Simultaneous transient expression assays of the trypanosomatid parasite *Leishmania* using $\beta$-galactosidase and $\beta$-glucuronidase as reporter enzymes

(Recombinant DNA; protozoan parasite; DNA transfection; Kinetoplastida)

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**SUMMARY**

We describe a transient transfection protocol for cultured *Leishmania major* promastigotes, utilizing *Escherichia coli* genes encoding $\beta$-galactosidase and $\beta$-glucuronidase inserted into an expression vector derived from the dihydrofolate reductase-thymidylate synthase locus. Less than 0.1 pg of either reporter enzyme can be detected with a simple fluorimetric assay, and transfection of 10 pg of either reporter construct yields activities at least 100-fold over background. Simultaneous introduction of both constructs showed that the activity of each reporter gene was unaffected by the presence of the other, allowing one reporter construct to serve as a control for experimental variability in test gene constructs containing the second reporter gene. These results show that it is feasible to apply transient expression assays to the identification of *cis*-acting elements of genes encoding nonabundant mRNAs in the genus *Leishmania*.

**INTRODUCTION**

Recently, methods for both transient and stable DNA transfection have been developed for trypanosomatid protozoans such as *Leptomonas seymouri* (Bellofatto and Cross, 1989), six species of *Leishmania* (Laban and Wirth, 1989; Kapler et al., 1990; Cruz and Beverley, 1990; Laban et al., 1990; LeBowitz et al., 1990; Coburn et al., 1991) and *Trypanosoma brucei* (Ten Asbroek et al., 1990; Clayton et al., 1990; Rudenko et al., 1990). The use of transient expression assays will greatly facilitate the analysis of many of the unusual aspects of gene expression in these organisms, such as trans-splicing and promoters mediating polycistronic transcription (Clayton, 1988). For transient expression assays the reporter gene encoding CAT has been widely utilized, as there is no endogenous CAT activity in these protozoans and there are several sensitive methods that allow assay of as little as 1 pg of CAT (Seed and Sheen, 1988).

It is often desirable to utilize more than one reporter gene, for example, to serve as a control of experimental variability. In other organisms, $\beta$Gal and Gus have been utilized as reporter enzymes, since cassettes encoding these enzymes are commonly available and sensitive fluorimetric assay methods have been developed (Braell, 1987; Shapira et al., 1983; Jefferson et al., 1986; Schmitz et al., 1990). We tested these genes in transient expression assays following their insertion into a *Leishmania* expression vector, pX (Fig. 1;
LeBowitz et al., 1990). Plasmid pX is derived from the region encoding DHFR-TS of *Leishmania major* and contains a selectable G418-resistance marker. Constructs bearing the *E. coli lacZ* gene inserted into the expression site of pX (pX-βGAL) have been used to develop stably transfected lines expressing high levels of βGal (LeBowitz et al., 1990; Coburn et al., 1991). In these lines, pX-βGAL transcripts were shown to use *Leishmania* sequences specifying the formation of mRNAs by 5' trans-splicing and 3' polyadenylation (LeBowitz et al., 1990; Coburn et al., 1991).

Transient expression assays of other trypanosomatid genes have used constructs derived from genes encoding abundant RNAs, such as tubulin or PARP. In contrast, the *Leishmania* sequences within pX originated from the DHFR-TS-encoding gene, which normally encodes non-abundant mRNAs (Washtien et al., 1985; Kapler et al., 1987; Kapler and Beverley, 1989). It was therefore of interest to test whether these constructs would generate sufficient activity to allow detection in transient expression assays.

**RESULTS AND DISCUSSION**

(a) Transient expression of βGal and Gus

We tested pX-βGAL (Fig. 1, top; LeBowitz et al., 1990) in transient expression assays, using electroporation as the method for introducing DNA. Significant βGal activity was obtained, increasing linearly with increasing amounts of transfected pX-βGAL DNA (Fig. 2A). In this experiment about 300 F.U. were obtained per µg DNA, while untransfected control cells revealed little if any endogenous βGal activity, about 10 F.U. The low background allowed the detection of activity from as little as 1 µg of DNA (Fig. 2A).

