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Temporal, Spatial and Tissue-Specific Expression of a Myogenin-*lacZ* Transgene Targeted to the *Hprt* Locus in Mice

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ABSTRACT

A *lacZ* transgene, expressed by the myogenin promoter, was introduced into the mouse hypoxanthine phosphoribosyltransferase (*Hprt*) locus by gene targeting in embryonic stem cells. Embryos between E10.5–E18.5 days were analyzed for expression of the transgene after staining for *β*-galactosidase activity. Transgene expression was restricted to the skeletal muscle lineages reflecting a similar temporal and spatial pattern previously demonstrated for the endogenous myogenin gene. Additionally, a second transgene, MC1tk, showed expression in 87% of the clones when targeted to *Hprt*. This strategy, called targeted transgenesis, provides control for analyzing promoter sequences and for comparing various transgenes expressed by the same promoter.

INTRODUCTION

The analysis of transgenic mice has been a common method to evaluate promoter sequences and gene-product function (13,25,33). Transgenes have been integrated into the genome of mammalian cells and animals by a variety of techniques, including pronuclear injection, viral integration and knock-ins. However, there are problems associated with each of these techniques that can complicate interpretation or limit application. Therefore, the development of other methods to introduce transgenes into mammalian cells and animals might improve control and broaden application.

Pronuclear injection has been the most commonly used method to introduce transgenes into mice, and the problems that arise from pronuclear injection have been well described (25). First, large 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 (9,10,36). Sec-

ond, variable levels and patterns of transgene expression are observed between founder mice because of concatemerization of the transgene and chromosomal positional effects (2). Third, mosaic expression of the transgene can occur due to position-effect variegation (28) or intrachromosomal recombination within the transgene concatemer (29). Transgenic mice derived from multiple founders must be analyzed and compared to control for these variables.

Transgenes can be introduced directly into 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, and DNA:lipopolyamine-mediated gene transfer has only been used for transient expression (12,31,33). In addition, stable transmission of the transgene through the germ line is difficult.

Embryonic stem (ES) cell technology has been used to produce transgenic mice. Promoterless transgenes have been integrated into a target locus by homologous recombination; a method

referred to as a “knock-in” (13,35). The goal is twofold: (i) to disrupt the target gene and (ii) to express the transgene within the target gene’s promoter. In addition, large fragments of DNA in a yeast artificial chromosome (YAC) clone have been introduced into the genome of ES cells (8). These approaches lessen the need to understand the promoter sequences necessary for regulated expression and were proven effective in analysis of the transgene product; however, they are ineffective for analysis of promoter sequences.

To address these problems, a procedure called targeted transgenesis (18) was developed. This procedure introduces a single copy of a transgene into a known location. The first reported attempt at targeted transgenesis was expression of a transgene targeted to the third exon of hypoxanthine phosphoribosyltransferase (*Hprt*) (22), in ES cells (32). The transgene was *lacZ*-driven by a promoter containing the polyoma virus enhancer and herpes simplex virus (HSV)-thymidine kinase (*tk*) promoter (called *MC1*; Reference 20). Unfortunately, variable expression was observed between different cell types, and transgenic mice were not generated.

More recently, a *bcl-2* transgene, expressed by the *b-actin* promoter, was targeted upstream of *Hprt* and shown to be uniformly expressed between different founder mice (4). However, expression at the cellular level was not studied.

In this report, targeted transgenesis was evaluated for temporal and tissue-specific expression at the cellular level using the myogenin promoter. Myogenin is a member of the *MyoD* family of basic-helix-loop-helix transcription factors (11) and is important for skeletal muscle development (14). Expression of myogenin is tightly restricted to the skeletal muscle lineage (5,24). Analysis of the myogenin promoter revealed that 1565 bp of sequences immediately preceding the myogenin translation initiation codon was sufficient to direct tissue-specific expression of *lacZ* (6). This transgene, *Myo1565lacZ*, was targeted to the second intron of *Hprt* in ES cells to test for tissue-specific expression. Transgenic mice were generated, and appropriate temporal, spatial and tissue-specific expression was observed in all transgenic embryos. In addition, expression of the HSV-*tk* gene by the *MC1* promoter (*MC1tk*) was tested for clonal repro-

