High-Throughput Inducible Expression of Transgenes at the Hprt Gene in Mouse Embryonic Stem Cells

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Overexpression and/or ectopic expression of gene products has led to important discoveries about gene product function; unfortunately, the expression of transgenes has been difficult and unreliable because of our poor understanding of transcription. Pronuclear injection has been the most common method to introduce transgenes into mouse embryos; however, the problems that arise from pronuclear injection are well described (1). First, chromosomal deletions, inversions, and duplications are frequently generated with transgene integration. These alterations could potentially mutate nearby genes, resulting in an unwanted phenotype that complicates analysis of the transgene product (2). Second, variable levels and patterns of transgene expression are observed between founder mice due to concatemerization of the transgene and chromatin position effects (3). Third, mosaic expression of the transgene may occur because of position-effect variegation (4,5), methylation (6), or intrachromosomal recombination within the transgene concatamer (7). Transgenic mice derived from multiple founders must be analyzed to control for these variables, substantially increasing the resources devoted to an experiment. Transgenes can also be introduced directly into cells or mouse embryos by a variety of techniques including retroviral and adenoviral-mediated gene transfer and DNA:lipopolyamine-mediated gene transfer. However, these procedures still present many of the same problems as pronuclear injection, in particular, sustained expression of the transgene.

To address these problems, a procedure has been developed termed targeted transgenesis (8). This procedure introduces a single copy of a transgene into a known location and relies on manipulation of embryonic stem (ES) cells in tissue culture. Different methods to achieve targeted transgenesis have been developed that include targeting a transgene to the hypoxanthine phosphoribosyltransferase (Hprt) gene (9) and to other loci, as reviewed by Jasim et al. (8). Hprt was chosen because it is a housekeeping gene, expressed in all cells, and functions in the purine salvage pathway. Thus, chromatin effects are likely minimal. In addition, Hprt is on the X chromosome and therefore is hemizygous in male cells. Disruption of Hprt can be selected in 6-thioguanine (TG) media and expression of Hprt can be selected in hypoxanthine-aminopterin-thymidine (HAT) media. This selection strategy enables high-throughput transgenesis by gene targeting. The fact that all transgenes are positioned at the exact same location in the genome controls for unknown variables of position effect that accompany random integration.

Previously, we have targeted transgenes to Hprt in mouse ES cells; this approach mutated the Hprt gene (9). Our data showed that a reporter was expressed in greater than 85% of Hprt-mutant ES cell clones. In addition, another reporter exhibited the correct temporal and tissue-specific expression pattern of the promoter in mouse embryos. Thus, targeted transgenesis was successful at the Hprt gene.

Here our goal is to establish a high-throughput method for inducible transgene expression in cells and mice with the tetracycline responsive system (10). The tet-responsive activator system is composed of the tet repressor fused to the activation domain of virion protein 16 (VP16) of herpes simplex virus. This transactivator, called tTA, stimulates transcription of a minimal promoter fused to tet operator sequences (tetO). The minimal promoter is the minimal immediate early promoter of the cytomegalovirus (mCMV) juxtaposed to the tetO sequences (tetO-mCMV). Inducible expression is achieved by the use of a derivative of tetracycline called doxycycline, which binds and inactivates tTA. Thus, tTA activates transcription, and doxycycline prohibits this activation.

A luciferase reporter (11), under the regulation of the tetO-CMV promoter, was tested for inducible expression when targeted to the Hprt gene. We
used recombinant luciferase, a 61-kDa monomeric protein from fireflies that catalyzes the oxidation of beetle luciferin with the concomitant production of light. Since luciferase is monomeric and does not require posttranslational modification, it serves as a reporter immediately after translation. In addition, luminescence decays rapidly so that accurate time points can be taken. The luciferase assay was performed using the Promega Luciferase Assay System (Promega, Madison, WI, USA).

