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Testing an "In-Out" Targeting Procedure for Making Subtle Genomic Modifications in Mouse Embryonic Stem Cells

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We have introduced a 4-bp insertion into the hypoxanthine phosphoribosyltransferase (*HPRT*) gene of a mouse embryonic stem (ES) cell line by using an "in-out" targeting procedure. During the in step, a homologous integration reaction, we targeted a correcting plasmid to a partially deleted *hprt*$^-$ locus by using an integrating vector that carried a 4-bp insertion in the region of DNA homologous to the target locus. *HPRT*$^+$ recombinants were isolated by direct selection in hypoxanthine-aminopterin-thymidine (HAT) medium. The HAT^r cell lines were then grown in medium containing 6-thioguanine (6-TG) to select for *hprt*$^-$ revertants resulting from the excision of the integrated vector sequences. The revertants were examined by Southern blot hybridization to determine the accuracy of this out reaction and the frequency of retaining the 4-bp modification in the genome. Of the 6-TG^r colonies examined, 88% had accurately excised the integrated vector sequences; 19 of 20 accurate revertants retained the 4-bp insertion in the resulting *hprt*$^-$ gene. We suggest a scheme for making the in-out targeting procedure generally useful to modify the mammalian genome.

Gene targeting, the method of using homologous recombination to modify the mammalian genome, can be used to introduce specific changes into cultured cells. By targeting the gene of interest in embryonic stem (ES) cells, these changes can be introduced into the germ lines of laboratory animals to study the effects of the modifications on whole organisms. During the gene targeting procedure, cloned DNA that is homologous to the target locus but has been modified in vitro to carry the intended change is introduced into tissue culture cells. The treated cells are then screened for accurate targeting to find those that have been properly modified.

Several schemes have been devised to aid in the isolation of the recombinants. A positively selectable helper gene is often included in the targeting vector to permit selection for cells that have taken up and expressed the introduced DNA. Typically, this selection results in a 1,000-fold enrichment for targeted cell lines (7, 10, 11, 20, 24). Addition of a second, negatively selectable helper gene allows for selection against random insertion events (15). The positive-negative selection scheme achieves an additional 10- to 1,000-fold enrichment for the homologous recombinants over the single positive selection procedure (4, 15). In both the single positive and the dual positive-negative selection systems, the positive selection element is introduced into the target locus in such a way as to interrupt or knock out the targeted region.

To create subtle modifications of the mammalian genome for fine-structure analyses or to mimic human genetic diseases resulting from small specific mutations, it is desirable to develop a targeting procedure that allows for easy identification of the recombinant cell lines (such as with the aid of selectable sequences) yet does not leave extraneous sequences in either the target or any other region of the genome in the final product. Recently, Steeg et al. (21) reported the introduction of a single base pair change into a mammalian genome without accompanying alterations. They targeted the RNA polymerase II gene in a mouse ES cell line in such a way that homologous recombinants, different from the parental line by only one or two base pairs, were directly selectable with α-amanitin. Zimmer and Gruss (27) described the insertion of 20 base pairs into the Hox 1.1 locus in mouse ES cells without using any selection. The DNA was introduced by nuclear microinjection, and the polymerase chain reaction was used to detect recombinants. To date, there have been no other reports of successful gene targeting using this procedure.

Another procedure for making subtle changes in loci which are not directly selectable was described for the yeast system by Scherer and Davis (17). They targeted the *HIS3* locus with an insertional plasmid carrying a mutated *his3* gene as well as the directly selectable *URA3* gene. Targeted recombinants, isolated by the URA3$^+$ phenotype, were expanded without selection then screened for the ura3$^-$ phenotype. They were able to isolate colonies that had lost all target vector sequences but now had only the mutated *his3* gene initially introduced into the genome by the targeted insertion event. As other yeast targeting techniques have been successfully transferred to the mammalian system, it should be possible to use this type of two-step selection scheme to make subtle changes in nonselectable loci in mammalian cells.

We have therefore tested the feasibility of using a two-step selection procedure in mouse ES cells to introduce a small change into a target locus. To this end, we targeted the mutant hypoxanthine phosphoribosyltransferase (*hprt*$^-$) gene in the cell line E-14TG2a (8) with an insertional plasmid capable of correcting the deletion mutation at this locus. The plasmid carried a 4-bp insertion in the region of DNA homologous to the target locus. *HPRT*$^+$ targeted cell lines were isolated by direct selection with hypoxanthine-aminopterin-thymidine (HAT) medium, expanded, and then selected with 6-thioguanine (6-TG) to recover *hprt*$^-$ revertants. The revertant colonies were examined by genomic Southern blot hybridization to confirm that the integrated vector DNA had been excised from the genome and to determine the frequency at which the intended 4-bp modifi-

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cation was obtained. Our results show that a subtle modification of the mouse *HPRT* gene can be achieved by using the two-step selection procedure. This "in-out" targeting procedure can be adapted to modify nonselectable loci with the aid of the *HPRT* minigene constructed by Reid et al. (16).

