Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 9736–9740, December 1990
Genetics

Development of a stable *Leishmania* expression vector and application to the study of parasite surface antigen genes

(DNA transfection/protozoan parasites/trans-splicing/β-galactosidase/extrachromosomal DNA)

JONATHAN H. LEBOWITZ†, CARA M. COBURN†, DIANE MCMAHON-PRATT‡, AND STEPHEN M. BEVERLEY†§

†Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115; and ‡Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT 06510

Communicated by Louis H. Miller, September 10, 1990 (received for review August 17, 1990)

ABSTRACT **Trypanosomatid protozoan parasites cause several important tropical diseases and have been a fertile ground for the discovery of molecular paradigms such as trans-splicing and RNA editing. Transfection-based methods for the study of these organisms have recently been developed, and we have now designed an expression vector, pX, which contains only 2.3 kilobases of *Leishmania* DNA and can be stably transfected with high efficiency. Genes encoding *Escherichia coli* β-galactosidase or a *Leishmania amazonensis* protective membrane glycoprotein (GP46A/M-2) were inserted into the pX expression site and transfected into *Leishmania major*, where they directed the synthesis of high levels of mRNAs formed by 5′ and 3′ processing events occurring predominantly at the sites used by the normal transcripts. Colony assays and immunoblot analysis showed that both proteins were produced; enzymatically active β-galactosidase comprised ≈1% of total protein. Sizes of the GP46A protein synthesized in transfected *L. major* or *L. amazonensis* were similar and differed from the predominant *L. amazonensis* GP46, suggesting that the GP46A gene may encode a variant GP46 family member. Because these vectors function efficiently in pathogenic species of *Leishmania*, pX will facilitate the genetic analyses of parasite proteins crucial for infectivity as well as the identification of cis-acting elements mediating transcription and replication.**

Trypanosomatid protozoans cause several diseases that have profound consequences for human health in tropical regions, such as sleeping sickness (*Trypanosoma brucei*), Chagas disease (*Trypanosoma cruzi*), and leishmaniasis (genus *Leishmania*). Immunological and biochemical studies have identified many molecules likely to play a role in the establishment and maintenance of the infectious cycle; however, in many cases the specific functions of these molecules are unknown. Although conventional genetic crosses are either difficult or impossible to conduct in trypanosomatids, recent advances now permit use of the powerful alternative approach of DNA transfection to conduct genetic tests of molecular function (1–6).

Our laboratory has focused on constructs derived from the circular 30-kilobase (kb) R region [bearing the dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene] amplified in certain lines of methotrexate-resistant *Leishmania major* (7). This DNA appears to contain all genetic elements normally required in cis for expression and replication (6, 7). In the prototypic construct pR-NEO, the DHFR-TS coding region has been replaced by a selectable marker conferring resistance to the aminoglycoside G418 [neomycin phosphotransferase (NEO)] (6). After efficient introduction into cultured *Leishmania* by electroporation, pR-NEO is maintained extrachromosomally and directs the synthesis of a hybrid NEO/DHFR-TS mRNA at nearly normal levels. This chimeric RNA arises from correct use of the normal DHFR-TS gene signals for polyadenylylation and trans-splicing that flank the inserted NEO gene.

Normally the *Leishmania* R region DNA present within pR-NEO is extensively transcribed, yielding at least 10 different polyadenylylated RNAs (8, 9). Because transcription of pR-NEO closely resembles that of the normal DHFR-TS locus, quantitative deletional studies of this region should be suitable for probing elements involved in transcription and processing. In this report we describe initial studies of smaller derivatives that retain functional equivalence with pR-NEO. These studies yielded a construct, pX, the structure and properties of which suggested it could function as an efficient general expression vector for any foreign gene. To test the utility of pX, we have characterized the expression of two foreign genes inserted into pX: *Escherichia coli* β-galactosidase, an enzymatically active reporter gene, and a surface antigen gene from *Leishmania amazonensis*, GP46A. GP46A is a member of a family of genes encoding the GP46/M-2 protein (ref. 10; S.M.B., Y. Traub-Cseko, K. Lohman, D.M.P., unpublished work), the expression of which is developmentally regulated and specific for the promastigote stage (11). Immunization of mice with GP46 has been shown to confer protection against subsequent challenge with parasites (12). The GP46 gene family is present in most species of *Leishmania*, including *L. major* (ref. 10; unpublished data); however, the availability of the *L. amazonensis* GP46-specific monoclonal antibody M-2 (13) allowed identification of the transfected protein in *L. major* in the absence of GP46-deficient mutants.