The experiment was designed as follows. First, ES cells [Lex1 cells derived from 129SvEvBrd mice (Stratagene, La Jolla, CA, USA)] were deleted for the third exon of Hprt using a vector that replaces exon 3 with a neomycin phosphotransferase selection cassette (neo) flanked by an I-Sce1 endonuclease site (12) to generate G418R (selection for neo expression) + TGR clones (Figure 1A). Second, a single G418R + TGR clone that exhibited the correct targeting pattern by Southern analysis was transfected with pTet-Off (BD Biosciences Clontech, Palo Alto, CA, USA), a vector that contains the tTA expressed by the CMV promoter. In addition, a puromycin N-acetyltransferase selection cassette (puro) was co-transfected with pTet-Off, since pTet-Off contains neo for selection and the TGR clone is already resistant to G418. Puromycin-resistant clones were isolated.

Figure 1. Targeted transgenesis strategy. (A) First step: Generation of a clone deleted for Hprt exon 3. Hprt sequences from a HindIII site to an EcoRI site that include neo (an I-Sce1 endonuclease site flanks neo). As described previously (14), this targeting vector (10 µg) was linearized with BamHI (cuts at the edge of homology) and electroporated into ES cells (1 × 10^7 cells in 1 mL PBS at 575 V/cm, 500 µF with a Bio-Rad gene pulser II; Bio-Rad Laboratories, Hercules, CA, USA) and selected in G418 (approximately 540 µg/mL active ingredient). After five days in only G418, TG (1 × 10^{-5} M) was added to select for targeted clones. Clones resistant to G418 and TG were expanded and confirmed by Southern analysis (EcoRI-restricted-DNA hybridized to a probe, exons 2 and 3 of Hprt) for deletion of exon 3 by gene replacement (GR). The wild-type (WT), vector insertion (VI), and GR patterns are shown. Gene replacement is desired and results in a single EcoRI-restricted fragment of 8 kb. Vector insertion is undesired and incorporates the entire vector into Hprt (14). (B) Second step: Generation of parental clones. The G418R + TGR clone that exhibited the GR pattern was expanded and co-electroporated with pTet-Off (10 µg uncut) and puro (10 µg uncut). Puromycin (3 µg/mL) was added 24 h later, and 20 puromycin-resistant colonies were picked one week later. (C) Third step: Generation of daughter clones. A luciferase reporter was targeted to Hprt by a second gene replacement event and has a tTA-mCMV promoter and a bovine growth hormone polyadenylation site (bpA) (15). The targeting vector (10 µg uncut) and the I-Sce1 endonuclease expression vector (30 µg uncut) were co-transfected into ES cells (as described in part A) (12). Alternatively, the targeting vector (10 µg cut at the edge of homology with KpnI) was transfected into ES cells. Clones of ES cells that corrected Hprt were selected in HAT (1 mM sodium hypoxanthine, 4 µM aminopterin, 0.16 mM thymidine) media. HATR colonies were picked and expanded while maintaining HAT selection. The I-Sce1 endonuclease improved the recovery of HAT-resistant clones by about 100-fold (about 100 compared to about 1 HAT-resistant colony per electroporation). Genomic DNA, isolated from HAT-resistant colonies, was restricted with EcoRI and hybridized to exons 2 and 3 from Hprt cDNA to confirm gene replacement (8-kb fragment is converted into 5- and 1.3-kb fragments). All HAT-resistant colonies were correctly targeted by gene replacement.
ed and expanded. These are the parental clones (Figure 1B). Third, for 20 parental clones, a luciferase reporter was targeted to Hprt. This vector contains the luciferase reporter in the second intron of Hprt. Upon recombination, Hprt exon 3 was restored, and these clones were selected in HAT medium. Recombination was facilitated using an I-Sce1 endonuclease expression vector that generated a DNA double-strand break at the I-Sce1 endonuclease site adjacent to neo in the Hprt gene (12). The use of the I-Sce1 endonuclease system increased the generation of HAT^R clones by about 100-fold (data not shown). These are the daughter clones, and they have normal morphology (Figure 1C).

The mean and range of luciferase activity were determined for 3–5 of the daughter clones generated from 10 parental clones. Table 1 shows the mean luciferase activity for each parental clone. Table 1 shows the mean luciferase activity for daughter clones generated from each parental clone. The counts are arbitrary. Luciferase activity was determined by subtracting the counts in the off state (plus doxycycline) from the counts in the on state (minus doxycycline).

