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Commentary

Targeted transgenesis

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The decade of the 1980s witnessed a revolution in mouse molecular genetics. At its start, the first transgenic mouse was created by nuclear injection of cloned DNA into fertilized mouse eggs (1). This built on earlier studies in which viruses carried DNA into mice (2). With nuclear injection of DNA, investigators rapidly reported germ-line transmission of transgenes (3, 4), expression of transgenes (5–7), and a phenotype associated with transgene expression (8). Subsequently, in a flurry of studies that have continued unabated, transgenic mice have been used to study a number of phenomena, including tissue-specific gene expression, oncogenesis, and developmental mutations (9). Since injected DNA primarily integrates at random locations in the genome, transgene insertion by microinjection is usually restricted to gain-of-function studies in the mouse.

By the end of the decade, the second major advance in mouse molecular genetics had occurred—the disruption of genes in mice. Key to this approach was the ability to grow pluripotential embryonic stem (ES) cells in culture (10, 11). If their pluripotency is maintained, ES cells can be returned to mouse embryos and contribute to all tissues of a mouse, including the germ line (12). ES cells were modified to carry foreign DNA into mice using standard gene transfer approaches (13, 14) and could also be manipulated to carry a selectable mutation into mice (15, 16). Most significant, however, was the application of gene targeting to these cells (17, 18), resulting in the creation of loss-of-function mutations in the mouse (19, 20). With gene targeting, the homologous integration of DNA into the genome, any cloned sequence can be altered in the genome. Several hundred "knock-out" mice have now been created (21–23; TBASE, http://www.gdb.org/Dan/tbase/tbase.html). These two approaches, gain-of-function transgenesis and gene targeting in ES cells, have now been combined, as reported in this issue of the *Proceedings* (24).

The report by Bronson *et al.* (24) describes the introduction of transgenes by gene targeting into the hypoxanthine phosphoribosyltransferase (*hprt*) locus of ES cells. The transgenes consist of a mouse *bcl-2* cDNA driven by either the chicken or human β-actin promoter. Gene targeting of transgenes controlled both the site and copy number of the transgene insertion. The results presented in this report are convincing that targeted integrations of a transgene, in contrast to random integrations, yield consistent expression levels of transgenes both in cell clones and in mouse tissues. Targeting transgenes to loci in ES cells, therefore, may be a much improved method for controlling their expression.

Standard Transgenesis

In contrast to the targeting technique used by Bronson *et al.* (24), the standard approach for the introduction of DNA into cells, including microinjection into fertilized mouse eggs, involves the random integration of DNA into the genome (Fig. 1). The mechanism of the illegitimate recombination events is not understood, but may be related to nonhomologous pathways that mammalian cells generally use to repair damaged DNA (25). Transgenes frequently integrate in tandem arrays consisting of a few copies to several hundred copies of the transgene. Although the integration is often in a single chromosomal locus, integrations occasionally occur into two chromosomal loci, requiring breeding to the next generation to segregrate the transgene sites. Integration may also occur after DNA replication or cell division, resulting in a founder mouse that is mosaic for the transgene. Germ-line and somatic tissues in such cases usually have a similar amount of mosaicism. However, founder mice occasionally do not transmit the transgene, suggesting that the germ line does not contain the transgene. In some cases, insertion mutations result from transgene integrations.

The two properties of transgene integration by standard approaches, the randomness of the site of integration and the variability in copy number, can have profound effects on transgene expression. Transgenes may express poorly or not at all, possibly due to the genomic location of integration—i.e., closed chromatin regions. Alternatively, high levels of transgene expression may be obtained, for example, in cases in which a large number of copies have integrated, although expression is frequently not correlated with copy number. Thus, to draw conclusions about transgene expression, it is often necessary to analyze several transgenic mouse lines to obtain a consensus.

Variation in transgene expression attributable to local chromatin effects at the site of integration has been controlled in some cases by the addition of cis-acting elements that can direct the formation of transcriptionally active domains. These elements include locus control regions, insulator elements, and matrix or scaffold attachment regions. The β-globin locus control region is perhaps the best-studied element of this type, giving position-independent, as well as copy number-dependent, expression to the human β-globin gene (26) in transgenic mice, as well as to heterologous genes in cell lines (27). More recent studies have identified other such elements that direct position-independent, copy number-dependent expression in multiple tissues (28).