METHODS AND MATERIALS

Molecular Constructs. Plasmids pS45NEOA and pS45NEOB were constructed by inserting the 3.6-kb *Sal* I fragment from pR-NEO (bearing the hybrid DHFR-TS/NEO gene) into the *Sal* I site of pBS*SK− (Fig. 1*A*). pBS*SK− is pBluescript SK(−) (Stratagene) in which the *Pvu* II fragments have been inverted and the *Pvu* II site next to the *Sst* I site has been lost. pX was obtained by deleting the 0.36-kb *Xma* I fragment 3′ of the DHFR-TS/NEO transcript region, adjacent to the polylinker site (Fig. 1*A*). pX-βGAL contains the 3.1-kb *Bgl* II fragment bearing the *E. coli* β-galactosidase-coding region of plasmid pJ3βgal (C. Cepko and J. Morgenstern, unpublished work), inserted into the unique *Bam*HI site of pX (Fig. 1*B*). pX-GP46A contains a 2.7-kb *Bam*HI fragment from λ clone 7A12 (10), bearing the GP46A-coding region, an intertranscript region, and the 5′ end of the flanking GP46B gene, inserted into the *Bam*HI site of pX (Fig. 1*B*). Plasmid pBS5′GP46A contains a 376-base-pair (bp)

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

Abbreviations: DHFR-TS, dihydrofolate reductase-thymidylate synthase; Dst RNAs, downstream RNAs; NEO, neomycin phosphotransferase gene.

§To whom reprint requests should be addressed.

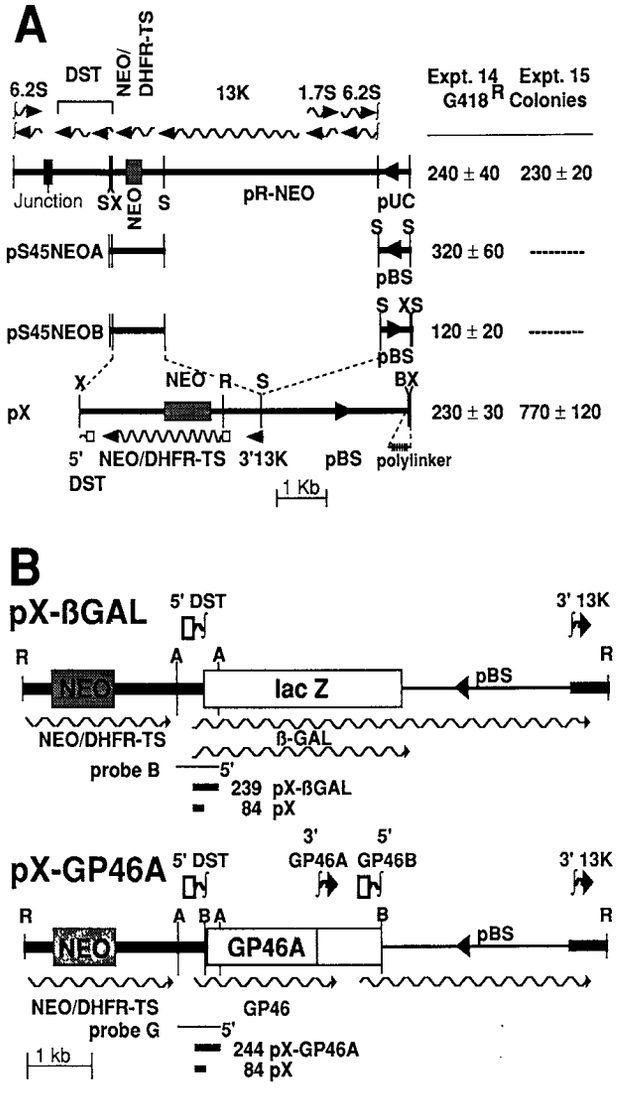

FIG. 1. Molecular recombinants introduced into *Leishmania*. (*A*) Structure of deletion plasmids and their plating efficiencies. pR-NEO is shown as a heavy line, linearized at a *Bgl* II site flanking the pUC vector; the junction region at which the original amplified DHFR-TS gene circularized is labeled (solid box) as is the NEO gene (stippled box). Relevant *Bam*HI (*B*), *Eco*RI (*R*), *Sal* I (*S*), and *Xma* I (X) sites are indicated. Major transcripts of the DHFR-TS gene or pR-NEO are depicted as wavy lines with arrows marking their orientation. Origins of plasmids pS45NEOA and pS45NEOB and pX are shown below the map of pR-NEO. Large arrows show orientation of the *lac* promoter within pBS*SK−, and the sites of miniexon addition and polyadenylylation are indicated by open squares and solid arrowheads, respectively. The recovery of G418-resistant ($G418^R$) colonies per 40 μg of transfected DNA is shown at right of each construct (average of three determinations). 13K, 13-kb RNA; 6.25, 6.2-kb overlapping RNAs; 1.75, 1.7-kb overlapping RNAs (8). (*B*) Maps of pX-βGAL and pX-GP46A. Miniexon addition and polyadenylylation sites flanking or carried by the inserted genes are shown above the maps as in *A*; orientation of the pBluescript vector is also shown as in *A*. Major transcripts identified in Figs. 4 and 5 are indicated by wavy lines. Relevant *Avi* II (*A*), *Bam*HI (*B*), and *Eco*RI (*R*) sites are indicated. Location of RNA probes B and G (synthesized from plasmids pBS5′βgal and pBS5′GP46A, respectively) are indicated by thin lines; the scale in this region has been expanded. Below them, black bars indicate locations and sizes of the predicted protected fragments, assuming correction use of the Dst (DST) miniexon addition site for trans-splicing for the indicated transfectants.

