Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 864–868, February 1991
Biochemistry

Homologous recombination in *Leishmania enriettii*

(protozoa/kinetoplastids/transfection/gene expression)

JAMES F. TOBIN, AVRAHAM LABAN, AND DYANN F. WIRTH*

Department of Tropical Public Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115

Communicated by Elkan Blout, October 29, 1990

ABSTRACT **We have used derivatives of the recently developed stable transfection vector pALT-Neo to formally demonstrate that *Leishmania enriettii* contains the enzymatic machinery necessary for homologous recombination. This observation has implications for gene regulation, gene amplification, genetic diversity, and the maintenance of tandemly repeated gene families in the *Leishmania* genome as well as in closely related organisms, including *Trypanosoma brucei*. Two plasmids containing nonoverlapping deletions of the chloramphenicol acetyltransferase (CAT) gene, as well as the neomycin-resistance gene, were cotransfected into *L. enriettii*. Analysis of the DNA from these cells by Southern blotting and plasmid rescue revealed that a full-length or doubly deleted CAT gene could be reconstructed by homologous crossing-over and/or gene conversion between the two deletion plasmids. Additionally, parasites cotransfected with pALT-Neo and pALT-CAT-S, a plasmid containing two copies of the chimeric α-tubulin–CAT gene, resulted in G418-resistant parasites expressing high levels of CAT activity. The structure of the DNA within these cells, as shown by Southern blot analysis and the polymerase chain reaction, is that which would be expected from a homologous exchange event occurring between the two plasmids.**

Homologous recombination is involved in the regulation of gene expression (1), the amplification of genes in response to stimuli (2–4), the generation of multigene families (5), the maintenance of homogeneity within these families (5, 6), and the generation of chromosomal rearrangements involved in genomic evolution (5, 6). Herein we report functional evidence for homologous recombination in the lower eukaryote *Leishmania enriettii*.

In the course of analyzing the structure of the DNA in *L. enriettii* stably transfected with pALT-Neo (7), we found evidence that the DNA existed as an extrachromosomal element made up of six to eight tandemly repeated units of the pALT-Neo plasmid. In addition to the major bands of hybridization corresponding to this structure, there were several bands of lesser intensity detected in the Southern blot (7). Based on the size and hybridization patterns of these bands, we suggested that these fragments would exist if a subset of the tandem repeats had undergone homologous recombination events. Cloning and sequence analyses have subsequently confirmed the identity of these bands (J.F.T. and D.F.W., unpublished results).

In this report, we test this hypothesis and show that homologous recombination occurs in promastigote forms of *L. enriettii*. We use an approach that has been extensively used in mammalian cells (8). We looked for homologous recombination by assaying for the reconstruction of a functional gene from two plasmids containing nonoverlapping mutations. Twenty-five percent of the identified products are recombinants. We also showed the formation of a dimeric plasmid containing neomycin-resistance (neo^r) and chloramphenicol acetyltransferase (CAT) gene sequences after cotransfection with separate plasmids containing the neo^r or CAT genes. We discuss the significance of homologous recombination with respect to the generation of extrachromosomal DNA elements (9) and the generation and maintenance of tandemly repeated genes in the *Leishmania* genome.

MATERIALS AND METHODS

Cells. Promastigote forms of *L. enriettii* were cultured in Schneider's *Drosophila* medium as described (10). Clones were isolated by limiting dilution in 96-well microtiter plates.

Transfections. Promastigotes were transfected by electroporation (7, 11). After selection in G418 (150 μg/ml) for 7–10 days, resistant parasites appeared at a frequency of $\approx 10^{-6}$. Individual cells or mixed populations of cells were isolated after 3 weeks and expanded. Plasmids were linearized by digestion with *Sac* I prior to transfection.

