Evaluation of tyrosinase minigene co-injection as a marker for genetic manipulations in transgenic mice

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ABSTRACT

The utility of tyrosinase minigene co-injection was evaluated as a visual marker for the generation and breeding of transgenic mice. In an evaluation of 39 transgenic founder animals and 44 transgenic lines five phenotypic patterns of pigmentation were consistently observed, including albino, dark, light, mottled and himalayan. In these studies co-injection of the tyrosinase minigene along with the transgene of interest (TOI) resulted in genomic integration of the two transgenes in 95\% of the F0 generation. Co-segregation of transgenes occurred in 94\% of doubly transgenic mice in the F1 generation, without dissociation in subsequent generations. All pigmented phenotypes proved useful for distinguishing homozygous from heterozygous F2 animals via backcross trials, while the light, mottled and himalayan phenotypes proved useful in visually discriminating between homozygous and heterozygous F2 animals. In addition, the light, mottled and himalayan phenotypes proved useful in determining segregation patterns of transgenes in the progeny of crosses between separate transgenic lines. Moreover, there appears to be a correlation between intensity of pigmentation and degree of expression of the co-injected TOI. These studies confirm that tyrosinase co-injection is a useful adjunct in transgenic mouse studies and can serve to reduce routine genetic validation of transgenic lines.

INTRODUCTION

Pigmentation systems in the mouse have been well described and characterized by classical genetic techniques (1). In recent years this characterization has been extended to the molecular level with the cloning of the gene for tyrosinase, the key enzyme in the production of melanin pigment (2–4). Genetic defects in the tyrosinase gene, located at the C locus in the mouse, result in albinism (5). Cloned mouse tyrosinase gene sequences have also been evaluated for their utility in the visual identification of transgenic mice in co-injection strategies (9,10), in order to circumvent the routine analysis of DNA required for the generation and maintenance of transgenic lines. We extend these studies by describing the utility of tyrosinase minigene sequence co-injection for the visual identification of heterozygote and homozygote F2 generation animals and for distinguishing the segregation patterns of transgenes in the progeny of crosses between transgenic lines. By means of the co-injection strategy we also address the question of predictability of the expression levels of a transgene using the intensity of pigmentation.

MATERIALS AND METHODS

Generation of transgenic founder mice and transgenic lines

Transgenic mice were generated by microinjection of single cell embryos using standard techniques (11). The FVB/N albino mouse line (12) was used as the source of embryos for the manipulations and for subsequent breeding trials. Manipulated embryos were re-implanted into the oviducts of pseudo-pregnant ICR females anesthetized with tribromoethanol (0.015 ml 2.5\% solution/g body wt) given by intraperitoneal injection (13). The tyrosinase minigene used has been previously described (6) and was microinjected at a concentration of 1 \( \mu \text{g/ml} \) in co-solution with one of six different transgenes (hereafter referred to as the transgene of interest or TOI), also at a concentration of 1 \( \mu \text{g/ml} \). Potential founder animals were weaned at 3 weeks of age and samples of tail tissue were taken for DNA analysis using standard protocols (11). All animal manipulations were carried out in accordance with institutional guidelines and were approved by a local ethics committee.

Establishing double genomic integration in the F0 generation

Mice in the F0 population were scored for tyrosinase minigene genomic integration by pigmentation or, in the case of non-pigmented mice, by PCR analysis for the tyrosinase minigene using a forward primer within exon 1 (5'-CTTITTTTCTACCTCAGTTAGC-3') and a reverse primer within exon 2 (5'-CGTATTGTTTCCCCCGGGT-3') (3). TOI integration was determined in all F0 mice by PCR analysis of the appropriate recombinant transgene using oligonucleotide primers to be described elsewhere (Methot et al., in preparation). Genomic amplification was performed in a 50 \( \mu \text{l} \) total volume consisting of 1 \( \mu \text{l} \) genomic...
Figure 1. Pigmentation phenotypes observed in F2 transgenic mice. All mice displayed are positive for the injected tyrosinase minigene as determined by PCR analysis.