Avi II fragment from pX-GP46A spanning the junction of pX and the 5′ end of the GP46A gene, inserted into the *Hin*cII site of pBluescript SK(−) (Stratagene); plasmid pBS5′βgal contains a 369-bp *Avi* II fragment from pX-βGAL spanning the junction of pX and the 5′ end of the β-galactosidase gene inserted into the same site.

Parasite Strains and Culture. The clonal CC-1 derivative of *L. major* strain LT252 (6) and the LTB0016 line of *L. amazonensis* were used for transfection. Methods for culture, electroporation, and plating of parasites have been described (6). Transfected lines of *L. amazonensis* were initially plated on medium containing 50 μg of G418 per ml and cultivated in 25 μg of G418 per ml.

Immunological Methods. Late logarithmic-phase cells (1 mg of protein per 10^8 cells; ref. 14) were collected and boiled in sample buffer (lacking mercaptoethanol for the GP46 expression experiments) and subjected to immunoblot analysis as described (15) using Immobilon-P membranes (Millipore). Immunoprecipitations with the M-2 antibody were done as described (11, 12).

***In Situ* Colony Assays.** β-Galactosidase was detected in colonies on the surface of agar plates by carefully overlaying the plate with 1% Sea-Plaque agarose (FMC) containing 0.5% SDS and 0.25% 5-bromo-4-chloro-3-indolyl β-D-galactoside; blue color became visible within a few hours. GP46 expression was detected in colonies by (*i*) pressing a nitrocellulose filter to the plate, (*ii*) removing and blocking the filter with blotto for 1 hr [blotto is 5% powered milk in TNE (10 mM Tris/50 mM NaCl/1 mM EDTA, pH 7.5)], (*iii*) incubating 90 min with the M-2 antibody in blotto, (*iv*) washing five times with TNE (5 min each), (*v*) incubating 90 min with goat anti-mouse IgG coupled to horseradish peroxidase in blotto, and (*vi*) development with diaminobenzidine in the presence of $CoCl_2$ (15); all steps were at room temperature with gentle agitation. Total colonies were detected by incubating nitrocellulose transfers with the phosphatase substrate bromochloroindolyl phosphate and nitro blue tetrazolium as described (15).

RNA Analysis. Isolation of polyadenylylated RNAs, Northern blot hybridization with randomly primed probes, and RNase protections were done as described (6, 16).

RESULTS

Deletional Analysis of pR-NEO. Deletions of pR-NEO were constructed and tested by quantitative plating assay for G418 resistance. A series of derivatives were tested, leading to the constructs pS45NEOA and pS45NEOB, which contained only 2.6 kb of leishmanial DNA (Fig. 1*A*). These constructs differed only in the orientation of the bacterial plasmid sequences and yielded G418-resistant colonies at a frequency similar to pR-NEO (Fig. 1*A*). Molecular karyotype analysis of several clonal lines revealed that the transfected DNAs were intact covalently closed circular molecules, present in ≈10–20 copies, as seen previously with pR-NEO (data not shown; ref. 6).

Relevant sequences in pS45NEO are (*i*) the 5′-flanking region of the DHFR-TS gene, encoding the 3′ end of an upstream 13-kb RNA and the 5′ end of the DHFR-TS mRNA, (*ii*) NEO, and (*iii*) the 3′-flanking region of DHFR-TS, encoding the 3′ end of the DHFR-TS mRNA and the 5′ end of two RNAs termed the downstream (Dst) RNAs (Fig. 1*A*; refs. 8 and 9). Because the Dst RNAs bear the trans-spliced miniexon (9), we reasoned that insertion of foreign genes near the *Sal* I site within the Dst RNA region would generate hybrid mRNAs bearing the miniexon analogous to the hybrid NEO/DHFR-TS mRNA. An ATG codon between the miniexon addition site of the Dst RNAs and the *Sal* I site was removed, bringing the Dst miniexon addition site within 80 bp of the pBluescript polylinker and yielding the plasmid pX (Fig. 1*A*). pX was also isofunctional with pR-NEO in terms of quantitative transfection efficiency (Fig. 1*A*), extrachromosomal localization, and copy number (10–20; data not shown).