Plasmid Constructions. Plasmid pALT-Neo-CAT-DR (deletion right) and pALT-Neo-CAT-DL (deletion left) were constructed by fusing the respective fragments of the CAT gene downstream of the α-tubulin intergenic sequence in pALT-Neo (Fig. 1 *A* and *B*). Briefly, plasmid pALT-Neo (7) was linearized with *Bam*HI and the restriction site was converted to a blunt end by treatment with the Klenow fragment of DNA polymerase I. To this DNA was ligated the 561-base-pair (bp) blunt-ended *Bam*HI–*Sty* I fragment from plasmid pALT1-1 (11) to produce pALT-Neo-CAT-DR. pALT-Neo-CAT-DL was constructed by ligating the blunt-ended 535-bp *Eco*RI–*Bam*HI fragment from pALT1-1 to the blunt-ended *Bam*HI-digested pALT-Neo. Plasmid pALT-CAT-S containing two copies of CAT gene was constructed in a stepwise manner. Plasmid pALT1-1 was linearized with *Sac* I and the restriction site was converted to a blunt end by treatment with the Klenow fragment of DNA polymerase I. This DNA was digested with *Xho* I, and the chimeric α-tubulin–CAT gene fragment was isolated by agarose gel electrophoresis. The fragment was ligated into the *Xho* I and *Hin*cII sites in the vector pBluescript. The second copy of the chimeric gene was isolated by agarose gel electrophoresis after digesting pALT1-1 with *Pst* I and *Sac* I. This fragment was ligated into the *Pst* I and *Sac* I sites of the plasmid above to form pALT-CAT-S.

Isolation of Recombination Products. Circular DNA was isolated from transfected *L. enriettii* by using the alkali treatment method as described (12) and digested with *Sac* I. This DNA (5 μg) was ligated under dilute conditions (13) to favor intramolecular ligation and introduced into *Escherichia coli* JM109 (14) by electroporation (15). Plasmids were isolated from the resulting ampicillin-resistant colonies and their structures were determined by digestion with *Bst*BI and *Sac* I followed by electrophoresis on a 1.7% agarose gel. The plasmids can be grouped into four main classes. One class represents the input plasmids DL and DR, a second class

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Abbreviations: neo^r, neomycin resistance; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction.

*To whom reprint requests should be addressed.

represents the wild-type full-length CAT plasmid, a third represents the doubly deleted plasmids, and the fourth is the "other" class. The structure of the class described as other is ambiguous due to the presence of *L. enriettii* genomic *Sac* I fragments that were presumably ligated into the plasmids during the cloning procedure.

CAT Assay. CAT was assayed using the diffusion assay as described (11, 16). Lysate (20 μl) was used for each CAT assay.

Polymerase Chain Reaction (PCR). PCR reactions were performed as described (17). The Neo primer corresponds to nucleotides 2485–2512 (top strand) of the neo^r gene in transposon Tn*5* (18) and the CAT primer corresponds to nucleotides 422–442 (bottom strand) of the yeast/*E. coli* chloramphenicol-resistance marker (19). Amplification was performed for 26 cycles (1 min at 94°C; 2 min at 50°C; and 3 min at 72°C) on an MJ Research thermocycler. During the final cycle the time of the elongation step at 72°C was increased to 6 min. The reaction mixture (10 μl) was analyzed by electrophoresis on a 1.7% agarose gel.

Southern Blot Analysis. DNA was isolated from parasites as described (20) and analyzed on a Southern blot. The blots were hybridized with a 360-bp radiolabeled [position 1785–2145 of the neo^r gene in Tn*5* (18)] neo^r-specific fragment of pALT-Neo or the *Bam*HI CAT-specific radiolabeled fragment from pALT1-1.

RESULTS

Functional Evidence for Homologous Recombination. Previous results (7) formed the basis for the hypothesis that homologous recombination was occurring between plasmids that were transfected into *L. enriettii*. To further test this hypothesis, two deletion plasmids were introduced into the parasite and analyzed for recombination. The plasmids are derivatives of the *L. enriettii* stable transfection vector pALT-Neo (7) and are shown in Fig. 1*B*. In addition to the selectable marker neo^r, the plasmids contain two nonoverlapping deletions of the bacterial CAT gene; pALT-Neo-CAT-DR contains a 234-bp deletion of the carboxyl terminus and pALT-Neo-CAT-DL contains a 260-bp deletion of the amino terminus. These plasmids were cotransfected into *L. enriettii* and the resulting G418-resistant cells were assayed for an intact CAT gene.

Fig. 1*C* and Table 1 show the results of the CAT assays and the Southern blots from six transfection experiments. Two types of G418-resistant cell lines were obtained after cotransfection of both deletion plasmids, those expressing high levels of CAT activity (cell lines 4A, 12, and 13) and those expressing low (cell line 11) or undetectable CAT activity (cell line 2A). Control transfections performed with pALT-Neo-CAT-DR or pALT-Neo-CAT-DL individually gave rise to G418-resistant cell lines expressing no detectable CAT activity (sample 10 and data not shown).