MATERIALS AND METHODS

Cell culture. The mouse ES cell line E-14TG2a was isolated as described previously (8, 25). Cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 15% heat-inactivated fetal calf serum (Flow) and 10 μM 2-mercaptoethanol (Sigma). The pluripotential nature of the ES cells was retained by supplementing each liter of growth medium with 10^6 U of recombinant human leukemia inhibitory factor, graciously supplied by N. Gough (Walter and Eliza Hall Institute, Melbourne, Victoria, Australia). Because feeder layers were not used, all culture dishes were coated with 0.1% sterile gelatin to ensure cell adhesion. HAT medium was standard culture medium supplemented with 120 μM hypoxanthine, 0.4 μM aminopterin, and 20 μM thymidine. 6-TG selection was carried out in standard medium containing 10 μM 6-TG. Cultures were incubated at 37°C in an atmosphere of 5% CO_2. They were checked periodically for mycoplasma contamination.

Vectors. Plasmid pNMR133 has already been described (6). It contains 5 kb of DNA identical to the exon 3 target region of the mouse *HPRT* gene, except for a 4-bp insertion that destroys a unique *Hin*dIII site and consequently generates a new *Nhe*I site in intron 2. It also carries the human *HPRT* promoter and exon 1 sequences (which have been shown to function in mouse cells) and the mouse exon 2 region.

Plasmid pNMR133D200 was derived from pNMR133 by removing a 200-bp *Bgl*II fragment from intron 2.

DNA preparation. Targeting vector DNAs were prepared by standard methods, omitting the CsCl purification, which we found unnecessary (unpublished results). All targeting DNAs were linearized by restriction enzyme digestion, using the manufacturers' recommended conditions, prior to electroporations. Digested DNAs were ethanol precipitated and resuspended in sterile TE buffer (0.05 M Tris, 0.001 M EDTA).

DNA transfers and selections. The vectors were introduced into the ES cells by electroporation (2). The cells were grown in 100-mm culture dishes (as described above) to a density of 1×10^7 to 2×10^7 cells per dish in nonselective medium. Cultures were trypsinized, centrifuged, and then resuspended in nonselective medium to a density of 4×10^7 to 10×10^7 cells per ml. A 0.5-ml sample of the cell suspension was added to each microfuge tube, and prepared DNA was then added to a final concentration of 5 nM. The cell-DNA mixtures were incubated on ice for 20 min, loaded into an electroporation chamber precooled on ice (length, 5 mm; cross section, 100 mm^2), and exposed to a 1-s electrical pulse from a 250-μF capacitor charged to 300 V. Cells were immediately removed from the chamber and plated into five 100-mm culture dishes. The plates had been prepared by gelatinization and contained 7 ml of nonselective medium. The cells were allowed to recover overnight. The next day, the number of colonies in each dish was determined by counting, and HAT selection was then applied.

Cultures to be assayed for the loss of *HPRT* function by selection in 6-TG were maintained under HAT selection for at least 1 month prior to the start of the assay in order to kill any accumulated *hprt*$^-$ cells. These cultures were trypsinized, counted, and then replated at a density of 0.5×10^7 to 1×10^7 cells per plate in nonselective medium. They were grown without selection for 3 or 4 days to allow spontaneous revertants time to purge residual *HPRT* transcripts or protein. Selection was then started by applying 6-TG medium.

All selections were maintained for 16 days, with feeding as necessary. Targeting and reversion frequencies were determined by counting the number of resistant colonies obtained for each experiment. Individual colonies were picked by using cloning rings into 24-well (1 ml per well) dishes and maintained under selection. Cultures were transferred to 60-mm culture dishes and then either harvested for genomic DNA preparation or transferred to 100-mm dishes for further expansion.

Genomic DNA preparation and characterization. DNA was prepared from expanded clones by using conventional procedures. Restriction enzyme digestions were done according to manufacturers' specifications, incubating overnight. After electrophoresis on 0.8% agarose gels, Southern blotting was done by standard techniques.