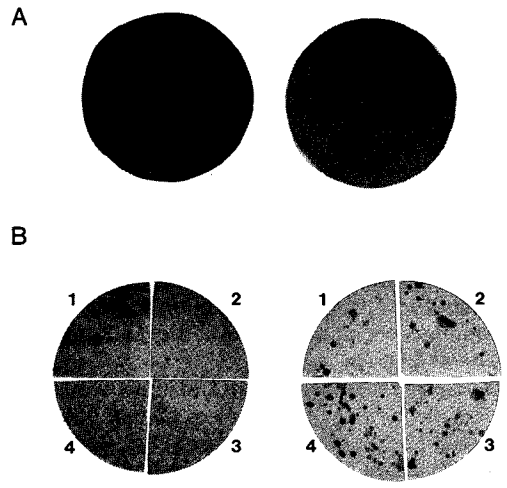

FIG. 2. Detection of foreign gene expression *in situ*. (*A*) β-Galactosidase activity. G418-resistant colonies were stained for β-galactosidase activity as described. Left plate, pX-βGAL transfectants; right plate, pX transfectants. Dots on right plate mark the location of colonies. (*B*) Colony immunoscreening for *L. amazonensis* GP46 expression. Nitrocellulose filters containing transferred *L. major* colonies transfected with pX-GP46 (*1*), pX-βGAL (*2*), pX (*3*), or no DNA (*4*; plated without G418) were developed for GP46 expression with the M-2 monoclonal antibody (left filter) or for total colonies (right filter). Representative quadrants from the four lines were assembled. Note that separate filters were used for immunoscreening and total colony visualization.

Expression of *E. coli* β-Galactosidase in *L. major*. The *E. coli* β-galactosidase gene was inserted into the expression site of pX, yielding pX-βGAL (Fig. 1*B*). pX-βGAL was efficiently introduced into *L. major* (1100 ± 80 vs. 860 ± 20 G418-resistant colonies per 40 μg of DNA for pX-βGAL vs. pX, respectively) and persisted as an extrachromosomal circular DNA with a copy number similar to pX (data not shown). An activity stain for β-galactosidase in *Leishmania* colonies showed activity in all pX-βGAL colonies but in none of the pX transfectants (Fig. 2*A*). Immunoblot analysis revealed the expected 116-kDa β-galactosidase polypeptide only in the pX-βGAL transfectants (Fig. 3*A*). To increase the copy number of pX-βGAL, cells were selected for increased G418 resistance (up to 128 μg/ml; ≈50 copies per cell). In these lines β-galactosidase increased, comprising up to 1% of total cell protein, and was readily seen in total cellular proteins separated by SDS/PAGE (Fig. 3*B*). β-Galactosidase activity assays of cellular extracts gave similar results, indicating that most protein was functionally active (data not shown).

Expression of the *L. amazonensis* GP46A Gene in *L. major*. The GP46A gene was introduced into the polylinker *Bam*HI site of pX, yielding the construct pX-GP46A (Fig. 1*B*). After introduction into *L. major* (1100 ± 30 G418-resistant colonies per 40 μg of pX-GP46A), pX-GP46A was maintained as an extrachromosomal circular DNA with a copy number similar to pX (10–20 copies; data not shown). With the *L. amazonensis* GP46-specific monoclonal antibody M-2, GP46A protein was detected in every colony after transfection with pX-GP46A but not in three control lines (Fig. 2*B*).

Immunoblot analysis with the M-2 antibody revealed a doublet with an apparent molecular mass of 42 kDa in lines transfected with pX-GP46A that was not present in control lines (Fig. 3*C*). Levels of this protein increased in transfected lines selected for increased G418 resistance; however, even at the highest G418 concentration tested (128 μg/ml, ≈50 copies of pX-GP46A per cell) the quantity of GP46 was only ≈1% of that seen in *L. amazonensis* (Fig. 3*C*) or ≈0.0003% of total *L. major* protein. Moreover, the *L. amazonensis* GP46 protein synthesized in *L. major* migrated faster than the endogenous 46-kDa GP46 synthesized in *L. amazonensis* (Fig. 3*D*). These results were confirmed with two other polyclonal antisera (data not shown).