DNA was extracted from these cell lines and analyzed for the presence of an intact CAT gene on a Southern blot. All of the cell lines contained either the 646-bp (cell lines 12 and 13) or the 620-bp (cell lines 2A, 4A, 10, and 11) fragment derived from the input plasmids that are most likely present as extrachromosomal tandemly repeated units (7). Cell lines that expressed high levels of CAT activity contain an additional 880-bp fragment corresponding to the full-length CAT gene (Fig. 1*C*; cell lines 4A, 12, and 13). In contrast, cell lines that expressed low or no detectable CAT activity contained an additional 386-bp band corresponding to the CAT double deletion gene (Fig. 1*C*, cell lines 2A and 11). Within the detection limits of the Southern blot, we were unable to obtain a cell line that contained both the 880- and 386-bp fragments. Control transfections performed with either pALT-Neo-CAT-DR or pALT-Neo-CAT-DL individually contained only the 646- and 620-bp fragments (Fig. 1*C*, sample 10, and data not shown). The presence of the two new fragments derived from sequences present in each plasmid,

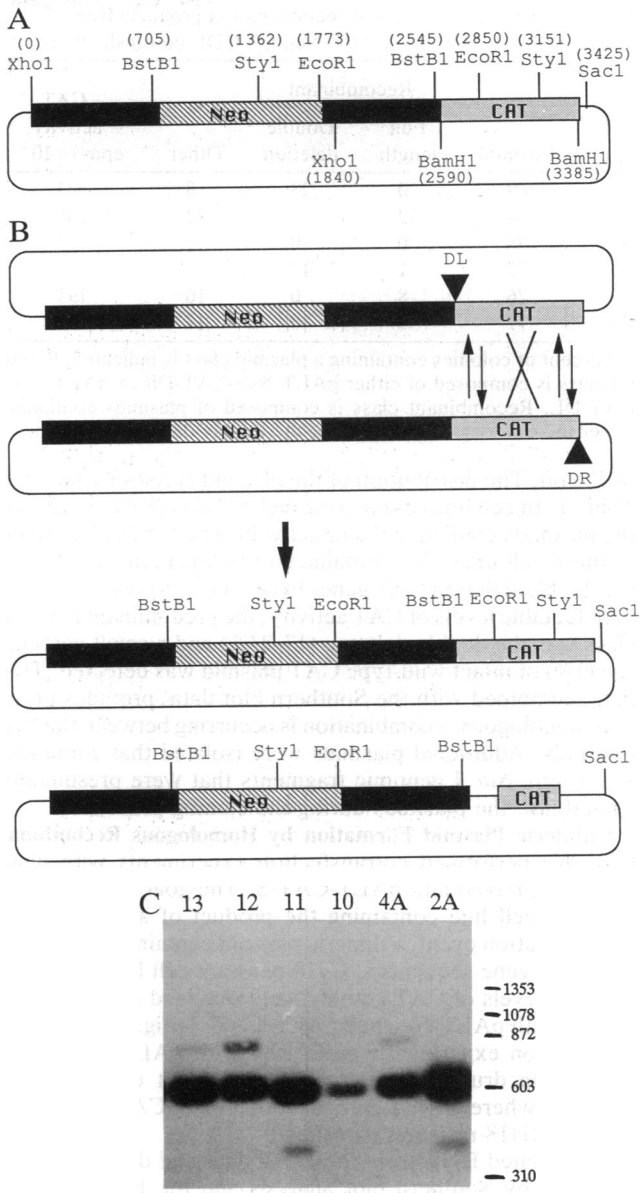

FIG. 1. Functional and structural analysis of homologous recombination. (*A*) Plasmid pALT-Neo-CAT. Numbers in parentheses refer to the position of the given restriction site in base pairs from the *Xho* I site. The pBluescript sequence (thin line) is 2913 bp. (*B*) A wild-type CAT gene and a doubly deleted CAT gene may be reconstructed from plasmids pALT-Neo-CAT-DL and pALT-Neo-CAT-DR by gene conversion in either direction (arrows) or by reciprocal exchange (×). (*C*) DNA was isolated from the same cultures, digested with *Bst*BI and *Sac* I, resolved on a 1.7% agarose gel, and analyzed by probing a Southern blot with the CAT-specific probe. Lanes: 2A, cotransfection with linearized pALT-Neo-CAT-DL and supercoiled pALT-Neo-CAT-DR; 4A, cotransfection with linearized pALT-Neo-CAT-DL and linearized pALT-Neo-CAT-DR; 10, transfection with pALT-Neo-CAT-DL; 11, cotransfection with linearized pALT-Neo-CAT-DL and supercoiled pALT-Neo-CAT-DR; 12, cotransfection with supercoiled pALT-Neo-CAT-DL and linearized pALT-Neo-CAT-DR; 13, cotransfection with linearized pALT-Neo-CAT-DL and linearized pALT-Neo-CAT-DR.

which cannot be obtained from either of the input plasmids alone, is strong evidence for homologous recombination between the two input plasmids.

Isolation of Recombination Products. To confirm the Southern blot data and to search for the presence of both recombination products within a single cell, DNA was isolated from the cells, digested with *Sac* I, and cloned into a recA$^-$ strain

Table 1. Characterization of recombination products from pALT-Neo-CAT-DR and pALT-Neo-CAT-DL cotransfections

Cell line	Parental	Recombinant: Full length	Recombinant: Double deletion	Other	CAT activity, cpm ($\times 10^{-3}$)
2A	69	0	23	8	3
4A	74	12	2	12	166
10	98	0	0	2	3
11	77	1	17	5	5
12	76	8	0	16	195
13	77	9	0	14	172

Percent of colonies containing a plasmid class is indicated. Parental class is composed of either pALT-Neo-CAT-DR or pALT-Neo-CAT-DL. Recombinant class is composed of plasmids containing either the full-length CAT gene or the doubly deleted CAT gene.

