

# Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes

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*Gene targeting—homologous recombination of DNA sequences residing in the chromosome with newly introduced DNA sequences—in mouse embryo-derived stem cells promises to provide a means to generate mice of any desired genotype. We describe a positive and negative selection procedure that enriches 2,000-fold for those cells that contain a targeted mutation. The procedure was applied to the isolation of *hprt*<sup>-</sup> and *int-2*<sup>-</sup> mutants, but it should be applicable to any gene.*

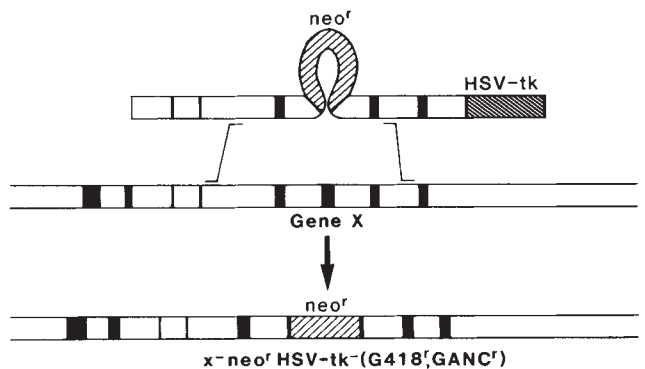
A GENERAL approach for producing mice of any desired genotype would involve the use of standard recombinant DNA techniques to introduce a desired mutation into a cloned DNA sequence of a chosen locus. That mutated sequence would then be transferred into an embryo-derived stem (ES) cell genome by gene targeting<sup>1,2</sup>. Microinjection of the mutant ES cells into mouse blastocysts could then be used to generate germ-line chimaeras<sup>3</sup>, and finally, interbreeding of heterozygous siblings would yield animals homozygous for the desired mutation.

A major technical difficulty in this approach has been the unavailability of practical methods for obtaining ES cell lines carrying desired but non-selectable, targeted mutations at loci of interest. Previous studies have shown that we could specifically inactivate the *hprt* (hypoxanthine-guanine phosphoribosyl transferase) locus in murine ES cells<sup>1</sup>. The *hprt* gene was chosen for these experiments because its location on the X chromosome meant that only one mutant copy was needed to yield a phenotype in male cells. Furthermore, *hprt*<sup>-</sup> ES cells could be directly selected by growth in the presence of the cytotoxic base analogue 6-thioguanine (6TG). The results from these experiments established that gene targeting in ES cells was feasible and defined some of the parameters that control the efficiency of this process. Most genes, however, are present as two copies in the genome, and a selectable cellular phenotype is not associated with the inactivation of the vast majority of these genes. Therefore, isolation of the rare ES cell in which a non-selectable gene has been inactivated by gene targeting must be accomplished by using a suitable enrichment and/or screening procedure.

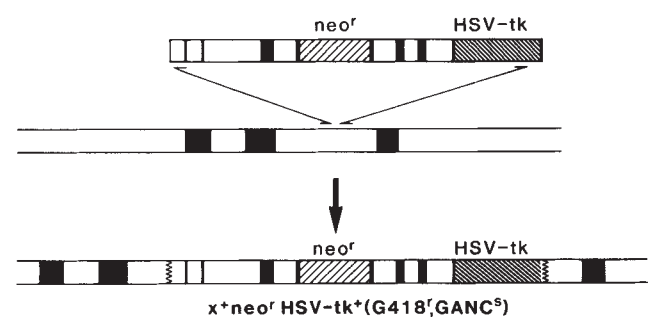
Here we describe a general method for isolating ES cells containing targeted mutations in any gene, regardless of its function. A cloned fragment of the gene must be available and the intron-exon boundaries within that fragment defined. No other information is required. The procedure that we have developed uses a positive selection for cells that have incorporated the targeting vector anywhere in the ES genome (that is, either at the target site or at random sites) and a negative selection against the cells that have randomly integrated the vector into their genomes. The net effect is to enrich for cells containing the targeted mutation. This procedure was used to isolate ES cells containing insertions in the *hprt* and *int-2* genes.