Probes. Two probes were used, a 250-bp *Rsa*I fragment from intron 3 and a 300-bp *Hin*dIII-*Xho*I fragment from the human cDNA which includes exons 3 to 6 but is specific for the mouse exon 3 element (6). Both probes hybridize to sequences present in the endogenous locus as well as on the targeting vectors. For each blot, 25 to 50 ng of purified fragment was radiolabeled with ^{32}P-dCTP by the random-primed oligonucleotide method, using a Boehringer Mannheim kit. Four-hour prehybridizations and overnight hybridizations were done in 50% formamide solutions at 42°C. Blots were washed to a stringency of 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) at 68°C. Washed blots were exposed to preflashed XAR-5 film at −70°C.

RESULTS

In step: the integration event. The first step in the two-step targeting procedure is a homologous integration event that incorporates vector DNA carrying the desired modification into the genome. We used the method of Doetschman et al. (6) to introduce into mouse ES cells an integrating targeting vector that carries a 4-bp insertion in the second intron of the *HPRT* gene. Either plasmid pNMR133 or plasmid pNMR133D200 (which has a 200-bp gap in the region homologous to the target locus) was electroporated into the male mouse-derived ES cell line E-14TG2a. This cell line, isolated by Hooper et al. (8) as a spontaneous mutation in culture, carries a nonreverting deletion of the promoter and first two exons of the nine-exon, 33-kbp, X-linked *HPRT* gene (25), rendering it phenotypically *hprt*$^-$. Both targeting vectors contain approximately 5 kb of DNA identical in sequence to the exon 3 target region of the *hprt*$^-$ gene except for the intended modification: a 4-bp insertion in intron 2 that destroys a unique *Hin*dIII site. They also carry the human *HPRT* promoter and exon 1 sequences and the mouse exon 2 region.

The homologous integration event generates a duplication of the 5-kb target region separated by the remainder of the vector sequences (Fig. 1). The duplicated regions are identical with the exception of the 4-bp insertion, identified by a missing *Hin*dIII site, that is located on the downstream repeat. This event restores the promoter and first two exons deleted from the locus, generating *HPRT*$^+$ targeted recombinants that can be directly selected with HAT-containing medium.

