A Telomere-mediated Chromosome Fragmentation Approach to Assess Mitotic Stability and Ploidy Alterations of *Leishmania* Chromosomes*

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We have used a telomere-associated chromosome fragmentation strategy to induce internal chromosome-specific breakage of *Leishmania* chromosomes. The integration of telomeric repeats from the kinetoplastid *Trypanosoma brucei* into defined positions of the *Leishmania* genome by homologous recombination can induce chromosome breakage accompanied by the deletion of the chromosomal part that is distal to the site of the break. The cloned telomeric DNA at the end of the truncated chromosomes is functional and it can seed the formation of new telomeric repeats. We found that genome ploidy is often altered upon telomere-mediated chromosome fragmentation events resulting in large chromosomal deletions. In most cases diploidy is either preserved, or partial trisomic cells are observed, but interestingly we report here the generation of partial haploid mutants in this diploid organism. Partial haploid *Leishmania* mutants should facilitate studies on the function of chromosome-assigned genes. We also present several lines of evidence for the presence of sequences involved in chromosome mitotic stability and segregation during cell cycle in this parasitic protozoan. Telomere-directed chromosome fragmentation studies in *Leishmania* may constitute a useful tool to assay for centromere function.

*Leishmania* is a kinetoplastid protozoan parasite that is endemic in several parts of the world and is responsible for considerable mortality and morbidity. The structure of *Leishmania* chromosomes is comparable to many other protozoa with a central core of single copy or tandemly arrayed genes and a large number of subtelomeric and telomeric repeats at chromosome ends (1–4). The physical map of the *Leishmania major* Friedlin genome has been recently established by cosmid fingerprinting (5) and the smallest *Leishmania* chromosome has been sequenced (4). The sequence of the rest of the parasite genome is progressing steadily with almost half of it present in the data bases. In eukaryotes, the functional elements known to be essential for segregation and transmission of chromosomes during cell division are origins of replication, centromeres, and telomeres. Although the structure-function and organization of the telomeres are relatively well known in *Leishmania* (3, 6) the presence of centromere and origins of replication are not yet established in any protozoan parasite. In Kinetoplastidae, telomeres are composed of tandem repeated copies of a 5'-TTAGGG-3' hexamer organized in arrays of variable length in which the guanine-rich strand runs 5'-3' toward the chromosome end (7–10), hence resembling human telomeres (11). A telomerase activity has been recently described in kinetoplastid parasitic protozoa including *Trypanosoma brucei*, *L. major*, and *Leishmania tarentolae* (12). A sequence that could resemble an autonomous replication origin has been documented in *T. brucei* (13). Artificial minichromosomes lacking centromere-like sequences have been constructed in trypanosomes (14, 15). However, these minichromosomes were unstable and they were rapidly lost when grown in the absence of selective pressure.

Modification of eukaryotic genomes by homologous targeting constitutes an important tool toward understanding genome structure and function. Several approaches including targeted gene disruption in Chinese hamster ovary cells (16) and embryonic stem cells (17), transposon insertions (18), rare cutting restriction endonucleases (19), the Cre recombination system (20), telomere-directed chromosome breakage (21–29), and the construction of yeast (30) and mammalian (31, 32) artificial chromosomes have been used in the last decade to manipulate eukaryotic genomes and assessing function and structure of several chromosomes within these organisms. In *Leishmania*, gene targeting is mediated almost exclusively by homologous recombination and it has been widely used during the last decade to study the function of individual genes (33–38). The use of homologous recombination to generate gross alterations within the *Leishmania* genome has not been explored yet.

We report here a telomere-mediated chromosome fragmentation approach to engineer large chromosomal truncations at defined positions within the *Leishmania* genome by homologous recombination. Our studies show that cloned telomeric and subtelomeric repeats of the kinetoplastid protozoan *T. brucei* are capable of inducing a chromosome-specific breakage and the seeding of new telomeres. Using this strategy we have addressed chromosome mitotic stability during cell division in this kinetoplastid parasitic protozoan and have generated large haploid deletions for facilitating functional studies in this diploid organism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections—** *L. tarentolae* TarII and *Leishmania donovani donovani* MHOM/IN/80/DD8 strains have been described previously (39, 40). Cells were grown in SDM-79 medium supplemented with 10% fetal bovine serum (Multicell, Wisent Inc.) and 5 μg/ml hemin.
Approximately 4 μg of linearized DNA fragments derived from the different targeting constructs were used to transfect Leishmania cells by electroporation as described (41). Transfectants were selected with 40 μg/ml G418 (Geneticin, Life Technologies, Inc.) or 160 μg/ml hygromycin B (CalBiochem) on SDM-agar plates (1%). Colonies resistant to G418 or hygromycin B were isolated after 10–15 days of growth at 29 °C.