The gene encoding Gus was inserted into the appropriate expression site of a modified version of pX differing only in the bacterial vector sequences (pX63NEO), yielding the plasmid pX63NEO-GUS (Fig. 1, bottom). Enzymatic assay of control untransfected *Leishmania* revealed little Gus activity (50 F.U.; Fig. 2B). Transfection with pX63NEO-GUS yielded significant Gus activity in transient assays, increasing linearly with increasing DNA (Fig. 2B). In this experiment about 210 F.U. per µg DNA were obtained, again allowing the detection of as little as 1 µg of DNA (Fig. 2B).

(b) Effect of experimental parameters: method of cell lysis, length of enzyme assay, carrier DNA

Two methods for lysing transfected *Leishmania* were evaluated for their convenience and ability to yield maximal enzyme levels: sonication or addition of detergent (0.1% SDS). The levels of βGal obtained were the same with these two methods (Table I). Gus activity was 22% less with the SDS lysis method than with sonication (Table I). However, extracts made by this method showed Gus activity that was linear with respect to the length of assay (Fig. 3B), showing that the presence of detergent did not lead to enzyme instability. The SDS lysis method is more convenient when it is necessary to assay many samples simultaneously, and was utilized in most of the studies in this work.

We next examined the effect of carrier DNA. First, varying amounts of pX-βGAL were transfected in the presence or absence of 20 µg of sonicated salmon sperm DNA. Only a 30% increase in βGal activity was obtained (Fig. 2A). Second, varying amounts of pX63NEO-GUS were transfected with sufficient salmon sperm DNA to maintain a constant amount of total DNA (60 µg). Only a
Fig. 2. Transient assay of βGal and Gus in Leishmania. L. major strain 252 (clone CC-1) was grown in M199 medium as described (Kapler et al., 1990). 4 x 10⁷ cells were electroporated in the presence of the indicated quantities of DNA using 2.25 kV/cm and the 500 μF capacitance setting of the BioRad Gene Pulser unit (Kapler et al., 1990). Varying amounts of reporter construct were electroporated in the presence or absence of sheared salmon sperm (carrier) DNA; for pX-βGAL, 20 μg of carrier DNA was included in all experiments (A), while for pX-63NEO-GUS the total amount of DNA was adjusted to 60 ng with carrier DNA (B). Following electroporation, cells were added to 10 ml M199 medium and allowed to grow for 20 h at 26°C. Cells were collected by centrifugation (1300 x g, 10 min), resuspended in 1.4 ml Hanks balanced salt solution and pelleted in a microcentrifuge (1 min). The washed pellets were suspended in 80 μl of buffer containing protease inhibitors, TPI buffer. Resuspended cells were lysed by addition of SDS to a final concentration of 0.1%. The cells were then mixed vigorously, incubated on ice for 15 min, and centrifuged at 4°C for 15 min in a microfuge. 80 μl of the supernatant were assayed for βGal (A) or Gus (B) activity by adding 320 μl of enzyme buffer (23 mM Tris·HCl pH 7.5/125 mM NaCl/2 mM MgCl₂/12 mM β-mercaptoethanol) containing either 0.3 mM 4-methylumbelliferyl β-D-galactoside (A) or 1 mM 4-methylumbelliferyl β-D-glucuronide (B). After mixing, the reactions were incubated at 37°C for 5 h. Reactions were terminated by the addition of 2 ml of 133 mM glycine/83 mM Na₂CO₃, pH 10.7. Fluorescence of the 4-methylumbelliferone product was measured in a Hoefer TKO 100 minifluorometer and is shown on the Y axis for βGal (A) and Gus (B); in these assays, 120 F.U. corresponds to 1 pmol product in a 2.4 ml reaction. 1 unit is defined as 1 μmol product formed/min at 37°C. The inset in B shows an expanded view of F.U. for the lower DNA amounts.

(c) Simultaneous transfection with both reporter genes

To ensure that the constructs expressing βGal and Gus functioned independently when introduced together in transient expression assays, we performed several experiments transfecting with the two reporter constructs simultaneously. Since the buffer utilized to assay these enzymes is the same, one cell extract was prepared and then divided for separate assay of the two activities.

*Leishmania* transiently transfected with pX-βGAL exhibited Gus activity comparable to that obtained in untransfected control cells (49 vs. 41 F.U.), much less than the 7000 F.U. obtained following transfection with pX63NEO-GUS (Table I). Transient assays of *Leishmania* transfected with pX63NEO-GUS revealed a low but significant level of βGal activity, about 5-times control values (128 vs 28 F.U.); this value was only 2% of the βGal activity obtained with comparable amounts of pX-βGAL (4800 F.U.; Table I). When pX-βGAL and pX63NEO-GUS were introduced simultaneously, the activities of βGal and Gus were similar to those obtained when the two plasmids were introduced separately (Table I). These experiments showed little cross-activity in simultaneous transient expression assays.

