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Introduction of Three Independent Selection Markers in *Leishmania*

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ABSTRACT

The pLE2SCX vector was developed for the stable expression of exogenous genes in the protozoan parasite *Leishmania.* The pLE2SCX construct contains three independent selection markers: herpes simplex virus thymidine kinase (HSV-TK), cytosine deaminase (CD) and streptothericin acetyltransferase gene (sat) in multiple cloning site, flanking by 5' and 3' untranslated regions of the previously cloned *Leishmania major* hexa-binding protein gene. Selection was based on resistance to the nourseothericin (Ns) which corresponds to sat gene. The two negative selection; HSV-TK and CD genes, make the transformed cell sensitive to ganciclovir (GCV) and 5-fluorocytosine (5-FC).The vector was introduced into *Leishmania* promastigotes by electroporation and maintained as circular form. The selected transfectants were not grown on media with GCV or 5-FC. Using two drug sensitive selectable markers together on a vector is a novel strategy in gene cloning in *Leishmania*. This stable transfection vector has allowed the permanently expression of several different exogenous genes at the same time in *Leishmania.* *Iran. Biomed. J. 7 (1): 13-18, 2003*

Keywords: HSV-thymidine kinase (HSV-TK), Cytosine deaminase (CD), ganciclovir (GCV), 5-fluorocytosine (5-FC), nourseothericin (Ns)

INTRODUCTION

Protozoan parasites of the genus *Leishmania* are the etiologic agents of a spectrum of human disease collectively referred to leishmaniasis. *Leishmania* is transmitted via the bite of a blood-feeding insect vector and is endemic in many tropical and subtropical areas of the world [1]. During the 1980s, a variety of kinetoplastid genes were cloned and their functions inferred from homology with genes from other organisms, location of the corresponding proteins or expression in heterologous systems. Before the availability of DNA transfection methodology, we could not analyze the function of kinetoplastid genes within the organisms. Since then, it has become possible to create and complement mutants, to over-express foreign proteins in the parasites, to knock out genes and even to switch off essential functions [2]. The breakthrough in transfection technology started with the expression of reporter genes after electro-poration of parasites with circular vectors and then a wide spectrum of methods for functional gene analysis were developed. [3].

Herpes simplex thymidine kinase (HSV-TK) has been the target for drugs such as acyclovir and ganciclovir (GCV) which are acyclic guanosine analogues converted to their monophosphate forms by HSV-TK. Cellular kinases metabolize these into di- and tri-phosphates. The triphosphate forms inhibit DNA-alpha-polymerase [4] and are in-corporated into DNA causing chain termination [5].

Cytosine deaminase (CD) is found in a variety of bacteria and fungi. It functions in the salvage pathway during nutritional stress to deaminate cytosine to uracil [6]. Cells expressing these enzymes are sensitive to the drug 5-fluorocytosine (5-FC) which is deaminated to 5-fluorouracil (5-Fu). This metabolite is then acted by cellular enzymes to produce 5-fluorouridine, 5-triphosphate and 5-fluoro-2-deoxyuridine, 5-monophosphate, which interfere with DNA and RNA synthesis, and lead to cell death [7].

The procedures for transfection of *Leishmania* and other kinetoplastid protozoans have been developed by electroporation [3, 8-10]. This study describes the development of expression vector that contains independent positive and negative selectable markers for transfection of *Leishmania.* This construct could be used as autonomously

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replicating expression vector or integrational form with genomic DNA of *Leishmania*. In present study, we introduced circular form of this construct in *Leishmania* and obtained sensitivity to two drugs; GCV and 5-FC and also resistance to nourseothericin (Ns) antibiotic. These drug-sensitive strains could be exploited for vaccine development.

