Stable Transfection of the Human Parasite Leishmania major Delineates a 30-Kilobase Region Sufficient for Extrachromosomal Replication and Expression

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To delineate segments of the genome of the human protozoan parasite Leishmania major necessary for replication and expression, we developed a vector (pR-NEO) which can be reproducibly introduced into L. major. This DNA was derived from a 30-kilobase extrachromosomal amplified DNA bearing the dihydrofolate reductase-thymidylate synthase gene, with the coding region for neomycin phosphotransferase substituted for that of dihydrofolate reductase-thymidylate synthase and a bacterial origin of replication and selectable marker added. G418-resistant lines were obtained at high efficiency by electroporation of pR-NEO (approaching \(10^{-4}\) per cell), while constructs bearing an inverted neo gene or lacking Leishmania sequences did not confer resistance. pR-NEO replicated in L. major and gave rise to correctly processed transcripts bearing the trans-spliced minixenon. Molecular karyotype analysis showed that in some lines pR-NEO DNA exists exclusively as an extrachromosomal circle, a finding supported by the rescue of intact pR-NEO after transformation of Escherichia coli. These data genetically localize all elements required in cis for DNA replication, transcription, and trans splicing to the Leishmania DNA contained within pR-NEO DNA and signal the advent of stable transfection methodology for addressing molecular phenomena in trypanosomatid parasites.

Trypanosomatid parasites are an important group of human pathogens that employ diverse mechanisms for survival and evasion of host defenses, thereby causing several severe diseases. These protozoans exhibit numerous unusual molecular and biochemical properties, including obligatory trans splicing of a 39-base 5′-leader sequence onto protein-coding RNAs (11), antigenic variation mediated by gene rearrangement (11, 18), RNA editing of mitochondrial transcripts (38), polycistronic transcription and pre-mRNAs (24, 33), and contain unique organelles such as the kinitoplast (37) and the glycosome (34). These novel features have attracted the interests of scientists from many diverse fields and have frequently served as paradigms for the study of higher eucaryotes. Unfortunately, attempts to dissect both the biological and molecular properties of trypanosomatid protozoa have been hindered because neither traditional genetic analysis nor DNA transfection-based methods have been routinely available (11, 15, 18).

One genetic approach that has been successfully utilized is the selection and analysis of mutants, especially in the human parasitic genus Leishmania. These organisms can employ many diverse mechanisms of resistance when exposed to drugs such as the antifolate methotrexate (MTX), including specific gene amplifications (8, 16, 19; unpublished data). In most instances, the amplified DNAs are extrachromosomal and circular and are present in up to 100 copies per cell (6, 8, 17, 20, 35a). These DNAs can be maintained indefinitely in the presence of drug pressure and for varying amounts of time in the absence of selection.

Because the circular amplified DNAs are maintained at high copy number and are associated with overexpression of encoded RNAs and proteins, we and others have proposed that the circular DNAs contain all elements required in cis for DNA replication and expression (6, 8, 16, 17, 27). However, it is possible that the amplified DNAs are transcriptionally silent but in some manner potentiate the expression of the chromosomal locus, perhaps by titration of trans-acting factors. A similar effect in trans has been observed in studies of the HSP70 locus in cultured mammalian cells (25). Moreover, owing to the wide variety of unique mechanisms often employed by protozoans in storing and expressing genetic information, nonconventional possibilities exist for the generation and maintenance of amplified DNAs in Leishmania major. For example, the amplified DNAs could be continually generated from a modified chromosomal locus. Examples of genetic systems involving programmed gene amplification include the formation of the macronucleus in ciliated protozoa (10, 12), ribosomal gene amplification in amphibians (30), or chorion gene amplification in Drosophila melanogaster (26, 39). Thus, the functionality of the amplified Leishmania DNAs was an open question.

To address these issues and other questions concerning trypanosomatid genetics, we have developed methods for stable DNA transfection. We were encouraged by recent reports indicating that the related species Leptomonas sp. and Leishmania enrietti were capable of transiently taking up and expressing exogenous DNA constructs containing genomic sequences fused to the coding region of the chloramphenicol acetyltransferase gene (3, 29). We used the amplified dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene contained within the extrachromosomal circular amplified R region (6) as the starting point for the construction of a DNA transfection vector, since a great deal is known about the protein-coding and transcriptional properties of this amplified DNA (7, 27; T. E. Ellenberger, Ph.D. thesis, Harvard University, Cambridge, Mass. 1989). How-

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ever, our previous studies have shown that transcriptional organization of the amplified R region is complex, exhibiting at least 10 RNAs including overlapping and antisense RNAs (27). Moreover, the locations of cis-acting elements required for transcription, processing, trans splicing, or replication have not been genetically defined for this or any other trypanosomatid gene. Accordingly, we sought to modify the amplified DNA minimally, assuming that these elements were indeed contained within the amplified R region. In this communication, we report the successful introduction of a modified amplified DNA construct into Leishmania major and demonstrate its ability to function autonomously within the parasite.