Variability in copy number integration in transgenic mice can also be tackled by the addition of cis-acting elements. The site-specific recombinase Cre can delete sequences between two *loxP* sites. While this approach is expected to be used widely in conjunction with gene targeting to delete gene segments in a tissue or temporally regulated manner, it may have utility in the reduction of transgene copy number. A *deleter* mouse that expresses the Cre recombinase during germ cell development (29) mated to a mouse that contains a single *loxP* site in each transgene copy in an array would be expected to give rise to progeny with a reduced number of transgene copies. Thus, starting with one transgenic line, it may be possible to generate several lines expressing a transgene at a variety of levels.

Gene Targeting and Targeted Transgenesis at *hprt*

Although random integrations of introduced DNA predominate, homologous integrations do occur, resulting in the

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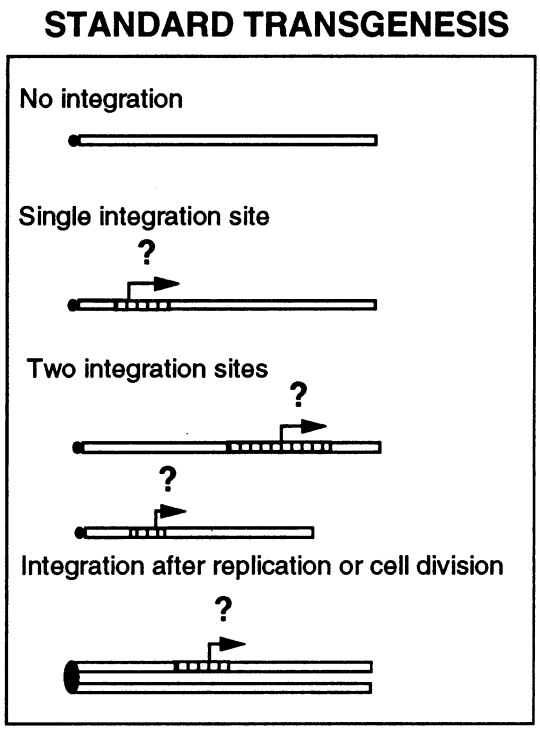

FIG. 1. Standard transgenesis by microinjecting DNA into fertilized eggs. Successful integration of the transgene may occur in up to 10–30% of the mice. The transgene usually integrates into a single chromosomal locus in the fertilized egg, although two independent integrations may occur or integration may occur after DNA replication or cell division. The transgene frequently integrates in head-to-tail tandem arrays consisting of one or a few copies to several hundred copies of the transgene, as indicated by the small boxes. One or more transgenes in the array may be expressed (question mark), depending on the chromatin context of the integration site.

introduction of DNA to a precise location in the genome. The first examples of gene targeting required extensive screening for the infrequent homologous events, which occurred at about 10^{-3} to 10^{-5} of total integrations, whether the locus was "natural" (30) or artificially constructed (31, 32). Since then, numerous examples in ES cells have generally yielded targeting frequencies of 10^{-2} or higher of total integrations. When enrichment schemes are incorporated into the selection, the percentage of targeted clones among selected clones can be enhanced (33). A targeting frequency of 10^{-2} of total integrations corresponds to an absolute targeting frequency of 10^{-5} to 10^{-6} of the transfected cell population, making drug selection essential for isolating targeted clones.

Targeting transgenes to the endogenous *hprt* gene, which is itself both a positive and negative selectable marker, is ideal. Because the *hprt* gene is located on the X chromosome and is hemizygous in XY ES cells, integrations that disrupt the gene can be directly selected for in appropriate media. Mice containing the disruption are viable (15, 16). Since phenotypes might be uncovered with disruption of this locus in some genetic backgrounds (34), Bronson *et al.* (24) used an *hprt*$^-$ cell line and selected for correction of the mutant *hprt* gene. The mutant cell line contains a 5′ gene deletion and was originally generated in 1987 (15), although more recent derivations of this mutant are available (ref. 35; B. Koller, personal communication). Gene targeting restored the *hprt* gene and placed the transgenes upstream of the *hprt* locus (Fig. 2*A*). The inserted transgenes, the chicken and human β-actin promoter-driven *bcl-2* genes, are 1.6 and 5.8 kb, respectively. The chicken β-actin promoter is 350 bp, whereas the human β-actin 5′ region is 4.5 kb, including 3 kb of promoter sequences, as well as an intron of 0.8 kb. Previous studies have concluded that inserts into targeting vectors ranging from 8 bp to 12 kb have no effect on targeting efficiency (36) .

