

LETTER

Gene-Trap-Based Target Site for Cre-Mediated Transgenic Insertion

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Summary: There is an increasing need for tissue-specific gene expression regulatory elements to study normal and disease development in the mouse. However, the cloning and characterization of these elements are time-consuming and costly. Thus, there is a particular need to be able to identify gene expression patterns without having to clone the promoter elements. Gene-trap strategies identify expression patterns assigned for endogenous genes using reporters, such as LacZ (Gossler *et al.*, 1989; Skarnes, 1990) or green fluorescent protein (GFP) (Ishida and Leder, 1999; Zheng and Hughes, 1999). The gene-trap vector randomly inserts into the genome and “steals” regulatory elements for the reporter. Here we describe an improved gene-trap strategy, which allows an efficient Cre recombinase-mediated insertion of any transgene into the trapped loci as a post-integrational modification and links the expression of the transgene to that of the reporter. *genesis* 26: 245–252, 2000. © 2000 Wiley-Liss, Inc.

Key words: Gene trap; Cre recombinase; transgene replacement; define site transgene insertion

INTRODUCTION

Regulatory elements of most mammalian genes are localized as dispersed sequences spanning several or several dozens of kilobases of chromosomal DNA. Therefore, their complete characterization and isolation usually represents an arduous task. The low number of cloned and characterized gene regulatory elements currently available has created a limitation in the application of new genetic strategies. Meanwhile, the need for a large variety of lineage and cell type-specific transgene expressions for gene function and cell lineage studies is increasing.

Gene-trap approaches applied in embryonic stem (ES) cells have no theoretical limitation in identifying any possible endogenous gene expression pattern (Evans *et al.*, 1997). The classical gene-trap vector (Gossler *et al.*, 1989) contains a splice-acceptor site immediately upstream of a reporter gene, followed by a selectable marker expression cassette. When this vector integrates

into an intron, a spliced fusion transcript between the reporter and the endogenous gene is generated under the control of endogenous transcriptional regulatory elements of the trapped gene. If the coding region of the reporter remains in-frame with the 5' coding sequence of the trapped gene, translation of the fusion transcript creates a fusion protein between the N-terminus of the gene product and the reporter. The bacterial β -galactosidase (*LacZ*) gene is the most commonly used reporter gene because its detection is simple, and as an enzyme, it is extensively tolerant to N-terminal extensions. Therefore, in most gene-trap insertions, simple histochemical staining for LacZ activity demonstrates the normal expression pattern of the trapped gene.

Cre recombinase of the P1 bacteriophage mediates both intramolecular (excision or inversion) and intermolecular (insertion or translocation) recombinations between loxP sites (Sauer, 1993) in any cellular environment including ES cells and all cell types in the mouse. In the past few years, the system is being more and more applied in ES cell-mediated genetics (Nagy, 2000) because it allows post-integrational modification of targeted genes and transgenes. It has opened possibilities for cell type-specific gene/transgene manipulations and for creation of any imaginable genome alterations in the mouse from subtle changes to large chromosomal aberrations.

We have developed a strategy that combines gene trapping with Cre/loxP site-specific recombination-mediated insertion of a transgene. The insertion is a second event after the first gene-trap integration that is detected and perhaps characterized with a LacZ reporter. This second step further extends the fusion transcript generated by the initial gene-trap insertion by an internal ribosomal entry site (Jang *et al.*, 1988) (*IRES*)-driven transgene. The resulting bicistronic transcript serves as a template for the translation of the original LacZ fusion

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protein and the introduced transgene-encoded protein. The two proteins are independent entities but translationally linked. Using the strategy described here, we are able to direct transgene expression by any endogenous regulatory machinery of trapped genes without cloning these regulatory elements. Furthermore, the transgene can be removed in situ in a cell type-specific manner in Cre recombinase transgenic mice. Thus, a gene expression library of ES cell clones can be banked to serve as a universal reagent pool for expressing transgenes in a promoter-specific manner.