The *int-2* proto-oncogene was first identified as a gene activated in mammary tumors of mice by the nearby insertion of the mouse mammary tumour virus<sup>4,5</sup>. The *int-2* gene codes for a protein with significant similarity to the fibroblast growth factor family<sup>6</sup>, and its expression is highly restricted during mouse development. *int-2* messenger RNAs are detectable at very low levels in ES cells (less than one copy per cell) and are induced following *in vitro* differentiation of ES cells along the extra-embryonic endodermal lineage<sup>7,8</sup>. *In situ* hybridization

## a Gene Targeting



## b Random Integration



**Fig. 1** The PNS procedure used to enrich for ES cells containing a targeted disruption of gene X. *a*, A gene X-replacement vector, that contains an insertion of the *neo<sup>r</sup>* gene in an exon of gene X and a linked *HSV-tk* gene, is shown pairing with a chromosomal copy of gene X. Homologous recombination between the targeting vector and genomic X DNA results in the disruption of one copy of gene X and the loss of *HSV-tk* sequences. Such cells will be *X*<sup>-</sup>, *neo<sup>r</sup>* and *HSV-tk*<sup>-</sup> and will be resistant to both G418 and GANC. *b*, Because non-homologous insertion of exogenous DNA into the genome occurs through the ends of the linearized DNA<sup>9-11</sup>, the *HSV-tk* gene remains linked to the *neo<sup>r</sup>* gene. Such cells will be *X*<sup>+</sup>, *neo<sup>r</sup>* and *HSV-tk*<sup>+</sup> and therefore resistant to G418 but sensitive to GANC. Open boxes denote introns or flanking DNA sequences, closed boxes denote exons and cross-hatch boxes denote the *neo<sup>r</sup>* or *HSV-tk* genes.

analysis of mouse embryo sections has shown that *int-2* is expressed specifically in the parietal branch of the endodermal lineage<sup>9</sup>. This analysis also showed that *int-2* transcripts are found in the embryo proper at 7.5–9.5 days of development: in mesodermal cells migrating through the primitive streak, in the neuroepithelium adjacent to the developing otocyst, and in the endoderm of the pharyngeal pouches. No *int-2* expression has been detected in the adult mouse. This diverse but highly restricted pattern of expression suggests that *int-2* may have several different functions during embryogenesis. To define these roles we have initiated a genetic analysis of *int-2* in the mouse.

## Strategy

In experiments designed to introduce an exogenous DNA sequence at a specific, target locus in the genome, the vast majority of transfected cells contain the exogenous sequence inserted at random loci. Figure 1 outlines our experimental design to enrich for those rare cells in which the exogenous DNA recombined with its chromosomal cognate. The recombinant vector used for these experiments is of the replacement class<sup>1</sup>. It contains 10–15 kilobases (kb) of DNA homologous to the target gene, *X*; a neomycin resistance (*neo*<sup>r</sup>) gene inserted into an exon; and a Herpes simplex virus thymidine kinase gene (*HSV-tk*) adjacent to the target homology. The purpose of using the *neo*<sup>r</sup> gene in the target vector is twofold: first, to disrupt the coding sequence of gene *X* and second, to act as a selectable marker (conferring resistance to the drug G418) for cells containing an integrated copy of the recombinant vector. To maximize the number of chromosomal integration sites that are capable of expressing *neo*<sup>r</sup>, we have used pMC1Neo, a *neo*<sup>r</sup> gene that was modified for efficient expression in ES cells<sup>1</sup>. The *HSV-tk* gene was similarly placed under the influence of the promoter/enhancer that controls the *neo*<sup>r</sup> gene.

The vector is designed so that when replacement of the endogenous *X* sequence by the exogenous one occurs via homologous recombination, the *HSV-tk* gene will not be transferred into the endogenous target (Fig. 1a). Exclusion of the *HSV-tk* gene during homologous recombination occurs because the *HSV-tk* gene represents a discontinuity in the incoming vector between homology and non-homology with the endogenous target sequence. Cell lines in which the targeting event occurred will therefore be *X*<sup>-</sup>, *neo*<sup>r</sup>, *HSV-tk*<sup>-</sup>. On the other hand, random integration of the target vector into the recipient cell genome should result in most cases, in cells that are *X*<sup>+</sup>, *neo*<sup>r</sup>, *HSV-tk*<sup>+</sup> (Fig. 1b) as we and others have shown that the majority of random insertions of exogenous, linearized DNA into the genome occurs through the ends<sup>10–12</sup>. Therefore, by selecting for cells containing a functional *neo*<sup>r</sup> gene (G418<sup>r</sup>) and against cells containing a functional *HSV-tk* gene (gancyclovir resistance, GANC<sup>r</sup>) we enrich for cells in which the targeting event had occurred.