MATERIALS AND METHODS

Construction of pR-NEO. (i) Insertion of a SpeI site upstream of DHFR-TS. The 2-kilobase (kb) EcoRI-EcoRV fragment from pLTS-S45 (7) containing the 1,560-base-pair (bp) protein-coding region of the DHFR-TS gene of L. major was excised and inserted into the EcoRI-EcoRV-digested vector pC19H (31) and designated pRrv20. The 2-kb EcoRI-HindIII fragment from this construct was then inserted into the EcoRI-HindIII sites of M13mp19. Using the 32-mer (5') CGGGTCCGACACTAGTCGAGATGTCCAGGG, an SpeI site was introduced 5 bp upstream of the initiation ATG codon of the DHFR-TS gene by the method of Taylor et al. (40), yielding the construct B1-Spe-9. Sequencing confirmed the expected sequence from the EcoRI to SpeI sites (see Fig. 7C).

(ii) Construction of the pR vector backbone. Plasmid pK300 containing the entire amplified R region (4) was digested with EcoRV, ligated to the oligonucleotide CACTAGT, cleaved with SpeI, and recircularized, thus introducing an SpeI site at position 1737. This plasmid (pK300-Spe) was digested with EcoRI in the presence of 100 μg of ethidium bromide per ml to obtain a population of singly-cut molecules, the ethidium bromide was removed, and the DNA was digested to completion with SpeI. Contour-clamped homogeneous electric field (CHEF) gel electrophoresis (2-s pulse time, 6 V/cm) was used preparatively to isolate the 30.6-kb SpeI-EcoRI fragment, which was then ligated to the 0.2-kb EcoRI-SpeI fragment from B1-Spe-9 and transformed into Escherichia coli. The desired recombinant was designated pK300-Spe-ΔDHFR. The pUC fragment of this plasmid was moved from the KpnI site to a BglII site, cleaved with SpeI, and recircularized, thus introducing an SpeI site was introduced 5 bp upstream of the SpeI site.

(iii) Construction of pR-NEO and pR-OEN. SpeI sites were added to the 0.9-kb MluI-BamHI fragment from pMClneo-POLYA containing the neo gene (Stratagene, La Jolla, Calif.), and this fragment was inserted into the Bluescript vector SK− (Stratagene), yielding the plasmids pSpe-NEOA and pSpe-NEOB (the neomycin phosphotransferase [NPT]-coding region is inserted in the same orientation from the lac promoter in pSpe-NEOA and in the opposite orientation in pSpe-NEOB). The 0.9-kb neo resistance cassette was isolated after digestion with SpeI and ligated with pR DNA which had previously been digested with SpeI and treated with phosphatase. The orientation of presumptive recombinants was confirmed by restriction mapping and DNA sequencing. pR-NEO carries the NPT-coding region in the same orientation as DHFR-TS, while pR-OEN carries it in the opposite orientation (Fig. 1B).

Cells. Leishmania is a digenetic trypanosomatid genus that resides as the spherical amastigote form within the phagolysosome of vertebrate macrophages and is transmitted as the flagellated extracellular promastigote form by phlebotomine sand fly vectors (44). All cells utilized were promastigotes derived from the LT252 line of L. major (9). Cells were grown in M199 medium supplemented with 10% fetal bovine serum, 100 μM adenine, 10 μM of heme per ml, and 40 μM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.4), 50 units of penicillin per ml, and 50 μg of streptomycin per ml. Clonal derivatives of this line and transfected lines were obtained by plating on M199 medium—1% agar plates containing 0.6 μg of bipotirin (Calbiochem-Behring, La Jolla, Calif.) per ml and 16 μg of G418 per ml as indicated. Filter lifts of Leishmania colonies were made by using a protocol developed by B. Sina in this laboratory (unpublished data). Disks of nitrocellulose or Gene-Screen Plus membranes were pressed onto a plate containing colonies; the filters were carefully peeled away and laid on filter paper soaked in 0.5 M NaOH—1.5 M NaCl for 10 min and then on filter paper soaked in 0.5 M Tris—1.5 M NaCl (pH 7.4) for 10 min, and then on filter paper soaked in 2× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaPO4, and 1 mM EDTA; pH 7.0). The DNA was then fixed to the filter by baking in vacuo or by UV cross-linking and subjected to hybridization analysis by standard methods.

Electroporation. Cells were grown to the late log phase (0.5×107 to 1.0×107/ml), collected by centrifugation, and suspended at a density of 1×109/ml in HEPES-buffered saline (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH2PO4, 6 mM glucose, pH 7.4 [36]) in an electroporation cuvette (Bio-Rad Laboratories, Richmond, Calif.) and incubated on ice for 10 min; 0.4 to 0.8 ml of cells was used. Chilled DNA (1 mg/ml in 10 mM Tris—1 mM EDTA, pH 7.4) was added, and then electroporation was immediately performed with a Bio-Rad Gene Pulser apparatus, using capacitances and voltages as indicated below. After electroporation, cells were incubated for 10 min on ice and transferred to 10 ml of drug-free medium. Selection in G418 (geneticin; Gibco Laboratories, Grand Island, N.Y.) was performed as described in the text.

Molecular karyotype analysis. Chromosomal DNAs were prepared in low-melting-temperature agarose plugs, and chromosomes were separated by CHEF gel electrophoresis (14) as described previously (4, 5). Gamma-irradiation analysis was performed with a 60Co source as described previously (5, 35).