RESULTS

Description of the Strategy

Our gene-trap vector contains the *En-2* splice acceptor (SA) immediately 5' of a *LacZ* coding region including a stop codon but lacking a polyA site. Immediately downstream is the *PGK-1* promoter flanked by two wild-type *loxP* sites in the same orientation, followed by the *neo* selectable marker gene carrying its own polyA signal. The gene-trap replacement strategy is depicted in Figure 1A and as follows:

Step 1: Following electroporation of the gene-trap vector, neomycin-resistant ES cells are selected in G418. Then clones are analyzed for LacZ activity in both undifferentiated and differentiated ES cells. The expression pattern of selected trapped genes is characterized in vitro or in vivo.

Step 2: The ES cell clone carrying a trapped gene with a desired expression pattern is transiently transfected with Cre recombinase to excise the *PGK-1* promoter driving the *neo* gene. The excision renders the cells G418 sensitive and leaves a single *loxP* site between the *LacZ* and *neo* coding sequences.

Step 3: The neo-sensitive ES cell clone is co-transfected with a Cre-recombinase expression vector and the "pop-in" plasmid. The latter consists of a *PGK-1* promoter 5' of a *loxP* site and an *IRES* sequence-driven transgene 3' of the same *loxP* site. The transient expression of Cre recombinase induces a trans-recombination event between the *loxP* site in the trapped locus and the *loxP* site of the "pop-in" vector, allowing the integration of the vector. Insertion of the "pop-in" vector G418 selection is used to select clones in which Cre-mediated insertion occurred. In these lines, the transcriptional activation of the trapped gene results in a bicistronic message of the *LacZ* fusion mRNA and the transgene,

which allows co-translation of LacZ and the transgene-encoded product.

Derivation of Gene-Trap Cell Lines

A total of 1,400 neo-resistant colonies were obtained following electroporation using the pNH94 plasmid of which 10⁴ clones exhibited LacZ staining in undifferentiated ES cells. The expressions varied from sparse (only a few blue cells among a few thousand) to strong overall (more than 98% LacZ-positive cells). Then the clones were characterized by Southern blotting for the trap vector integration, and those carrying single copy and single site of integration of the pNH94 vector (60% of the lines) were identified. Two clones, 2.22 with mosaic/moderate and 2.C1 with strong LacZ expression, were selected for further studies (Fig. 1B).

Cre-Mediated Excision of the PGK Promoter

The 2.22 and 2.C1 lines were independently expanded and submitted to transient Cre expression using our circular pCAGGS-nlsCre plasmid. Randomly picked colonies were tested for the removal of the *PGK-1* promoter by PCR and Southern blot analysis (Fig. 1C). The excision occurred in both lines at a similar (50%) efficiency. All the clones, exhibiting the expected 2.0-kb Southern band, lost neo resistance shown by G418 selection. For further experiments, one subline from each parental line was selected and designated as 2.22/8 and 2.C1/B6, respectively. The 2.C1/B6 subline exhibited the same strong LacZ expression as the C1 parental line (Fig. 2B, D), whereas the 2.22/8 subline interestingly showed stronger LacZ staining than the parental one (Fig. 2A, C). This result was attributed to the *neo* gene expression itself, which has been shown to mediate a negative effect on the expression of adjacent genes (Artelt *et al.*, 1991; Rijli *et al.*, 1994).

Cre-Mediated Insertion of an IRES-Transgene Cassette

A pop-in vector was constructed, as described above, using a human alkaline phosphatase (*bAP*) gene as a transgene (pNH99). The circular "pop-in" vector was co-transfected with the circular pCAGGS-nlsCre plasmid at different ratios into the 2.22/8 and the 2.C1/B6 sublines. Surprisingly, some neo-resistant colonies were obtained when only the "pop-in" or the Cre expression plasmids alone were electroporated (Table 1). In these cases, however, no proper Cre-mediated insertion oc-