DNA (500 ng/µl), 5 µl Taq buffer at 10x concentration (BIO/CAN Scientific), 4 µl dNTP (2.5 mM each), 1 µl forward and reverse oligonucleotide primers (100 ng/µl) and 2.5 U Taq polymerase (BIO/CAN Scientific). Cycling was performed in an Ericomp thermal cycler (San Diego, CA) and included a denaturation step for 3 min at 94°C followed by 30 cycles of annealing for 2 min at 57°C, elongation for 2 min at 72°C and denaturation for 1 min at 94°C, after which there was a final elongation step for 10 min at 72°C. An aliquot of 20 µl/reaction was size fractionated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. DNA from non-transgenic animals was amplified as a reaction control. DNA samples were considered positive for a particular transgene if a band of the predicted size in the test sample was present with no amplification occurring in the control sample. For the tyrosinase transgene the amplified DNA fragment migrated at 495 bp. Endogenous genomic tyrosinase sequences do not amplify under the PCR conditions chosen.

Levels of co-expression

Ten transgenic lines were studied to determine if a correlation exists between the level of expression of the tyrosinase minigene and the TOI. Expression of the tyrosinase minigene was determined by visually classifying the different pigmentation phenotypes. Expression of two different TOIs, human prorenin (hProren or TOI A) and human angiotensinogen (hAogen or TOI B) targeted to the mouse pituitary, were evaluated by RNase protection assay using mouse proopiomelanocortin (mPOMC) as an internal control. The RNA was isolated from single pituitaries by the acid/guanidium/thiocyanate/phenol/chloroform method (14). To prepare labeled RNA probes hProren from nt 401 to 650 of the cDNA, hAogen from nt 178 to 377 of the cDNA and mPOMC from nt 578 to 680 of exon 3 were subcloned in the Bluescript II KS+ plasmid (Stratagene, San Diego, CA). RNase protection assays were carried out with the Promega Riboprobe® Gemini System (Promega Corp., Madison, WI) according to the manufacturer’s protocol. Unquantitated total RNA from each single pituitary was hybridized with 5 x 10³ c.p.m. ³²P-labeled RNA probes of either hProren or hAogen and with 5 x 10³ c.p.m. ³²P-labeled RNA probes of mPOMC overnight at 45°C in hybridization buffer containing 80% formamide. Yeast RNA (5 µg) and RNA from non-transgenic mouse pituitaries (unquantitated) were hybridized under the same conditions as negative controls and RNA products from in vitro transcription of hProren and hAogen were also hybridized under the same conditions as positive controls. Protected fragments after RNase digestion were separated on a 6% polyacrylamide–7 M urea denaturing gel by electrophoresis and were then exposed to photographic film (Reflection™, DuPont) for 5 days at -80°C using intensifying screens.

RESULTS

Pigmentation phenotypes

In the 39 transgenic founder animals and 44 transgenic lines analyzed five discernible pigmentation phenotypes were observed in the F2 and subsequent generations. These could be reproducibly classified as albino, dark, light, mottled and himalayan (Fig. 1). Dark pigmentation proved to be the most frequent phenotype observed, accounting for 32% of tyrosinase transgenic mice, while mottled was the least frequent phenotype observed, accounting for 9% of tyrosinase transgenic mice (Table 1A). An expressing (i.e. pigmented) phenotype was observed in 82% of tyrosinase transgenic lines, while a non-expressing (i.e. albino) phenotype was observed in 18% of lines.

Table 1A. Occurrence of pigmented phenotypes in transgenic mice using tyrosinase minigene co-injection

<table>
<thead>
<tr>
<th>Tyrosinase transgenic phenotypes</th>
<th>No. of lines with a given phenotype or genotype/total no. of lines analyzed</th>
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<tbody>
<tr>
<td>Albino</td>
<td>8/44 (18%)</td>
</tr>
<tr>
<td>Dark</td>
<td>14/44 (32%)</td>
</tr>
<tr>
<td>Light</td>
<td>12/44 (27%)</td>
</tr>
<tr>
<td>Himalayan</td>
<td>6/44 (14%)</td>
</tr>
<tr>
<td>Mottled</td>
<td>4/44 (9%)</td>
</tr>
</tbody>
</table>

Numbers in brackets represent percentage of tyrosinase positive lines showing a given phenotype or genotype.