The different size of the GP46 protein specified by pX-GP46A in *L. major* could reflect differences in processing of this protein in a heterologous species. However, inspection of the immunoblot shown in Fig. 3*D* revealed that the GP46A protein synthesized by pX-GP46A-transfected *L. major*

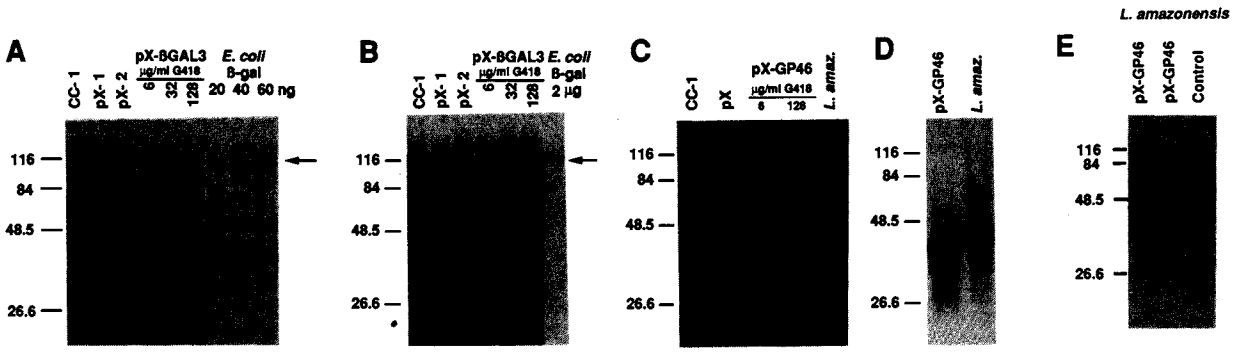

FIG. 3. Analysis of foreign proteins synthesized in transfected *Leishmania*. Molecular mass markers (in kDa) are at left of *A–E*. (*A*) *E. coli* β-galactosidase synthesized in pX-βGAL-transfected *L. major*. Immunoblot analysis of proteins from 10^6 cells of transfected lines of *L. major* was performed by using rabbit anti-β-galactosidase (Cappel Laboratories) as the primary antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG as the secondary antibody. The samples were as follows: CC-1, untransfected *L. major*; pX-1 and pX-2, two lines transfected with pX; pX-βGAL-3, a line transfected with pX-βGAL and grown in 6, 32, or 128 μg of G418 per ml, respectively, and authentic *E. coli* β-galactosidase (β-gal). Position of β-galactosidase is marked by an arrow. Results for the pX-βGAL-4 line were identical to those for pX-βGAL-3 and are not shown. (*B*) Total proteins from pX-βGAL-transfected *Leishmania*. Proteins from 2×10^7 cells (130 μg of total protein) from lines described in *A* were electrophoretically separated on SDS/PAGE and stained with Coomassie blue for total protein. *E. coli* β-galactosidase standard (2 μg) is marked by an arrow. (*C*) *L. amazonensis* GP46A expressed in *L. major*. Immunoblot analysis of transfected *L. major* was done using M-2 monoclonal as primary antibody and ^{125}I-labeled anti-mouse IgG (Amersham) as secondary antibody. The samples were CC-1, untransfected *L. major*; pX, pX-transfected *L. major*; pX-GP46, *L. major* line pX-GP46A-6 transfected with pX-GP46A, grown in 6 or 128 μg of G418 per ml as indicated; *L. amaz.*, untransfected *L. amazonensis*. Another pX-GP46A transfectant gave similar results and is not shown. Proteins from 3×10^7 cells (*L. major*) or 3×10^5 (*L. amazonensis*) were analyzed. (*D*) Immunoprecipitation of GP46A from *L. amazonensis* or pX-GP46A-transfected *L. major*. Protein from 1×10^8 cells was subjected to immunoprecipitation with the M-2 antibody and eluted and subjected to immunoblot analysis as described in Fig. 3*A*, except that the primary antibody was a rabbit polyclonal antibody against *L. amazonensis* GP46 (13). pX-GP46, pX-GP46A-transfected *L. major*; *L. amaz.*, *L. amazonensis*. (*E*) Expression of pX-GP46A in transfected *L. amazonensis*. Immunoblot analysis was done using the M-2 monoclonal antibody as described in *C*, using protein from 3×10^5 cells. pX-GP46, two different lines of *L. amazonensis* transfected with pX-GP46A; control, untransfected *L. amazonensis*.

comigrated with a minor 42-kDa GP46 protein additionally identified by the M-2 antibody in *L. amazonensis*. To test whether this protein could be the product of the GP46A gene, pX-GP46A was introduced into *L. amazonensis*. Immunoblot analysis revealed that the pX-GP46A-transfected *L. amazonensis* possessed elevated levels of the 42-kDa protein (Fig. 3*E*). This result suggested that the transfected GP46A gene encodes a variant form of the GP46 family.

Northern (RNA) Blot Analysis of Hybrid RNAs. Analysis of polyadenylylated RNAs isolated from the transfected lines described above suggested that, in general, *Leishmania* sequences normally directing 5′ and 3′ termini formation continued to do so in the context of the pX vector. Hybridization with a NEO-specific probe revealed that all transfected lines expressed a 2.4-kb hybrid transcript arising from correct use of the normal DHFR-TS processing sites flanking the NEO-coding region (Fig. 4*A*; ref. 6). Origins of the less abundant or larger transcripts are not known, and they may arise from incomplete or aberrant processing. Hybridization with the bacterial vector probe (pBS) showed that pX directed synthesis of a 3.4-kb RNA (Fig. 4*D*), the size expected for one using the normal 5′ terminus of the Dst RNA, proceeding through pBS, and terminating at the normal 3′ terminus of the 13-kb RNA (Fig. 1*B*).