Homologous recombination involving transfected DNAs has been shown in two studies in *Leishmania* (Cruz and Beverley, 1990; Tobin et al., 1991). With an active recombination system, recombination between cotransfected constructs could restore activity to an otherwise nonfunctional test plasmid. To test this possibility, we utilized a test plasmid which in transient expression assays produces only 3% as much βGal activity as pX-βGAL, p67βGALΔMlu
### TABLE I
Cotransfection of two reporter genes

<table>
<thead>
<tr>
<th>Electroporated DNA</th>
<th>Lysis method</th>
<th>Enzyme activity (F.U.)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>βGal</th>
<th>Gus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<tr>
<td>Expt. 1:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pX63NEO-GUS + pX-βGAL</td>
<td>Sonication</td>
<td>4800 ± 20</td>
<td>7700 ± 120</td>
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<td>pX63NEO-GUS + pX-βGAL</td>
<td>SDS</td>
<td>5000 ± 170</td>
<td>6000 ± 350</td>
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<td>Expt. 2:</td>
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<td></td>
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<tr>
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<td>SDS</td>
<td>130 ± 8</td>
<td>7000 ± 45</td>
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<tr>
<td>pX63NEO-GUS + pX-βGAL</td>
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<td>4900 ± 370</td>
<td>7500 ± 30</td>
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<td>pX-βGAL</td>
<td>SDS</td>
<td>4800 ± 60</td>
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<td>pX67/βGALΔMlu</td>
<td>SDS</td>
<td>180 ± 20</td>
<td>40 ± 4</td>
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<td>pX67/βGALΔMlu + pX63NEO-GUS</td>
<td>SDS</td>
<td>320 ± 15</td>
<td>6500 ± 760</td>
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<td>no DNA</td>
<td>SDS</td>
<td>28 ± 2</td>
<td>41 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Plasmids pX-βGAL, and pX63NEO-GUS are described in Fig. 1. Plasmid pX67/βGALΔMlu is described in section c.

<sup>b</sup> L. major cells were transfected with 10 μg of the indicated plasmids, collected and assayed for the enzymatic activity as described in the legend to Fig. 2, except that 160 μl of TPI buffer was utilized to resuspend the cell pellets; 80 μl of the resulting extract was assayed for each enzyme. When only one construct was transfected 10 μg of carrier DNA was included to maintain a constant total DNA amount. SDS lysis was performed as described in the legend to Fig. 2. For sonication, the SDS addition step was omitted. Instead, a microcentrifuge tube containing 80 μl of TPI was placed in a cup horn sonicator filled with ice water (Branson sonifier, model 250) and sonicated for 30 s (constant duty cycle, setting 9). Each assay was performed in duplicate.

![Fig. 3](image_url)

**Fig. 3.** Time dependence of βGal (panel A) and Gus (panel B) activities in transient assays. Untransfected L. major (■, □) or cells cotransfected with 10 μg of pX βGAL and pX63NEO-GUS (▲, △) were examined. Cells were lysed with SDS in 160 μl TPI buffer, and 80 μl aliquots were assayed separately for the two enzyme activities as described in the legend to Fig. 2.

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**d) Comparison with other reporter genes**

These studies show that βGal and Gus are sensitive reporter enzymes for transient expression assays following DNA transfection of *Leishmania*. For both constructs, as little as 1 μg can be detected while 10 μg yields activity at least 100-fold over background. These two reporter enzymes are thus comparable to the more commonly utilized CAT enzyme. However, both βGal and Gus have several advantages, including increased sensitivity. It is
possible to measure less than 0.1 pg βGal in a 5 h assay (assuming a specific activity of 600 units βGal/mg βGal). The substrates are not radioactive and are relatively inexpensive, the same cell extract can be utilized for both assays, and quantitation is simple. Both enzymes are stable, allowing the use of detergent lysis methods to rapidly and conveniently prepare multiple samples. Finally, for both enzymes several histochemical stains are available, which may allow quantitation of enzyme activity at the cellular level in the future as reported previously for cultured mammalian cells (Nolan et al., 1988).

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REFERENCES


