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Investigation of Coelectroporation as a Method for Introducing Small Mutations into Embryonic Stem Cells

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We have investigated coelectroporation as a method for introducing minor genetic changes into specific genes in embryonic stem cells. A selectable marker (*neo*) and a targeting replacement vector designed to insert a 4-bp insertion into exon 3 of the mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene were coelectroporated into embryonic stem cells and selected in G418 and 6-thioguanine (6-TG). HPRT-negative clones were obtained at a frequency of approximately 1 per 520 G418^r clones. Southern analysis and the polymerase chain reaction were used to demonstrate that 3 of 36 of the 6-TG-resistant clones had the desired 4-bp insertion without any other disruption of the HPRT locus. Initial studies indicated that the other 33 6-TG-resistant clones probably resulted from the targeted integration of a concatemer containing both the targeting construct and the selectable *neo* gene.

The advent of embryonic stem (ES) cell technology and the ability to introduce genetically altered cells into the mouse germ line open up a field of endeavor previously denied mouse geneticists (3, 16). To date, targeting of specific genes in ES cells has been achieved by using homologous recombination of a targeting construct containing a selectable marker (8, 10, 13, 18, 23, 28; for reviews, see references 2 and 4). This has been necessary since the frequency of the desired recombination event is between 10^{-5} and 10^{-6} per ES cell electroporated (23). Positive selection for DNA integration reduces the number of clones to be screened to manageable numbers. The intent is usually to create a null allele; however, the integration of an extraneous promoter, enhancer, and polyadenylation signal within the coding sequences of the targeted gene may have other unpredictable effects on the gene which will make the phenotype hard to interpret. It is therefore highly desirable to be able to introduce small mutations, perhaps single nucleotide changes, without the presence of a selectable marker in the targeted gene. Small mutations introduced into the germ line would also be useful for creating exact animal models of human genetic disease (11, 12).

Two methods for introducing small mutations without selectable markers have been reported elsewhere. Zimmer and Gruss (29) used microinjection of ES cells. Although targeting occurred at a frequency of 1 per 150 cells injected, to our knowledge these experiments have not been repeated, nor have the microinjected stem cells been successfully introduced into the germ line. The "hit-and-run" method described by Hasty et al. (6) and a similar "in-out" method described by Valancius and Smithies (27) have been shown to be successful in two genes, Hox 2.6 and hypoxanthine-guanine phosphoribosyltransferase (HPRT), with the caveat that the selectable marker must be expressed after integration at the targeted locus. Expression of a selectable marker may not be possible for all targets in the genome. We have, therefore, used coelectroporation to introduce a selectable marker and a targeting vector on separate DNA molecules such that the selectable gene is located in a genomic location different from that of the targeting event. Coelectroporation with two selectable markers has been shown to be possible, although the frequency of coexpression varied between 23 and 100% in different laboratories (1, 14, 25, 26). Shulman et al. coelectroporated a *neo* gene and targeting vector designed to correct a mutation in the immunoglobulin μ heavy chain in hybridomas and found a minimum of sixfold enrichment in the frequency of cells with immunoglobulin M function restored by homologous recombination after selection with G418 (20). The exact nature of the integration events and whether coelectroporation would be useful for targeted recombination are the subjects of this investigation. Ideally, the targeting vector would undergo a homologous replacement-type recombination event at the desired locus and a selectable *neo* gene would integrate randomly in another location. Assuming that the nonhomologous event is random, the chance of this being so closely linked that it is not conveniently separated by meiosis (1 centimorgan [cM]) is less than 0.05% in the mouse genome of 2,000 cM. Thus, the second integration site could easily be separated from the desired targeted event (99.95% probability) once the targeted clone was introduced into the mouse germ line. We have targeted the HPRT gene in male ES cells, as successful homologous recombination confers resistance to 6-thioguanine (6-TG) and thus all recombination events at the locus can be recovered and characterized. We find that 8% of the targeted events do have the desired structure. The remaining targeted events appear to be due to the integration of concatemers at the locus. In a nonselectable gene, the correctly targeted clones could be identified by polymerase chain reaction (PCR) with one primer spanning the mutation and the other in genomic sequences outside the targeting vector. Coelectroporation allows the introduction of a small nonselectable mutation into a genomic target and is the only method described to date which would allow targeting of genes in which a selectable marker fails to be expressed at the targeted locus.