of *E. coli*. The distribution of the plasmid classes is shown in Table 1. In cell lines expressing high CAT activity, 8–12% of the plasmids contained the intact wild-type CAT gene. Some of these cell lines also contained a small percentage, 2%, of the double-deletion CAT gene. In cell lines expressing low or undetectable levels of CAT activity, the predominant plasmid class was the double deletion (17–23%) and a small percentage (1%) of intact wild-type CAT plasmid was detected. This data, combined with the Southern blot data, provides proof that homologous recombination is occurring between the two plasmids. Additional plasmids were isolated that contained *L. enriettii Sac* I genomic fragments that were presumably ligated into the plasmids during the cloning procedure.

Chimeric Plasmid Formation by Homologous Recombination. We performed cotransfection experiments with plasmids pALT-Neo and pALT-CAT-S. The goal was to try to obtain a cell line containing the product of a homologous recombination event, a dimeric plasmid containing both neo^r and CAT gene sequences. G418-resistant cell lines expressing high levels of CAT activity were obtained after cotransfection with pALT-Neo and pALT-CAT-S (Fig. 2*A*). Control transfection experiments performed with pALT-Neo alone resulted in drug-resistant cells that did not express CAT activity, whereas transfection with pALT-CAT-S did not result in G418-resistant parasites.

We isolated DNA from these cell lines and determined its structure by Southern blot analysis and the PCR. Fig. 2*B* shows a representative Southern blot of DNA extracted from cell lines cotransfected with linearized pALT-Neo and supercoiled pALT-CAT-S. Digestion of the DNA with *Eco*RI yields four bands, of 5290, 4600, 1060, and 540 bp when probed with a CAT-specific radiolabeled probe (lane 1) and a single 5290-bp fragment when probed with a neo^r-specific probe (lane 7). Digestion with *Bam*HI results in a 800-bp CAT-hybridizing fragment (lane 2) and a 5550-bp neo^r-hybridizing fragment (lane 8). Digestion with *Sty* I yields four bands that hybridize with CAT (lane 3), fragments of 4600 and 4550 bp that cannot be separated from one another under the conditions of this experiment but can be seen by pulse-field gel analysis (data not shown), a 1750-bp fragment, and a 1600-bp fragment. When hybridized with the neo^r probe a major band of 4600-bp fragment and a minor band of 5550 bp are detected (lane 9). This data is consistent with the structure shown in the model in Fig. 3*A*. The hallmark feature of the structure is the junction formed between the neo^r and CAT gene, typified by the 1750-bp CAT-hybridizing *Sty* I fragment. This band is not detected in the Southern blot probed with neo^r since the neo^r probe does not hybridize with this portion of the neo^r gene. We confirmed the existence of this junction by amplifying it using the PCR with an oligonucleotide primer that hybridized to the 3′ end of the neo^r gene and a second primer that hybridized to the 5′ end of the