As Herpes infections are a delicate human condition, researchers have actively sought nucleoside analogues that would selectively block viral propagation as a result of their specific metabolism by the viral thymidine kinase<sup>13–16</sup>. Such drug design is possible because the substrate requirements of the *HSV-tk* protein are less stringent than those of the cellular tk enzyme. We tested two such nucleoside analogues, acyclovir and gancyclovir, for their cytotoxic effects on ES cells that contain a functional *HSV-tk* gene. Gancyclovir proved to be more effective. In Fig. 2, we compare the colony forming efficiency of ES cells with two *HSV-tk*<sup>+</sup> ES cell lines as a function of gancyclovir concentration. It is apparent that over the concentration range tested, 10<sup>-8</sup>–10<sup>-5</sup> M, gancyclovir is not cytotoxic to the parental ES cell line but selectively kills ES cells containing *HSV-tk*.

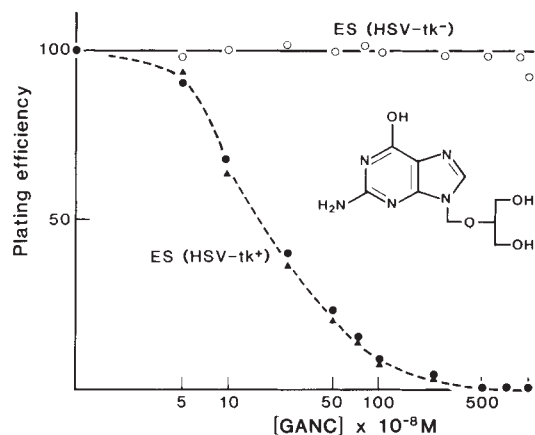
## Enriching for *hprt* targeting events

The feasibility of using the experimental design depicted in Fig. 1 to enrich for cells in which a targeting event has occurred was

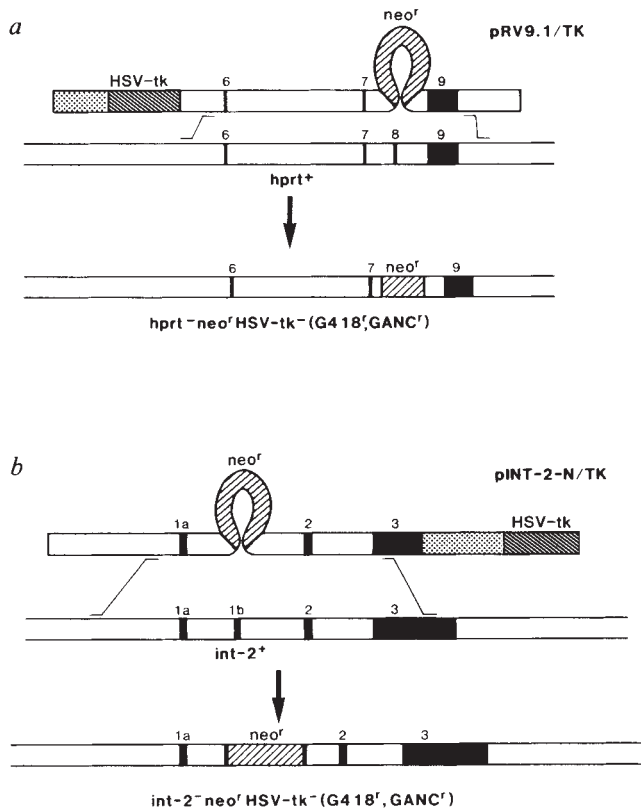
first tested by using the endogenous *hprt* gene as the target sequence. Using this gene, we can directly compare the results obtained by direct selection for *hprt*<sup>-</sup> with those obtained by using the enrichment procedure.