We isolated three independent *HPRT*$^+$ cell lines by selec-

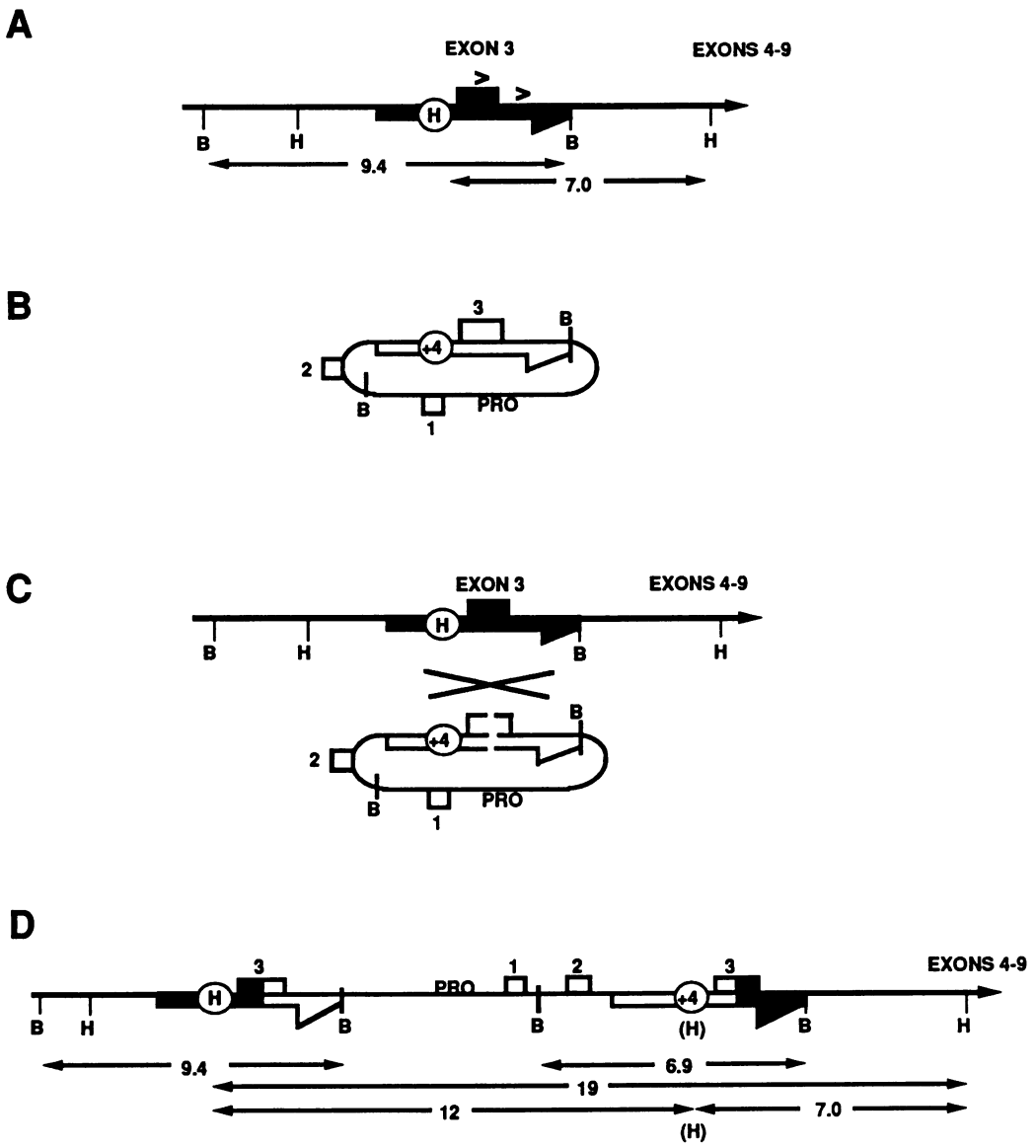

FIG. 1. The integration reaction. (A) The *hprt*$^-$ locus found in cell line E-14TG2a. The 5-kb target region is denoted by the thick black arrow. >, Regions used as probes. These sequences are also present in the targeting vectors. (H), The *Hin*dIII site that has been destroyed in the targeting vectors. (B) The pNMR133 targeting vector (12 kb). +4 denotes the 4bp insertion carried by this plasmid; it destroys the *Hin*dIII ((H)) site. Plasmid pNMR133D200 is identical to this plasmid except that a 200-bp *Bgl*II fragment has been removed from the region of homology. The open arrow denotes the region of DNA homologous to the target locus. PRO, Promoter; 1, 2, and 3, exons. (C) The homologous integration reaction with pNMR133 linearized at the *Xho*I site in the middle of the *HPRT* sequences. (D) The resulting *HPRT*$^+$ recombinant locus. The 5-kb duplicated regions (the large half-black, half-open arrows) are composed of both target locus and vector-derived sequences. B, *Bam*HI; H, *Hin*dIII.

tion in HAT medium at an average frequency of 2.8×10^{-6} per electroporated cell and then confirmed that these cell lines were targeted by genomic Southern blot hybridization. The blots were probed either with a 250-bp *Rsa*I fragment from intron 3 or with a 300-bp *Hin*dIII-*Xho*I fragment from the human cDNA that specifically hybridizes to the mouse exon 3. Both probes hybridize to sequences found in the genome as well as on the targeting vectors (Fig. 1A). All of the cell lines examined contained the expected recombinant locus depicted in Fig. 1D, indicating that a single copy of the targeting vectors had integrated into the E-14TG2a *hprt*$^-$ gene.

Cell lines A and C hybridized to the single 19-kb *Hin*dIII fragment expected for a simple insertion of the 12-kb vector into the 7-kb endogenous fragment. Cell line D hybridized to two *Hin*dIII fragments, the endogenous 7-kb and the vector 12-kb fragments. This cell line, generated with plasmid pNMR133D200, has lost the 4-bp insertion as a consequence of the integration event (see reference 6), so that revertants obtained from this line could not be properly modified. However, it was used in the excision experiments (see below) since it could still generate useful information about the frequency and accuracy of the excision reaction. *Bam*HI digestion of all recombinants revealed the expected 9.4-kb