Table 1B. Integration patterns observed in transgenic mouse lines generated by co-injection of tyrosinase and a transgene of interest (TOI)

<table>
<thead>
<tr>
<th>Integration patterns of transgenes</th>
<th>No. of lines with a given phenotype or genotype/total no. of lines analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double co-integration</td>
<td>5/39 (13%)</td>
</tr>
<tr>
<td>Single integration tyrosinase</td>
<td>2/44 (5%)</td>
</tr>
<tr>
<td>Co-segregation tyrosinase and TOI transgenes in F1</td>
<td>31/33 (94%)</td>
</tr>
</tbody>
</table>

Double co-integration refers to multiple chromosomal loci for both the tyrosinase and TOI transgenes. Numbers in brackets represent percentage of tyrosinase positive lines showing a given phenotype or genotype.

The pigmented phenotypes can be classed as differing both quantitatively and qualitatively. Quantitatively, a range of
Figure 2. Using pigmentation as a marker of TOI homozygosity. Mice co-injected with the tyrosinase minigene and a TOI were inbred to the F2 generation and scored for being negative (−−), heterozygous (+−) or homozygous (+++) for the TOI using DNA dot blots of tail DNA. Results demonstrate a good correlation between pigmentation intensity and TOI content with inbreeding of the light (A), himalayan (B) and light mottled (C) phenotypes. The breeding pair is at the left of the panel, with the male at the extreme left.

pigmentation was seen which varied in intensity from albino (transgenic for tyrosinase but no detectable expression), to light (light brown evenly distributed pigmentation, the dorsal aspect usually being slightly darker than the ventral aspect of the body), to dark (dark gray evenly distributed pigmentation). Qualitatively, pigmentation patterns included: even distribution, showing dark gray to light brown coloration, as mentioned; mottled distribution, showing patchy or variegated distribution of pigmentation similar to the chinchilla (c<sup>th</sup>) phenotype (11) and as described previously in transgenic studies (7,9,15); himalayan distribution, showing darkly pigmented ears and tail with lightly pigmented body. The mottled class could further be subdivided into true mottled and double integration phenotypes (6), depending on the phenotype of the F1 progeny. Mottled and himalayan phenotypes also displayed differences in pigmentation intensities when comparisons between lines were made, however, within a given line and for a single integration site the pigmentation pattern remained constant from one generation to the next. Also, although the intensity of pigmentation within a mottled line was constant, the exact pattern of the mottled phenotype was not the same from one animal to another within a line. It is noteworthy that after analysis of 44 separate transgenic lines no pigmentation patterns in addition to the five described here were observed.

Establishing co-integration, co-segregation patterns and F2 generation mice

At 6–8 weeks of age founder mice shown to be transgenic for the TOI by DNA analysis or for the tyrosinase minigene by pigmentation were mated with non-transgenic FVB/N animals to generate F1 mice and transgenic lines. Integration of both the tyrosinase and TOI transgenes in F0 mice was most commonly seen, with single integration of tyrosinase occurring only 5% of the time (see Table 1B). Double sites of integration (as evidenced by segregation of different pigmentation patterns in the F1 generation) were seen, but were not common, accounting for 13% of founder animals; when this happened each pattern of pigmentation was subsequently considered to represent a separate transgenic line.

To establish co-integration of the two microinjected transgenes their segregation patterns in the F1 generation were studied. Pigmented F1 animals from each transgenic line, as well as albino littermates, were analyzed via PCR for the presence (or absence) of the TOI. Co-integration at a single site was assumed to have occurred if the pigmented animals tested positive for the TOI and the albino animals tested negative for the TOI. Of a total of 33 lines analyzed 31 (i.e. 94%) showed co-segregation of pigmentation and TOI in the F1 generation. In all crosses subsequent to the F1 generation (>100) where co-segregation was established we have seen no dissociation of pigmentation and the TOI.