MATERIALS AND METHODS

Plasmids. PGKneobpA (Fig. 1) (21) was cut with *Xho*I prior to electroporation, which gave a 1.4-kb *neo* fragment and a 2.9-kb pKS vector fragment. The targeting plasmid (RV6.8XP) consists of 6.8 kb of the mouse HPRT gene

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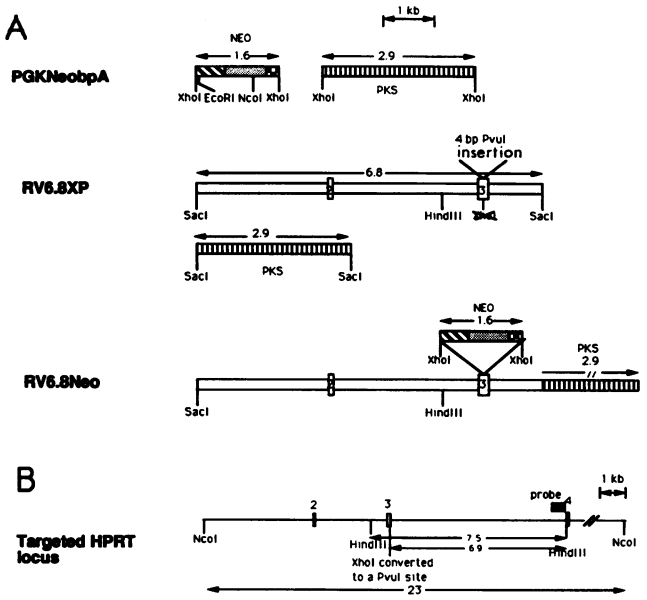

FIG. 1. Vectors used for coelectroporation and gene targeting. (A) Open boxes represent HPRT sequences with exons numbered. Vectors are diagrammed as they were cut for electroporation. (B) Structure of the targeted locus. The *Xho*I site in exon 3 is converted to a *Pvu*I site by RV6.8XP. The probe used in Fig. 4 and 5 is a *Pst*I-*Hin*dIII fragment which is located outside the targeting vector.

(exons 2 and 3) located on two genomic *Eco*RI fragments (15), with a 4-bp insertion in exon 3 created by filling in *Xho*I sticky ends, generating a *Pvu*I site. This mutation has been previously shown to generate resistance to 6-TG. In addition, a control construct (RV6.8Neo) in which PGKneobpA was inserted into the *Xho*I site of exon 3 was also included to compare coelectroporations to conventional positive selection vectors. All constructs were linearized or excised with *Sac*I prior to electroporation and are diagrammed in Fig. 1.

Electroporation. CCE ES cells (3) were expanded on STO/N/L feeder cells, trypsinized, washed once in phosphate-buffered saline (PBS), and resuspended at a concentration of 10^7 cells per 0.9 ml of PBS. DNA was prepared by the detergent lysis method (17), linearized, extracted with phenol-chloroform, precipitated with ethanol, resuspended in a volume of 25 μl of 0.1× Tris-EDTA, and incubated with the cells for 5 min at room temperature in a Bio-Rad electroporation cuvette. Cells were electroporated with a Bio-Rad Gene Pulser equipped with a capacitance extender at 230 V and 500 μF. The cells were plated at 10^7 cells per 10-cm plate on mitomycin-treated feeder layers. G418 (GIBCO) was added to the medium 24 h after electroporation at a concentration of 180 μg of active ingredient per ml. 6-TG was added to the medium 5 days postelectroporation at a concentration of 10^{-5} M. The cells from one electroporation were selected in G418 without 6-TG. Fourteen days after electroporation, colonies surviving in 6-TG were expanded and genomic DNA was prepared for Southern analysis.

Southern analysis. Genomic DNA was isolated from ES cells and digested with 5 U of the appropriate enzyme per microgram of DNA. Southern analysis was performed by standard methods (22). Field inversion gel electrophoresis was performed by using a 0.7% agarose gel in 0.5× Tris-borate-EDTA containing 50 ng of ethidium bromide per ml and a programmable power inverter (PPI-200; MJ Research). Electrophoresis was performed by using program 2 at 7.8 V/cm for 24 h to achieve maximum separation in the 20- to 35-kb range. Once electrophoresis was completed, the gels were treated as described above for DNA transfer and filter hybridization.