**Construction of Chromosome Fragmentation Vectors**—The pTRY-NEO-TEL and pPTR1YNEO-TEL fragmentation vectors (see Fig. 1) were made as follows. The 1.8 kb PstI-EcoRI fragment (TEL) from vector pT4 (7) containing 50 perfect tandem telomeric repeats of the hexanucleotide TTAGGGHII plus a 1.5 kb of subtelomeric repeats of T. brucei was first cloned into the PstI-EcoRI sites of pSP72 (Promega). Then, the NEO gene cloned downstream of a 92-bp synthetic polypyrimidine stretch necessary for the maturation of NEO transcript was extracted as a SmaI-EcoRI vector from pSPYNEO (42) and inserted into the EcoRI site of the vector. Vector pYNEO-TEL was further digested with BglII and ligated either to a 1.2-kb BamHI-BglII fragment carrying the L. donovani trypanothione reductase (TR) gene or to a 400-bp BamHI-BglII fragment containing the second half of the L. tarentolae PTR1 gene to yield pTRYNEO-TEL and pPTR1YNEO-TEL, respectively. The fragmentation vector pTRcNeOa-TEL (Fig. 1) was constructed following these three cloning steps. First, to change the orientation of the (TG), repeats, the 1.5-kb fragment containing the telomeric and two of the 50 repeats of T. brucei was extracted by PstI digestion and re-introduced into the EcoRI site of pSP72. Then the L. donovani TR gene was introduced into the BamHI site of this vector as a BamHI-BglII fragment. Finally, the NEoa expression cassette isolated from vector pSL1180-neOa (43) as a EcoRV-SmaI fragment was inserted into the EcoRI site of the above vector. Maturation of the NEO transcript in this cassette is undertaken by the intergenic region of the α-tubulin gene (αIR or α) (44). To construct the fragmentation vector pPTR1-neOa-TEL (see Fig. 1), we have replaced the TR gene in pTRcNeOa-TEL by the L. tarentolae PTR1 gene delivered from vector pPTR1-neOa-TEL (see Fig. 1), in which the PTR1 coding sequence was made by polymerase chain reaction was cloned into the EcoRI-BglII sites of pSP72. The PTR1 gene in pTRcNeOa-TEL was cloned as a SmaI-SmaI fragment into the XhoI-SmaI sites of the vector filled in with Klenow DNA polymerase I. To construct vector pYHYG-SceI, the 18-bp recognition sequence of the yeast endonuclease I-SceI was first introduced into the KpnI site of pSP72 and then the hygromycin phosphotransferase gene (HYG) derived from pSPHYG (42) was cloned as a BamHI-BglII fragment into the BamHI site of this vector. The I-SceI recognition site was made as described by Tamar et al. (45). Expression of the HYG gene in vector pYHYG-SceI is driven by a synthetic polyuridylic acid stretch of 92 bp including an AG-spliced acceptor site (42). To make vector pTRYHYG-SceI, the BamHI-BglII HYG-SceI expression cassette was introduced into the unique BamHI site of the L. tarentolae PTR1 gene, vector of part p40.8 (41). To target sequence X located at 3.9 kb up-BamHI from this cassette is undertaken by the intergenic region of the PTR1 gene. A PGPA-specific probe was made as described (49). A probe containing some 50 tandem telomeric repeats of the chromosome 5′-CCCTAA-3′ and a 1.5 kb of subtelomeric sequences of T. brucei (TEL) isolated from plasmid pT4 (7) was used in this study. A 500-bp polymerase chain reaction fragment corresponding to the subtelomeric repeat of T. brucei was used as a probe to distinguish between endogenous and cloned telomeric DNA. Probe B562 corresponds to a 6.8-kb BamHI-BamHI fragment derived from cosmids B562 mapped on L. donovani infantum chromosome 5 (45). Probe X corresponds to a 3.9-kb Xhol-ClaI fragment located upstream of the L. donovani TR gene. Probe Y corresponds to a 1.7-kb BamHI-HindIII fragment located at ~10 kb downstream of the L. donovani HYG gene and it is part of vector pMAC40 (39). To evaluate genome ploidy in the different transfectants subjected to chromosome fragmentation events (see Figs. 8 and 9), we have quantified hybridization intensities of different chromosome-specific genes by densitometric analysis of the Southern blots and filters using a PhosphorImager with the ImageQuant 3.1 software. Internal standards corresponding to single copy Leishmania genes were used for this analysis.