RNA isolation and S1 nuclease and primer extension analysis. Total cellular RNA was prepared by lysing parasites in 5 M guanidinium isothiocyanate followed by precipitation of RNA with 4 M lithium chloride (13). Poly(A) RNA was prepared by chromatography on oligo(dT)-cellulose (1). Northern (RNA) blotting was performed as described previously (27). Double-stranded DNA probes were radioactively labeled with [32P]dCTP by random priming (20).

S1 nuclease mapping was performed as previously described (28) with 2 μg of total cellular poly(A) RNA and the 597-bp NarI-XhoI DNA restriction fragment shown in Fig. 7B and labeled at the NarI 5' end with T4 polynucleotide
kinase and [γ-32P]ATP. Protected fragments were separated on 4% acrylamide–8 M urea gels. Primer extension was performed with 2 μg of poly(A)+ RNA and annealed with 5 pmol of oligonucleotide primer (labeled at the 5' end with T4 polynucleotide kinase) for 3 h at 55°C in 20 μl of 80 mM KCl–80 mM Tris (pH 8.3). The volume was adjusted to 50 μl, final concentrations of 50 mM Tris (pH 8.3), 50 mM KCl, 6 mM MgCl₂, 10 mM dithiothreitol, 500 μM each dATP, dGTP, dCTP, and dTTP, 80 μg of actinomycin D per ml, and 16 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) were added, and the mixture was incubated for 1 h at 42°C. The reaction mixture was extracted once with a 1:1 phenol/chloroform mixture and precipitated with ethanol. The precipitate was suspended in 6 μl of 10 mM Tris (pH 7.4)–10 mM EDTA, incubated with 50 μg of RNase A per ml for 10 min at 67°C, and subjected to electrophoresis as for S1 analysis. The oligonucleotide primer GGGATTCGGATC/CATCAGAGCAAGCCGATGTCTG contained a 13-nucleotide 5' extension encoding EcoRI and BamHI restriction sites and 22 nucleotides of NPT-coding sequence (nucleotide positions 108 to 87; the 5' end of this oligonucleotide corresponds to position +121 of pR-NEO).

PCR amplification. Primer extension was performed as described above, except that the oligonucleotide used was not radioabeled. cDNA from 0.2 μg of poly(A)+ RNA was subjected to polymerase chain reaction (PCR) amplification with the Neo oligonucleotide described above and the oligonucleotide GGAATTCGGATC/AACGCATATAAGTTACTAG. The reaction product was used as a template for 14-nucleotide extension with EcoRI and BamHI sites and nucleotides 5 to 23 of the L. major minixion (J. Miller, submitted for publication). Amplification was performed with 2 U of Taq polymerase (Perkin-Elmer-Cetus, Norwalk, Conn.) in a 40-cycle reaction consisting of 39 cycles of 2 min at 55°C, 2 min at 72°C, and 1 min at 94°C, followed by 1 cycle of 2 min at 55°C and 7 min at 72°C in an Eriocmp programmable cycle reactor. The smaller Neo–DHFR-TS PCR product was recovered from a 3% NuSieve agarose–1% ME agarose gel (FMC Corp., Philadelphia, Pa.), incubated with T4 DNA polymerase in the presence of all four deoxynucleotide triphosphates, digested with BamHI, and cloned into the BamHI site of pUC13mp19, and the sequence was determined by the dideoxy method. The 5'-end sequence of the normal DHFR-TS gene was similarly determined, using the oligonucleotide GCCGTGCTGATATCGAG (nucleotide positions 114 to 97) in conjunction with the minixion primer.

RESULTS

Strategy for design of transfection vector pR-NEO. Most DHFR-TS amplifications encompass a specific 30-kb segment of chromosomal DNA (the R region, map positions +8.5 to −21.5 kb; Fig. 1A) (6, 8, 21; unpublished data) which is rearranged and joined to form an extrachromosomal circular DNA. In some lines, different rearrangement sites are utilized, which can be interpreted as delimiting a core region required for DHFR-TS expression and maintenance as a circular DNA. Specifically, the D7B-R1000 line (8, 23) lacked the segment of DNA between positions −16 and −21.5 kb of the prototypic R region (Fig. 1A). Accordingly, we inserted a pUC vector in this region (Fig. 1B). Assuming that the DHFR-TS coding region only provided protein-coding information, we replaced it with a unique SpeI restriction site, yielding the vector backbone pR (Fig. 1B). The neo gene encoding NPT, which confers resistance to amino-glycosides such as G418 (2), was inserted into the SpeI site of pR in either the DHFR-TS orientation (pR-NEO) or the opposite orientation (pR-NEO; Fig. 1B).