Hybridization with a β-galactosidase probe identified abundant transcripts of 9-, 6.6-, and 3.6-kb in pX-βGAL-transfected lines that were not present in control lines (Fig. 4*B*); the 9- and 6.6-kb transcripts were also identified by the pBS probe (Fig. 4*D*). The 6.6-kb transcript agrees with the size predicted for a chimeric RNA using known signals within leishmanial DNA—i.e., starting at the 5′ terminus of the Dst RNAs, traversing the β-galactosidase and pBS sequences, and terminating at the 3′ end of the 13-kb RNA (Fig. 1*B*).

Polyadenylylated RNAs from *L. major* lines transfected with pX-GP46A displayed a major 2.4-kb and a minor 3.4-kb transcript when probed with a *L. amazonensis* GP46-specific probe under conditions that precluded hybridization to *L. major* GP46 mRNA (Fig. 4*C*). Neither transcript has the size predicted for a chimeric transcript (5.9 kb) analogous to that seen with pX or pX-βGAL. However, the 2.7-kb *Bam*HI fragment inserted into pX-GP46 encodes the normal 3′ end of GP46 mRNA in *L. amazonensis* and the 5′ end of GP46B gene (Fig. 1*B*; ref. 10). The 2.4-kb fragment is the size predicted for an RNA beginning at the 5′ end of the Dst RNA and ending at the *L. amazonensis* GP46A 3′ end, and a 3.4-kb fragment is the size predicted for a transcript initiating at the 5′ end of the GP46B gene, traversing the bacterial vector sequences, and terminating at the 3′ end of the 13-kb RNA. Accordingly, the pBS vector probe identified the 3.4-kb RNA as the most abundant RNA within the pX-GP46 transfectants (Fig. 4*D*). These data suggest that the *L. major* transcriptional apparatus can recognize *L. amazonensis* sequences that direct 3′- and 5′-end formation.

Mapping the 5′ Termini of Hybrid RNAs by RNase Protection. To confirm that the Dst RNA miniexon addition site was correctly utilized, nuclease protection studies were employed using fragments spanning the Dst miniexon addition site and the 5′ ends of β-galactosidase or GP46A (probes B and G, respectively; Fig. 1*B*, Fig. 4*F*). When poly(A)$^+$ RNA from pX-βgal transfectants was analyzed with probe B, a protected fragment of 235 nucleotides was observed (Fig. 4*F*, lane 3), in good agreement with the 239 nucleotides expected for a hybrid RNA using the Dst miniexon addition site. When poly(A)$^+$ RNA from pX-GP46A-transfected *L. major* was analyzed with probe G, a protected fragment of ≈245 nucleotides was seen (Fig. 4*F*, lane 2), agreeing well with the 244 nucleotides expected. When poly(A)$^+$ RNA from pX transfectants was analyzed with either probe B (Fig. 4*F*, lane 4) or probe G (data not shown), protected fragments of ≈80 nucleotides were seen, compared with the 84-base size predicted (Fig. 1*B*; ref. 9). These data demonstrate that hybrid RNAs encoding β-galactosidase, GP46A, or the 3.4-kb pX transcript are correctly processed at their 5′ ends.

DISCUSSION

We have shown that pX meets a number of criteria appropriate for a general vector for stably introducing and express-