F1 male and female pigmented, TOI-positive transgenic mice were mated to produce F2 generation mice. To determine whether mice of the F2 generation were heterozygous or homozygous for the tyrosinase minigene these mice were scored visually for degree of pigmentation and tested genetically by performing backcross breeding to non-transgenic albino FVB/N mice. As can be seen in Figure 2A–C, those mice with the heaviest pigmentation in the F2 generation from crosses within either the light, himalayan or mottled phenotypes are homozygous for the tyrosinase minigene. Analysis of 23 F2 mice for TOI gene copy number by dot blot analysis confirmed that the most heavily pigmented mice in these crosses were also homozygous for the TOI (data not shown).
Intensity of pigmentation was not a reliable indicator of homozygosity, however, in lines displaying the dark pigmentation phenotype (data not shown). Nevertheless, backcross of all the tyrosinase-expressing pigmentation patterns can be used as a reliable visual indicator of homozygosity (Fig. 3A and B).

Crosses between transgenic lines

Crosses between transgenic lines were performed to evaluate the utility of tyrosinase as a visual marker for double TOI transgenics. Animals in the F1 generation which had been demonstrated to be transgenic for both tyrosinase and one of the TOI transgenes were crossed and the resulting progeny scored for pigmentation intensity and presence of the differing TOIs. Figure 4 shows the pigmentation patterns of the parents as well as offspring of the crosses light × mottled pigmented (Fig. 4A), himalayan × mottled pigmented (Fig. 4B) and mottled (light) × mottled (light) pigmented lines (Fig. 4C). Results demonstrate that segregation of the various TOIs correlates directly with the type and intensity of pigmentation seen in the offspring, i.e. pigmentation resembling either of the parents contained the appropriate parental TOI, dark mice contained both TOIs and albino mice contained neither TOI. As with the prediction of homozygosity, in crosses involving either two lines with dark pigmentation patterns or one dark and one light pigmentation pattern it was not possible to predict double TOI transgenic mice.
Table 2. Relative expression of the tyrosinase minigene and two different TOIs

<table>
<thead>
<tr>
<th>Pigmentation phenotype</th>
<th>Relative intensity of pigmentation</th>
<th>Relative level of TOI expression</th>
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<tbody>
<tr>
<td>A. Comparison of pigmentation and TOI A expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>Himalayan</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Mottled</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Himalayan</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Light</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Albino</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Albino</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. Comparison of pigmentation and TOI B expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mottled</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Dark</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Albino</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Expression of the tyrosinase minigene is divided into two categories; qualitative, including the pigmentation phenotype (i.e. albino, dark, light, mottled and himalayan), and quantitative (as displayed by + and − signs), to define the relative pigmentation intensity of the different lines. Level of expression of the TOIs, quantitated by RNase protection assay, is displayed in the right-hand column.

Levels of co-expression

Correlation between the level of expression of the tyrosinase minigene and the TOI was studied in 10 transgenic lines. Table 2 shows the relative pigmentation intensity compared with the relative level of expression of two different TOIs as determined by RNase protection assay. Overall there was a qualitative correlation between the appearance of pigmentation and TOI expression: three tyrosinase minigene-non-expressing (i.e. albino) lines did not express the TOI, while seven tyrosinase minigene-expressing (i.e. dark, light, mottled and himalayan) lines expressed the co-injected TOI at variable levels. Furthermore, for TOI A there appeared to be a quantitative correlation between the degree of pigmentation, as determined arbitrarily by comparison between lines, and the level of expression of TOI A, as determined by RNase protection assays (see Table 2A). However, this correlation did not hold for TOI B, where TOI B expression was highest for the lesser pigmented of the two mouse lines tested (Table 2B). In total, for nine of 10 transgenic lines tested there appeared to be a general correlation between intensity of pigmentation and TOI expression.

DISCUSSION

The use of tyrosinase genes in an albino genetic background in transgenic mice has been proposed as a useful technique for screening for transgene integration and segregation (9,10). We have now extended these studies to document the utility of the tyrosinase marker gene in screening for homozygous mice in an F2 generation and in screening for doubly transgenic animals in crosses made between independent transgenic lines. We have also tested whether the intensity of pigmentation in transgenic animals could serve as an indication of the level of expression of the co-injected TOI.