FIG. 1. DHFR-TS gene and R region of L. major and design of pR-NEO. (A) Map and features of the chromosomal R region. The restriction map for the chromosomal R region is adapted from reference 6. The open box represents the coding region of the DHFR-TS enzyme (7), and the hatched bars correspond to the locations of repeated sequences that recombine to form the R-region amplification in the R1000-3 and other MTX-resistant lines (6; unpublished data). The locations of BgIII (B) and KpnI (K) restriction sites are shown, and the map positions (in kilobases) relative to the start codon of the DHFR-TS gene are shown at the top of the figure. Above the restriction map, the locations of poly(A)+ RNAs transcribed from this region are shown by wavy lines, with arrows denoting the direction of transcription (27). Below the restriction map are shown the segments amplified in the R1000-3 and other amplified lines (R1000 [6, 8, 21; unpublished data]) and the D7B-R1000 line (D7B-R1000 [8, 23]). (B) Map of the pR vector backbone and pR-NEO and pR-ÖEN derivatives. The map of the circular pR vector backbone is shown, linearized at the hatched boxes. pR contains all the segments between the hatched boxes in the map of panel A, except that (i) the DHFR-TS-coding region has been replaced by an SpeI restriction site, juxtaposing bases −5 and +1771, and (ii) a pUC sequence has been inserted into the BgIII site B₄, giving rise to an additional BgIII site (the orientation of the lac promoter of the pUC vector is the same as DHFR-TS). In the plasmid pR-NEO, the neo gene cassette from pÍpe-NEO was inserted into the SpeI site, with the NPT-coding region in the same orientation as DHFR-TS; in pR-ÖEN, the NPT-coding region is in the opposite orientation. The neo gene cassette derives from pMCneo-POLYA (Stratagene) (41) and contains 34 bp of the HSV tk 5' region (lacking the TATA element [32]), an 18-bp synthetic translation initiation sequence fused to the NPT-coding region (2), and 55 bp containing a functional HSV tk polyadenylation sequence (45). See the Materials and Methods for details of these manipulations.
Transfection of pR-NEO: G418 resistance. Electroporation of DNA into *L. major* was performed as described in the Materials and Methods, using a Bio-Rad Gene Pulser apparatus, 4 × 10^7 to 4 × 10^8 cells suspended in HEPES-buffered saline, voltage gradients of 2,250 to 3,500 V/cm, and capacitances of 25 to 500 μF. These conditions were selected because they resulted in significant cell lethality, 50% or greater, which from studies in other organisms signals conditions appropriate for the introduction of foreign DNAs (36).

Following electroporation, 1 day was allowed for expression of the resistance gene before imposing drug selection. As the DHFR-TS mRNA is not abundant (27, 28, 42) and transfected genes frequently are expressed less well than endogenous genes, we anticipated low levels of expression of the Neo–DHFR-TS hybrid mRNA. We employed a gradual stepwise selection, beginning at 2 μg of G418 per ml and progressing through three 1:10 dilutions of cells into 4 μg/ml and then 8 μg/ml (protocol A). The gradual nature of this selection is shown by the facts that the 50% effective concentration for G418 action was 2 μg/ml and that mock-transfected cells continued to grow in this selection protocol until the second passage in 8 μg of G418 per ml. In contrast, pR-NEO recipients could be maintained indefinitely in 8 μg of G418 per ml. In later experiments, we found that electroporated cells could be passaged directly at a 1:10 dilution into 8 μg of G418 per ml (protocol B), a protocol that mock or control plasmid-transfected cells could not survive for one passage. Although all electroporations with the ranges of voltage and capacitance listed above yielded G418-resistant *L. major*, 2,250 V/cm and 500 μF yielded initial cultures that grew and attained stationary phase more rapidly, and we refer to these as our standard electroporation conditions.

Electroporation under the standard conditions followed by selection with protocol B with plasmids bearing only the neo gene cassette inserted in the *E. coli* plasmid vector in either orientation (pSpeNEOa, pSpeNEOB), pR, or pR-OLEN failed to yield G418-resistant lines. Mixed transfections of these DNA preparations in combination with pR-NEO DNA successfully yielded G418-resistant lines, showing that the nonfunctional DNA preparations were not themselves inhibitory. These data indicate that G418 resistance after electroporation requires *Leishmania* sequences and a correctly oriented neo gene.

Quantitative plating studies of a clonal derivative of the LT252 line (CC-1) electroporated in the presence of various amounts of pR-NEO DNA and grown for 1 day in drug-free medium revealed that many independent G418-resistant colonies were obtained (Fig. 2A and C); no colonies were observed in platings of mock- or control-transfected cells (Fig. 2B). The colonies obtained were shown to be *L. major* by subculture and microscopic examination, and filter lift hybridizations showed the presence of *neo*-specific sequences in each colony (data not shown). Transfectants were obtained at efficiencies of up to 62 colonies per μg of pR-NEO DNA, with up to 1,150 colonies recovered per plate (Fig. 2D); this latter value corresponds to a total cellular transfection frequency of 2.8 × 10^-7 (7 × 10^-3 for cells surviving electroporation). The efficiency of transfection was reduced fourfold at higher DNA levels but was not saturated at the highest DNA level tested (88 μg). The shape of this curve suggests that there are two different routes for transfection, one of higher efficiency and saturable; whether this reflects different routes of DNA entry or subpopulations of cells (perhaps due to the cell cycle) is currently unknown. To date, we have performed more than 50 independent electroporations of pR-NEO DNA into *L. major*, all of which successfully yielded G418-resistant lines.

pR-NEO DNA in transfected *L. major*. We discuss here the results obtained from four independent experiments transfecting pR-NEO: experiment 1, with the LT252 line, 25 μF, 3,000 V/cm, and selection protocol A; experiments 2 and 3, under standard electroporation conditions and with selection protocol B; and experiment 2-25, with 25 μF, 3,500 V/cm, and selection protocol B; the last three experiments employed the clonal CC-1 line as the recipient. The lines arising from these transfections are referred to by the experiment, whether they are a population of cells (pool) or clonal derivatives obtained by plating, and in parentheses is the final G418 concentration (micrograms per milliliter) in which they were propagated, e.g., E1-pool(8) or E2-25-B1(8).