Table 1. Mean and Range of Luciferase Activities for Daughter Clones

<table>
<thead>
<tr>
<th>Parental Clone No.</th>
<th>Mean Activity</th>
<th>Range of Activity</th>
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<tbody>
<tr>
<td>1</td>
<td>6980+/−15854</td>
<td>155–29321</td>
</tr>
<tr>
<td>2</td>
<td>47+/−20</td>
<td>16–50</td>
</tr>
<tr>
<td>3</td>
<td>690+/−694</td>
<td>47–1322</td>
</tr>
<tr>
<td>4</td>
<td>89+/−53</td>
<td>28–167</td>
</tr>
<tr>
<td>5</td>
<td>3653+/−2884</td>
<td>451–8778</td>
</tr>
<tr>
<td>6</td>
<td>795+/−335</td>
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</tr>
<tr>
<td>7</td>
<td>257+/−227</td>
<td>53–711</td>
</tr>
<tr>
<td>8</td>
<td>179+/−91</td>
<td>34–309</td>
</tr>
</tbody>
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Luciferase activities were determined for daughter clones generated from 10 parental clones. Three to five daughter clones were observed from each parental clone. The mean counts with standard errors and the range of counts are shown for daughter clones generated from each parental clone. The counts are arbitrary.

Table 1 shows the mean luciferase activity for daughter clones generated from 10 parental clones. In addition to constitutive expression and promoter-regulated expression, targeted transgenesis at the Hprt gene is suitable for induced expression as shown for the tetO-CMV promoter expressing the luciferase reporter.

REFERENCES

Microtubule arrays in zebrafish (Danio rerio) embryos are vital to many developmental processes. Besides their obvious role in mitosis, microtubules are required for epiboly (1-3), furrow formation (4), and the cohesion of post-cytokinesis blastomeres (5). As transport lines for regulatory substances and maternal mRNAs, microtubules are also required for axis determination (6) and symmetric and synchronous cleavage (7). The study of microtubule function in both fixed and live embryos is consequently important for understanding the molecular mechanisms underlying numerous developmental processes.

In fixed preparations, immunofluorescence microscopy allows the comparison of microtubule structures in embryos at different stages or comparison of mutant or developmentally modified embryos to wild-type embryos fixed at the same age. Microtubule arrays in thick samples such as embryos are notoriously difficult to preserve. The dynamic nature of the microtubule polymer and sensitivity to calcium make microtubules extremely labile. Furthermore, fixations that work well to preserve microtubules in one cell type or sample may work poorly in others.

Several fixation methods are routinely used, and there is only anecdotal support for one method over the other. Most of these methods have been adapted from Xenopus protocols, and the anatomy of the zebrafish embryo possesses unique features that may prevent direct extrapolation of these methods. Here we report a comparison of the common fixation methods and make recommendations for the analysis of microtubules in different cell types of the early embryo.

We focus on the visualization of microtubules in three areas of zebrafish embryos: (i) the enveloping layer of cells (EVL), (ii) the deep cells that lie beneath EVL cells, and (iii) the yolk syncytial layer (YSL) (Figure 1). The deep cells of the early embryo will form the tissues of the embryo-proper, while EVL cells are epithelial and form a single-cell covering over the entire embryo. Both the EVL and deep cells sit upon and surround a multinucleated syncytium, the yolk cell. Visualization of these specific domains is valuable in different areas of developmental research.

The embryo develops within a clear sack called the chorion. If the chorion is not removed before fixation, then it must be removed at a later stage for antibody penetration in immunohistochemistry. After fixation, the chorion can be easily removed with forceps. Before fixation, a limited enzymatic pronase digestion can remove the chorion. We tested multiple fixation methods and tested whether removal of the chorion before fixation had any effect.

We examined two common fixation procedures: a simple paraformaldehyde (PFA) fixation and a fixation using microtubule stabilization buffer (MSB). PFA is a widespread fixative used on many different sample types and has been used to prepare zebrafish embryos for immunohistochemistry of many epitopes (5,8), in situ hybridization, and a combination of these two techniques (9). The MSB fixative was originally developed for Xenopus (10) and was later used with slight modifications for zebrafish embryos (1,11). We examined these two fixatives both with and without intact chorions during fixation. We also tested embryos that were permeabilized with Triton® X-100 following de-