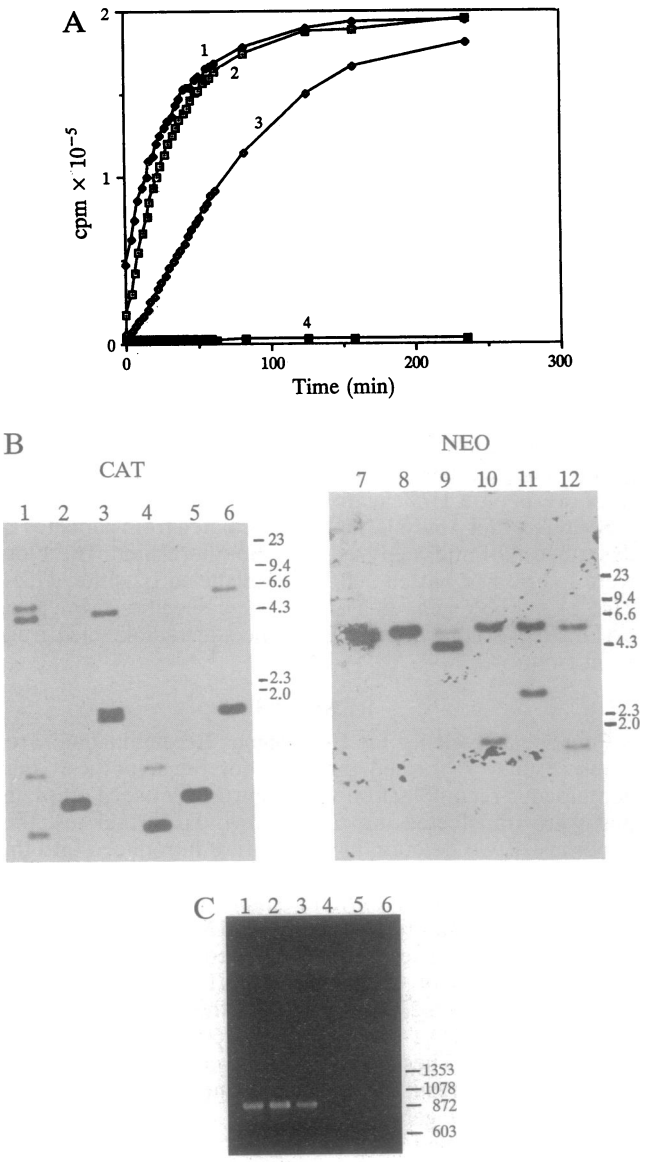

FIG. 2. Detection of recombination products. (*A*) CAT assays were performed with 1.0 μl of purified CAT enzyme (0.25 unit/μl) or with 20 μl of cell extract. Cell line 8 (curve 1) was cotransfected with linearized pALT-Neo and supercoiled pALT-CAT-S, cell line 9 (curve 2) was cotransfected with supercoiled pALT-Neo and linearized pALT-CAT-S, and cell line pALT-Neo (curve 3) was transfected with pALT-Neo. Curve 4 is 0.25 unit of CAT. (*B*) DNA was isolated from cell lines 8 (lanes 1–3 and 7–9) and 9 (lanes 4–6 and 10–12), digested with *Eco*RI (lanes 1, 4, 7, and 10), *Bam*HI (lanes 2, 5, 8, and 11), or *Sty* I (lanes 3, 6, 9, and 12), resolved on a 0.8% agarose gel, and analyzed on a Southern blot with a CAT-specific probe (CAT) or a neo^r-specific probe (NEO). (*C*) A PCR of DNA isolated from cell lines 8 and 9 and clones derived each cell line. Lanes: 1, cell line 8; 2, clone 8B8; 3, clone 8C7; 4, cell line 9; 5, clone 9B8; 6, clone 9C8.