Southern blot hybridization of *BglII*-digested DNAs from transfected lines with a *neo*-specific probe identified the expected 9.4-kb fragment only in cells transfected with pR-NEO and growing in G418 (Fig. 3A); the results of five representative clonal lines are shown). Hybridization of the same blot with a *pR*-NEO probe (Fig. 3B) revealed fragments unique to the endogenous R region (7 kb and two poorly resolved larger fragments) and fragments shared by both pR-NEO and the endogenous R region (3.4 and 12.5 kb), with all these fragments being present in all lines. Additionally, the pR-NEO-transfected lines exhibited novel fragments derived exclusively from pR-NEO (9.4, 3.8, and 2.7 kb). Scanning densitometry of lines grown in 8 μg of G418 per ml revealed that the pR-NEO copy number was about 2 relative to the endogenous DHFR-TS gene in lines derived by selection protocol A and about 10 to 12 in lines derived from three different experiments by selection protocol B; the copy number was the same in pools and clonal derivatives from the same experiment.

It is surprising that the copy number of pR-NEO obtained in lines derived by protocol A in experiment 1 was about fivefold less than those derived by protocol B in experiments 2 and 3, since all lines were grown in the same final drug concentration (8 μg/ml). The pooled samples were analyzed as soon as possible after growth in G418 was established, suggesting that the difference is a primary event and must therefore arise from differences in the electroporation conditions or between gradual versus one-step selection protocols. This difference in copy number was also maintained through two rounds of cloning on agar plates (approximately 50 cell doublings). If the results with protocol A are interpreted to mean that only two copies of pR-NEO are required to confer drug resistance, these observations suggest that the transficients derived by protocol B have maintained excess copies of pR-NEO during this period. It remains to be determined whether the copy number will decline over a prolonged period, however.

In mixed transfections, e.g., pR-NEO plus pR-OLEN, only pR-NEO-specific sequences were revealed in the G418-resistant lines by Southern blotting with appropriate enzymes and probes (data not shown). This provided no evidence for stable cotransfection.

To determine whether the copy number of pR-NEO could be increased, we selected the E1-pool(8) line for resistance to 128 and then 500 μg of G418 per ml. Analysis of these lines by Southern blotting and densitometry revealed that the copy number of pR-NEO increased from 2 in the E1-pool(8) line to 20 to 25 in the E1-pool(12) line and to 50 in E1-pool(500) line (Fig. 3C), showing that cells bearing elevated copy numbers can be readily obtained. We did not
determine whether the increase in copy number results from selection of cells that initially received high numbers of pR-NEO during electroporation or from subsequent amplification of pR-NEO within a cell.

Replication of transfected DNA. Initial data indicated that the pR-NEO sequences could be maintained within transfected L. major for many cell doublings in the presence of G418, suggesting that pR-NEO was replicating. To test this, we examined the methylation state of the pR-NEO DNA within L. major, since DNA in E. coli is methylated at the A of GATC sequences by the dam methylase, whereas Leishmania DNA is unmethylated (unpublished data). Replication of the transfected DNA was assayed with restriction enzyme isoschizomers that are differentially sensitive to this modification: DpnI, requiring methylated DNA, and MboI, requiring unmethylated DNA. As expected, the input transfecting pR-NEO DNA was sensitive to DpnI and resistant to MboI, while the total genomic DNA of the E1-pool(128) line was resistant to DpnI and sensitive to MboI (Fig. 4A). Southern blot hybridization with a neo-specific probe revealed that the pR-NEO DNA within the E1-pool(128) line uniformly showed the same sensitivity as total genomic DNA and thus was unmethylated (Fig. 4B). Similar data were obtained with other transfected lines and the enzymes BclI and XbaI, which are also sensitive to the dam methylation (data not shown). These findings are presumptive evidence for replication of pR-NEO within L. major.