In Fig. 3a we illustrate the *hprt* targeting vector used for these experiments. It represents the previously described *hprt* replacement vector pRV9.1 (ref. 1) to which we have added the *HSV-tk* gene at the 5' end. The linearized DNA was introduced into ES cells by electroporation and the cells were then subjected to different growth conditions (see Table 1 legend). The results of these experiments are summarized in Table 1. Of the cells, 50–60% survived electroporation. The number of G418<sup>r</sup>, G418<sup>r</sup>-6TG<sup>r</sup> and G418<sup>r</sup>-GANC<sup>r</sup> colonies per 10<sup>6</sup> survivors was approximately 10<sup>4</sup>, 5 and 4 respectively. DNA samples from six of the G418<sup>r</sup>-6TG<sup>r</sup> and 24 of the G418<sup>r</sup>-GANC<sup>r</sup> cell lines were analysed by Southern transfer. As expected, all of the G418<sup>r</sup>-6TG<sup>r</sup> cell lines were *hprt*<sup>-</sup> as a result of homologous recombination between the targeting vector and the endogenous *hprt* gene. None of these cell lines contained *HSV-tk* sequences. Thus, as intended, these sequences were eliminated from the incoming vector during the homologous recombination events. Of the 24 G418<sup>r</sup>-GANC<sup>r</sup> cell lines analysed, 19 contained the targeted mutations in the endogenous *hprt* gene. These cell lines were also 6TG<sup>r</sup> and lacked *HSV-tk* sequences. Each of the original colonies was of independent origin (that is, obtained from separate electroporation experiments and on separate plates). From these data we can conclude that the enrichment procedure was successful: the majority of G418<sup>r</sup>-GANC<sup>r</sup> ES cell lines contained targeted disruptions of the *hprt* gene even though we did not directly select for the *hprt*<sup>-</sup> phenotype.

In Fig. 4 we compare the *hprt* Southern transfer pattern of the parental ES cell line with that of two cell lines electroporated with pRV9.1/TK and subjected to either G418 plus 6TG selection (line 40-9n), or G418 plus GANC selection (line 42-6j). The DNA from both of the latter two cell lines is altered at the



**Fig. 2** The effect of gancyclovir on the plating efficiency of ES cells and two derivative, *HSV-tk* containing, ES cell lines. ES cells ( $2 \times 10^4$ ) (○-○) or two derivative ES cell lines (●-● or ▲-▲), each containing a single copy of the *HSV-tk* gene, were plated on 100-mm petri plates containing Dulbecco's modified Eagle's medium, 10% fetal calf serum and the indicated concentration of GANC. The cells were incubated at 37°C in 7% CO<sub>2</sub>. Every three days the cells were fed with fresh medium. After nine days, the plates were stained with Giemsa and the number of colonies per plate determined. The normalized number of colonies from the plates without GANC is represented by 100. Each point represents data obtained by averaging the results from three plates. *HSV-tk*<sup>+</sup> cells were obtained by electroporating a plasmid containing the *neo*<sup>r</sup> gene and the *HSV-tk* gene into the ES cells and selecting for G418<sup>r</sup> cells. The *HSV-tk* gene contained the same promoter/enhancer present in the *neo*<sup>r</sup> gene of pMC1Neo (ref. 1). The insert illustrates the structure of GANC.

