Dominant Positive and Negative Selection Using Luciferase, Green Fluorescent Protein and β-Galactosidase Reporter Gene Fusions

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The introduction of exogenous DNA sequences into cultured cells is a cornerstone in the study of mammalian gene expression. For most cell types, the efficiency of stable gene transfer is usually quite low, which mandates the use of selectable markers to isolate a population of transfected cells with the desired phenotype. In simple gene expression experiments, only one type of selectable marker is required. Positive selectable markers such as the bacterial neo and hph genes facilitate the direct selection for cells that are resistant to the cytotoxic effects of the antibiotics, G418 and hygromycin B, respectively (1,12). Conversely, negative selectable markers facilitate the elimination of the cells in which they are expressed.

The most popular negative selectable marker is the herpes simplex virus thymidine kinase gene, tk, which sensitizes cells to the toxic effects of the nucleoside analogue gancyclovir (11,15). In more complex gene transfer experiments such as in the use of gene, promoter or enhancer traps, it becomes necessary to include multiple marker genes. For example, fusions between the bacterial lacZ reporter gene and the selectable neo marker (2), the hph and tk selectable markers (7) or the neo and tk selectable markers have been developed for use in enhancer or gene trapping experiments. Here, we describe the further development of gene fusions between the tk and neo selectable markers and the bacterial lacZ, jellyfish gfp and firefly luc reporter genes.

The gene fusions were constructed using cDNAs derived from the previously described pPNT (14), pβgeo (2), pGL3-Control (Promega, Madison, WI, USA), and pEGFP-C1 (Clontech Laboratories, Palo Alto, CA, USA) vector constructs, which encode the HSV tk and bacterial neo genes, a lacZ-neo fusion gene, the enhanced luc and gfp reporter genes, respectively. A tkneo gene fusion was generated using PCR amplification in conjunction with vector-specific primers to eliminate the endogenous stop codon in the tk gene of pPNT through the incorporation of a BamHI site. The amplified product was cloned as an EcoRI-BamHI fragment into the corresponding EcoRI-BamHI sites of pβgeo, which effectively replaces the lacZ-portion of the lacZ-neo gene fusion. The tkneo fusion gene was then subcloned as a HindIII-NotI fragment into the pEFG3 expression vector, and this construct was designated pEF-tkneo (Figure 1). We incorporated the gfp cDNA by subcloning the corresponding reading frame as a blunt-ended NheI-BglII fragment from pEGFP-C1 into the blunt-ended BamHI site to generate pEF-tkgfneo.

The tklucneo gene fusion was generated using PCR amplification in conjunction with vector-specific primers to introduce PacI restriction endonuclease sites into the 5′-UTR and to eliminate the endogenous stop codon in the luc gene of pGL3-Control, which was then subcloned into the BamHI site of pEF-tkneo to generate pEF-tkβgeo (Figure 1). The lacZ gene was incorporated into the tkneo gene fusion by subcloning the corresponding reading frame as a BamHI fragment from pβgeo into the BamHI site of pEF-tkneo to generate pEF-tkβgeo (Figure 1). The gfp cDNA was incorporated into the BamHI site of this construct to generate pEF-TβGN (Figure 1). As positive control plasmids for our experiments, we subcloned the individual luc, gfp and lacZ genes into the pEF1 vector (Invitrogen, Carlsbad, CA, USA) to generate pEF-Luc, pEF-GFP and pEF-βGal, respectively (Figure 1).

The activity of the reporter gene components in each of the five fusion genes was assayed following transient transfection into HEK293 (human embryonic kidney) cells. Cells were grown in DMEM supplemented with...
Benchmarks