MATERIALS AND METHODS

Plasmid constructs. **I)** The thymidine kinase gene of herpes simplex type-1 virus was obtained from vector pET23d:75 (a kind gift from Dr. M. Black) by PCR amplification using the following primers: 5'-ACT AGT GGT ATG GCT TCG T-3' and 5'-TCT AGA TGT TTC AGT TAG C-3' that contain the SpeI and XbaI restriction sites, respectively (underlined). This gene is a derivative of the wild type HSV-TK gene that was identified in a screen following random mutagenesis of the putative nucleoside-binding region. The 1100-bp PCR product was digested by SpeI and HindIII, and then cloned into pGEM9zf resulted in plasmid called "pGEMtk75" (Fig. 1A).The intergenic region of the α-tubulin gene of *Leishmania* contained sequences required for gene expression in *Leishmania* [11, 12]. The intergenic region between repeated genes contains the correct RNA processing signals for polyadenylation of upstream gene and a splice leader addition to the downstream gene [13, 14]. This region was chosen because it was highly expressed in both *Leishmania* life stages [14]. The intergenic α-tubulin region was obtained by PCR amplification on *L. major* genomic DNA using primers based on the published DNA sequence of *L. donovani* α-tubulin [12]. The upstream primer 5'-CGC AAG CTT GGT ACA CTC GTG CCG CGC -3' and downstream primer 5'-TCT AGA GGC TGA AAA AGA AGA AAG AGG GG –3' that contain the HindIII and XbaI restriction sites, respectively (underlined) were used for PCR amplification (Fig. 2). The 900-bp PCR product of *L. major* α-tubulin intergenic region was cloned at downstream of HSV-TK gene in pGEMtk75 and named "pGEMtk-tu"(Fig. 1B).

The 5'-UTR of *L. major* hexa-binding protein (HEXBP)gene [14] was cloned in SpeI site, upstream of HSV-TK in pGEMtk-tu and resulted in plasmid named "pGEM (5'-utr-tk-tu)" (Fig. 1C).

II) CD gene was the second suicide gene or negative selectable marker for our gene cassette. *Saccharomyces cervisiaea* CD gene was obtained by PCR amplification from yeast genomic DNA using primers based on the published DNA sequence [6, 15]. The primers 5'-GCG ACT AGT TAC CAC TGT CCC CCT TAC CGT and 5'-GCG AAG CTT GAT GAG TGG TTA TAG AAG TTT were used for amplification. The amplification conditions were as follow: 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C and the last cycle 1 min at 94°C, 2 min 55°C and 7 min 72°C. The 500-bp PCR product of the CD gene, with SpeI/HindIII restriction sites (underlined), was cloned in upstream of α-tubulin intergenic region in pGEM-tu (Fig. 1D). In the pBSK(-) plasmid, Ns resistance gene*;* strepthotricin acetyltransferase (sat) [15] was cloned in SpeI/EcorV and then 3'-UTR of *L. major* HEXBP gene [14] was subcloned downstream of sat gene in EcorV/SalI restriction sites. The fragment contained CD and α-tubulin intergenic region exised by SpeI/XbaI enzymes from pGEM(cd-tu) and subsequently subcloned at the upstream of sat gene in pBSK (-). This plasmid named pBSK-(cd-tu-sat-3'utr) (Fig. 1E).

Development of pLE2SCX vector. The fragment containing; "5'-UTR-Tk-α-tubulin" from pGEM-(5'utr-tk-tu) was digested by SpeI/XbaI and subcloned at upstream of CD gene in SpeI site in pBSK (cd-tu-sat-3'utr). The resulted plasmid called pLE2SCX (tk-cd), that is a novel construct and carries 3 independent selection markers (Fig. 3). The total size of our vector (inserts and pBSK vector) is 9.8 kb.

Sequencing of construct. The 5' end of each gene in each construct was sequenced to define in-frame sequence by Sanger method [16] and was done using oligonucleotides matching to the upstream and downstream of each gene. The final construct also was sequenced using pBSK (M13) primers.

Cell line, culture and transfection. *L. major* MHOR/IR/76 strain was obtained from Razi Institute (Iran). The strain was grown in M199 medium (Gibco, BRL, Germany) supplemented with 10% FCS [Life Technology Inc.] and 50 IU/ml of penicillin and 50 µg/ml of streptomycin. Log phase promastigotes were transfected by electro-poration and cultured on semi-solid M199 medium supplemented with Ns (Clonat Co., Germany) [9]. For transfection we used Bio-Rad electroporation unit with 0.2 cm cuvette. Cells resuspended in 200 µl electroporation buffer and the pLE2SCX construct added to cells before transfection [10].

Colonies appeared on plates after 5 to 10 days and then transferred to fresh media with Ns as regular culturing.