Figure 1. Targeting *Myo1565lacZ* to *hprt*. Diagram of the vector and *Hprt* allele. Thick line, homology to *Hprt* in vector. Thin line, genomic *Hprt*. Wavy line, plasmid backbone. Gray line, deleted *Hprt* sequences in the targeting vector. Arrows point to transcriptional orientation. Size of *Eco*RI-restricted fragments are shown in kb. X represents the crossover junction. Southern analysis: DNA, extracted from the yolk sac, was digested with *Eco*RI, separated by electrophoresis, blotted and hybridized to exons 2 and 3 of *Hprt*. Lane 1, non-transgenic embryo; lanes 2–4, transgenic embryos.

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ducibility of expression when targeted to *Hprt*. For clones with *MC1tk* targeted to *Hprt*, 87% exhibited sufficient expression for sensitivity to 1-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl)-5-iodouracil (FIAU).

MATERIALS AND METHODS

Construction of Insertion Vector to *Hprt* with Myo1565lacZ (IVH-MyoZ)

A *SacI-EcoRI* fragment that contains exons 2 and 3 was used for *Hprt* (22) homology. Myo1565lacZ (6) was inserted into the *NheI* and *HindIII* sites of *Hprt* intron 2, removing about 2.3 kb of this intron. The positive-selection cassette, *PGKneobpA*, was positioned in the bacterial plasmid, pTZ (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The vector was linearized in the *Hprt* sequences at a unique *XhoI* site.

Construction of Insertion Vector to *Hprt* with HSV-1 Thymidine Kinase (IVH-tk)

A *SacI-EcoRI* fragment that contains exons 2 and 3 was used for *Hprt* homology. *MC1tk* (20), derived from the HSV-*tk* gene was inserted into the plasmid backbone (pTZ) of the targeting vector. The positive-selection cassette, *PGKneobpA*, was positioned in exon 3 of *Hprt* at the *XhoI* site. The vector was linearized in the *Hprt* sequences at a unique *NheI* site.

Gene Targeting with IVH-MyoZ and IVH-tk

AB1 ES cells were grown on a monolayer of mitotically inactivated SNL 76/7 STO (21) cells in M15 (15% fetal calf serum [FCS; HyClone Laboratories, Logan, UT, USA], Dulbecco's modified Eagle medium [DMEM; Life Technologies, Gaithersburg, MD, USA], 10^{-4} M β -mercaptoethanol, 2 mM L-glutamine, 49.5 U/mL penicillin and 38.8 μ g/mL streptomycin). Cells were trypsinized and resuspended in phosphate-buffered saline (PBS;

Figure 2. Whole-mount staining of transgenic embryos. (A and B) day-E10.5 embryo. (C and D) day-E11.5 embryo. (E and F) day-E13.5 embryo. Scale bars: (A) 50, (B) 20, (C) 80, (D) 40, (E) 200, (F) 80 μ m.

Ca²⁺, Mg²⁺-free) for transfection with IVH-MyoZ cut with *Xho*I or IVH-tk cut with *Nhe*I (10 µg DNA electroporated in 10⁷ cells/mL PBS at 230 V, 500 µF using a Gene Pulser[®] System [Bio-Rad, Hercules, CA, USA]). One electroporation was plated onto a single 10-cm plate. G418 (180 µg active ingredient/mL M15) was added 24 h later to select for transfected cells, and 6-thioguanine (TG; 10⁻⁵ M final concentration) was added five days later to select for targeted cells. Eleven days after the electroporation, G418-resistant (G418^R) plus TG-resistant (TGR) clones were picked for Southern analysis.

Southern Analysis

Determination of gene targeting at *Hprt* has been previously described for both insertion and replacement vectors with a variety of probes (17,38). Genomic DNA was extracted from ES cells for Southern blot analysis by the micro-extraction procedure (26). Digested DNA, separated by electrophoresis through a 0.7% gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) was transferred to a Hybond[®]-N+ filter (Amersham Pharmacia Biotech) and hybridized to random-primed labeled probe. For clones transfected with IVH-MyoZ, *Eco*RI was used for restriction, and *Hprt* exons 2 and 3 isolated from cDNA cut with *Hinc*II and *Hpa*II was used for probe (22). For IVH-tk, *Bam*HI was used for restriction, and a 400-bp fragment from intron 1 cut out with *Bam*HI to *Xba*I was used for probe (38).