**RESULTS**

**Leishmania Chromosome Fragmentation Vectors**—The ability to engineer large chromosomal alterations by gene targeting mediated by homologous recombination in Leishmania cells would be useful for analyzing chromosome function. To generate a defined chromosomal breakage at internal genomic sites, we have therefore constructed a series of chromosome fragmentation vectors that could direct a cloned telomeric DNA to a specific position within the Leishmania genome. All these fragmentation constructs are acenric (centromeres have not been characterized yet in protozoan parasites) and contain cloned telomeric DNA from T. brucei (7), the neomycin phosphotransferase (NEO) marker for selection and a region of homology to allow targeted integration into the parasite genome by homologous recombination. The direction of chromosome breakage is dependent on the orientation of telomeric repeats in the fragmentation vectors, which are cloned in a 5′ to 3′ TTAGGG orientation to serve as a template for the telomerase. The cloned telomeric DNA is part of a 1.8-kb fragment derived from the kinetoplastid protozoan parasite T. brucei that contains 300 bp of the telomeric hexamer repeat (TTAGGG), and 1.5 kb of subtelomeric sequences (7) (Fig. 1). The maturation of the NEO transcript in these vectors is driven either by a synthetic stretch of 90 pyrimidines with an AG-spliced acceptor site (Y) (42) or from the intergenic region of the α-tubulin gene (α) (44) (see Fig. 1). In all cases, the NEO gene is oriented toward the cloned telomeric DNA to be better expressed as already reported for T. brucei artificial minichromosomes (14, 15). We have chosen to target two distinct loci, the trypanothione reductase (TR) gene of L. donovani (36, 50) mapped on chromosome 5 and the L. tarentolae pteridine reductase 1 gene (PTR1), part of the H locus (41, 51) mapped on chromosome 23. The fragmentation vectors were designed in such a way to delete sequences from both parts of the chromosome breakage site depending on the 5′-3′ orientation of the guanine-rich strand with respect to each chromosomal end (see Fig. 1).

**Telomere-directed Chromosome Fragmentation in Leishmania**—We first introduced the cloned telomeric DNA into the L. donovani TR gene on chromosome 5 by transfecting pTRY-NEO-TEL and pTRcNeOa-TEL linear fragmentation vectors (see Fig. 1) to delete either one or the other arm of chromosome 5 flanking the TR target locus. The chromosomal position of the TR gene has been estimated at 120 and 400 kb from the telomeres using an in vitro chromosome fragmentation approach mediated by the endonuclease I-SceI (45). The outcome of a fragmentation event depends upon the orientation of the stretch of targeting DNA along the Leishmania chromosome. If the TR gene is oriented toward the 5′-3′ arm of the chromosome, the fragmentation event using the pTRYNEO-TEL linearized construct (Fig. 1) should produce a 400-kb truncated chromosome hybridizing to the NEO probe (Fig. 2A). If present in the opposite orientation, the same construct should generate a truncated chromosome fragment of 120 kb (Fig. 2A). Hybridization of Leishmania chromosomes resolved by CHEF electrophoresis with a TR probe indicated a specific

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1 The abbreviations used are: kb, kilobase(s); bp, base pair(s).
Telomere-mediated Chromosome Fragmentation in Leishmania

breakage at the level of the TR locus and the generation of a 400-kb truncated chromosome in the transfectants (Fig. 2A). This engineered minichromosome hybridized to the NEO probe (Fig. 2A) strongly suggesting that the TR gene is oriented toward the right telomere of chromosome 5. Only one allele has been targeted upon G418 selection hence explaining the hybridization of the intact 520-kb chromosomal allele with the TR probe in the transfectant, as the Leishmania genome is diploid (Fig. 2A).