Drug sensitivity assay. To measure the cytotoxicity of GCV and 5-FC to the transfected *L. major* promastigotes, the MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide: thiazol blue) colorimetric assay was used [17]. This assay involves the conversion of MTT by living cells to formazan, the concentration of that can be measured spectrophotometrically. Promas-tigotes were grown at a concentration of 5×10^5/ml. Cells were seeded (10^4/well) into 96-well plates (Costar, Corning) and incubated in the presence of different concentrations of GCV and 5-FC. After 72 h incubation at 27˚C, 10 µl of MTT (5 mg/ml) was added to wells and cells were further incubated at 37°C for 4 h. Following incubation, the medium was removed and the converted dye (formazan) was solubilized with 150 µl of acidic isopropanol (0.1 N HCl in absolute isopropanol). The absorbance of the converted dye was measured at 570 nm, with background subtraction at 630-690 nm.

RESULTS AND DISCUSSION

As described in materials and methods, development of final construct was done in sequential stages and all resulted plasmids have been shown in Figure 1. Construct pLE2SCX vector obtained by integration of the fragment "5'-UTR-tk-α-tubulin" (Fig. 1C) at upstream of CD gene in pBSK (Fig. 1E).

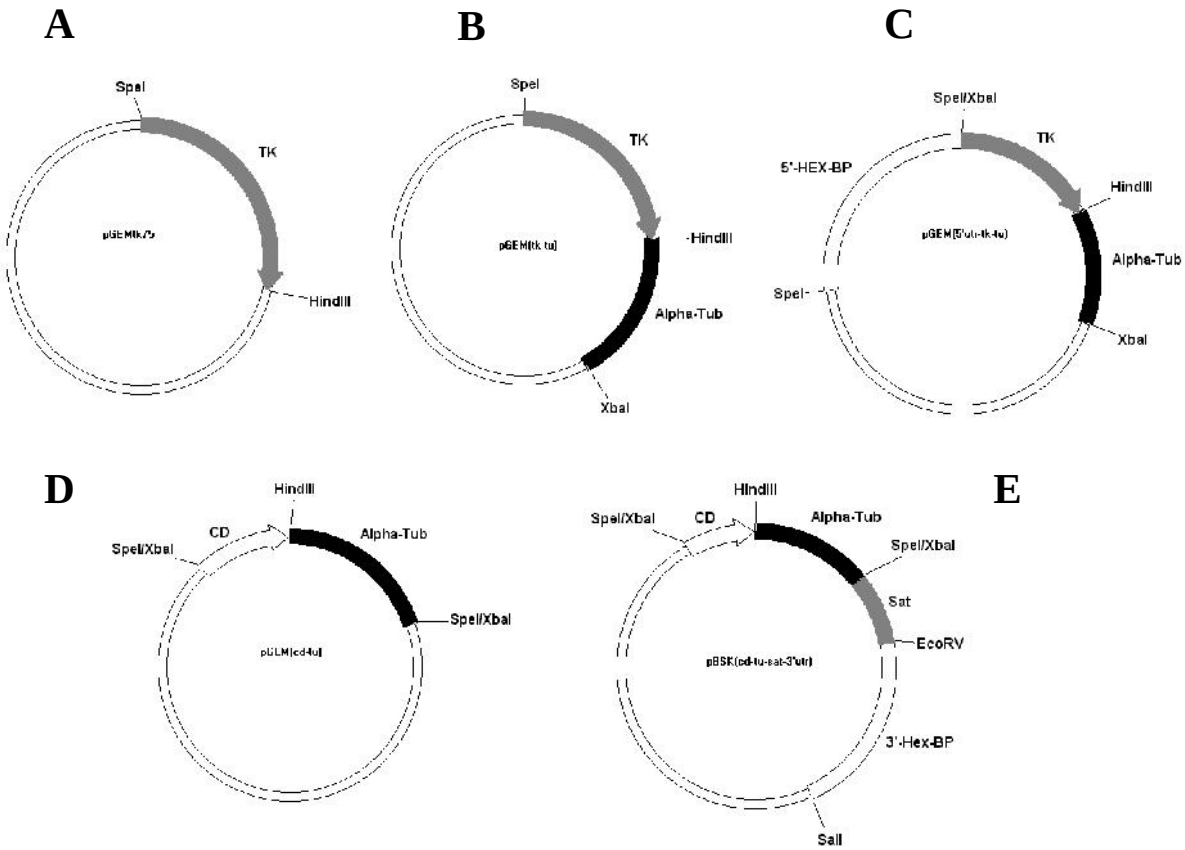

Fig. 1. Schematic diagram of developed constructs for generating pLE2SCX expression vector. **(A)**, pGEM9zf was digested and TK gene cloned in SpeI/HindIII site; **(B)**, an intergenic region from the *L. major* α –tubulin, tandem array was amplified by PCR. The 850-bp PCR product was cloned in pUC19 and digested by XbaI/HindIII (not shown), the digested fragment was subcloned in downstream of TK, to generate pGEM(tk-tu); **(C)**, the 5'-UTR of HEXBP (white frag.) was cloned at upstream of TK gene to make pGEM(5'-utr-tk-tu); **(D)**, in another pGEM9zf plasmid the CD and α-tubulin genes were cloned in indicated sites; **(E)**, The Ns^R gene (sat) obtained from pLEXsat by digestion and subcloned in pBSK(-). Then, 3'-UTR of HEXBP was cloned at downstream of sat gene. The (cd-α-tubulin) fragment digested by SpeI/XbaI and subcloned at upstream of sat gene in pBSK(D) to generate pBSK(cd-tu-sat-3'utr).