FIG. 1. Three-step strategy to utilize endogenous "trapped" gene expression for any transgene. **(a)** Step 1: structure of the gene-trap vector and its integration into an intron; step 2: Cre recombinase-mediated removal of the promoter driving the *neo* selectable marker; step 3: Cre-mediated insertion of a circular plasmid delivering a transgene immediately downstream of *LacZ*. The transgene is transcriptionally joined to *LacZ* and independently translated from the viral internal ribosome entry site (*IRES*). **(b)** Selection for single-site and copy integration among the gene-trap lines by Southern blotting of *EcoRV*-digested genomic DNA. Lanes 1, 3, 5, and 7 show multiple copy and site integrations of the gene-trap vector. **(c)** Assay to detect Cre-mediated excision of *neo* driving promoter. Lane 1 shows mosaic clone with incomplete excision as opposed to lanes 3 and 5 with complete excision and lanes 2, 4, 6, and 7 with nonremoval of the promoter. This latter band was observed in the Southern blot analysis on DNA of the parental (pre-Cre) cell line as well (data not shown). **(d)** PCR detection of Cre-mediated "pop-in" into the trap insertion.

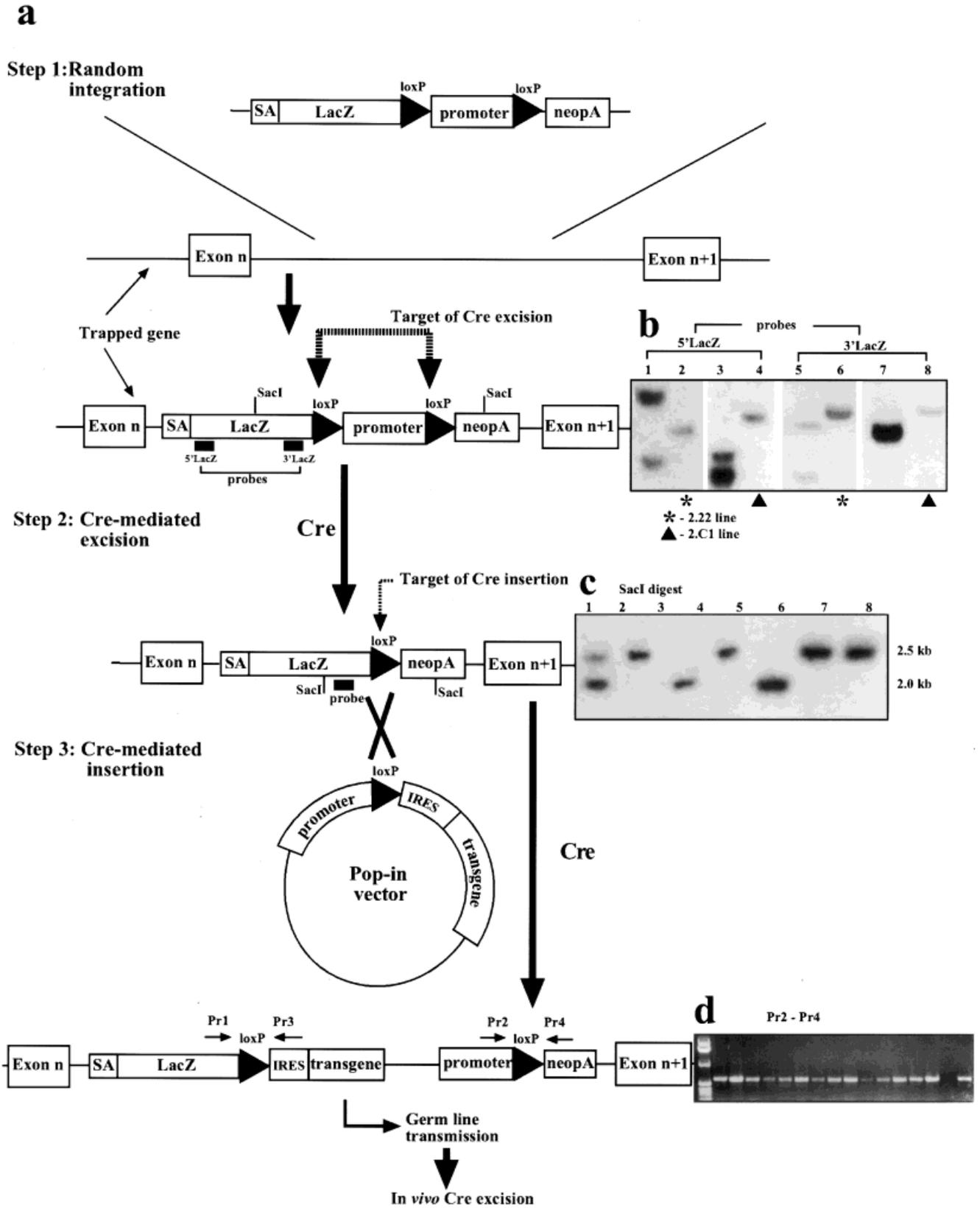


FIG. 1

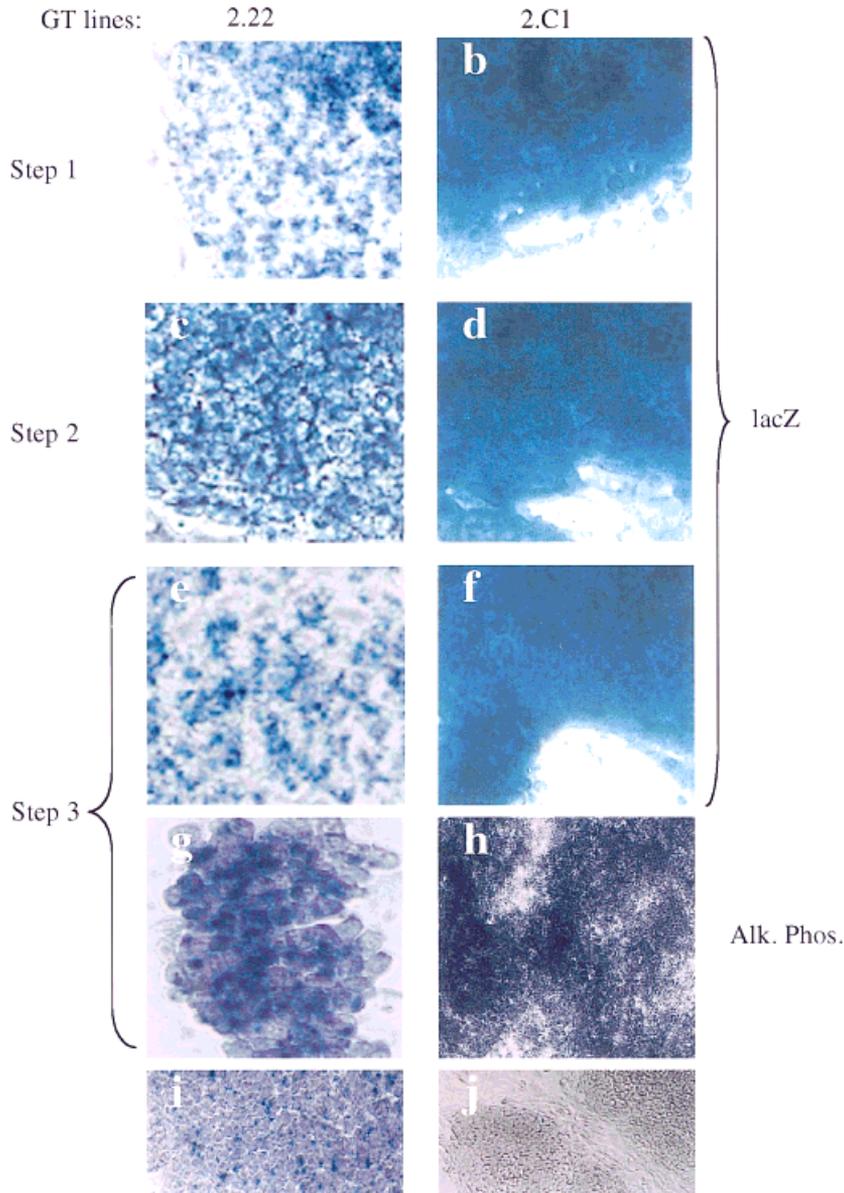


FIG. 2. Expression of the LacZ and hAP reporters by the trapped gene in two cell lines (2.22 and 2.C1) in the different steps of the gene-trap “pop-in” strategy. (a, b) LacZ expression in the primary gene trap (step 1). (c, d) LacZ expression after removal of the promoter-driving neo (step 2). LacZ (e, f) and hAP (g, h) expression after Cre-mediated insertion of the “pop-in” vector (step 3). (i) Double-stained step 3 cell line. (j) hAP staining remained negative on cell line in which no “pop-in” occurred.