Once we determined the orientation of the TR gene on chromosome 5, we made a fragmentation vector to generate a 120-kb truncated chromosome. Upon the homologous integration of pTRαNEO-TEL vector into the TR locus, a specific chromosome breakage has occurred, which resulted in a truncated 120-kb chromosomal fragment hybridizing to the TR and NEO probes (Fig. 2B). Similar experiments were carried out with the second target locus, the PTR1 gene, on chromosome 23 of L. tarentolae. The chromosomal position of PTR1 gene was estimated by the endonuclease I-SceI-mediated chromosome fragmentation approach to be at 140 and 650 kb from the telomeres (45). Homologous integration of the pPTR1αNEO-TEL (Fig. 1) linearized vector into the PTR1 locus resulted in chromosome fragmentation as shown in the filter hybridization of Fig. 3A. The generation of a 650-kb truncated chromosomal fragment hybridizing to PTR1 and NEO probes suggests that the PTR1 gene is oriented toward the long 650-kb arm of chromosome 23 (Fig. 3A). As expected, the integration of the pPTR1βNEO-TEL vector generated a 140-kb fragment hybridizing to the NEO probe (Fig. 3B). Hence, our results indicate that the integration of cloned telomeric DNA from T. brucei into defined internal chromosomal locations within the Leishmania genome has produced specific chromosome breakage and the generation of truncated minichromosomes carrying telomeric repeats at both sides (Figs. 2 and 3). All the fragmentation vectors used for the above studies carry a single region of homology from 0.4 to 1.2 kb and once linearized they generate one double-strand break that could be used for targeting by homologous recombination. Targeting most likely occurred through homologous recombination by a single crossover event at the level of the targeted genomic sequence (Figs. 2 and 3). The T. brucei telomeric DNA did not integrate into pre-existing internal telomeric repeats that could possibly surround the TR and PTR1 loci, as suggested from sequence data (data not shown).

**De Novo Formation of Telomeres in the Truncated Leishmania Chromosomes**—Our results presented in Figs. 2 and 3 show that linear fragmentation vectors carrying cloned telomeres at one end, once integrated by homologous recombination into the targeted loci, can induce defined chromosomal truncations and generate chromosomal fragments with added telomeres at the break site. To determine whether the cloned T. brucei telomeric DNA can seed the formation of a new telomere, the length and heterogeneity of the terminal restriction genomic fragments in the different classes of transfectants (Figs. 2 and 3) were examined. Restriction fragments of known size that harbor either the T. brucei cloned 1.8-kb telomeric and subtelomeric repeats (Fig. 4, A and B) or the telomeric DNA together with the NEO marker and/or the targeted gene (Figs. 4, A-C, and 5) were compared by Southern blot hybridization. In all cases, the length of the telomeric repeats at the end of the truncated chromosomal fragments was higher than the one in the original fragmentation vectors extended from few hundred to more than 3,600 nucleotides depending on the transfectant and on the fragmentation event (Figs. 4 and 5). Indeed, XbaI genomic fragments of 12 kb (Fig. 4A) and 9.5 kb (Fig. 4B), and a PstI 3-kb fragment (Fig. 4C) have hybridized to a NEO probe instead of the expected 10.5-, 8.0-, and 2.5-kb fragments, respectively (lower panel, Fig. 4, A-C). Moreover, Southern blot hybridization of the transfected clones digested with enzymes that delimit the cloned telomeres using a telomere-specific probe indicated fragments which were smaller in length than the average telomere size observed in wild-type cells (Fig. 4, lower panel).
A-C). These results suggest that the cloned telomeric DNA can be used as a substrate for further telomere elongation upon chromosome truncations. Although hybridization of the Clal-digested fragments derived from clones 1 to 3 with a telomere-specific probe has revealed a set of heterogeneously sized fragments (Fig. 4, A and B), hybridization of the XbaI digests of the same clones also harboring the telomeric repeats to the gene-specific TR, PTR1 and NEO probes indicated a relatively discreet pattern, especially for clone 1 (Fig. 4, A and B). Further hybridization of the same blots to a T. brucei subtelomeric subtelomeric repeat probe which can distinguish added telomeres at the end of the truncated chromosomes from the endogenous ones (Fig. 4, A-C) showed that only the bands at the range of 3.8 kb (Fig. 4A) and 3–3.4 kb (Fig. 4B) were recognized (data not shown) hence supporting the results obtained with the gene-specific probes. The remaining small telomere-hybridizing fragments correspond most likely to spontaneously shortened telomeres derived from various chromosomes (see “Discussion”). Similarly, no high variation in the length of the de novo seeded telomeres was observed while analyzing clones derived from the same transfectant (Fig. 5A and data not shown). The lack of highly heterogeneous profile in the length of the de novo seeded telomeres in clones 1 to 4 is discussed below (see “Discussion”).

Telomere size can change depending upon environmental or developmental conditions (for review, see Ref. 52). We have therefore investigated whether the size of the newly seeded telomeres in the transfectants varies throughout the parasite's cell cycle. *L. donovani pTrNEoTEL* cells harboring a truncated 120-kb chromosome (Fig. 2B) were selected at log-, midlog, early stationary, and late stationary phase and their DNA was digested with XbaI and analyzed by Southern hybridization using a NEO probe. The length of the newly seeded telomeres was significantly increased by ~2 kb when parasites were grown at early stationary phase and shortened back to normal size when cells stopped dividing (Fig. 5B). The differences in size observed between the endogenous telomeres and the construct-associated telomeres, the use of the cloned telomeres as a template for telomere elongation as suggested from the systematic increase in the length of telomeric repeats upon genomic integration and chromosome breakage, and the demonstration that the cloned telomeres are dynamic structures presenting an increase or a decrease in length in response to culture conditions support that the latter have seeded the formation of a new telomere at the site of the chromosome break (Figs. 4 and 5).