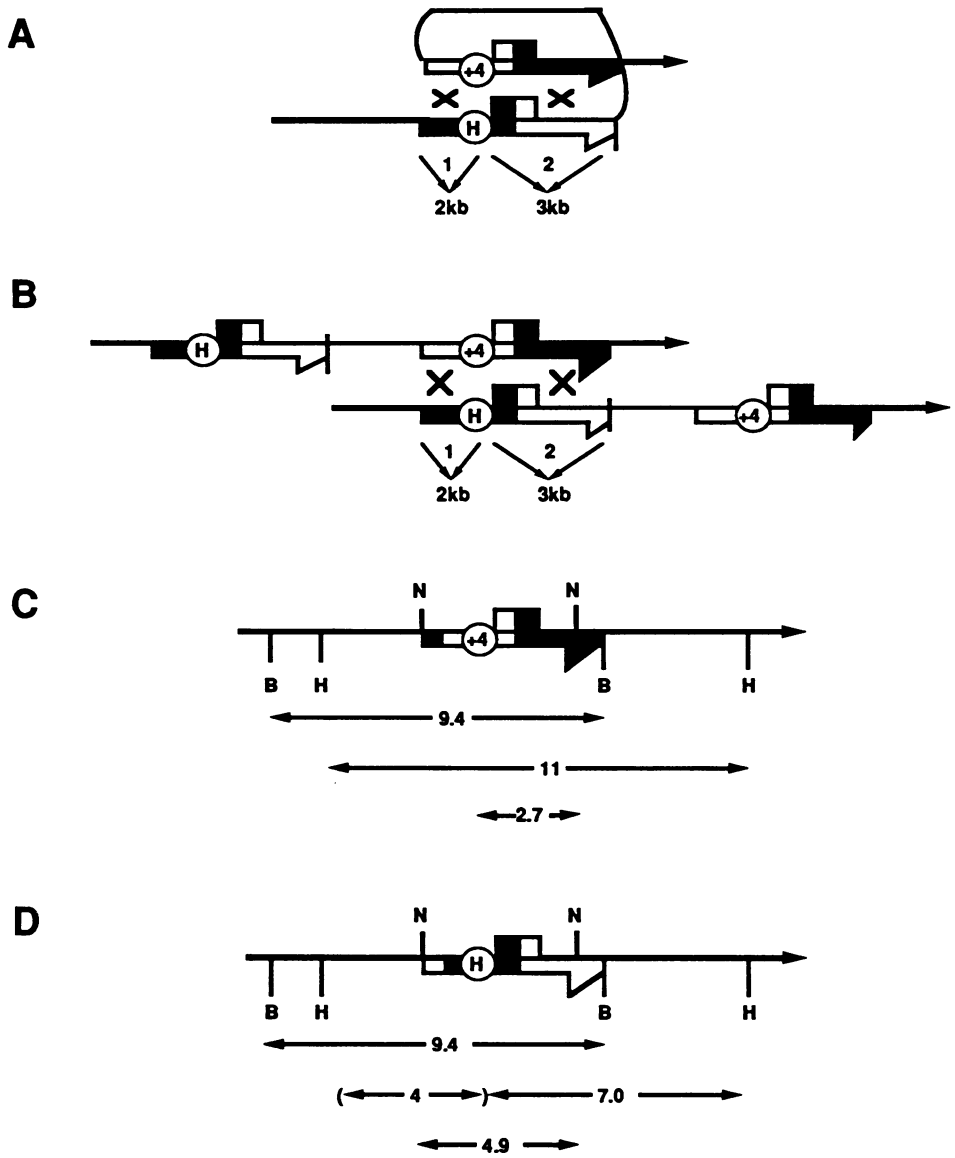

FIG. 2. The excision reaction. (A) Homologous excision via intrachromosomal recombination between the 5-kb duplications. Crossover in region 1 (2 kb) retains the 4-bp insertion in the excision product; crossover in region 2 (3 kb) removes the 4-bp insertion. (B) Homologous excision via unequal sister chromatid exchange. One of the resulting chromosomes (the excision product) contains a single copy of the duplicated region. Regions 1 and 2 are as in panel A. (C) The desired product: the *hprt*$^-$ locus now containing the 4-bp insertion. (D) The other possible *hprt*$^-$ locus: the E-14TG2a starting locus (Fig. 1A). The 4-kb fragment in parentheses does not hybridize to our probes; it is included in this figure to clarify the origin of the 11-kb band in panel C. B, *Bam*HI; H, *Hin*dIII; N, *Nhe*I.

endogenous band and the 6.9-kb vector-derived band. No extraneous bands could be detected, confirming that all of the recombinants carried single-site, single-copy insertions of the vector DNAs (see Fig. 3). In addition to these three lines, one more cell line, B, generated previously in the laboratory (6) was used in the excision studies (see below). This line carries the same recombinant locus found in lines A and C.

Out step: the excision event. The second step in the two-step targeting procedure is a spontaneous event that excises from the genome the vector sequences that integrated in the first step. A homologous recombination event between the regions duplicated during the in reaction can occur by either intrachromatid recombination (Fig. 2A) or unequal sister chromatid exchange (Fig. 2B). A crossover event in the 2-kb region 1 will leave the 4-bp insertion in the genome; crossing over in region 2, which is 3 kb, will excise the 4-bp modification along with the vector sequences (Fig. 2A) or move it to the (*HPRT*$^+$) triplicated chromosome (Fig. 2B). Either way, the excision event removes the vector-derived promoter and first two exons, causing a reversion to the *hprt*$^-$ phenotype. Such revertants can be selected with the nucleoside analog 6-TG.