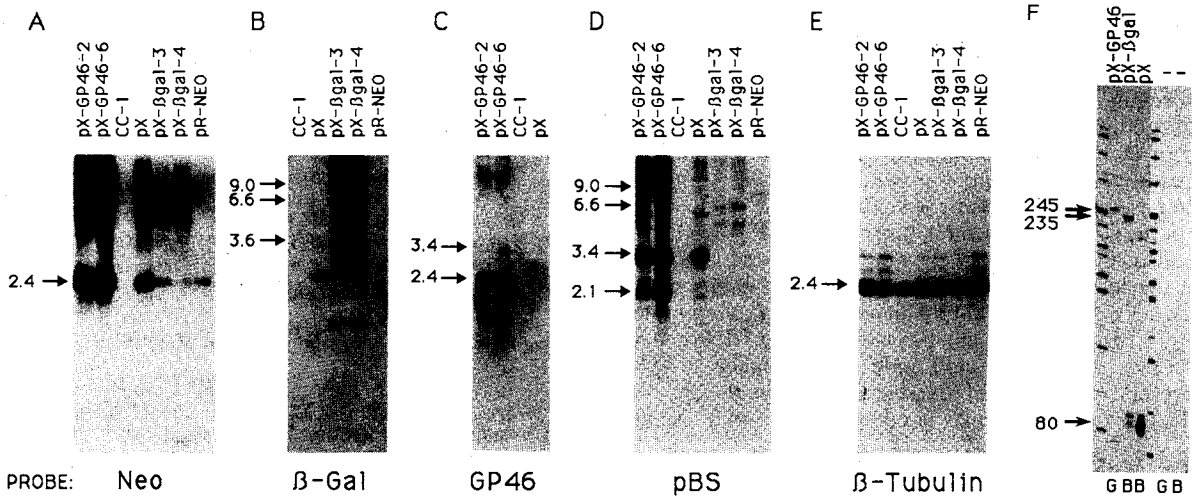

FIG. 4. Transcripts from *L. major* transfected with pX and its derivatives. Northern blot analysis of RNAs synthesized in transfected *L. major* was done with ≈200 ng of poly(A)$^+$ RNA per lane. The sources were as follows: CC-1, untransfected *L. major*; pX, pX transfectant; pR-NEO, pR-NEO transfectant; pX-GP46-2 and -6, two different pX-GP46A transfectants; pX-βGAL-3 and -4, two different pX-βGAL transfectants. Cells were grown in medium containing 6 μg of G418 per ml, except the pX-GP46A transfectants, which were grown in 128 μg of G418 per ml and CC-1, which lacked G418. Hybridization probes were as follows: NEO, 0.9-kb *Spe* I fragment containing the NEO fragment from pSpe-NEOB (6) (*A*); β-GAL, 1.9-kb *Hpa* I–*Nde* I fragment from pX-βGAL containing *E. coli* β-galactosidase (*B*); GP46, 2.7-kb *Bam*HI fragment containing the *L. amazonensis* GP46A gene from pX-GP45A (*C*); pBS, plasmid pBluescript KS$^-$ (*D*); β-tubulin control, 3.8-kb *Hin*dIII fragment from pLTβ-1 containing the *L. major* β-tubulin (17) (*E*). Arrows indicate position and sizes (kb) of transcripts discussed. (*F*) Mapping of 5′ termini of chimeric RNAs in *L. major* by RNase protection. RNase protection was done with ≈200 ng of polyadenylylated RNA from the lines described above and hybridization probes depicted in Fig. 1*B*. A dash (–) indicates that no RNA was added. Unlabeled lanes contain molecular mass marker (*Msp* I-digested pBR322 labeled with [^{32}P]dCTP by treatment with Klenow fragment). Arrows mark location of the protected fragments, the sizes of which are indicated in nucleotides.

ing foreign genes in *Leishmania*. (*i*) It can be introduced into *Leishmania* promastigotes with high efficiency, where it confers G418 resistance. (*ii*) pX is maintained episomally, allowing recovery back into *E. coli* as a shuttle vector (6). (*iii*) pX provides signals directing the synthesis and correct processing of polyadenylylated RNAs. (*iv*) High levels of protein expression can be achieved, either directly or after increased G418 pressure. To complement these features of pX, we have developed methods that allow rapid detection of pX-encoded proteins directly in *Leishmania* colonies, either by immunostaining or by histochemical enzyme stains *in situ*. In combination these methods permit the rapid introduction and identification of molecular constructs expressing inserted genes and visualization of the results of manipulations on existing constructs.

The utility of a general expression vector such as pX is illustrated by our studies of the GP46 gene family of *L. amazonensis*. These data suggest that the GP46A gene may encode a variant protein similarly processed in the two species. That variant GP46 genes exist was first revealed by Southern blot analysis; unlike many gene families in trypanosomatids, GP46 genes show significant divergence (ref. 10; S.M.B., Y. Traub-Cseko, K. Lohman, and D.M.P., unpublished work). Additionally, in *L. amazonensis* GP46 is a surface glycoprotein anchored by a glycerol–phosphatidylinositol linkage (ref. 10; L. Rivas, L. Kahl, and D.M.P., unpublished work), and the transfected GP46A protein could differ in one or more of these modifications. In preliminary studies we were unable to detect GP46 on the surface of transfected *L. major*, which could reflect a difference in cellular localization arising from structural differences. Future studies to definitively determine the structure and location of the GP46A gene product will be aided considerably by use of pX-GP46A-transfected *Leishmania* and the species-specific M-2 antibody.