**Mitotic Stability of Truncated Chromosomes in Leishmania Transfectants upon Telomere-associated Chromosome Fragmentation**—So far, telomere-associated chromosome fragmentation in yeast and in mammalian somatic cells was used mainly for mapping studies and for generating artificial chro-
mosomes to assess centromere function (for reviews, see Refs. 31 and 53). To assess the mitotic stability of Leishmania-truncated chromosomes during cell division, a clone from each individual transfectant (Figs. 2 and 3) was subdivided in two subclones, one maintained with G418 selection and the other grown for several generations in the absence of drug selection. The stability of the truncated chromosomes was addressed by pulsed field electrophoresis and hybridization studies. The integration of the pTRYNEO-TEL fragmentation vector has induced a chromosome breakage at the level of the TR locus resulting in two chromosomal fragments, one of 400 kb containing the cloned telomeres at the left end and the other of 120 kb with no telomeres at the right end (Figs. 2A and 6A). The 120-kb fragment was lost very rapidly as even after the first passage (~8–10 generations) it was not detectable in the cells as indicated by hybridization studies with probe X located upstream of the TR gene (Fig. 6A). This was an expected result as broken chromosomes without telomeres are often very unstable (53, 54). The 400-kb truncated chromosome fragment with functional telomeres at both termini was, as anticipated, maintained in the presence of G418 selection. When cells were grown in the absence of drug pressure for more than 200 passages (at least 1800 generations), the stability of this 400-kb chromosomal fragment was not affected (Fig. 6A). The extremely high fidelity of segregation of this truncated chromosome strongly suggests that sequences required for maintenance and segregation should be present within this part of chromosome 5. To assess whether any chromosomal fragment harboring functional telomeric repeats at both ends can also be stably maintained during the parasite cell division, we have analyzed the mitotic stability of the 120-kb truncated chromosome generated upon integration of vector pTRYNEO-TEL into TR locus. This chromosomal fragment was maintained as far as the G418 selection was present but, in contrast to what seen with the 400-kb fragment (Fig. 6A), it was rapidly lost upon removal of the drug pressure (Fig. 6B). The 400-kb chromosomal fragment without telomeric repeats at one chromosome end, generated following fragmentation in this transfectant, was also lost as confirmed by hybridization with probe B562 (Fig. 6B). These data further support the presence of sequences important for mitotic stability only within the long 400-kb arm of chromosome 5 (Fig. 6A).

We have further extended our studies to other chromosomes and Leishmania species using the same telomere-mediated chromosome fragmentation approach. We generated new truncated chromosomal fragments derived from chromosome 23 of L. tarentolae and tested their mitotic stability in the absence of drug selection. The integration of PTR1aNEO-TEL cassette at the PTR1 locus produced a 650-kb truncated chromosome with the cloned telomeres at the right end, which was maintained in the presence of G418 selection (Figs. 3 and 7A) and an unstable 140-kb fragment missing telomeres at one end that was rapidly lost as shown by hybridization studies using a telomere-specific probe (Fig. 7A). The 650-kb fragment, part of chromosome 23, has been stably inheriting from cell to cell for more than 70 passages in culture without any drug pressure...
This suggests that sequences important for mitotic stability are likely to be present within this 650-kb fragment. The possibility that the remaining 140-kb fragment of chromosome 23 might also contain such sequences was examined by assessing its mitotic stability during a number of cell divisions. However, this fragment was not maintained further in the cell upon the removal of the drug as shown by hybridization studies with the PTR1 probe (Fig. 7B). Moreover, the 140-kb fragment was initially present in multiple copies (5–7) (Fig. 7B and data not shown) that were rapidly lost by culturing the parasite in the absence of drug selection for ~10 passages. Chromosomal instability is often associated, among other things, with gene amplification (55, 56). These results indicate that specific parts of Leishmania-truncated minichromosomes demonstrate a high mitotic stability that ensures their retention and segregation during cell division suggesting the presence of “centromere-like” sequences within these chromosomes. Our results also show that when these “centric” fragments lack telomeric repeats at one of their termini they cannot be healed by the addition of new telomeres as has been seen in other systems (see “Discussion”).