curred into the gene-trap loci. When the two vectors were co-electroporated, the majority (>90%) of the neo-resistant colonies were found to be generated by Cre-mediated insertion into the trapped gene (Fig. 1D). The highest number of colonies was obtained when the concentration of the “pop-in” plasmid was 20 μ g and the Cre expression plasmid was equal to or above 10 μ g DNA per electroporation (Table 1).

Expression of the “Pop-In” Gene

After the Cre-mediated insertion of the “pop-in” vector, the cells co-expressed LacZ and hAP. The LacZ staining was similar in the “pop-in” lines and in the parental lines moderate for 2.22 and strong for 2.C1 sublines (Fig. 2E, F), demonstrating that the newly introduced *IRES* se-

quence did not compromise the expression of the preceding *LacZ* gene. The level of hAP expression was comparable to that of LacZ (Fig. 2E–H), showing that the expression level of the *IRES-hAP* correlated with the strength of the trapped promoter predicted by LacZ expression. Moderate staining performed on 2.22 sublines allowed double staining for the two reporters (Fig. 2I). Clones, which became neo resistant without proper Cre-mediated insertion of the “pop-in” vector, did not express the hAP reporter (Fig. 2J).

DISCUSSION

Site-specific recombinases are now integrated parts of genome alteration strategies, which have been quickly

Table 1
Efficiency of Cre Recombinase-Mediated Insertion Into Gene-Trap Sites Containing a Single loxP Site

| Pop-in plasmid/Cre plasmid per electroporation | Number of ES cells electroporated | Number of neo ^R colonies | Number of neo ^R colonies analyzed by PCR for insertion | Cre-mediated insertion occurred |
|--|-----------------------------------|-------------------------------------|---|---------------------------------|
| 20 μg/20 μg ^a | 5 × 10 ⁶ | 115 | 58 | 53 |