Our studies did reveal quantitative differences in RNA and protein expression directed by the pX constructs bearing different inserts and in different species. Per transfected gene copy, pX-βGAL- and pX-GP46A-transfected *L. major* contained comparable amounts of chimeric RNAs encoding β-galactosidase and GP46A with correct 5′ termini (Fig. 4). In contrast, ≈0.1% as much GP46A protein was detected in pX-GP46A-transfected *L. major* or ≈1–5% the levels of total GP46 normally seen in *L. amazonensis* (Fig. 3). This discrepancy could arise from differences in conformation or stability of the GP46A protein in *L. major*. Another source could be differences in the translatability of the chimeric GP46A mRNA, perhaps arising from the 5′- and 3′-noncoding regions of the GP46A fragment within pX-GP46A. Regardless of the cause, the reduced levels were specific for *L. major* because the levels of GP46A directed by pX-GP46A in *L. amazonensis* were 20- to 100-fold higher than in *L. major* (per gene copy). Assuming that most *L. amazonensis* GP46 genes (20–24 copies per diploid cell) code for the major 46-kDa GP46 protein, the level of expression per gene copy of pX-GP46A in *L. amazonensis* is comparable to that arising from the endogenous genes.

Our data and previous work (5, 6) show that transcriptional signals in *Leishmania* are modular because when fragments spanning intertranscript regions are joined to inserted sequences, they continue to specify correct sites of 5′- and 3′-end formation. These findings are notable because consensus-sequence elements that mark RNA-processing sites in other organisms are generally cryptic in *Leishmania* and other trypanosomatids (9, 18, 19). Because pX remains isofunctional with the original amplified R region, the small pX-derived constructs narrow the search for functional genetic elements in the DHFR-TS gene region considerably, from the original 30-kb-amplified extrachromosomal region down to just 2.3 kb of *Leishmania* DNA. At least one promoter, two sites for trans-splicing and polyadenylylation, and a replication origin must reside in pX.

In summary, vectors such as pX open the way for the functional analysis of many *Leishmania* genes by transfection-based approaches. We have recently shown that pR-NEO, pX-βGAL, or pX-GP46A can be introduced with high efficiency and can direct protein synthesis in all four of the pathogenic species complexes of *Leishmania* (C.M.C., K. M. Otteman, T. McNeely, S. Turco, and S.M.B., unpublished work) and developed methods for modifying chromosomal genes by homologous gene targeting (20). In combination, these techniques will provide a powerful set of tools for probing a broad range of parasitological questions.

We thank C. Cepko for providing the β-galactosidase casette and D. Dobson, T. Kristie, and L. Panton for comments. This work was supported by National Institutes of Health Grants AI-21903 and AI-29646 to S.M.B. and AI-23004 to D.M.P.. J.H.L. is a Leukemia Society of America Special Fellow, and S.M.B. was a Burroughs–Wellcome Scholar in Molecular Parasitology.

1. Bellofatto, V. & Cross, G. A. M. (1989) *Science* **244,** 1167–1169.
2. Clayton, C. E. (1990) *Mol. Cell. Biol.* **10,** 3036–3047.
3. Rudenko, G., LeBlancq, S., Smith, J., Lee, M. G. S., Rattray, A. & van der Ploeg, L. H. T. (1990) *Mol. Cell. Biol.* **10,** 3492–3504.
4. Laban, A. & Wirth, D. F. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9119–9123.
5. Laban, A., Tobin, J. F., de Lafaille, M. A. C. & Wirth, D. F. (1990) *Nature (London)* **343,** 572–574.
6. Kapler, G. M., Coburn, C. M. & Beverley, S. M. (1990) *Mol. Cell. Biol.* **10,** 1084–1094.
7. Beverley, S. M., Ellenberger, T. E., Iovannisci, D. M., Kapler, G. M., Petrillo-Peixoto, M. & Sina, B. J. (1988) in *The Biology of Parasitism*, eds. Englund, P. T. & Sher, A. (Liss, New York), pp. 431–448.
8. Kapler, G. M. & Beverley, S. M. (1989) *Mol. Cell. Biol.* **9,** 3959–3972.
9. Kapler, G. M., Zhang, K. & Beverley, S. M. (1990) *Nucleic Acids Res.*, in press.
10. Lohman, K. L., Langer, P. J. & McMahon-Pratt, D. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8393–8397.
11. McMahon-Pratt, D. & David, J. R. (1982) *Mol. Biochem. Parasitol.* **6,** 317–327.
12. Champsi, J. & McMahon-Pratt, D. (1988) *Infect. Immunol.* **52,** 3272–3279.
13. Kahl, L. P. & McMahon-Pratt, D. (1987) *J. Immunol.* **138,** 1587–1595.
14. Ellenberger, T. E. & Beverley, S. M. (1987) *J. Biol. Chem.* **262,** 10053–10058.
15. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
16. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, ed. Nolan, C. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed.
17. Huang, P. L., Roberts, B. E., McMahon-Pratt, D., David, J. R. & Miller, J. S. (1984) *Mol. Cell. Biol.* **4,** 1372–1383.
18. Clayton, C. E. (1988) *Genet. Eng. (London)* **7,** 1–56.
19. Borst, P. (1986) *Annu. Rev. Biochem.* **55,** 701–732.
20. Cruz, A. & Beverley, S. M. (1990) *Nature (London)*, in press.