Extrachromosomal localization of pR-NEO in transfecants. Separation of transfecant chromosomes by pulsed-field electrophoresis followed by Southern blot hybridization with a neo-specific probe revealed the presence of DNAs exhibiting pulse-time-dependent mobility and migration outside the apparent migration path of the linear chromosomes, two hallmarks of circular DNA (4, 22, 23). These circular DNAs were seen only in the pR-NEO-transfected lines such as the clonal derivatives E2-A4(8), E2-A6(8), E3-C4(8), and E3-D1(8) (Fig. 5A; the circular DNA is marked by an arrow; data for other pulse times are not shown). The patterns for lines derived from experiment 1 were more complex and will be discussed elsewhere. Weak hybridization was also observed to the sample well, probably reflecting the occurrence of nicked or relaxed circular DNAs which are trapped in the well in pulsed-field electrophoresis (4), but no other hybridization was evident (Fig. 5A). This suggested that integration of pR-NEO into a chromosome had not occurred. Hybridization with a pR-NEO probe similarly identified the circular DNAs in addition to the 500-kb wild-type DHFR-TS chro-

FIG. 2. Plating assay of transfection of L. major. Cells (4 × 10^7) of the CC-1 line of L. major LT252 were electroporated with various amounts of pR or pR-NEO DNA as indicated (500 µF, 2,250 V/cm), grown for 1 day in drug-free medium, and plated on M199 agar plates containing 16 µg of G418 per ml. (A) pR-NEO DNA (88 µg). Heterogeneity in colony size is commonly observed in platings of wild-type cells on drug-free plates (data not shown). (B) pR DNA (50 µg). (C) pR-NEO DNA (11 µg). (D) Quantitative plating efficiency. Shown are numbers of colonies and the calculated transfection efficiency as a function of the amount of pR-NEO DNA electroporated. The means of three replicate electroporations for each DNA amount are shown, with standard deviations of colony numbers being 5% or less of the mean.
mosome, whose size and levels were the same in the transfectant and parental lines (Fig. 5B).

To confirm the presence and measure the size of the circular pR-NEO DNA within the transfected *L. major*, we used gamma-irradiation analysis to introduce double-strand breaks and thereby form discrete linear DNAs (5, 35, 43). Separation of irradiated DNAs by pulsed-field electrophoresis followed by Southern blot hybridization with a neo-specific probe revealed discrete linear DNAs whose appearance depended on irradiation (Fig. 5C; compare with Fig. 5A). In most lines, the circular DNAs detected by this approach were of the same length as the input pR-NEO DNA, 32 kb; however, in several lines, such as the E1-pool(8), larger DNAs were observed, corresponding to oligomers of the 32-kb unit (Fig. 5C). Irradiated DNA from clonal derivatives of this pool revealed unit-length DNAs, showing that this pool was a heterogeneous population with respect to oligomer length (Fig. 5C).

The presence of circular DNAs was also indicated by reintroduction of transfected pR-NEO DNA back into *E. coli*. Chromosomal DNA preparations from several transfec-
tants were transformed into *E. coli*, yielding ampicillin-resistant colonies which contained intact pR-NEO DNA (data not shown). This additionally demonstrated the ability to shuttle pR-NEO between species.

**Transfected pR-NEO is transcribed and gives rise to a correctly processed hybrid Neo–DHFR-TS mRNA.** Northern blot analysis with a neo-specific probe identified a 2.4-kb RNA in poly(A)+ RNA prepared from the E1-pool(128) line (Fig. 6A, lane 2); no hybridization was observed to RNAs prepared from the wild-type (data not shown) or the MTX-resistant R1000 (Fig. 6A, lane 1) lines. Significantly, 2.4 kb is the size predicted for a hybrid Neo–DHFR-TS mRNA, assuming use of the processing sites of the normal 3.2-kb DHFR-TS mRNA (Fig. 6B). Correspondingly, a hybridization probe specific for the 3′ nontranslated region of the wild-type DHFR-TS mRNA (probe 3′NT in Fig. 6B) identified the new 2.4-kb mRNA in the E1-pool(128) line and the endogenous 3.2-kb wild-type DHFR-TS mRNA in both lines (Fig. 6A, lanes 3 and 4).

Since the 3′NT probe has comparable extents of homology to both the hybrid Neo–DHFR-TS and endogenous DHFR-TS mRNAs, densitometric scanning was used to quantitate the relative abundance of these RNAs. The 2.4-kb Neo–DHFR-TS mRNA was only 50% as abundant as the 3.2-kb

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**FIG. 3.** Southern blot analysis of transfected *L. major*. Genomic DNAs from the indicated lines were digested with *Bgl*II and separated on 0.8% agarose gels; pR-NEO refers to the input plasmid DNA. Molecular weight markers (in kilobases) are shown on the left. (A) neo-specific hybridization probe (0.9-kb NPT-containing fragment purified from pSpe-NEOB; see Materials and Methods). Faint hybridization is seen to the 2.7-kb Bluescript vector sequence owing to contamination of the neo fragment. (B) pR-NEO hybridization probe (entire pR-NEO plasmid, depicted in Fig. 1B; same blot as in panel A). (C) Amplification of pR-NEO sequences. E1-pool(8) was selected for growth in 128 and then 500 μg of G418 per ml, and the DNA was analyzed as in panels A and B. The pR-NEO hybridization probe was used.