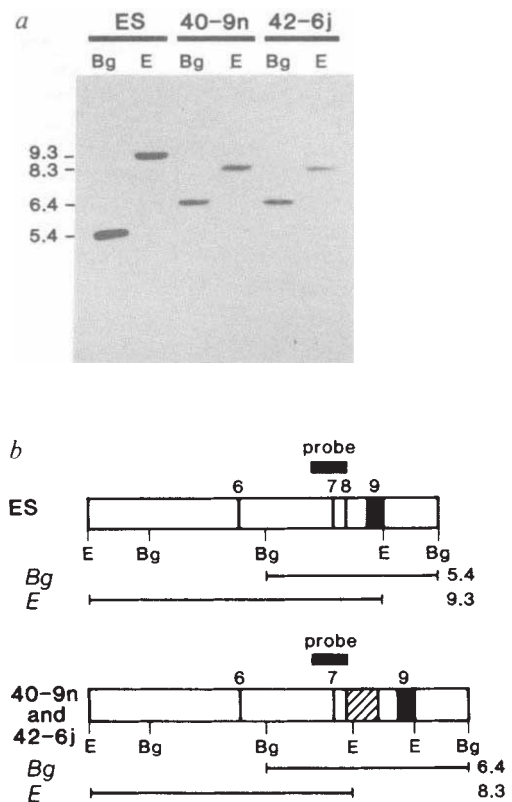


**Fig. 3** Targeted disruption of the *hpert* locus (a) or the *int-2* locus (b) by the replacement vectors pRV9.1/TK or pINT-2-N/TK respectively. a, pRV9.1/TK contains 9.1 kb of DNA from the 3' end of the murine *hpert* gene, exons 6 to 9, with the eighth exon disrupted by the *neo<sup>r</sup>* gene from pMC1Neo (ref. 1), and the *hpert-neo<sup>r</sup>* sequences ligated to the HSV-*tk* gene. After homologous pairing between the linearized targeting vector and the genomic *hpert* gene, a recombination event replaces the endogenous *hpert* sequences with sequences that contain the *neo<sup>r</sup>* insertion. The HSV-*tk* gene is not transferred to the genome. The cell line produced by this recombination event will be *hpert*<sup>-</sup>, *neo<sup>r</sup>* and HSV-*tk*<sup>-</sup> and will thus be resistant to both G418 and GANC. Stippled boxes denote plasmid sequences. The *int-2* replacement vector, pINT-2-N/TK was constructed starting with a 10-kb *int-2* genomic clone containing exons 1a, 1b, 2 and part of 3 in pAT153 (ref. 4). The *neo<sup>r</sup>* gene from pMC1Neo was inserted at the *Apa*I site in exon 1b, the first protein-coding exon, disrupting it, and creating pINT-2-N. The HSV-*tk* gene, with the same enhancer-promoter as the *neo<sup>r</sup>* gene of pMC1Neo, was inserted adjacent to pINT-2-N at its 3' end to create pINT-2-N/TK. Homologous recombination between the vector and genomic *int-2* sequences results in the disruption of one copy of the *int-2* gene and the loss of the HSV-*tk* gene. These cells are *int-2*<sup>-</sup>/*int-2*<sup>+</sup>, *neo<sup>r</sup>* and HSV-*tk*<sup>-</sup>, and thus resistant to both G418 and GANC.

*hpert* locus as predicted for a targeting event. Digestion of parental ES DNA with *Bgl*II and *Eco*RI isolates sequences homologous to the *hpert* probe on fragments of 5.4 kb and 9.3 kb in length, respectively; the patterns of 40-9n and 42-6j are quite different, with fragments of 6.4 kb and 8.3 kb respectively. Because the *neo<sup>r</sup>* gene has no *Bgl*II site, the size of the genomic *Bgl*II fragment is increased by the length of the *neo<sup>r</sup>* insert (~1 kb). However, an *Eco*RI site near the 5' end of the *neo<sup>r</sup>* gene reduces the genomic *Eco*RI fragment by ~1 kb (see Fig. 4b).

### Targeting into the *int-2* gene

Figure 3b illustrates the vector used to target a mutation to the chromosomal *int-2* gene. This targeting vector, pINT-2-N/TK,