The four HAT^r cell lines described above were used to study the excision (out) reaction. They all carry essentially the same *HPRT* locus: a duplication of 5 kb separated by 7 kb of plasmid-derived unique sequence (Fig. 1D). ES cell line D-3 (5), which carries the wild-type *HPRT* locus, was

TABLE 1. Frequency of the out reaction

Cell line	Cells plated (10^7)	Colonies 24 h post (10^6)[a]	6-TG^r colonies[b]	Reversion frequency[c]
A	2.90	3.2	23	7.9×10^{-7}
B	2.2	3.2	13	5.9×10^{-7}
C	4.2	6.4	14	3.3×10^{-7}
D	3.8	4.0	56	14.7×10^{-7}
Total	13.1		106	8.1×10^{-7}
D-3 (control)	5.6	4.8	0	$<1.8 \times 10^{-8}$

[a] Number of colonies counted the day after replating, reflecting a 10 to 20% plating efficiency (see text).
[b] Number of colonies counted after 2 weeks of 6-TG selection.
[c] Number of 6-TG^r colonies obtained per plated cell.

used as a control in these experiments to determine the spontaneous rate of mutation from *HPRT*$^+$ to *hprt*$^-$ at the normal locus. The experiments were performed as described in Materials and Methods. The day after replating, the number of colonies observable in each dish was determined by counting. Typically, ES cells form one colony for every 5 to 10 cells plated (Table 1). This is due to their propensity to form aggregates, not to a high death rate. That is to say, each colony found the day after replating is composed of 5 to 10 individual cells. Although this aggregation may interfere with our 6-TG selections as a result of metabolic cross-feeding (9), it cannot be avoided (see Discussion).

The number of 6-TG^r colonies obtained for each line examined and calculated reversion frequencies are listed in Table 1. As shown, all four HAT^r lines initially generated by gene targeting reverted to the *hprt*$^-$ phenotype at similar frequencies, averaging 8×10^{-7} 6-TG^r colonies isolated for every HAT^r cell plated. Control cell line D-3, which carries the wild-type *HPRT* locus, failed to produce any 6-TG^r colonies from 5.6×10^7 cells plated. Thus, the spontaneous mutation frequency at the *HPRT* locus, for cells preselected with HAT, is less than 1.8×10^{-8}. This result is consistent with the rate of 1.5×10^{-8} per cell generation reported for the locus by Caskey and Kruh (3).

Several of the individual 6-TG^r colonies were analyzed further by genomic Southern blot hybridization, using the *HPRT*-specific probes described above. The number of colonies examined from each line and a summary of the results obtained from the Southern blot hybridizations are presented in Table 2. Representative Southern blots are presented in Fig. 3.

Of a total of 26 6-TG^r colonies examined, 23 (88%) had executed the out reaction and accurately excised the integrated vector sequences from the genome, as determined by the genomic Southern blots. They all revealed a single 9.4-kb *Bam*HI band upon hybridization, the size predicted

TABLE 2. Accuracy of the out reaction

Cell line	Colonies examined	Accurate revertants[a]	Revertants with 4-bp insert[b]
A	3	2	2/2
B	9	7	6/7
C	11	11	11/11
D	3	3	NA
Total	26	23	19/20

[a] Number of colonies containing the expected *hprt*$^-$ locus.
[b] Number of *hprt*$^-$ colonies that retain the 4-bp insertion presented as a fraction of the number of accurate revertants obtained.
NA, Not applicable; this cell line does not carry the 4-bp insertion.