PCR. Exon 3 of HPRT was amplified by using two 24-mer oligonucleotide primers as diagrammed in Fig. 7. The PCR consisted of 250 ng of genomic DNA, 400 μM each primer, 200 μM deoxynucleoside triphosphates, and 2.5 U of *Taq* polymerase in a 50-μl volume of Perkin-Elmer Cetus PCR buffer. Reaction mixtures were denatured at 94°C for 4.5 min immediately prior to initiation of the PCR in a Perkin-Elmer Cetus DNA thermal cycler. Thirty cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 2 min were used to amplify a 1.5-kb fragment. The 1.5-kb fragment amplified from clones 21, 22, 25, and 32 and CCE wild-type cells was gel purified, restricted with either *Pvu*I or *Xho*I, and analyzed on a 1% agarose gel. The restricted DNA was transferred to GeneScreen Plus (Dupont) and probed with two oligonucleotides (one which hybridized with the large fragment and one which hybridized with the small fragment) end labelled with [γ-^{32}P]ATP.

RESULTS

Determination of the frequency of integration of two DNA fragments in different genomic loci after electroporation. Previous investigators had reported that, in contrast to microinjection or calcium phosphate precipitation, electroporation most commonly resulted in the integration of a single copy rather than a concatemer (1). For coelectroporation to be effective, we required optimum conditions for recombination at two different genomic loci. ES cells were electroporated with an efficiently expressed *neo* gene and selected in G418. Electroporations were conducted as described in Materials and Methods by using amounts of PGKneobpA varying from 12.5 to 200 μg (4.3 to 68 pmol) per electroporation. To control for nonspecific toxicity at high DNA concentrations, an additional set of electroporations were done in which the quantity of PGKneobpA varied but the total quantity of DNA in all samples was 200 μg. The number of G418-resistant colonies increases as the *neo* DNA concentration increases to 100 μg and then reaches a plateau at approximately 2,000 G418^r colonies per electroporation (data not shown). There is no apparent toxicity at even the highest levels of DNA tested. The saturation of the curve is not due to limiting culture conditions, as similar results are obtained at plating densities of 1×10^6, 3×10^6, and 6×10^6 per 10-cm plate. The fact that the number of G418^r colonies does not increase at high DNA concentrations suggests that only a certain percentage of the total cell population is susceptible to DNA entry into the cell and/or stable incorporation of exogenous DNA. We also investigated the nature of the integration events at different DNA concentrations. For this experiment, ES cells were electroporated with concentrations of between 2.5 and 82 μg (0.82 to 29 pmol) of PGKneobpA DNA, with the number of G418^r colonies being determined (Fig. 2A) and genomic DNA being isolated from 10 randomly selected clones at each concentration. To determine the number of different integration sites, genomic DNA was digested with *Bam*HI, an enzyme which does not cut in either the *neo* gene or the vector fragment and should thus give distinct size fragments for each integration site when probed with both *neo* and pKS (Fig. 2C). From the *Bam*HI digest, 84% of the 56 clones tested had a single integration site and the remaining 16% had two integration