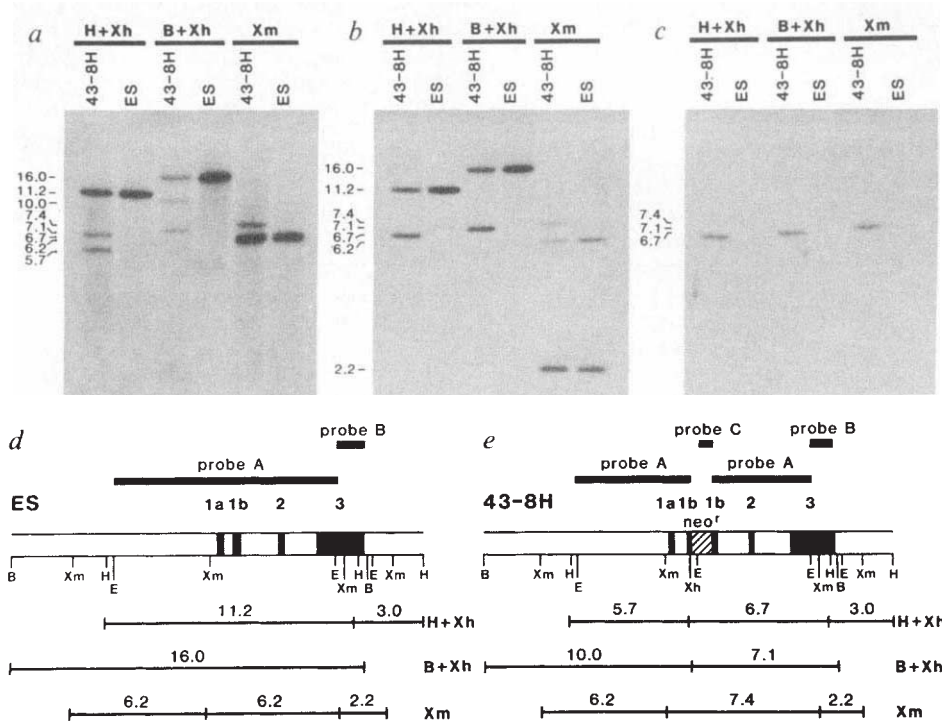


**Fig. 4** Southern transfer demonstration of gene targeting by the targeting vector pRV9.1/TK. ES refers to the DNA from the parental, wild-type ES cell line, 40-9n refers to DNA from a transfected cell line resistant to G418 plus 6TG, and 42-6j refers to DNA from a transfected cell line selected for G418<sup>r</sup> plus GANC<sup>r</sup>. The length of the fragments is in kb. b, A schematic representation of the Southern transfer data. The top map represents the 3' end of the *hpert* gene from ES cells; the bottom map represents the *hpert* gene from a targeted cell line. Beneath each gene is shown the length and position of restriction fragments hybridizing to the probe. *Bg*, *Bgl*II; *E*, *Eco*RI. The very light hybridization signal observed in 40-9n and 42-6j at the parental position (that is, 5.4 kb and 9.3 kb) comes from the feeder cells upon which the ES cells are grown<sup>1</sup>.

**Methods.** ES cells were transfected with pRV9.1/TK and selected for G418<sup>r</sup> plus 6TG<sup>r</sup> (line 40-9n) or G418<sup>r</sup> plus GANC<sup>r</sup> (line 42-6j) as described in the Table 1 legend. Following the isolation of individual cell lines, DNA was purified and digested with restriction endonuclease. DNA (7 µg) was electrophoresed through agarose, transferred to nitrocellulose and hybridized to a <sup>32</sup>P-labelled probe from the murine *hpert* gene<sup>1</sup>.

was linearized as shown, introduced into ES cells by electroporation, and subjected to one of three growth conditions. The results from these experiments are summarized in Table 1. It is evident that G418 plus GANC selection resulted in a 2,000-fold enrichment for ES cells containing a disrupted *int-2* gene. Approximately one in 40,000 G418<sup>r</sup> colonies contained an *int-2* mutation. Of the 81 independent G418<sup>r</sup>-GANC<sup>r</sup> cell lines tested, four (5%), contained a mutant *int-2* gene.

DNA from cells resistant to G418 plus GANC was subjected to Southern transfer analysis to assay for the presence of the targeted disruption in one copy of the *int-2* gene. In Fig. 5 we illustrate Southern transfer analysis of one of the G418<sup>r</sup>-GANC<sup>r</sup> cell lines, 43-8H, in which one of the two *int-2* autosomal genes was disrupted by the *neo<sup>r</sup>* gene. Parental ES DNA that was digested with *Hind*III plus *Xho*I, *Bst*EII plus *Xho*I or *Xmn*I alone and hybridized with the 10-kb genomic *int-2* probe, shows the expected fragments of 11.2, 16 and 6.2 kb doublet respec-



**Fig. 5** Southern transfer analysis of DNA from ES and 43-8H cells. Top: the DNA samples and the enzymes with which they were digested are indicated above each lane. The membranes were hybridized with a 10-kb *EcoRI* fragment from *int-2* (Probe A, a), a 1.3 kb *int-2* flanking DNA fragment not present in the targeting vector (Probe B, b) or a 0.6-kb *PstI* to *BamHI* neo fragment from pMC1Neo (Probe C, c). a, Digestion of DNA from ES cell line 43-8H revealed new fragments compared with the digestion pattern of DNA from the parental ES cell line. Digestion with *HindIII* plus *XhoI* generates new fragments of 6.7 and 5.7 kb; *BstEII* plus *XhoI* digestion generates new fragments of 10 and 7.1 kb; and *XmnI* digestion generates a new fragment of 7.4 kb. b, As expected, probe B hybridizes with a *HindIII* plus *XhoI* fragment of 11.2 kb, a *BstEII* plus *XhoI* fragment of 16 kb, and *XmnI* fragments of 6.2 and 2.2 kb in both the parental and 43-8H cell lines. Additional DNA fragments of 6.7 kb (*HindIII* plus *XhoI*), 7.1 kb (*BstEII* plus *XhoI*) and 7.4 kb (*XmnI*) that hybridize with probe B were detected in 43-8H. d, e, Restriction maps of the parental ES DNA and the targeted DNA (43-8H) in the vicinity of *int-2* are depicted. Open boxes represent introns or flanking DNA, closed boxes indicate the positions of *int-2* exons 1a, 1b, 2 and 3. The *neo<sup>r</sup>* gene is denoted with a cross-hatched box. The position of restriction sites used in the analysis are indicated with the letters B (*BstEII*), E (*EcoRI*), H (*HindIII*), Xh (*XhoI*) and Xm (*XmnI*). Above each map are boxes showing the map positions of the probe fragments used in the hybridization analysis and below each map is a schematic depiction of the expected fragments from each restriction enzyme digestion.