CAT gene. Fig. 2*C*, lanes 1–3, shows that a 913-bp fragment that hybridizes with the α-tubulin intergenic sequence (data not shown) is amplified from these cell lines. The 5550-bp minor fragment detected by the neo^r probe in the *Sty* I digestion most likely represents pALT-Neo DNA that has not recombined with pALT-CAT-S.

Representative Southern blots of DNA from cells cotransfected with supercoiled pALT-Neo and linearized pALT-CAT-S are also shown in Fig. 2*B*. Digestion with *Eco*RI yields three fragments of 4810, 1060, and 540 bp when hybridized with the CAT probe (lane 4) and two fragments, 5550 and 1800 bp, when hybridized with the neo^r probe (lane

10). Digestion of the DNA with *Bam*HI gives rise to a 800-base fragment hybridizing with the CAT probe (lane 5), and fragments of 5550 bp and 2600 bp when hybridized with the neo^r probe (lane 11). *Sty* I digestion results in a 5500-bp fragment and 1650/1600-bp fragments when hybridized with the CAT probe (lane 6), and 5500- and 1650-bp fragments when hybridized with the neo^r probe (lane 12). This data is consistent with the structure shown in the model in Fig. 3*B*. The hallmark feature of this structure is generation of a CAT–neo^r junction with the CAT genes located upstream of the neo^r gene. This structure can be easily detected since the insertion of the CAT gene upstream of the neo^r gene will generate a new 2600-bp *Bam*HI fragment and a new 1800-bp *Eco*RI fragment that hybridize with the neo^r probe. The difference between this structure and the one described above (Fig. 3*A*) is that the CAT genes are located upstream of the neo^r gene. Therefore, the PCR with the two primers described above will not give rise to an amplified product as shown in Fig. 2*C* (lanes 4–6).

DISCUSSION

Our cotransfection studies with plasmids containing nonoverlapping deletions in the CAT gene have provided evidence that homologous recombination occurs in *L. enriettii*. After cotransfecting parasites with pALT-Neo-CAT-DL and pALT-Neo-CAT-DR, we obtained cells that expressed either high levels of CAT activity or no CAT activity. Southern blot analysis demonstrated that cells expressing high levels of CAT activity had reconstructed the full-length CAT gene, whereas cells expressing no CAT activity contained the doubly deleted CAT gene. These results were confirmed by isolating plasmids from each cell line and determining their structures by restriction endonuclease mapping. In cell lines expressing high levels of CAT activity a large percentage of the plasmids contained the full-length wild-type CAT gene and a small percentage contained the doubly deleted CAT gene. In cells expressing low or undetectable CAT activity the converse was true.

Two types of homologous recombination mechanisms could explain the results presented here. The recombination event could be a simple reciprocal crossing-over within the 301-bp region lying between the two deletions. This would produce a dimeric plasmid containing two copies of the CAT gene, the full-length wild-type gene and the double deletion. It is also likely that a nonreciprocal exchange (gene conversion) could have occurred in which one of the deletion plasmids donates sequence information to the other. The products of a gene conversion mechanism are two plasmids, one containing either the wild-type full-length CAT gene or the double deletion (depending on the direction of the conversion event) and a second plasmid containing the single deletion. We favor the latter interpretation since we do not detect the double deletion and the full-length CAT gene in the same copy number within a cell line. These experiments do not, however, eliminate the possibility that the mechanism of recombination is reciprocal crossing-over followed by selective loss of one of the products.

The cotransfections with pALT-Neo and pALT-CAT-S provide evidence for chimeric plasmid formation by homologous recombination resulting in a multimeric plasmid con-