Hybridization of CHEF and Southern blots of selected clones derived from different transfectants subjected to chromosome fragmentation (Figs. 2 and 3) to a number of single copy chromosome-specific probes suggest that the truncated chromosomes with the seeded telomeric DNA are present at one copy per cell with the only exception of the 140-kb truncated fragment of chromosome 23 shown to be present in multiple copies (Fig. 7B and data not shown). The presence of the stably inherited truncated chromosomal fragments at one copy per cell further supports the possibility that they harbor “centromere-like” sequences.

**Effects of a Telomere-mediated Chromosome Fragmentation on Parasite Genome Ploidy: Generation of Partial Leishmania Haploid Mutants**—In other eukaryotic systems, gross genomic alterations upon chromosome fragmentation events often produce aneuploid cells (57). We have tested whether a similar situation is taking place in Leishmania transfectants subjected to a telomere-associated chromosome fragmentation. Two approaches were used to assess ploidy in these transfectants. These include an hybridization with a number of single copy genes flanking the site of chromosome breakage combined to a PhosphorImager analysis and an enzymatic approach based on the genomic integration of the endonuclease I-SceI unique site at regions surrounding chromosome truncations followed by *in vitro* chromosome digestion permitting evaluation of the copy number of chromosomal alleles. These studies were done in transfectants grown in the absence of drug selection for several passages, represented here as revertants (Rev), that have in principle maintained or lost truncated chromosomal fragments depending on the experiment. We have first examined whether chromosome fragmentation in pTRYNEO-TEL transfectant has yielded a partial haploid strain containing only one copy of the 120-kb fragment of chromosome 5 (Fig. 8A, left panel). Comparative hybridization of a *PstI* digest between pTRYNEO-TEL RevA and wild-type using probe X suggested that this sequence is present at one copy in the transfectant (see Fig. 8A, middle panel). By hybridizing the same DNA with a TR probe,
we depicted two fragments of 3.4 and 2.4 kb corresponding to the gene copies present in the intact chromosomal allele and in the targeted allele, respectively (Fig. 8A, middle panel). The hybridization intensities of the various digestes suggest that sequence X in the deleted 120-kb fragment, is present at one copy (Fig. 8A, middle panel). These results were confirmed by PhosphorImager analysis using the PTR1 single copy gene probe as an internal standard (legend of Fig. 8). Integration of the I-SceI recognition sequence into sequence X just upstream of the TR locus and further digestion with the I-SceI endonuclease (Fig. 9A, middle panel) should yield a transfectant haploid for chromosome 23. I-SceI-mediated chromosome fragmentation and hybridization with a PTR1 probe indicated the presence of a second allele for chromosome 23, in addition to the one targeted and digested with I-SceI endonuclease (Fig. 9B, right panel). These data support a chromosome duplication event as seen also in RevB strain (Fig. 8B).