**Table 1** Gene targeting into the *hprt* and *int-2* genes

Vector	Number of cells surviving electroporation	Number of G418 <sup>r</sup> colonies	Number of G418 <sup>r</sup> -6TG <sup>r</sup> colonies	Number of G418 <sup>r</sup> -GANC <sup>r</sup> colonies	Fraction of G418 <sup>r</sup> -GANC <sup>r</sup> colonies containing targeting events
pRV9.1/TK	$1.3 \times 10^7$	$1.5 \times 10^5$	64*	48	19/24
pINT-2-N/TK	$1.5 \times 10^7$	$1.6 \times 10^5$	—	81	4/81

Linearized DNA, as depicted in Fig. 3, was introduced into ES cells by electroporation. Aliquots of cells were then subjected to one of three (pINT-2-N/TK) or four (pRV9.1/TK) growth conditions: non-selective medium to assess the total number of cells surviving electroporation; G418 medium to assay the fraction of survivors transformed by the *neo<sup>r</sup>* vector; G418 plus 6TG medium (pRV9.1/TK only) to select directly for cells simultaneously containing the *neo<sup>r</sup>* gene but lacking a functional *hprt* gene; and G418 plus GANC medium to enrich for, but not directly select for, cells that contain a functional *neo<sup>r</sup>* gene as a result of targeting into the *hprt* or *int-2* genes. ES cells were transfected by electroporation with  $25 \mu\text{g ml}^{-1}$  of linearized pRV9.1/TK or pINT-2-N/TK. The conditions for electroporation, cell culture, selection for G418<sup>r</sup>, G418<sup>r</sup>-6TG<sup>r</sup> or G418<sup>r</sup>-GANC<sup>r</sup> cell lines were as previously described<sup>1</sup> except that in G418-GANC medium, 6TG was substituted by  $2 \times 10^{-6}$  M GANC (gift of Syntex Research). Targeting events were identified by Southern transfer analysis (see Figs 4 and 5).

\*To evaluate the number of G418<sup>r</sup>-6TG<sup>r</sup> colonies, 1/8 of the cells surviving electroporation were grown in G418, 6TG medium. We obtained eight independent G418<sup>r</sup>-6TG<sup>r</sup> colonies. The value shown ( $8 \times 8$ ) was normalized to the total number of cells surviving electroporation.

tively (Fig. 5a). DNA from ES cell line 43-8H that was digested with the same enzymes, reveals fragments the same size as those from the parental line (that is, derived from the wild-type copy of *int-2*), but also reveals new fragments derived from the mutagenized copy of *int-2* (Fig. 5a). Each of these additional fragments is the size predicted from a homologous recombination event between the targeting vector and one copy of the

wild-type *int-2* gene (see Fig. 5d, e and legend). In addition, these new bands are of the same intensity as those derived from the unmutagenized chromosome, suggesting that the cell line is a pure population, heterozygous at the *int-2* locus.

The structure of the mutant allele was further confirmed by hybridizing separate copies of the blot in Fig. 5a with two probes: B, a 3' flanking *int-2* DNA fragment not present in the

targeting vector (Fig. 5b) and C, a DNA fragment derived from the *neo*<sup>r</sup> gene (Fig. 5c). Hybridization with probe B revealed additional fragments in the digest of the 43-8H cell line not present in the parental one (Fig. 5b and legend). This result is only consistent with an event in which the DNA from the *neo*<sup>r</sup>-containing targeting vector (which has no sequence homology to probe B) has replaced one copy of the wild-type *int-2* gene. Finally, the *neo*<sup>r</sup> DNA fragment (probe C) hybridizes only with DNA from 43-8H cells, and the hybridizing fragments align precisely with the unique fragments that were also detected with probe B (Fig. 5c).

In summary, the map positions of 10 restriction sites that cover approximately 20 kb of genomic DNA surrounding the *neo*<sup>r</sup> insertion in the *int-2* gene of 43-8H were confirmed by Southern transfer analysis. Several additional enzymes, including *Eco*RI which delimits the ends of the *int-2* sequences in the target vector, have been used to extend the above results (data not shown). In none of the four *int-2*<sup>-</sup>/*int-2*<sup>+</sup> cell lines have we detected any deviations from the pattern predicted for a homologous replacement of chromosomal *int-2* sequences with DNA from the targeting vector. This suggests that the targeting event did not induce any gross rearrangements of the target DNA other than the *neo*<sup>r</sup> insertion.