| 20 μg/10 μg ^b | 5 × 10 ⁶ | 116 | 36 | 33 |
| 20 μg/5 μg ^b | 5 × 10 ⁶ | 57 | ND | ND |
| 20 μg/1 μg ^b | 5 × 10 ⁶ | 40 | 12 | 12 |
| 1 μg/20 μg ^b | 5 × 10 ⁶ | 0 | — | — |
| 20 μg/0 μg ^b | 5 × 10 ⁶ | 46 | 46 | 0 |
| 0 μg/20 μg ^b | 5 × 10 ⁶ | 20 | 20 | 0 |

^aMean of three experiments.

^bOne experiment.

ND: not determined.

Insertion determined by PCR analysis.

developing after the advent of embryonic stem cells. By combining different tools, there are few limitations left concerning the creation of imaginable gene expression and genome alterations. We are not only able to knock out a gene of our particular interest (Mansour *et al.*, 1988), but we can also create a large range of changes, such as point mutations (Nagy *et al.*, 1998), isoform knockouts (Carmeliet *et al.*, 1999), large genomic deletions (Ramirez-Solis *et al.*, 1995), and inversions and translocations (Smith *et al.*, 1995; Zheng *et al.*, 1999). Furthermore, using Cre recombinase-mediated excisions, we can post-insertionally modify transgenic sites either in vivo or in vitro, which allows lineage-specific or inducible gene knockouts or transgene activation/inactivation (Nagy, 2000). Among these techniques, the Cre-mediated site-specific insertion has been the slowest to develop due to unique characteristics of Cre action. Namely, although recombination between a loxP site in a circular plasmid and a loxP site placed into the genome creates an insertion, it also results in two loxP sites in cis position flanking the inserted sequence. Then the introduced segment can be immediately subjected to Cre-mediated intramolecular excision, which is much more efficient than an intermolecular insertion event. Obviously, the equilibrium favors the excised over the inserted state by order of magnitudes.