**DISCUSSION**

We have constructed a number of linear fragmentation vectors carrying telomeric DNA at one end and a double-strand break at the region of homology to induce internal chromosome-specific breakage and the deletion of the chromosomal part that is distal to the site of integration. We have shown here that the integration of cloned telomeric DNA from the kinetoplastid *T. brucei* into defined positions of the *Leishmania* genome can induce chromosomal breakage and seed for the formation of a new telomere. We have also presented several lines of evidence for the presence of sequences involved in chromosome mitotic stability and segregation in this parasitic protozoan. Moreover, we have examined the maintenance of ploidy in parasites harboring truncated chromosomes and reported the generation of a partial haploid mutant for a defined chromosomal region. The use of telomere-mediated chromosome fragmentation to generate gross alterations and large deletions within the parasite genome has not yet been explored. Targeted breakage of yeast and human chromosomes mediated by the integration of cloned telomeric DNA has been successfully reported previously (22–25, 27–29, 53). Introduction of a piece of a cloned telomeric DNA into a yeast or human chromosome seeds the formation of a new telomere and causes the chromosome to break (21–23). Gene targeting in *Leishmania* occurs exclusively by homologous recombination (58, 59). Directed targeting of the linear fragmentation vectors, designed for this study, into the *Leishmania* genome has probably occurred by a single-strand repair model involving the formation of one homologous junction at the level of the region of homology with one double-strand break recombinogenic free end and another that involves an illegitimate junction at the level of the cloned telomeric DNA. One-sided invasion mechanisms for homologous recombination have been previously described in other systems (60–62). However, we cannot exclude the possibility of a double reciprocal recombination mechanism involving a concatamer of linear vector molecules.
De Novo Telomere Formation upon Chromosome Fragmentation Events in Leishmania—Work using cloned human telomeric DNA containing 0.5–1 kb of 5'-TTAGGG-3' repeats can seed telomere formation when re-introduced into a variety of mammalian cells, including mouse embryonic stem cells (23, 25, 26). It seems that the mechanism by which correctly oriented (5'-TTAGGG-3')ₙ arrays seed new telomeres involves their recognition and extension by telomerase (63, 64). Telomerase activity has been recently identified in kinetoplastid parasitic protozoa (12). Here we demonstrate that homologous integration of cloned T. brucei telomeric DNA into defined positions of the Leishmania genome could provide a substrate for the elongation reaction, hence seeding the formation of a new telomere. The significant increase in the length of the cloned telomeric DNA, its regulation upon culture conditions, and its capacity of forming functional telomeres that can ensure the stability of the truncated chromosomes (Figs. 6 and 7) support the formation of new telomeres with hexamer repeats added during telomere replication (Figs. 4 and 5). The estimated growth rate of the seeded telomeric DNA on these truncated chromosomes agrees with previous data on the growth of natural chromosomes in T. brucei (7, 15, 65, 66). As much as 3.5 kb was seen to be added to telomeres of T. brucei in a process that appears gradual and continuous and was calculated to result in the addition of 6–10 bp per end per cell division (7, 65, 66). We have obtained similar extension from 0.5 to 3.6 kb upon integration of the cloned telomeric DNA into the Leishmania genome (Figs. 4 and 5 and data not shown). Although the size range of telomeres in most organisms appears fixed, in some organisms it can change depending upon environmental or developmental conditions (65, 67). Our studies suggest that telomerase activity in Leishmania is probably higher at early stationary phase of growth as we have observed a significant increase in the telomere size at this stage of the parasite's cell cycle (Fig. 5B). It is well known in other systems that telomerase activity is regulated by the cell cycle and that telomere elongation appears to start in late S-phase coinciding with the action of telomerase (68–70).

Our studies show a lack of highly heterogeneous profile in telomere length upon telomere seeding (Fig. 4). This could be explained by the gradual shortening of telomeric DNA in clones 1 to 4 due to the high number of cell divisions in these transfectants (more than 100 passages). Indeed, comparative hybridization studies between a transfectant with low number of passages and clone 1 demonstrate a clear shortening of the telomere length by ~1.5 kb in the latter strain (data not shown). It has been reported in Tetrahymena thermophila that in cells subjected to hundreds of cell divisions, less heterogeneity in telomeric DNA length was observed due to gradual shortening of telomeric repeats (67). Shorter telomeres grow faster and can take over in continuous growing cells. Although telomere shortening has been often associated to aging and to limiting cell proliferation, recent data support that other factors with the presence or absence of active telomerase being the
critical one could also be involved (71).

Mitotic Stability of Truncated Leishmania Chromosomes—
For the stable maintenance and segregation of chromosomes during cell cycle, functional elements such as telomeres, centromeres, and origins of replication are essential. In yeast as well in mammalian cells, a chromosome becomes unstable after loss of a telomere (53, 72). Normally, the absence of a centromere causes acentric fragments that are lost immediately during division in a wide variety of organisms (73, 74). Several reports on telomere-directed chromosome breakage to produce functional minichromosomes in yeast (22, 30) and in humans (25, 27–29) also support the above requirements for chromosome stability and none of these studies have detected acentric fragmentation products. Our studies have shown that in Leishmania, chromosomal fragments lacking telomeres at one end were lost very rapidly even when they contained putative sequences ensuring their retention and segregation during cell division (Figs. 6 and 7). An acquired telomere from another chromosome by gene conversion was not seen in our system. Indeed, no telomeric repeats were added de novo by telomerase onto nontelomeric Leishmania chromosomal truncated fragments. This healing process although relatively rare, has been seen in other systems, such as in Tetrahymena and human chromosomes (75–77).