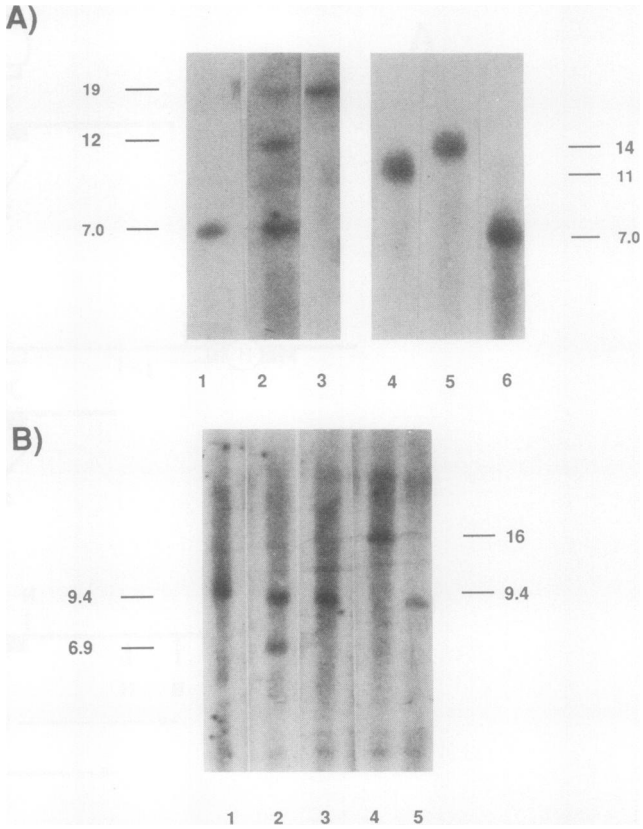

FIG. 3. Representative Southern blots. (A) *Hin*dIII-digested genomic DNAs hybridized to the exon 3 probe. Lanes: 1, starting cell line E-14TG2a (*hprt*$^-$); 2, HAT^r cell line without the 4-bp insertion (*HPRT*$^+$); 3, HAT^r cell line with the 4-bp insertion (*HPRT*$^+$); 4, correctly modified *hprt*$^-$ gene (with the 4-bp insertion); 5, unusual 6-TG^r revertant (see text); 6, accurate *hprt*$^-$ revertant that has lost the 4-bp insertion. (B) *Bam*HI-digested genomic DNAs hybridized to the 250-bp intron 3 probe. Lanes 1 and 2 are as in panel A, and lanes 3 to 5 are as lanes 4 to 6 in panel A.

for a simple homologous excision event. This is the same *Bam*HI fragment found in the parental E-14TG2a *hprt*$^-$ locus. *Hin*dIII digestion of the revertant DNAs is expected to reveal one of two bands upon hybridization: either an 11-kb fragment, if the crossover occurs in region 1 (Fig. 2A and B) and the 4-bp insertion introduced by the in event is retained, or a 7-kb fragment, if the crossover occurs in region 2 and the modification is removed from the *hprt*$^-$ genome.

Of the 23 out revertants examined, 20 were derived from *HPRT*$^+$ cell lines that carried the 4-bp insertion initially introduced by the targeted integration event; 19 of these colonies contain the single 11-kb *Hin*dIII fragment, indicative of the accurate excision event which retains the 4-bp insertion. Thus, these 19 colonies have been correctly modified by the in-out targeting procedure. Only 1 of these 20 revertant colonies lost the 4-bp modification, as determined by the presence of a 7-kb *Hin*dIII fragment. Therefore, 95% of the accurate revertants which could have retained the 4-bp insertion did so. The three remaining colonies which were found to have been generated by the out reaction were derived from the HAT^r cell line D. As this cell line does not carry the 4-bp modification, the revertants revealed only the 7-kb *Hin*dIII band upon hybridization.

To confirm that the 11-kb *Hin*dIII band characterizing our accurately modified *hprt*$^-$ revertants retained the 4-bp insertion initially introduced by the targeting vector, we digested two of the genomic DNAs with *Nhe*I. The 4-bp insertion introduced to destroy the *Hin*dIII site in the original targeting vector generates a unique *Nhe*I site. In the case of the revertants that have retained the 4-bp insertion (11-kb *Hin*dIII), *Nhe*I digestion will generate a 2.7-kb band which hybridizes to our probes. In the case of the revertants which have lost the insertion (7-kb *Hin*dIII), a 4.9-kb band will result (Fig. 2). The accurately modified revertant does contain a 2.7-kb *Nhe*I fragment which hybridizes to our probe, confirming the presence of the 4-bp insertion, and the revertant which has lost the 4-bp insertion reveals a 4.9-kb fragment upon hybridization (data not shown).

The other three colonies examined were found to contain aberrant *hprt*$^-$ loci that did not arise by the predicted homologous excision reaction. They contained a single 14-kb *Hin*dIII fragment and a 16-kb *Bam*HI fragment that hybridized to our probes. These fragments failed to hybridize to a plasmid-specific probe (data not shown), indicating that the target vector sequences have been excised from the genome. Since the bands are not the expected sizes, these colonies were probably generated by an alternate excision reaction. Because they account for only 12% of the 6-TG^r colonies obtained, we did not examine them further.