Three strategies have been developed to overcome this problem. The first utilizes a strict positive selection system that renders a cell resistant to a selective agent only if insertion occurred. The idea here is to introduce a silent, promoterless resistance gene placed after a single loxP site into the genome that marks the future insertion site. Due to the lack of a promoter, the selectable marker is not expressed. If Cre is expressed transiently in the presence of a loxP site containing circular vector, the recombinase introduces the plasmid into the loxP-determined site of the genome. If this insertion, by design, delivers a promoter for the selectable marker, clones retaining the insertion can be isolated (Fukushige and Sauer, 1992). The second means of Cre-mediated insertion utilizes the inability of the Cre recombinase to tolerate certain nucleotide changes in its recombining

loxP sites. Here, the loxP that is placed into the genome is mutant in one of the nucleotides at its 5' end and the loxP site of the inserting plasmid is mutant at its 3' end. Recombination between the two single-mutant loxP sites inserts the required DNA element but also creates wild-type and double-mutant loxP sites around the insertion. Since the cis recombination efficiency is much lower between these sites than it was between the single mutants, the insert remains at a reasonable frequency (Albert *et al.*, 1995; Araki *et al.*, 1997). The third strategy takes advantage of an altered lox site designated as lox511. Homotopic recombination between two lox511 or between two wild-type loxP sites occurs at a high efficiency. Heterotopic recombination, however, is much less efficient. Both loxP and lox511 sites are placed at the genomic insertion site; the "pop-in" vector also has these sites flanking the desired insertion sequence. Cre-mediated insertion occurs by the efficient homotopic recombination (Soukharev *et al.*, 1999).

In this study, we combined the positive-selection strategy described above with gene trapping. This approach provides a method to "pop in" any exogenous DNA into a loxP site inserted into a trapped locus. As proof of principle, we inserted an IRES-human Alkaline Phosphatase gene (*IRES-hAP*) cassette immediately downstream of the *LacZ* reporter coding region of the primary gene-trap insertion. The modified insertion resulted in co-expression of the *LacZ* and the hAP reporters under an endogenous gene's regulation. A single neo-positive selectable marker was used for both detecting the integration of the trap vector into the ES cell genome and for selecting the Cre-mediated insertion into the trap site. This strategy requires three consecutive cloning steps to reach the desired trapped gene alteration, which allows the expression of any transgene under the control of an endogenous gene's regulation. The three steps do not cause a limitation in germline transmission of the end product cell line if cautious measures are taken after each subcloning to preserve good developmental potential (Nagy *et al.*, 1993). The intermediate ES cell clones, in which the neo-driving, loxP-flanked promoter was removed, became G418 sensitive. When the Cre expres-

sor and the "pop-in" plasmids were co-introduced into these cells, more than 90% of the neo-resistant colonies underwent the expected Cre-mediated insertion, delivering a promoter for the *neo* gene. Intriguingly, at a lower efficiency, we obtained neo-resistant colonies when only one of the two plasmids was electroporated into the cells. In these cases no insertion occurred into the trap site, therefore the *neo* selectable marker remained promoterless. A possible explanation is that due to the lack of the polyA signal after the *LacZ* gene, the promoterless *neo* gene was included into the mRNA produced by the trapped locus, which could result in a trace amount of neo expression. When a large number of cells were placed back into G418, the cells died, except those that had undergone a similar event that operates when homozygous knockout ES cells are derived from heterozygous cells with high concentration G418 selection (Mortensen *et al.*, 1992). Further studies are required to prove this hypothesis and to show that at least some of these clones are in fact homozygous gene-trap lines.

