experiments do not rule out the possibility that DNA replication initiates elsewhere in the plasmid. The kDNA minicircle elements could engender plasmid stability by enhancing the frequency of replication or causing efficient plasmid segregation at mitosis. In either case, the plasmid must be maintained in the nucleus not only because the cell expresses the hygromycin-resistance gene but also because cell fractionation experiments have indicated no detectable pTbo-1 in the mitochondria (data not shown).

Sequences responsible for autonomous replication in *Saccharomyces cerevisiae* have been studied thoroughly, with

the finding that a specific 11-nucleotide consensus sequence (WTTTATRTTTW, where W = A or T and R = A or G) (21), a replication enhancer (22), and an undefined A+T-rich region (23) are required for full *ARS* activity. It is likely that these sequences serve a dual purpose; replication factors bind to the specific consensus site (24–26), and the free energy of DNA helix unwinding is controlled broadly by the sequence composition of the undefined (nonconsensus) regions (27). Bent DNA is an important element of replication enhancers in yeast (28) and is also seen in other prokaryotic and eukaryotic origins of DNA replication (29, 30). It may be required in replication for the destabilization of the DNA helix or the binding and positioning of specific factors, such as ABF-I and OBF-1 in yeast (31, 32).

The *ARS* elements of yeast and minicircles are similar in that both specific consensus sequences and broad sequence composition could be important in their role as replication origins. Although the mechanism of replication of kDNA minicircles in mitochondria has been partially elucidated, there is little information about how these sequences might function as *ARS* elements in the nucleus of *T. brucei*. In related trypanosomatids such as *Leptomonas* and *Leishmania*, bacterial plasmids are readily maintained in an extrachromosomal state (12, 33, 34). This is either due to the fortuitous inclusion of specific *ARS* elements in those recombinant plasmids or to a lessened requirement for specific *ARS* sequences in those genera. It is interesting to note that minicircles have an *ARS*-like activity in *T. brucei*, as some trypanosomatid minicircles are also capable of serving as *ARS* elements in yeast (35, 36). In yeast, this property is presumably dependent on the bent DNA, A+T-rich segments, and chance occurrence of the 11-nucleotide yeast consensus sequence (present for example in TbmiSM1, but not TbmiB10). Replication of pTbo-1 and pTbmi-b10 in trypanosome nuclei may depend on sequence characteristics that are similar to that of yeast *ARS* elements, with the UMS and the UMS-binding protein (if present) possibly fulfilling a role analogous to the 11-nucleotide yeast consensus sequence and its binding factors. Whether the UMS-binding protein is present in nuclei is uncertain; however, the significant affinity of UMS-binding protein for telomere sequences in trypanosomatids (9) suggests a possible role in both mitochondrion and nucleus.

Krysan *et al.* (37) have shown that in human cells, autonomous replication of a plasmid was inefficient unless approximately six copies of representative human sequence were included. Similarly, the observation that multimers of pTbo-1 and pTbmi-b10 are recovered from *T. brucei* could reflect a requirement for multiple kDNA *ARS* elements for plasmid stability. Alternatively, plasmids may be subject to a size requirement for stable maintenance—a possibility consistent with the large monomeric plasmids and small multimeric plasmids previously reported (19, 20). The intermediate level of pTbo-1 stability in the absence of drug selection could be due to a centromere-like function in the plasmid or a tethering of the plasmid to a nucleoplasmic or chromosomal structure. Lower than expected rates of loss in the absence of drug selection have also been noted in plasmids maintained as monomers (20).

Our observation that minicircle DNAs from *T. brucei* have an *ARS*-like activity in nuclei is an important tool for developing molecular genetic approaches to studying the processes of gene expression and DNA replication. Given that the kDNAs appear to be unique extrachromosomal elements, the generality of their use as *ARS* sequences not only in the Kinetoplastidae but in other protozoan organisms as well should be assessed.

We thank Jay Martin for technical assistance, Drs. Aïda Metzenberg, Robert Metzenberg, Beat Blum, and members of the Agabian laboratory for helpful advice or review of the manuscript. S.M. was supported by a National Research Service Award fellowship (GM13842). This work is supported by grants to N.A. from the John D. and Catherine T. MacArthur Foundation, and National Institutes of Health Grant AI21975. N.A. is a Burroughs Wellcome Scholar of Molecular Parasitology.