**FIG. 4.** Replication of pR-NEO in *L. major*. (A) Genomic DNA from the E1-pool(128) line or pR-NEO plasmid DNA was digested with *MboI* (M) or *DraI* (D) and separated on a 2% agarose gel. Molecular weight markers (in kilobases) are shown on the side. (B) Southern blot analysis of the E1-pool(128) lanes from panel A with the neo-specific probe (see the legend to Fig. 3A).
DHFR-TS mRNA in the clonal E1-B1(8) and E1-C6(8) lines (Fig. 6A, lanes 5 and 6). In contrast, the Neo-DHFR-TS mRNA was fivefold more abundant than the DHFR-TS mRNA in the E1-pool(128) line (Fig. 6A, lane 3). As discussed earlier, the pR-NEO DNA was 2-fold amplified in the E1-B1(8) and E1-C6(8) lines and 20-fold amplified in the E1-pool(128) line. These data show that the relative abundances of the hybrid Neo-DHFR-TS mRNA per pR-NEO gene copy (0.25) were comparable to that observed for DHFR-TS mRNA (0.19) in the R1000-3 line (15-fold mRNA increase, 80-fold R-region amplification [16, 27, 28]), although the R1000 line exhibits less mRNA per DHFR-TS gene copy than the wild-type line.

We next determined whether the hybrid Neo-DHFR-TS mRNA was correctly processed at the 5' end. S1 nuclease analysis with a probe specifically labeled within neo-derived sequences revealed protected fragments of 405 and 530 nucleotides from E1-pool(128) poly(A)+ RNA (Fig. 7A, panel S1; see Fig. 7B for a map of this region and the location of S1 probes), while no protected fragments were observed with R1000 poly(A)+ RNA (data not shown). These products map to positions −267 and −392 in pR-NEO, which correspond to positions −231 and −356 in the endogenous DHFR-TS gene and agree with those previously mapped for the normal DHFR-TS mRNA, positions −245 and −375 (Fig. 7B) [28]. In both the DHFR-TS and hybrid Neo-DHFR-TS mRNAs, the smaller S1 products were quantitatively more abundant (Fig. 7A and reference 28). Primer extension analysis with an oligonucleotide located within the neo sequence yielded two products of 430 and 560 nucleotides with E1-pool(128) but not R1000 RNA (Fig. 7A, panel PE), with the smaller product being more abundant. These products map to positions approximately 40 nucleotides beyond those identified by S1 nuclease analysis (Fig. 7B), suggesting that these RNAs contain an additional 5' extension corresponding to the miniexon sequence of L. major. Accordingly, PCR amplification of cDNA derived from RNA of the E1-pool(128) line with the neo-specific oligonucleotide used in the primer extension analysis (Fig. 7A) and an oligonucleotide from the L. major miniexon sequence yielded products whose sizes agreed with that obtained by primer extension (data not shown). DNA sequencing of the smaller PCR product showed that it contained the miniexon sequence and the 5’ nontranslated region of the DHFR-TS gene fused to the NPT-coding sequence as expected and that the site of addition of the miniexon was identical for the Neo– DHFR-TS hybrid and wild-type DHFR-TS mRNAs (Fig. 7C).

In total, these data demonstrated transcription of the transfected pR-NEO DNA and correct processing of a hybrid Neo–DHFR-TS mRNA, employing the sites present in pR-NEO that are normally used by the endogenous DHFR-TS gene.

Northern blot analysis with a pUC plasmid DNA probe identified in transfected lines three RNAs of 5.0, 5.5, and 9.0 kb (Fig. 6A, lane 8) that were absent in wild-type (data not shown) and R1000 (Fig. 6A, lane 7) lines; the region into which the pUC DNA was inserted is normally transcribed into two 6.2-kb opposite-strand poly(A)+ RNAs (Fig. 1A) (27). This suggested that transcription was occurring across the inserted pUC sequences, since the size of the largest of...
these RNAs corresponded to the predicted size for a hybrid RNA containing both pUC and the 6.2-kb RNA sequences (Fig. 6B). Hybridization with a probe located to the left of the pUC insertion site (probe 6A, Fig. 6B) identified three RNAs of 2.8, 6.2, and 9 kb (Fig. 6A, lane 9), while a probe located to the right of the pUC sequences (probe 6B, Fig. 6B) identified four RNAs of 5.5, 6.2, and 9 kb (Fig. 6A, lane 10). The location and sizes of the 5.5-, 6.2-, and 2.8-kb RNAs relative to the normal pattern of transcripts from this region suggests the possibility that these RNAs arise from the introduction of a signal in the vicinity of the left end of the pUC DNA, although whether the smaller RNAs arise from processing of the larger 9-kb hybrid RNA or by another mechanism is unknown. No alterations in the size of other RNAs transcribed from regions shared by both the R region and pR-NEO were observed (13 kb, 1.7 kb, and the downstream RNAs [27]), other than an elevation in level in the E1-pool(128) line consistent with that observed for the Neo-DHFR-TS hybrid mRNA described above.

**DISCUSSION**

We showed that a molecular construct (pR-NEO) derived from the amplified circular DHFR-TS gene of MTX-resistant *L. major* can be successfully introduced into wild-type cells by electroporation, thereby conferring G418 resistance. Quantitative plating studies demonstrated that the efficiency of transfection of this 32-kb DNA approaches $10^{-4}$ per cell, which compares well with efficiencies obtained in electroporations of cultured mammalian cells (36). It seems likely that the efficiency can be increased since we have not systematically optimized these protocols. Given the high transfection efficiency of pR-NEO, it is surprising that our previous attempts to introduce the native circular R-region amplified DNA into *Leishmania* species by electroporation followed by MTX selection did not succeed, since similar protocols succeed in cultured mammalian cells (unpublished data). This may be due to peculiarities of the effects of MTX on *Leishmania* species undergoing drug selection or to the possible existence of targets other than DHFR-TS (Ellenberger, Ph.D. thesis).