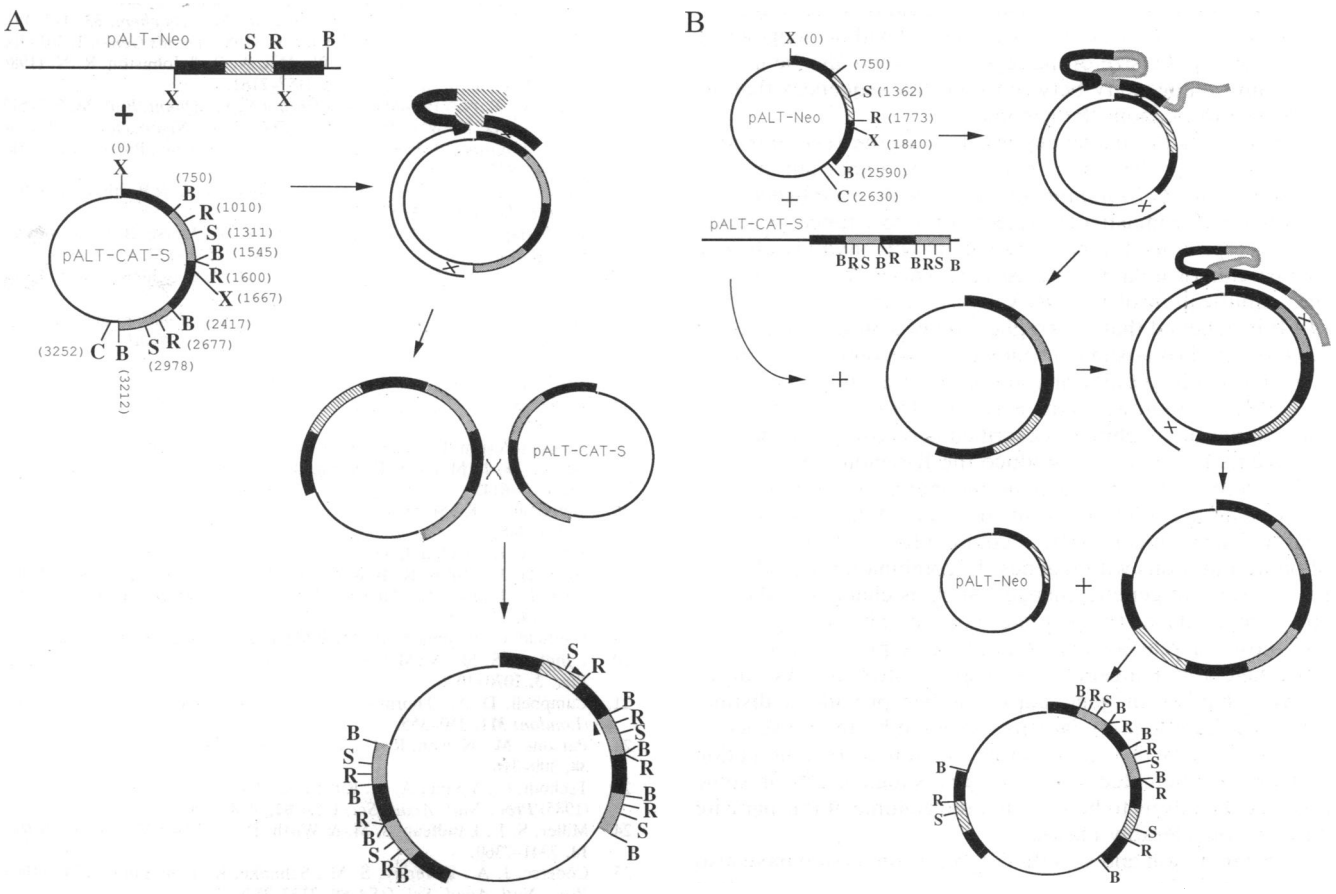

FIG. 3. Models for recombination between pALT-Neo and pALT-CAT-S. Mechanisms for generating the multimeric plasmids obtained after cotransfection of *L. enriettii* with linearized pALT-Neo and supercoiled pALT-CAT-S (*A*) or with supercoiled pALT-Neo and linearized pALT-CAT-S (*B*). Thin line, pBluescript sequence (2913 bp); solid thick sections, α-tubulin intergenic sequence; hatched sections, neo^r sequence; cross-hatched sections, CAT sequences. R, *Eco*RI; S, *Sty* I; B, *Bam*HI; X, *Xho* I; C, *Sac* I. Arrowheads, locations of primers used for the PCR. Numbers in parentheses refer to the position of the given restriction site in base pairs from the *Xho* I site.

taining neo^r and CAT gene sequences. Fig. 3 shows a likely mechanism involving multiple recombination events that could generate these multimers. When cells are cotransfected with linearized pALT-Neo and supercoiled pALT-CAT (Fig. 3*A*), a homologous exchange produces the dimeric plasmid containing the neo^r sequence upstream of the CAT genes in pALT-CAT-S. A subsequent exchange may occur between homologous sequences present in the newly formed dimeric plasmid and in plasmid pALT-CAT-S to produce the final product. We cannot distinguish this product from the coexistence of the dimeric plasmid and pALT-CAT-S. However, there is no selectable marker on pALT-CAT-S and it may not be stable as a separate plasmid. We do not know how many copies of the repeated unit each multimer contains. The final DNA structure in cells cotransfected with linearized pALT-CAT-S and supercoiled pALT-Neo could be generated by a similar mechanism (Fig. 3*B*).