Table 1. Analysis of Selectable Reporter Gene Fusions in HEK293 Cells

<table>
<thead>
<tr>
<th>Construct</th>
<th>G418 Colonies&lt;sup&gt;a&lt;/sup&gt;</th>
<th>G418&lt;sup&gt;R&lt;/sup&gt; Colonies&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Relative β-gal Activity&lt;sup&gt;b,c&lt;/sup&gt;</th>
<th>Relative Luciferase Activity&lt;sup&gt;b,c&lt;/sup&gt;</th>
<th>Relative GFP Activity&lt;sup&gt;b,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEF-GFP</td>
<td>220</td>
<td>150</td>
<td>1.2</td>
<td>-</td>
<td>720</td>
</tr>
<tr>
<td>pEF-βGal</td>
<td>164</td>
<td>147</td>
<td>180</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>pEF-tkneo</td>
<td>286</td>
<td>0</td>
<td>0.7</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>pEF-tkgeo</td>
<td>174</td>
<td>0</td>
<td>200</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>pEF-tkgfneo</td>
<td>187</td>
<td>0</td>
<td>0.6</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>pEF-TβGN</td>
<td>185</td>
<td>0</td>
<td>103</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>pEF-Luc</td>
<td>118</td>
<td>64</td>
<td>-</td>
<td>920</td>
<td>-</td>
</tr>
<tr>
<td>pEF-tkno</td>
<td>98</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>pEF-tklucneo</td>
<td>63</td>
<td>1</td>
<td>-</td>
<td>52</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Independent colonies per P100 culture dish.

<sup>b</sup>Cells were washed once in PBS, harvested, and lysed using a proprietary detergent formulation according to manufacturer’s protocols (Reporters Lysis Buffer, Promega, Madison WI, USA). The luciferase and β-gal activities in the lysates were determined by assaying duplicate dilutions using commercially available luminescent (Luciferase Assay System, Promega) and chemiluminescent (Galacto-Light Plus, Tropix, Bedford, MA, USA) substrates. Light yield from the reactions was detected using a tube format luminometer with autoinjector (Lumat LB9507, EG&G Wallac, Gaithersburg, MD, USA). The GFP in the lysates was determined by assaying duplicate dilutions directly in a fluorescent plate reader using an FITC filter set (Fluorescence Concentration Analyzer, IDEXX Laboratories, Westbrook, ME, USA).

<sup>c</sup>Corrected for differences in transfection efficiency by including 5 ng of either pGL3-Control (top series) or pCMVβ (bottom series) (Clontech Laboratories) in each reaction.

<sup>d</sup>not done.

controls, indicating that the neo component of each of the fusion proteins retained its function (Table 1). With the exception of a single “breakthrough”, the transfectants selected in G418 and gancyclovir yielded no colonies for any of the fusion genes. Because there was no effect on the number of colonies obtained in the pEF-Luc, pEF-GFP and pEF-βGal control transfections that do not express tk, this indicates that the tk component of each of the fusion proteins also retained its function (Table 1).

The stable clones were assayed for β-gal activity by histochemical staining, which showed that the reporter gene activity of each fusion construct was similarly retained in all of the clones examined (Figure 2). However, only about 10% of the clones examined expressed detectable green fluorescent protein (GFP). This finding is consistent with our previous data from the transient assays for GFP, which suggests that although every clone may express the fusion gene, the reduced GFP signal will only be detectable in those that express it at high levels. Interestingly, the subcellular localization of the fusion proteins differs somewhat, with the tkgfneo fusion yielding a diffuse cytoplasmic signal similar to the non-fusion GFP control, and the TβGN fusion yielding an intense punctate signal suggestive of incorporation into inclusion bodies (Figure 2 and References 3 and 13). Although expression levels are similar between the clones, GFP from the TβGN fusion is easier to detect because of a significant enhancement of signal intensity.

The selectable reporter fusion genes we have developed are well suited to serve as qualitative and quantitative probes of promoter or enhancer strength. They also provide a simple mechanism to eliminate false positive clones in gene, promoter, and enhancer trapping strategies (2) by virtue of their ability to be both positively and negatively selected. In a related application, we are currently using a “floxed” version of the tklucneo fusion gene as a selectable marker in conjunction with the tetracycline-regulated transactivator expression system (4–6), where it has proven useful in the identification of stable transfectants that have a high-level of “on” and low-level “off”
expression following modulation of promoter activity by doxycycline (manuscript in preparation).

REFERENCES


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