Recently, the mutant loxP strategy to mediate insertion described above was combined with the gene trapping (Araki *et al.*, 1999). Obviously, lox511 homotopic recombination should work as well. The pros and cons are well balanced among the three different ways of solving the problem of the Cre-mediated introduction of a DNA segment into a defined site of the genome. This balance makes none of them superior to the others. Combining these strategies with gene trapping provides a method to "steal" endogenous gene regulation for transgene expression. Gene-trap insertions frequently mutate the trapped genes. Most of these mutations, however, are compatible with heterozygous normality. Therefore, heterozygotes can be used for transgene expression after Cre-mediated insertion described in this study. This method provides an additional useful and unique strategy to rescue the gene trap or targeted mutation by "popping in" the cDNA of the trapped gene (Tucker *et al.*, 1996; Saga, 1998). This approach leaves two wild-type, fully functional *loxP* sites around the rescuing cDNA. Therefore, only this strategy provides an in situ, cell lineage-specific restoration of the mutation by crossing with lineage-specifically expressed Cre recombinase transgenic lines. This approach would provide a method to perform conditional or lineage-specific gene-trap mutational analysis.

Gene-trap strategies were originally designed for detecting (trapping) genes, which were expressed in undifferentiated ES cells. However, the development and use of ES cell in vitro differentiation assays provide a means to trap genes that are not expressed in undifferentiated ES cells (Forrester *et al.*, 1996; Stanford *et al.*, 1998). These methodologies make a large proportion of genes accessible for trapping and therefore eliminate a limitation of the presented gene-trap replacement strategy.

MATERIALS AND METHODS

Construction of the Gene-Trap Vector

The pPT1 plasmid (Hill and Wurst, 1993) was used as starting material. A *HindIII-XbaI* fragment containing the Engrailed-2 (*En-2*) intron followed by the *En-2* splice acceptor (SA) site and the bacterial β -galactosidase gene (*LacZ*) was excised to remove the polyadenylation site (polyA) of *LacZ* as well as the *PGK-1* promoter-*neo*-polyA cassette from this original plasmid. A promoterless neomycin selection marker carrying its own polyA site was then inserted 3' of the *LacZ* gene. Finally, a cassette containing a *PGK-1* promoter flanked by two identically oriented wild-type *loxP* sites derived from the pBS65 plasmid (Sauer *et al.*, 1987) was inserted between the stop codon of the *LacZ* gene and the 5' end of the *neo* cassette. The resulting plasmid was called pNH94 (Fig. 1).

Generation of the Trapped Clones

The pNH94 plasmid was linearized 5' to the *En-2* intron at the *HindIII* site, and 20 μ g/cuvette DNA were electroporated in R1 ES cells (Nagy *et al.*, 1993) as previously described and neo-resistant clones were established. Screening for β -galactosidase expression was performed in 96-well plates on 1,400 neo-resistant colonies. To identify single-copy integrations, genomic DNA was prepared from the 104 clones exhibiting LacZ activity. They were digested with *EcoRV* and analyzed by Southern blotting using a probe encompassing the *NcoI-ClaI* fragment from the *LacZ* gene (5' probe). In the case of single-copy and single-site integration, the hybridization was expected to reveal a single band of at least 3 kb. Candidate clones were rescreened using an independent probe encompassing the *SacI-EcoRI* fragment from the *LacZ* gene (3' probe), which was expected to identify single bands greater than 6.6 kb in size.