Since the *hprt* locus is widely expressed in the mouse, Bronson *et al.* (24) expected that it would be a favorable transgene expression site. The β-actin *bcl-2* transgenes targeted to the *hprt* locus were expressed in each of the ES cell clones analyzed. The five clones with the human β-actin promoter expressed the transgene at similar levels to each other and at consistently higher levels than the five clones with the chicken β-actin promoter. Examination of tissue expression in the mouse gave similar results. The transgenes were expressed in a variety of tissues, with the human β-actin promoter yielding higher expression levels than the chicken promoter. The higher expression from the transgene with the human 5′ region is due either to higher levels of transcription or to the presence of the intron (37). Expression of the adjacent, reconstructed *hprt* gene was also higher in transgenics containing the human β-actin promoter than in those containing the chicken promoter. Interestingly, expression of the *bcl-2* transgenes in tissues was generally as high or higher than the endogenous *bcl-2* gene. Random integrations, in contrast to the targeted integrations, resulted in a broad range of expression of the introduced *bcl-2* gene in 12 ES cell clones that were analyzed, from apparently undetectable levels to high levels of expression. The variation obtained in these random ES integrants likely reflects the variation that would have been obtained in random integrations in transgenic mice. Thus, in comparison to standard transgenic approaches, targeted transgenesis significantly reduces the number of transgenic lines that need to be analyzed. The use of a "drop in" targeting cassette at the 5′ region of the *hprt* locus is likely to be valuable for future analyses of transgene expression and the dissection of regulatory sequences involved in gene expression.

As in the current report using ES cells, targeting a gene to a defined location in the genome of Chinese hamster ovary cells led to highly reproducible expression levels. A *lacZ* gene, driven by the human β-actin promoter, was targeted to either of two loci in the genome by Cre/*loxP*-mediated site-specific recombination (38). The loci were determined by prior random integrations of a promoterless *neo* gene containing a *loxP* site into the genome. Recombination of a plasmid consisting of the β-actin/*lacZ* gene, a *loxP* site, and a promoter was easily selected by activation of the promoterless *neo* gene. Multiple independent clones were obtained in which the *lacZ* gene had integrated into either of the two *loxP–neo* loci. Highly reproducible expression of the *lacZ* reporter was seen among the various clones, with a standard deviation of less than 10%. The absolute level of expression depended on which of the two loci was targeted and what orientation the *lacZ* gene had upon insertion into the chromosome.

Predicting the expression of transgenes, even targeted ones, can still be uncertain, however. Targeting a *lacZ* reporter gene to the third exon of *hprt* was notable for variable expression that was both orientation and cell type-dependent among the targeted clones (39). The promoter driving *lacZ* in this case was a hybrid of polyoma virus and *tk* sequences. We have observed similar variability in expression of a *pgk–lacZ* reporter targeted to the *pim-1* locus (M.E.M. and M.J., unpublished). As with *lacZ* targeted to the *hprt* locus, ES cell clones in this case also demonstrated variegated β-galactosidase staining patterns. Promoter occlusion effects could be responsible in both cases for the variegated staining patterns, since it has been documented that neighboring promoters can influence each other (40–42). In the current study, the *bcl-2* transgenes are targeted upstream of *hprt* (Fig. 2*A*). Such a design may be sufficient to avoid promoter occlusion effects. However, since *bcl-2* expression was monitored by Northern analysis, rather than by expression within individual cells, it is difficult to draw a firm conclusion in this regard.