In our construct, the two drug sensitivity genes (TK and CD) are flanked by specific *L. major* sequence corresponding to the 900-bp intergenic region of the α-tubulin gene (Fig. 2). As shown previously in *Leishmania*, such intergenic fragments from loci composed of tandemly repeated genes provide sufficient information required for polyadenylation and trans-splicing for expression of the upstream and the downstream genes [13, 18].

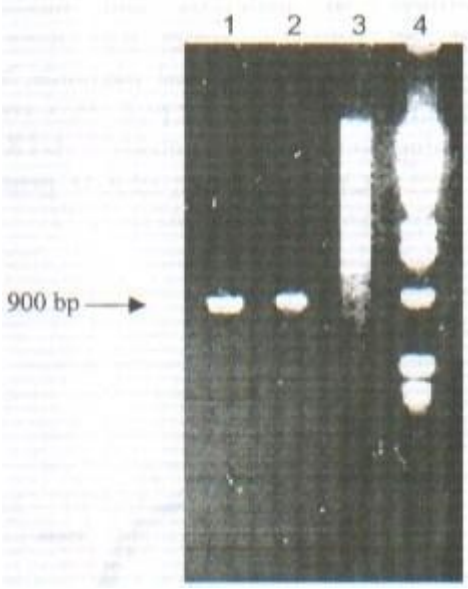

Fig. 2. PCR amplification of α-tubulin intergenic region of *L. major*. Lane 1 and 2, PCR product of α-tubulin intergenic region (900 bp band); Lane 3, negative control; Lane 4, 1 kb marker.

Better expression can often be obtained if the reporter gene is followed by a 3'-untranslated regions (3'-UTR) from highly expressed gene. Because polyadenylation and trans-splicing are coupled, the 3'-UTR should be followed by a second trans-splicing signal [12]. Promoters and RNA processing signals from higher eukaryotes do not function at all in trypanosomatids. Processing and expression are most efficient when 5'- and 3'-segments from the homologous species are used [2]. To demonstrate the expression of exogenous genes in *Leishmania*, the final construct (Fig. 3) used for transfection, the transfectants were plated onto media containing Ns. The growth rate of the transfectants was not inhibited at the highest concentration of Ns (400 μg/ml) and therefore it may be possible to attain higher levels of expression from pLE2SCX construct by further increasing the concentration of selective drug in the growth media [15]. This observation implies that pLE2SCX vector is maintained as circular molecules. Similar increase in copy number-linked expression levels has been observed for pLEX [15] and for the pX and pTEX kinetoplastid expression vector constructs [10, 19].

In order to determine whether the HSV-TK/GCV and CD/5-FC [20-22] negative selection systems inhibit the growth of promastigotes, the transfectants and mock transfected cells (electroporated in the absence of added DNA) were expanded in media with each drug (ganciclovir and 5-FC). Transfected promastigotes did not grow on solid M199 media containing 50 μM ganciclovir or 40 μM 5-FC. This shows that the TK, CD and sat genes, were expressed in *L. major* transfectants and all were sensitive to GCV and 5-FC compared to the control lacking the pLE2SCX.