Multiple tandemly repeated genes are a common element of gene structure in *Leishmania* sp. and in other kinetoplastids. Most of the genes that have been characterized in these organisms are present as tandem repeats (20–24). Homologous recombination may have played a role in the generation of these multigene families. These families may have resulted from the overreplication of a primordial gene followed by recombination to generate the tandem repeats. Recombination mechanisms may also be involved in maintaining the integrity and copy number of tandemly arranged repeated genes. Smith (5) has argued that multiple cycles of unequal crossing-over could maintain the sequence homogeneity of such families. In addition, gene conversion between repeated genes could also act as a correction mechanism (6). In contrast to crossing-over, a gene conversion mechanism could be used to remove sequence diversity where it is detrimental without lengthening or shortening the size of the repeats. It could also be used to maintain homogeneity between repeated sequences that are dispersed throughout the genome.

The enzymatic machinery involved in the recombination processes described here may also be important in drug resistance in *Leishmania* sp. It is well established that *Leishmania* promastigotes selected for resistance to antifolates overproduce the bifunctional enzyme thymidylate synthetase-dihydrofolate reductase (25). This is accomplished by the amplification of the DNA sequence encoding this gene (9). It is believed that overreplication of a 30-kilobase region present on chromosome 4 followed by recombination of the free ends leads to the formation of the circular extrachromosomal element known as the R-region DNA (26). Perhaps the recombination machinery described here also participates in the events necessary to produce the R-region DNA.

The demonstration of genetic recombination in *L. enriettii* has potential evolutionary significance. A large amount of genetic variation cannot be accounted for by the occurrence of point mutations within genes. Recombination would allow for a variety of genetic changes, such as changes in the size and complexity of the genome, copy number changes, and chromosomal rearrangements, that could provide a selective advantage for a digenetic parasitic protozoan. As shown above, changes in gene copy number provide a distinct selective advantage to parasites stressed by the presence of antifolate drugs. Thus, the large amount of recombination that we have detected in *L. enriettii* (as high as 25% in some instances) leads us to believe that the genome of this parasite may be considerably plastic.

Recombination and specifically gene conversion have also been implicated in programmed gene rearrangements that give a selective survival advantage to *Trypanosoma brucei*, a close relative of *L. enriettii* (1). This parasite evades the host immune defense mechanisms by continually changing its surface coat. The surface coat is mainly composed of a single protein, the variant-specific surface glycoprotein (VSG) (27). It is believed that new VSG genes are activated by the transposition of a VSG basic copy into a telomeric expression site, where it can be actively transcribed. Indirect evidence supports the hypothesis that the transposition occurs by a unidirectional gene conversion event with the gene at the expression site being replaced by the silent copy (28).

The identification of homologous recombination also raises the possibility of gene targeting in *Leishmania*. This technology has been extensively exploited in yeast and mammalian cells to systematically alter their genomes to study the function of a particular gene (29). We note that due to the tandemly repeated nature of the *Leishmania* genome, gene disruption may be limited to the few known single-copy genes in this organism. However, the tandemly repeated nature of the genome may be an asset in that the number of target sites for homologous recombination is increased.

In summary we have shown that somatic homologous recombination occurs in promastigote forms of *L. enriettii*. We suggest that the enzymatic machinery mediating these exchange events might play an intimate role in gene regulation, gene amplification, genetic diversity, and the maintenance of tandemly repeated gene families. These processes may provide the parasite with a selective survival advantage during its complex life cycle.

We thank Maria Lafaille for her comments. This work was supported by grants from the John D. and Catherine T. MacArthur Foundation and the National Institutes of Health (AI-21365) to D.F.W. D.F.W. is a Burroughs Wellcome Scholar in Molecular Parasitology. J.F.T. was supported by National Research Service Award.

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