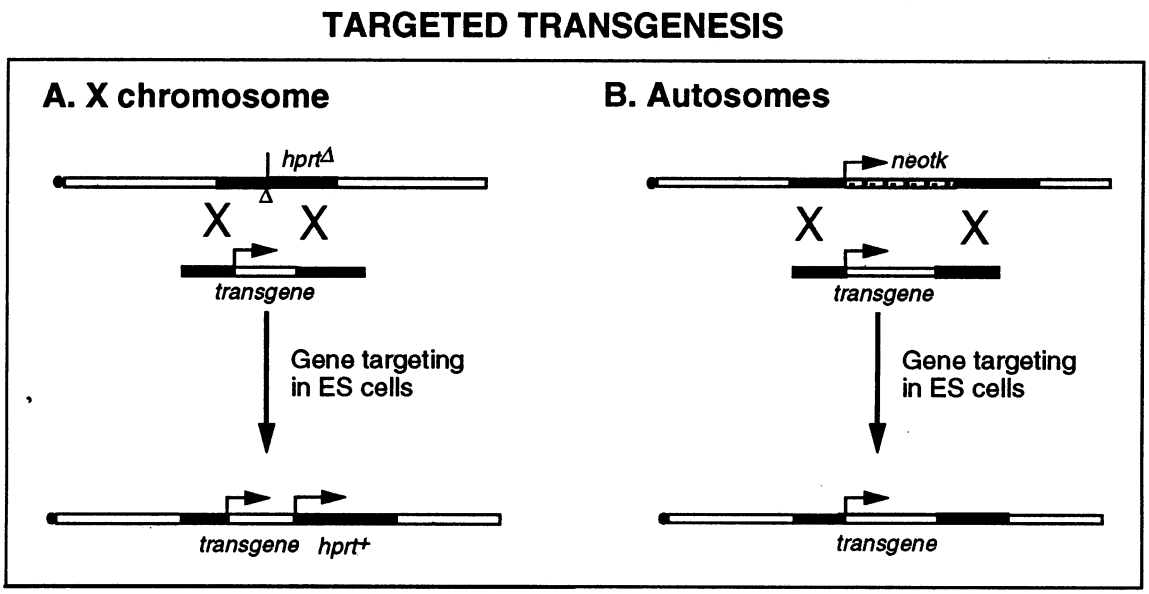

FIG. 2. Targeted transgenesis by integration of a transgene in ES cells using gene targeting. Black bars represent the homologous regions for recombination. (*A*) Gene targeting to the X chromosome is readily selected at the *hprt* locus. In this case, the starting ES cells are *hprt*$^-$ due to deletion of the 5′ end of the gene. Targeting restores a functional *hprt* gene and inserts a functional transgene upstream of *hprt* (see ref. 24 for details). *Hprt*$^+$ cells are selected in hypoxanthine/aminopterin/thymidine (HAT) medium. *Hprt*$^-$ cells can be selected in medium containing 6-thioguanine. (*B*) Repetitive gene targeting of transgenes to autosomes can be directly selected at a locus in which the *tk* (thymidine kinase) marker has been incorporated. The *tk* marker is introduced in a first round of targeting (not shown) with a neomycin phosphotransferase gene (*neo*), selecting for G418^R colonies and then screening for those that have incorporated the drug marker genes at the target locus. Subsequent rounds of targeting at the locus are selected by loss of the *tk* marker in medium containing nucleotide analogs such as gancyclovir.

Since the *hprt* gene is on the X chromosome, transgene expression at this locus is subject to random X-inactivation. The *bcl-2* transgene reported in the current study in homozygous transgenic females appeared to express at a similar level, rather than twice that level seen in hemizygous transgenic males, suggesting that it was indeed subject to X-inactivation. Expression of the transgene in all cells of the female, therefore, requires the generation of homozygous females. Studies in hemizygous females may permit more sophisticated analyses of transgene effects, for example, to determine if transgene expression is deleterious to certain tissue types, as suggested in this report.

Targeted Transgenesis at Other Loci

To avoid the complication of X-inactivation, transgenes could also be targeted to autosomes by using a "drop in" cassette approach similar to that used at *hprt*. Endogenous loci that target easily warrant examination as potential transgene expression sites. At expressed loci, promoterless targeting vectors can been used to enrich for homologous integrations, since most random integrations do not allow expression of a crippled selectable marker (43). Applying this approach in ES cells to both the *pim-1* and *oct-4* loci, 85% of selected clones have been found to be gene targeted (44, 45). The addition of transgenes to the *pim-1* targeting vectors does not change this frequency (M.E.M., P. J. Romanienko, and M.J., unpublished). Targeting at certain other loci, for reasons that are not clear, can be selected at high efficiency, including Rb (46), activin/inhibin βB (47), and olfactory marker protein (P. Mombaerts, personal communication). The selectable marker introduced with the transgene could be subsequently excised from the locus in the targeted cells with transient Cre expression.

Alternatively, modified selection schemes could ease the detection of transgene targeting to other sites (48–50). For example, in the first round of targeting, both positive and negative selectable markers (e.g., *neo* and *tk*, respectively) can be targeted to a desired chromosomal locus. Although this first event would need to be screened for using standard methods, screening for transgene targeting to the locus in subsequent rounds would be facilitated by selecting for loss of the *tk* marker (Fig. 2*B*). Use of this double-replacement strategy for the introduction of mutations in loci has demonstrated that as many as 10% of *tk*$^-$ clones are correctly targeted (49). Since *tk* expression in postmeiotic germ cells can lead to infertility in the male (51), the cells targeted in the first round may not be useful for generating mice.