To date, we have maintained pR-NEO in cells under G418 pressure for more than 200 cell doublings; in these lines, the pR-NEO gene copy number remained at similar levels (unpublished data), suggesting that dilution of nonreplicating pR-NEO DNA was not taking place. Correspondingly, methylation analysis confirmed that pR-NEO replicates within *L. major*. In several lines, pR-NEO is maintained exclusively as an extrachromosomal circular DNA, and in these there is no evidence for integration into any chromosome, including DHFR-TS gene which contains more than 29 kb of sequence homology with pR-NEO (Fig. 1). It is therefore not surprising that we did not observe cotransfection of nonfunctional molecular constructs when mixed with pR-NEO, as stable cotransfection in cultured mammalian cells requires covalent joining of the cotransfected DNAs followed by integration into a chromosome.

Current data indicate that transcription from pR-NEO occurs in a manner that accurately reflects normal transcriptional processes. First, successful transfection depended on *Leishmania* DNA sequences and required that the neo gene within the pR backbone be in the same orientation as DHFR-TS. Second, pR-NEO-transfected *L. major* expressed a hybrid Neo–DHFR-TS mRNA whose size corresponded to that predicted for an RNA that utilizes the normal DHFR-TS-processing sites present in pR-NEO. Accordingly, S1 nuclelease protection, primer extension, and sequence analysis of cDNAs indicated that the *Leishmania* minixenon sequence was added to the 5' end of the hybrid Neo–DHFR-TS mRNA, at precisely the sites utilized in the normal DHFR-TS transcript (28). The accuracy of this process is emphasized by the fact that both minixenon addition sites were utilized in relative proportions similar to that seen for the normal DHFR-TS gene (28). Third, preliminary studies of a construct bearing the chloramphenicol acetyl-transferase gene correctly inserted into the SpeI site of the plasmid pR showed low levels of enzyme activity in transient assays, and it seems likely that G418 resistance conferred by pR-NEO is mediated by synthesis of NPT. Fourth, on a per gene copy basis the level of expression of the hybrid
Neo–DHFR-TS mRNA appeared to be similar to that observed for the DHFR-TS gene in amplified lines obtained by MTX selection. This suggested that expression directed by pR-NEO is occurring in a quantitatively normal manner. Fifth, pR-NEO contains regions that encode other R-region transcripts, and correspondingly, lines bearing high levels of pR-NEO DNA such as E1-pool(128) showed elevated levels of these transcripts. Moreover, a 9-kb RNA corresponding in size to that predicted for a hybrid pUC-6.2-kb RNA was found. Thus, the pattern and abundance of stable RNAs in pR-NEO-transfected lines were consistent with utilization of normal transcriptional and/or processing signals located within the pR-NEO DNA.

The neo gene cassette within pR-NEO derives from the plasmid pMC1neo-POLYA and contains portions of the herpes simplex virus (HSV) thymidine kinase (tk) gene in addition to the NPT-coding region. The 5′ end of the neo cassette employed contains 30 bp of functionless 5′-flanking sequence from the HSV gene and a synthetic translation initiation sequence (32, 41). However, the 3′-flanking end contains a functional HSV polyadenylation signal (45). This sequence is specifically not recognized in L. major since we did not observe the predicted 1.3-kb hybrid Neo–DHFR-TS mRNA (Fig. 6). These studies demonstrate a fundamental difference in the cis-acting elements required for polyadenylation in trypanosomatids and higher eucaryotes and are consistent with the failure to detect consensus eucaryotic polyadenylation elements in many trypanosomatid genes.

The fact that pR-NEO is capable of autonomous extrachromosomal replication unambiguously demonstrates the functionality of the Leishmania sequences present within this DNA. This argues that the original extrachromosomal circular amplified R-region DNA also replicates autonomously and is not continually generated from a modified chromosomal locus. Similarly, the ability of pR-NEO to efficiently direct synthesis of a hybrid Neo–DHFR-TS...
mRNA suggests that increased DHFR-TS expression in amplified MTX-resistant lines is directly mediated by a gene dosage effect. Thus, this work established the functional characteristics of the previously described DHFR-TS (R-region) amplifications.

The data presented in this paper signal the advent of methodologies and vectors suitable for the stable introduction of DNAs into human parasitic protozoa. Molecular dissection of expressing constructs such as pr-NEO will allow a more precise localization of the cis-acting elements required for transcription, processing (including minioexon addition), and replication, in conjunction with the application of methods for transient assays of expression and replication. Moreover, our results showing the formation of hybrid transcripts containing pUC-derived sequences suggest that it will be possible to introduce other genes of interest into appropriate locations within the pr-NEO vector and obtain transcription and, potentially, translation. Finally, since the efficiency of transfection is high, it is now possible to contemplate transfection-based approaches for the identification and analysis of genes responsible for many interesting and unique features of the parasite infectious cycle.

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