Several patterns of phenotypic expression using tyrosinase minigenes in transgenic mice have been previously described (6,9,15,16). In general these observations were made on small numbers of transgenic lines and it was not clear as to the reproducibility or frequency of these phenotypes. In analyzing 39 founder animals and the resulting 44 transgenic lines using a tyrosinase transgene we have observed five discernible and repeating pigmentation phenotypes: albino, dark, light, mottled and himalayan. An important conclusion to be derived from this work is that only a limited repertoire of pigmented phenotypes is generated by tyrosinase gene expression in these animals and that these arise with a fairly predictable frequency.

One critical question in these studies is whether the tyrosinase minigene can serve as a reliable marker for integration and segregation of the TOI in transgenic mice. Consistent with previous reports (9,10), we have found high levels of co-integration of the tyrosinase and TOI transgenes, although in 5% of founder animals only one transgene was present and in 13% of the founder animals there were multiple integration sites for either or both of the transgenes. Our analysis of co-segregation suggests that in the F1 generation co-segregation of the tyrosinase and TOI transgenes is highly favored, but not absolute. For these reasons it remains important to verify segregation of the TOI with pigmentation in the F1 generation.

We compared direct visual identification of heterozygous versus homozygous animals via pigmentation markers with genetic backcross analysis and dot blot DNA hybridization methods. Visual identification of the heterozygote or homozygote condition for transgenes proved generally useful for light, himalayan and mottled phenotypes, which showed a quantitatively darker phenotype for homozygous animals when compared with heterozygous animals. This was only evident after appearance of hair and generally required heterozygous littermates for comparison. Direct visual identification of heterozygosity versus homozygosity was not useful for dark or albino phenotypes. In comparison, the utility of pigmentation was demonstrated for all tyrosinase-expressing pigmented phenotypes for the identification of homozygous versus heterozygous F2 transgenic animals via backcross analysis. Thus tyrosinase pigmentation is both reliable and generally useful as an alternative to DNA analysis in scoring homozygosity in transgenic mice.
The use of pigmentation as a marker proved particularly useful in identifying transgenic segregation patterns resulting from crosses between two independent transgenic lines. In crosses between lines with light, himalayan or mottled phenotypes the transgenic genotype of the resulting offspring, including double integration transgenic animals, could be predicted by the pigmentation phenotype, where the double integration site transgenic animals were more darkly pigmented than either the parental lines or their single integration site siblings. In crosses between a dark line and a more lightly pigmented line segregation of the light pigmentation-conferring transgene was possible, but integration sites producing a dark phenotype could not be distinguished visually from double integration sites.

One rather surprising finding of the current study was that the intensity of pigmentation served as a fairly reliable indicator of the level of expression of the co-injected TOI. As the TOIs used in this study were under the control of a pituitary-specific promoter (growth hormone; data not shown) and expression of the tyrosinase minigene is in melanocytes, the simplest explanation for this finding is that the transgenes have inserted in chromatin domains that are either constitutively ‘open’ (in the case of pigmented, TOI-expressing animals) or permanently ‘closed’ (in the case of albino, TOI-non-expressing animals) in both of the appropriate cells types. These results suggest that albino mice can be routinely excluded from further analysis in transgenic colonies derived by this method, whether from lack of transgene integration or expression. The additional limited correlation between the intensity of pigmentation and the degree of TOI expression seen in most of the pigmented animals suggests that pigmentation might be used as a preliminary screen for mice with high expression levels, particularly when large numbers of animals are developed and used in the F0 generation for studies. We do not presently know whether the same finding would apply to all TOIs, although Overbeek et al. (9) have reported the absence of expression of a TOI engineered for kidney expression in an albino mouse positive for tyrosinase minigene integration. In conclusion, while co-injection with visible marker genes such as tyrosinase in transgenic mouse studies will not eliminate the need for DNA analysis to establish the presence of the TOI, under the proper conditions it can significantly reduce the need for routine DNA analysis in established transgenic lines and may be useful for estimating relative levels of transgene expression.

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