Generation of Transgenic Mice

Transgenic mice were generated by injecting targeted ES cells into day-E3.5 C57BL/6 embryos as previously described (3).

Whole Mount Stain

Tissues were isolated and stained for β-galactosidase (β-gal) activity as described (7). Embryos at day E10.5–E13.5 were stained as whole mounts. Limbs from day-E18.5 embryos were dissected, skinned and stained.

Histology

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)-stained hind limbs were fixed in 10% buffered formalin, processed and embedded in paraffin using standard procedures (19). Sagittal sections (6-µm thick) were cut and lightly stained with nuclear Fast Red[®].

MC1tk Expression in ES Cell Clones Targeted with IVH-tk

G418^R + TGR ES cell clones transfected with IVH-tk were tested for *MC1tk* expression by survival in a base analogue, 0.2 mM FIAU. ES cell clones that express HSV-*tk* will die after metabolizing FIAU, while those clones that fail to express HSV-*tk* will survive; FIAU is lethal to cells only after it is metabolized. G418^R + TGR clones were picked and expanded on a 96-well plate for five days and then replica-plated onto two 96-well plates. One plate was selected in FIAU, and the other was not. Cell growth was visualized for five days.

RESULTS

Generation of Myo1565lacZ Transgenic Mice

An insertion vector, IVH-MyoZ, was used to introduce Myo1565lacZ into the *Hprt* locus. *Hprt* was chosen as the integration site for four reasons. (i) Deletion of *Hprt* does not cause a phenotype in mice unless they are predisposed to an adenine phosphoribosyltransferase deficiency (37). (ii) *Hprt* is a member of the purine salvage pathway and expressed in all cell types at low levels; therefore, it is a house-keeping gene, and chromosomal positional effects should be less a factor as for other loci. (iii) Selection for gene targeting is efficient because *Hprt* is single-copy in male cells, and its disruption can be selected in tissue culture with a toxic base analog, 6-thioguanine (TG). Finally, because *Hprt* is on the X-chromosome, it is subject to X-inactivation. This permits evaluation of the transgene product for cell autonomy by comparing mice that express the transgene in all cells (males and homozygous females) to mice that express the

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transgene in only a fraction of cells (hemizygous females).

Myo1565lacZ was introduced into *Hprt* intron 2 by gene targeting (Figure 1). The targeting vector, IVH-MyoZ, contained sequences homologous to *Hprt* that flanked the reporter (Myo1565lacZ) and a positive-selection cassette (PGKneobpA) outside the *Hprt* sequences. The vector was linearized within the *Hprt* homology at the *Xho*I site to make an insertion vector. Cells were transfected by electroporation and selected in G418 for integration into a chromosome and in TG for targeting to *Hprt*. Only one G418^R + TGR clone survived out of three transfections. The G418^R + TGR clone was analyzed by Southern analysis to confirm targeting, which resulted in the appropriate bands for an insertion event (13, 7 and 1.3 kb). However, the increased intensity of the 13-kb band compared with the 5-kb band indicated that two to three copies of the vector integrated into this site, perhaps a consequence of limiting target homology (16). Thus, targeting frequency would likely be improved by lengthening the homology that flanks the double-strand break. It is unlikely that two to three copies of the target vector would affect tissue specificity; therefore, the targeted clone was used to generate chimeric mice after injection into C57BL/6 embryos. The chimeric males were bred to C57BL/6 females to generate transgenic mice.