The (*int-2*<sup>-</sup>/*int-2*<sup>+</sup>) ES cell lines that we have isolated are capable of differentiating *in vitro* and of efficiently generating chimaeric mice following their introduction into recipient blastocysts. We are therefore encouraged that they will be capable of forming germ line chimaeras. The offspring of such chimaeras could then be interbred to assess the phenotypic consequences of the homozygous *int-2*<sup>-</sup> condition.

## Discussion

We have described an experimental design for isolating ES cells that contain targeted modifications of any endogenous gene, irrespective of the function of that gene. This procedure uses a combination of a positive selection, growth in the presence of G418, for cells containing the input recombinant vector, and a negative selection, growth in the presence of GANC, against cells containing random integrations of the incoming recombinant vector. The net effect of these two selections is to enrich for cells in which the targeted homologous recombination event has occurred. The procedure has been applied to the isolation of cell lines that contain targeted disruptions of the *hprt* and *int-2* genes. In each case, the positive-negative selection (PNS) procedure resulted in a 2,000-fold enrichment of cells that contained homologous versus non-homologous integrations of the targeting vector. In the case of *hprt*, as the ratio of homologous events to random integration events was approximately one in two thousand, nearly every G418<sup>r</sup>-GANC<sup>r</sup> colony that survived PNS treatment (19/24) contained a disrupted *hprt* gene.

Using a similar targeting vector, the relative and absolute frequencies of homologous recombination at the *int-2* locus were 20-fold lower than those at the *hprt* locus. This lower targeting frequency may reflect the lower level of *int-2* expression in ES cells (that is, less than one transcript per cell)<sup>7-9</sup>. The level of expression and the chromatin environment surrounding the target gene could influence the targeting efficiency at that

locus by influencing accessibility to the recombination machinery and/or by influencing the expression of the *neo*<sup>r</sup> gene, within that environment. Arguing against the second point, all of the ES cell lines that contain mutant *int-2* genes are robust in G418 medium, even though *int-2* expression is barely detectable in ES cells.

At this stage it is difficult to predict how much the targeting frequency will vary from locus to locus. However, for genes that are recalcitrant to targeted disruption, the enrichment factor for the PNS procedure may be increased from  $2 \times 10^3$  to  $4 \times 10^6$  by inserting into the targeting vector two HSV-*tk* genes, one at each end. The reason that a higher enrichment was not obtained with the single HSV-*tk* constructs is that some of the HSV-*tk* genes are mutated during transfection (unpublished results). In the PNS procedure we demand that the cells acquire a functional *neo*<sup>r</sup> gene. By placing two HSV-*tk* genes at opposite ends of the target vector, it becomes more likely that one will survive the transfection procedure unimpaired. If the frequency of one HSV-*tk* acquiring a deleterious mutation during transfection is one in  $2 \times 10^3$  molecules, then the frequency of inactivating both HSV-*tk* genes should be one in  $4 \times 10^6$  molecules.

It was somewhat surprising to find that placing a large block of non-homology, such as an HSV-*tk* gene, at both ends of the linearized targeting vector does not reduce its ability to participate in homologous recombination with the cognate chromosomal gene (K.R.T. and M.R.C., unpublished results). This observation at first appears paradoxical. On the one hand, linearized vectors are much better targeting substrates<sup>12</sup>. On the other hand, blocking both ends of the linearized vector with non-homologous DNA does not reduce its targeting efficiency. These results indicate that linearizing the target vector does not increase its targeting efficiency by providing homologous ends for invasion of the target gene. Rather, linearization may convert the targeting vector into a better topological substrate for participation in homologous recombination with the chromosomal gene.

## Conclusion

We have demonstrated that the PNS procedure effectively enriched for ES cells containing targeted disruptions of either the *hprt* or *int-2* locus. The same procedure has also been successfully applied to isolate ES cells containing a mutant mouse homoeobox gene, *hox1.2* (D. Kostic and M.R.C., unpublished results). This procedure is general and requires little knowledge of the target locus. We believe that the procedure should be applicable to any gene. It has worked for a gene that is expressed in ES cells at very low levels, *int-2*, but it remains to be determined whether the procedure is applicable to genes with no detectable expression in ES cells.

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