1. Agabian, N. (1990) *Cell* **61**, 1157–1160.
2. Simpson, L. (1986) *Int. Rev. Cytol.* **99**, 119–179.
3. Feagin, J. E., Abraham, J. M. & Stuart, K. (1988) *Cell* **53**, 413–422.
4. Blum, B., Bakalara, N. & Simpson, L. (1990) *Cell* **60**, 189–198.
5. Englund, P. T. (1979) *J. Biol. Chem.* **254**, 4895–4900.
6. Ferguson, M., Torri, A. F., Ward, D. C. & Englund, P. T. (1992) *Cell* **70**, 621–629.
7. Robinson, D. R. & Gull, K. (1991) *Nature (London)* **352**, 731–733.
8. Rauch, C. A., Perez-Morga, D., Cozzarelli, N. R. & Englund, P. T. (1993) *EMBO J.* **12**, 403–411.
9. Tzfati, Y., Abeliovich, H., Kapeller, I. & Shlomai, J. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6891–6895.
10. Eid, J. & Sollner-Webb, B. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2118–2121.
11. Cunningham, I. (1977) *J. Protozool.* **24**, 325–329.
12. Kapler, G. M., Coburn, C. M. & Beverley, S. M. (1990) *Mol. Cell. Biol.* **10**, 1084–1094.
13. Milhausen, M., Nelson, R. G., Parsons, M., Newport, G., Stuart, K. & Agabian, N. (1983) *Mol. Biochem. Parasitol.* **9**, 241–254.
14. White, T. C., Fase-Fowler, F., van Luenen, H., Calafat, J. & Borst, P. (1988) *J. Biol. Chem.* **263**, 16977–16983.
15. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
16. Koller, B. & Delius, H. (1984) *Cell* **36**, 613–622.
17. Österlund, M., Luthman, H., Nilsson, S. V. & Magnusson, G. (1982) *Gene* **20**, 121–125.
18. Ray, D. S. (1989) *Mol. Cell. Biol.* **9**, 1365–1367.
19. tenAsbroek, A. L. M. A., Mol, C. A. A. M., Kieft, R. & Borst, P. (1993) *Mol. Biochem. Parasitol.* **59**, 133–142.
20. Patnaik, P. K., Kulkarni, S. K. & Cross, G. A. M. (1993) *EMBO J.* **12**, 2529–2538.
21. Broach, J. R., Li, Y., Feldman, J., Jayaram, M., Nasmyth, K. A. & Hicks, J. B. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **47**, 1165–1173.
22. Walker, S. S., Francesconi, S. C. & Eisenberg, S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4665–4669.
23. Campbell, J. L. & Newlon, C. S. (1991) in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energetics*, eds. Broach, J. R., Pringle, J. R. & Jones, E. W. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 41–146.
24. Hofmann, J. F. & Gasser, S. M. (1991) *Cell* **64**, 951–960.
25. Kuno, K., Kuno, S., Matsushima, K. & Murakami, S. (1991) *Mol. Gen. Genet.* **230**, 45–48.
26. Schmidt, A. M. A., Herterrich, S. U. & Krauss, G. (1991) *EMBO J.* **10**, 981–985.
27. Natale, D. A., Umek, R. M. & Kowalski, D. (1993) *Nucleic Acids Res.* **21**, 555–560.
28. Williams, J. S., Eckdahl, T. T. & Anderson, J. N. (1988) *Mol. Cell. Biol.* **8**, 2763–2769.
29. Anderson, J. N. (1986) *Nucleic Acids Res.* **14**, 8513–8533.
30. Zahn, K. & Blattner, F. R. (1985) *Nature (London)* **317**, 451–453.
31. Buchman, A. R., Kimmerly, W. J., Rine, J. & Kornberg, R. (1988) *Mol. Cell. Biol.* **8**, 210–225.
32. Walker, S. S., Francesconi, S. C., Tye, B.-K. & Eisenberg, S. (1989) *Mol. Cell. Biol.* **9**, 2914–2921.
33. Bellofatto, V. & Cross, G. A. M. (1989) *Science* **244**, 1167–1169.
34. Laban, A., Tobin, J. F., Curotto de Lafaille, M. A. & Wirth, D. F. (1990) *Nature (London)* **343**, 572–574.
35. Davison, J. & Thi, V. H. (1982) *Curr. Genet.* **6**, 19–20.
36. Teixeira, S. M. R., Franscino, A. C. S., Galembeck, E. V., Azevedo, M. D. O. & Filho, S. A. (1986) *Gene* **44**, 171–175.
37. Krysan, P. J., Smith, J. G. & Calos, M. P. (1993) *Mol. Cell. Biol.* **13**, 2688–2696.
38. Sherman, D. R., Janz, L., Hug, M. & Clayton, C. (1991) *EMBO J.* **10**, 3379–3386.