Myo1565lacZ Expression is Specific to Skeletal Muscle Lineage when Targeted to *Hprt*

Myogenin expression is limited to the skeletal muscle lineage (5,24). Transcripts are first detected in the myotome by day E8.5 and in the limb buds by E11.5, with expression greatly declining around birth. Transgene expression was evaluated between E10.5–E18.5. Either yolk sacs (E10.5–E13.5) or tails (E18.5) were removed for genotyping by Southern blot analysis (Figure 1) to identify transgenic and non-transgenic embryos. β -gal expression was analyzed in these embryos by whole-mount staining in X-gal. Non-transgenic embryos failed to stain blue ($n = 22$); however, all transgenic embryos ($n = 18$)

stained blue in a pattern restricted to the skeletal muscle lineage for all time points observed. Staining was first observed in the myotome of day-E10.5 embryos (Figure 2, A and B), which progressed to the intercostal region by day E11.5 (Figure 2, C and D). Staining was also observed in the limb buds and developing facial musculature (Figure 2, E and F). β -gal activity was observed at the cellular level by histological sections of limbs from day-E18.5 embryos. Staining was clearly restricted to skeletal muscle and was not present in bone or other tissues (Figure 3). Therefore, expression of Myo1565lacZ targeted to *Hprt*, recapitulates the temporal and spatial expression pattern of the endogenous *myogenin* gene.

Expression of HSV-*tk* Transgene Targeted to *Hprt*

Transgene expression can vary from clone to clone for a variety of reasons, including position-effect variegation and mutation. The effects of clonal variation of transgene expression, targeted to *Hprt*, were examined. An HSV-*tk* gene expressed by the *MCI* promoter (*MCI*tk), was targeted to the second in-

tron of *Hprt*. *MCI*tk was chosen to test transgene expression because HSV-*tk* metabolizes FIAU to a product that is toxic to mammalian cells. Therefore, cells expressing *MCI*tk can be identified by their sensitivity to FIAU.

*MCI*tk was introduced into *Hprt* intron 2 by gene targeting (Figure 4). The targeting vector (IVH-*tk*) contained sequences homologous to *Hprt*, *MCI*tk and PGKneobpA. The vector was linearized in *Hprt* homology at the *Nhe*I site. Cells were transfected by electroporation and selected in G418 for chromosomal integration and in TG for targeting to *Hprt*. G418^R + TGR cells were analyzed by Southern blot analysis to confirm targeting (Figure 4). Targeting can occur by either an insertion event (insertion of the entire vector into *Hprt* due to a single crossover) or a gene-replacement event (replacement of endogenous *Hprt* sequences with vector-derived *Hprt* sequences due to crossovers at both sides of PGKneobpA). Thus, *MCI*tk is incorporated into *Hprt* only after vector insertion. Using Southern analysis, 39 G418^R + TGR clones were targeted by vector insertion, and seven were targeted by gene replacement.

Figure 3. Histological sections of hind leg at day E18.5. (A) Tibialis anterior muscle (TAM) attached to bone; scale bar, 40 μ m. (B) Increased magnification of orange inset shown for Panel A; scale bar, 170 μ m.

These clones were replica-plated onto microwells in media with and without 0.2 μ M FIAU. All clones grew in media without FIAU. Additionally, all seven clones targeted by gene replacement and five clones targeted by vector insertion grew in media with FIAU. By contrast, 34 clones targeted by vector insertion did not grow in media with FIAU. Therefore, of the clones targeted with *MC1tk* to *Hprt*, 87% expressed the transgene at sufficient levels to confer lethality when grown in media with FIAU.

DISCUSSION

A transgenic approach was used to introduce a *lacZ* cassette into the *Hprt* gene under the regulation of myogenin regulatory sequences. The *lacZ* transgene was expressed in a similar temporal, spatial and tissue-specific pattern as the endogenous *myogenin* gene. This demonstrates, to our knowledge, for the first time, the correct tissue-specific expression pattern for a transgene targeted to *Hprt*. Additionally, *MC1tk*, targeted to *Hprt*, was expressed at sufficient levels to confer sensitivity to FIAU for 87% of the clones. Thus, transgene expression was frequent but not absolute.

The level of staining observed for *Hprt*-targeted transgenic embryos appeared less intense than previously reported for transgenic mice generated by pronuclear injection (7); this might indicate reduced transcription. Transcription levels might be higher for mice generated by pronuclear injection than by targeted transgenesis due to the large number of transcriptional units that integrate into a chromosome or to integration into a locus favorable for transcription. Thus, it might not be appropriate to target transgenes with weak promoters to *Hprt*.