The extremely high stability of the 400- and 650-kb truncated chromosomal fragments, part of chromosomes 5 and 23, respectively, that have been maintained in the absence of selective pressure for more than 1800 and 700 generations strongly suggests the presence of sequences that are involved in chromosome replication, retention, and segregation when the cell divides. All fragmented chromosomal products that lack these “putative functional sequences” were lost in the absence of drug selection, even when flanked by functional telomeres (Figs. 6 and 7). In Trypanosomes, artificial minichromosomes of 10 and 13 kb containing a selectable marker, a number of tandemly linked genes, and telomeric and subtelomeric repeats at both ends have been constructed (14). However, these were only maintained stably in the absence of drug selection for 20 generations. Patnaik and collaborators (15) have also reported the construction of artificial minichromosomes for T. brucei by adding telomere and subtelomeric sequences to pT13-11 plasmid known to contain sequences important for its replication (13). These minichromosomes were also rapidly lost after 7 generations in more than 50% of the transfected population in the absence of selective pressure (15). These previous reports support also the thesis that for a chromosome to be mitotically stable in Leishmania, it should contain additional sequences likely to act as centromeres. In other systems, if the newly seeded chromosomal end is associated with a centromere, then a stably truncated chromosome will be the end result with the displaced acentric fragment being lost from the cell (31). This is indeed what we have observed in the case of the 400- and 650-kb fragments of chromosome 5 and 23, respectively (Figs. 6A and 7A). These truncated centric minichromosomes were stably inherited at one copy in 100% of the cells tested (Figs. 6 and 7, and data not shown). We are

![Diagram](image-url)
currently working on the 400-kb region of *L. donovani* chromosome 5 for identifying the sequences conferring mitotic stability. Our goal is to use artificially induced chromosome truncations as a mean to define a minimal size chromosome that retains the mitotic properties of a normal chromosome. The knowledge gained from such systematic analysis should allow the identification of components important for proper segregation and replication of *Leishmania* chromosomes and the construction of *Leishmania* artificial chromosomes to assay for centromere function as it has been used in other systems (32, 78) and to also study the regulation of gene expression in these organisms.

**Ploidy Alterations in Leishmania Cells Subjected to Targeted Chromosome Fragmentation**—To address ploidy alterations in cells submitted to targeted chromosome fragmentation, the karyotypes of the different transfectants with respect to loss or gain of genetic material distal or proximal to the sites of break were analyzed by Southern blot hybridization using a number of chromosome-specific single copy markers and by CHEF analysis using the I-SceI in vitro-mediated chromosome fragmentation approach. Our results indicated that generally large chromosomal deletions of 400–650 kb or deletion of the whole chromosomal allele were not permissive (Figs. 6B and 9B) probably because some of their gene content is essential for parasite survival and needs to be preserved in two copies. The maintenance of ploidy is essential for the survival of a given species. Mechanisms exist to ensure that during mitosis daughter cells receive identical sets of chromosomes. Mechanisms should also exist to ensure maintenance of ploidy in *Leishmania* after DNA damage, such as, a double-strand break generated upon chromosome fragmentation. In the case where smaller chromosomal deletions of 120–140 kb were generated, two types of response with respect to ploidy maintenance were obtained. To compensate for the loss of the 140-kb region of chromosome 23, the parasite became trisomic with two intact chromosomal copies in addition to the 650-kb truncated fragment (Fig. 9A). The stable maintenance of the 650-kb truncated chromosome despite the fact that the cell is diploid for chromosomal copies in addition to the 650-kb truncated fragment has numerous advantages for studying genome function in this parasite either by simplifying gene knockout strategies for this diploid organism and/or for assessing the function of more than one genes within large chromosomal regions. The mechanism(s) by which the parasite maintains ploidy upon chromosome breakage events has not been inves-
Telomere-mediated Chromosome Fragmentation in Leishmania

FIG. 9. Alterations in the genome ploidy of L. tarentolae transfectants carrying large chromosomal deletions mediated by experimentally induced chromosome fragmentation events. A, RevC is expected to be partial haploid for the 140-kb part of chromosome 23 (left panel) (see also Fig. 7A). Southern blot of L. tarentolae wild-type (lane 1) and RevC (lane 2) genomic DNA digested with XbaI (X-BglII) and hybridized to the PTR1 probe. Southern blot of L. tarentolae wild-type (lane 3) and RevC (lane 4) genomic DNA digested with HindIII and hybridized to PGPA probe (right panel). The copy number of the PGPA (lanes 3/4: 3215/4023) and PTR1 (lanes 1/2: 1412/12854, for the 9.5-kb band) gene probes was also confirmed by PhosphorImager analysis using TR (lanes 3/4: 934/870) as a single copy gene standard. Values between parentheses are those measured by PhosphorImager (see “Experimental Procedures”). B, integration of the HYG-SceI cassette into the PTR1 locus of RevD strain (expected to be haploid for chromosome 22 after the loss of the 650- and 140-kb fragments) (left panel) (see also Fig. 7B). Chromosomes were digested with I-SceI endonuclease, resolved by CHEF electrophoresis, and hybridized to PTR1 and HYG probes (right panel). The RevD transfectant became diploid for chromosome 23.

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