In addition to targeting transgenes containing their own promoters, gene targeting has been used to drive heterologous gene expression from endogenous promoters. *LacZ* has been frequently targeted into loci to serve as a tag to monitor the expression of the locus (36, 45, 52–58). Running the *lacZ* gene from an endogenous promoter appears to result in reliable expression of the gene. Considering the complexity of promoter regulatory elements, targeting transgenes for expression from endogenous promoters offers a significant advantage over trying to reconstruct complex regulatory interactions within transgene constructs. An elegant application of placing one gene under the control of another's regulatory elements was the replacement of the *Engrailed-1* coding region with the *Engrailed-2* coding region by gene targeting (55). This "knock-in" mutation could rescue the mutant phenotype of the *Engrailed-1* "knock-out," demonstrating the ability of one gene to substitute for its homolog when its expression pattern was altered to its homolog's. The study may have been difficult or impossible to accomplish by using standard transgenic approaches. Variations of this approach include targeting a transgene into an endogenous locus to restrict its expression to a certain cell type (59), to replace a mouse gene with a human one (50, 60), and to create a fusion oncogene (61).

Although the insertion of heterologous genes into endogenous loci has primarily been used in conjunction with the disruption of the endogenous gene, the identification of an internal ribosome entry site that functions in mammalian cells suggests an alternative to gene knockouts for achieving highly regulated expression of transgenes (45, 57, 58). Transgene expression can be coupled to the expression of an endogenous

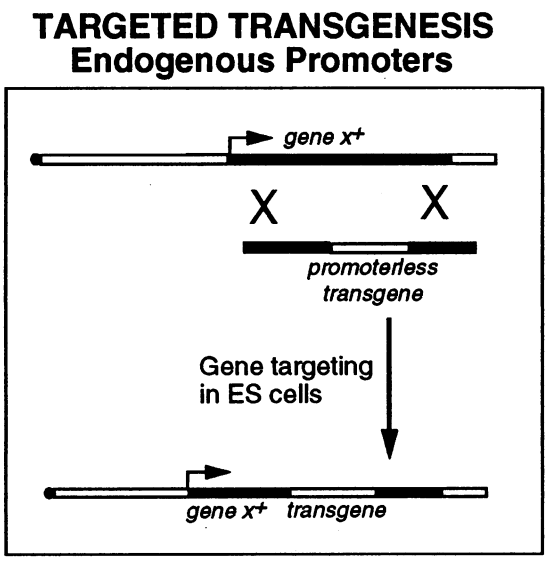

FIG. 3. Targeted transgenesis in which the transgene is expressed from an endogenous promoter. In the example shown, the endogenous gene is not disrupted. This can be accomplished by constructing a dicistronic transcription unit in which an internal ribosome entry site is incorporated upstream of the transgene. The selectable marker incorporated into the targeting vector (not shown) can remain in the locus or be excised by Cre/*loxP*-mediated recombination (see ref. 62).

gene, without disrupting the endogenous gene, by the creation of a dicistronic locus (Fig. 3; refs. 45 and 62).

Targeted transgenesis, as reported by Bronson *et al.* (24), is one of a number of recent developments that refine the ability to manipulate mammalian genomes. As mentioned above, Cre/*loxP*-mediated site-specific recombination, in conjunction with gene targeting, is expected to be used widely in the future to modify genes in a tissue or temporally regulated manner (63). The Cre/*loxP* system has also been utilized to engineer chromosomal translocations (64–66). Recent discoveries that double-strand breaks at a target locus can stimulate gene targeting two to three orders of magnitude (25, 67, 68) suggest that it may be possible to accomplish gene targeting in the absence of drug selection. Using the I-*Sce*I endonuclease to introduce double-strand breaks, absolute targeting efficiencies of 10^{-2} to 10^{-3} of transfected cells have been achieved in ES cells (C.R. and M.J., unpublished), including at the *hprt* locus (G. Donoho, M.J., and P. Berg, unpublished). Other advances in mouse molecular genetics include strategies for inducible gene expression (69, 70) and large-scale screens for identifying insertional mutations (71). Studies that incorporate these advances, and those described in this issue of the *Proceedings* (24), will allow for highly innovative explorations into mouse molecular genetics.

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