The positive-selection cassette did not appear to alter regulation of the myogenin promoter. However, there are reports that suggest the positive-selection cassette alters transcription of nearby genes (23,27). The Cre/Lox system could be used to remove selection cassettes to ensure altered expression of the transgene does not occur (1,30). Alternatively, the use of a positive-selection cassette can be avoided by utilizing

a targeting vector that corrects for a preexisting *hprt* mutation in ES cells as previously described (4).

Either the Myo1565lacZ or the *MC1tk* transgene can be removed from *Hprt* by intrachromosomal recombination. However, this recombination event is infrequent and was undetected in the Myo1565lacZ transgenic embryos. If selection is applied (15), transgene deletion is detectable in tissue culture cells. However, this event was not detected in any of the 34 clones that express *MC1tk* while being grown in microwells, which demonstrates the rarity of this event.

Targeting transgenes to specific chromosomal positions offers several advantages over pronuclear injection. (i) Gene targeting reliably alters the genome in a specific location without generating unwanted mutations (17,39). Targeted cells are screened by Southern blot analysis to ensure the fidelity of the targeted locus. (ii) The level of expression can be controlled by selection for transgene number and location. All transgenes to be compared are in the same location and under the same positional effects. In certain instances, consistent level of expression is critical for comparing one transgene to another. (iii) Chimeric embryos and adults can be analyzed if a transgene produces a toxic protein that prevents transmission through the germ line. The extent of chimerism is the only variable. (iv) Because of X-inactivation, a mosaic expression pattern is produced in hemizygous females when the transgene is located on the X-chromosome (34). The X-chromosome is randomly inactivated in the embryo; therefore, only 50% of cells will produce the transgene product.

By taking advantage of X-inactivation, the transgenesis technique described here can be utilized to create mosaic mice. Figure 5 summarizes a scheme to discriminate cells expressing a transgene product from non-expressing cells. Compound heterozygous female mice are generated. These mice carry different transgenes on each X-chromosome. The transgene-of-interest (TOI) is targeted to one X-chromosome, and a *lacZ* cassette is targeted to the other. Both TOI and *lacZ* cassette are under the same transcriptional regulatory sequences. Due to X-inactiva-

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Figure 4. Targeting *MC1tk* to *hprt*. Diagram same as for Figure 1. Southern analysis: DNA extracted from ES cell clones, digested with *Bam*HI, separated by electrophoresis, blotted and hybridized to a 400-bp fragment from intron one release by a *Bam*HI-*Xba*I digest (*Bam*HI-*Xba*I probe [bxp]). Lanes 1 and 3 are vector-insertion events (generated by a single crossover), and lane 2 is a gene-replacement event (generated by two crossovers, one on each side of PGKneobpA). Lane 4 is wild-type.

Figure 5. A scheme to identify mosaic expression of transgene targeted to *Hprt*. In a double-hemizygous female, the TOI is located on one X-chromosome, and the *lacZ* transgene (Z) is located on the other X-chromosome. The same promoter expresses both TOI and *lacZ*. The active X is green, the inactive X is red. Vertical arrows signify transcription; vertical blocked lines signify no transcription. (A) A cell with the TOI on the active X-chromosome (TOI expressed) and *lacZ* on the inactive X-chromosome (*lacZ* silent). This cell does not stain blue in X-gal. (B) A cell with the TOI on the inactive X-chromosome (TOI silent) and *lacZ* on the active X-chromosome (*lacZ* expressed). This cell stains blue in X-gal.

tion, cells will express only one of the transgenes in a given cell. Therefore, cells that express the *lacZ* transgene do not express the TOI and stain blue after X-Gal staining. Conversely, cells that express TOI do not express *lacZ* and do not stain blue after X-Gal staining.

Cells that stain blue (express *lacZ* but not TOI) can be compared to cells that do not stain blue (express TOI but not *lacZ*). Comparing the distribution and number of blue cells to non-staining cells can be helpful when analyzing expression of dominant or gain of function proteins, especially if those proteins are toxic to the cell or inhibit cellular proliferation. In such a case, only blue-staining cells would survive or proliferate. Additionally, comparing blue-staining cells to non-staining cells permits investigation of cell autonomous (effects limited to within the cell expressing the TOI) and cell nonautonomous (effects not limited to within the cell expressing the TOI) functions of the transgene product.

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