Cre-Mediated Excision

The clones 2-22 and 2.C1 were subjected to electroporation with 20 μ g/cuvette of the circular pCAGGS-*nl*sCre plasmid (Nagy, 2000), allowing transient expression of the Cre recombinase. Cells were plated at low density (100 cells per ml in a 10-cm dish) to enrich for single cell-derived colonies. Since a Cre-mediated excision should remove the promoter of the *neo* gene, the subclones were replica-plated then screened in G418 for loss of neo resistance. Complete excision of the promoter was confirmed by PCR and Southern blot analysis. In the latter, genomic DNA was digested with *SacI* and probed with a 3' *LacZ* probe. Bands of 2.5 kb and 2.0 kb were expected from nonexcised and excised clones, respectively. For PCR, the forward primer was chosen from the 3' end of the *LacZ* gene (primer 1: 5'-CAT-CTG-CTG-CAC-GCG-GAA-GAA-GG-3') and the reverse primer from the 5' end of the coding sequence of the *neo* gene (primer 4: 5'-CGA-ATA-GCC-TCT-CCA-CCC-AAG-C-3'). In case of excision, these two primers are expected to

amplify a 507-bp band as opposed to a 1,027-bp band representing the pre-excision state. Partial excision resulted in a mosaicism in the clones, which was detected by the presence of both bands. After careful diagnosis of the promoter excision, one subline of each clone was randomly selected (designated as 2.22/8 and 2.C1/B6, respectively).

Cre-Meditated Insertion

Different concentrations, up to 20 $\mu\text{g}/\text{cuvette}$, of both pCAGGS-nlsCre and an insertion plasmid, pNH99, were co-electroporated into the neo-sensitive clones 2.22/8 and 2.C1/B6. The insertion plasmid pNH99 consisted of a *PGK-1* promoter inserted into the pBS65 (Sauer *et al.*, 1987) plasmid 5' to the *loxP* site. An IRES sequence from the encephalomyocarditis virus (Jang *et al.*, 1988) was cloned immediately 3' of the *loxP* site, followed by a human Alkaline Phosphatase (*hAP*) reporter gene (DePrimo *et al.*, 1996) (Fig. 1). The two plasmids were electroporated as circular forms. G418 selection (180 μg of active G418/ml) was applied two days after electroporation and maintained until individual neo-resistant colonies appeared. The colonies were randomly picked and replica plated. One replica plate was frozen while the others were subjected to DNA analysis and to LacZ or hAP staining. For PCR analysis, two sets of primers were used: the first set consisted in primer 1 (see above) as the forward and primer 3 (5'-GAT-ATC-AAG-CTT-ATC-GAT-ACC-GTC-G-3') from the 5' end of the *IRES* sequence as the reverse primer. They amplified an approximately 300-bp band when insertion occurred. The other set was composed of a forward primer corresponding to sequence from the *PGK-1* promoter (primer 2: 5'-CCT-CGC-ACA-CAT-TCC-ACA-TCC-3') and primer 4 (see above) as a reverse primer. This set of primers amplified a 618-bp DNA fragment if the *PGK-1* promoter-*neopA* cassette was reconstituted. Further proof of proper insertion of the pNH99 plasmid was obtained by Southern blotting analysis of the genomic DNA, which was digested with *Clal*. Hybridization with the 3' *LacZ* probe resulted in a 2.3-kb band. Insertion at the 3' side was only verified by PCR.

Staining for LacZ and hAP

For LacZ analysis, cells were rinsed in PBS, fixed in 0.2% glutaraldehyde, 2mM EGTA, 2mM MgCl_2 at room temperature for 5 min, then washed three times in wash solution (Lobe *et al.*, 1999) at room temperature for 10 min each. Staining was performed overnight at 37°C in 0.02% X-Gal, 5mM $\text{K}_3\text{Fe}(\text{CN})_6$, 5mM $\text{K}_4\text{Fe}(\text{CN})_6$, 2mM MgCl_2 in PBS. hAP staining was performed as follows: After fixation and washing, the endogenous alkaline phosphatase was heat inactivated by incubation in PBS at 70°C for 30 min. Samples were then rinsed in PBS, washed in 100 mM Tris-HCl pH9.5, 100 mM NaCl, 10 mM MgCl_2 for 10 min, then stained with BM Purple AP substrate (Boehringer) at room temperature overnight. The stained samples were washed extensively in PBS, 0.1% Tween-20, 2 mM MgCl_2 .

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