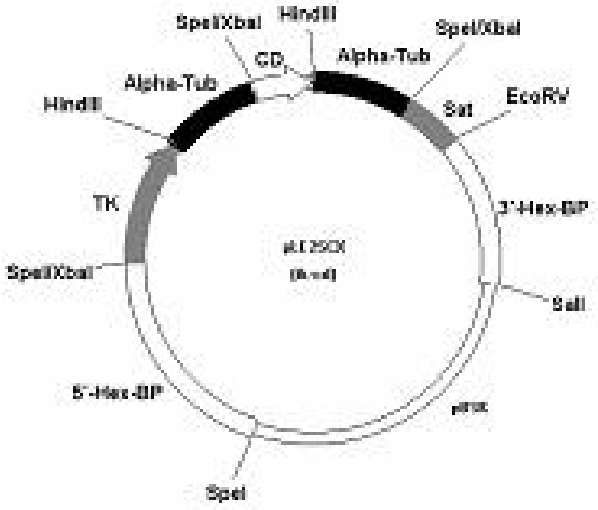

Fig. 3. Schematic diagram of pLE2SCX vector. The pBSK vector backbone is shown in narrow white, the HEXBP5' and 3' UTR are in wide white, the α -tubulin intergenic region is black and the negative selectable marker genes are shown as stippled(TK) and white (CD) arrows, the sat positive marker gene is stippled. Promastigotes were transfected with circular pLE2SCX construct by electroporation exactly as previously described (2.25Kv/cm, 500 μF [9]).

Cytotoxic effect of GCV and 5-FC on L. major promastigotes expressing the HSV-TK and CD genes. To test whether the GCV or 5-FC affects transfected promastigotes, we used MTT assay (described in Materials and Methods). Cells were cultured on 96-well plates in the presence of different concentration of each drug. Following GCV treatment for 72 h a non-toxic dose for the cells (35 μM), the growth of promastigotes was inhibited and caused cell death (with estimated EC_{50} of 25 μM) (Fig. 4). To test the cytotoxicity of 5-FC, different concentrations of drug were used.

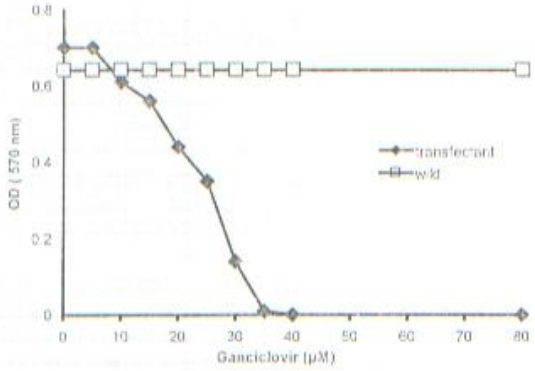

Fig. 4. The cytotoxic effect of ganciclovir on *L. major* promastigotes; transfectant and wild type. Promastigotes were grown in the presence of various concentrations of ganciclovir. After 72 h of incubation, the number of viable cells was obtained by measuring the absorbance at 570 nm (MTT assay).

The 30 μM of 5-FC caused cell death in culture media (two fold the EC_{50}) (Fig. 5). The controls, mock-trans-fected promastigotes, were not affected.

LeBowitz *et al.* [21] and Muyombwe *et al.* [22] described the expression of the HSV-TK gene in *L. major* for the creation of GCV-sensitive strains. We showed that, under control of α-tubulin intergenic region of *L. major* the HSV-TK and CD genes could be expressed. As expected, the expression of the HSV-TK and yeast CD genes in transfected promastigotes resulted in sensitivity to nucleoside analogues, such as the anti-herpes drug GCV[22] and the anti-fungi drug 5-FC [20]. However, because of using mutant HSV-TK that is 40-fold more sensitive than native TK gene, the sensitivity of our strain is more than previous reports.

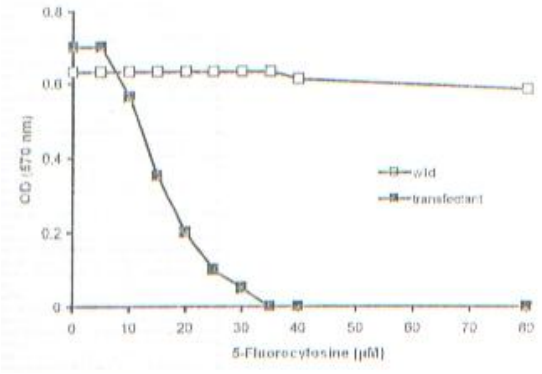

Fig. 5. MTT colorimetric assay for evaluation of drug toxicity on promastigotes. Transfected, and wild type *L. major* were seeded on 96-well plates (5×10^4 cells/ml) and incubated with 5-FC at different concentrations. After 72 h of incubation, the number of parasites was obtained by measuring at 570 nm. The curve shown here is the result of one experiment which has been repeated four times with essentially identical values.

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