DISCUSSION

We have succeeded in modifying the genome of a mouse ES cell line, introducing a 4-bp insertion, by using a two-step in-out targeting procedure. The average frequency of the in reaction was found to be 2.8×10^{-6}, in agreement with other reports for gene targeting in murine systems (6, 7, 10, 20, 21, 23, 24). The frequency of the out reaction, 8×10^{-7} per HAT^r cell plated, is approximately 30% that of the in reaction. This frequency is 40-fold higher than the spontaneous mutation rate at the normal *HPRT* locus. Of the 6-TG^r colonies isolated, 88% had accurately excised the target vector sequences from the genome.

The mechanism of the out reaction, the excision event which generates 6-TG^r colonies, is likely to be either intrachromatid recombination between the 5-kb duplicated regions resulting from the insertion event (12–14) or an unequal sister chromatid recombination event between these same regions (19, 22). Both modes are depicted in Fig. 2. Our current information does not allow us to distinguish between them. However, it is of considerable interest that 95% of the 6-TG^r colonies examined from the HAT^r lines carrying the 4-bp insertion retained this insertion in the final locus. Because the modification is located 2 kb from the 5′ end of the duplicated region and 3 kb from the 3′ end, one would expect a 3:2 ratio in favor of losing the insertion if a simple crossover, occurring randomly along the length of the repeat, were responsible for the excision event. As this ratio is not observed, the 2-kb region may contain a hot spot for recombination.

Thompson et al. (25) have also examined reversion of an *HPRT*$^+$ targeted recombinant cell line that was generated in much the same way as ours. They similarly found that the target vector sequences had been excised from the genome in those cells which spontaneously reverted to the *hprt*$^-$ phenotype. Intrachromosomal recombination events occurring between direct repeats generated by gene targeting at the immunoglobulin Mu heavy-chain locus have been examined by Baker (1). The downstream repeat contained a 2-bp deletion relative to the upstream region. The majority of the recombinants were found to be gene conversion products; the excision products that did arise contained the longer (upstream) and shorter (downstream) repeats with equal frequency. This observation probably indicates that the bias we observed toward retaining the 4-bp insertion is gene specific rather than genome wide.

It has been shown that metabolic cross-feeding can interfere with 6-TG selections (9); therefore, the reversion frequency that we determined may be an underestimate. Because the ES cells always form aggregates upon plating, we could not eliminate the possibility of such cross-feeding. When using the in-out targeting procedure in other cell lines, it may be useful to plate HAT^r cells at a lower density to minimize the potential of revertant loss due to such cross-feeding.

An important part of our protocol (as described in Materials and Methods) was that the ES cells were not grown on feeder layers. Instead, the pluripotential nature of the cells was retained by supplementing the growth medium with purified human leukemia inhibitory factor (18, 26). This modification greatly simplifies the two-step selection procedure. Otherwise, the ES cells would have to be grown successively on two different feeder layers possessing opposite *HPRT* genotypes, requiring an adaptation stage between the selections. Also, 6-TG selection occurs slowly, requiring feeder-grown cultures to be passaged. We were able to simplify the selection procedure by eliminating this passage requirement. We were also able to isolate single clonal populations of the revertants, allowing for a more accurate determination of the reversion frequency.

The rationale for performing our experiments was to determine the feasibility of using an in-out targeting procedure to modify the mammalian genome. We have shown that both the integration and excision events occur accurately and at frequencies sufficient for use in a two-step targeting technique. Although we tested the two-step procedure in the directly selectable *HPRT* locus, the same procedure should be adaptable to modify nonselectable loci in an *hprt*$^-$ cell line by using the *HPRT* minigene described by Reid et al. (16).

The minigene would be carried on an integrating targeting vector, thereby allowing selection to be used for both the integration and excision events. Homologous recombinants are likely to be found after the in step at a frequency of 1 in 1,000 HAT^r cells, this being the ratio of transformed to targeted cells reported previously (20, 24). The targeted cell lines can then be identified by the polymerase chain reaction (see reference 27, for example). Including a small gap in the region of homology on the insertional vector provides a convenient primer binding site, since all gaps are repaired during the homologous insertion event (25a). Excision-derived *hprt*$^-$ revertants are likely to be found after the out step at a frequency of nearly 1 in 10^6 per targeted cell line.

The two-step selection scheme permits an enrichment for homologous recombinants carrying the desired modification, but the selectable sequences are not present in the final product. This in-out targeting procedure has the potential to be used in a variety of situations to create subtle modifications of the mammalian genome, a necessary prerequisite for fine-structure genetic analyses in mammalian systems.

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