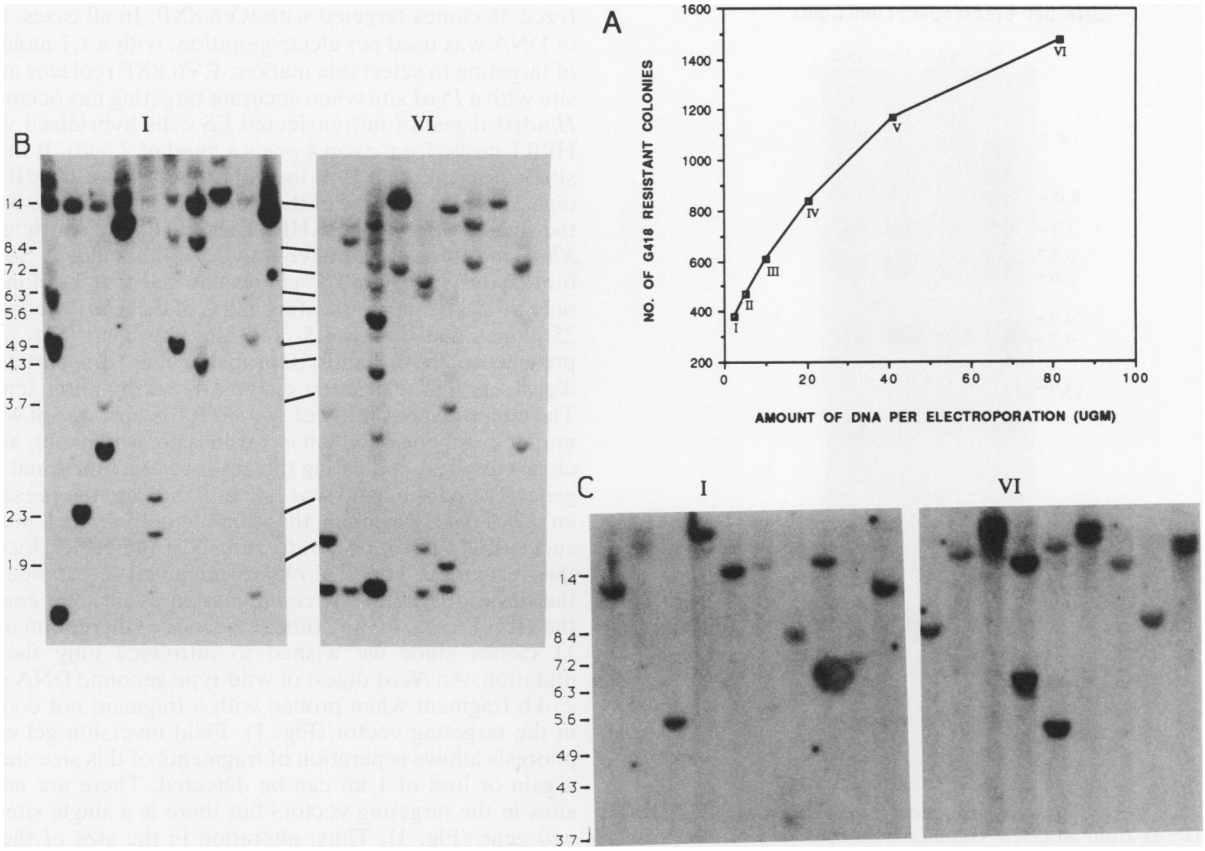

FIG. 2. Nature of the integration events after electroporation. Individual colonies were isolated from each of the six electroporations with PGKneobpA shown in the graph (A) and examined by Southern analysis. μGM, micrograms. (B) Southern analysis of representative clones from electroporations I (2.5 μg) and VI (81.6 μg). DNA was digested with *Eco*RI (which cuts once within the vector [Fig. 1]) and probed with the *neo* coding sequence. Marker sizes are illustrated on the left. Lines show positions of the same markers on the filter on the right. (C) Southern analysis of the same clones as in panel B, cut with *Bam*HI (which does not cut in the vector) and probed with *neo* and pKS.

sites. The average number of integration sites did not increase with increasing DNA concentrations. The average numbers of integration sites per clone for each DNA concentration were as follows: I, 1.0; II, 1.3; III, 1.1; IV, 1.0; V, 1.3; and VI, 1.2 (Fig. 2). The same genomic samples were also digested with *Eco*RI and probed with the *neo* coding sequence (Fig. 1). This enzyme cuts the 1.4-kb *neo* gene once, 25 bp from the 5′ end of the promoter, such that each *neo* integration should give a hybridizing band whose size depends on the proximity of the next *Eco*RI site in the genome. Multiple bands were commonly observed at all DNA concentrations (Fig. 2B), suggesting that multiple copies of the *neo* gene were integrating, usually at a single site. In addition, genomic DNA from four pools of several thousand clones, each from electroporations with different quantities of DNA, was prepared and digested with *Eco*RI (Fig. 3). When this pooled DNA was probed with *neo*, bands were detected, indicating either that certain sites in the genome were favored for integration events or that concatemers were being integrated. Bands were detected at 1.7, 3.4, 4.6, 6.0, 7.2, and 8.7 kb. Assuming that occasionally the *Eco*RI site at the 5′ end of the *neo* construct is destroyed during ligation of the *neo* and pKS fragments to form concatemers, the observed bands can be accounted for by various combinations of the 1.7-kb *neo*-containing fragment and the 2.9-kb vector fragment integrating as a concatemer. Concatemers do form and integrate even at the lowest DNA concentration tested (2.5 μg, [0.82 pmol]), so decreasing the DNA concentration is unlikely to be of benefit in optimizing targeting.

Importantly, 16% of the clones have integrated DNA at two genomic locations and thus it is possible for a cell to randomly integrate one sequence and homologously recombine another. We anticipated that we could target a nonselectable mutation to one gene and enrich for those cells by selection in G418.

Targeting the HPRT locus by using coelectroporation. A selectable marker (PGKneobpA) and a targeting construct (RV6.8XP; Fig. 1) were coelectroporated into CCE ES cells at various molar ratios of targeting to selectable constructs and total DNA concentrations. In each case, one electroporation was selected in G418 alone and the rest were selected in G418 and 6-TG. Control experiments in which PGK neobpA alone was electroporated gave no 6-TG-resistant colonies in 1.8×10^4 G418^r clones. In cases in which more than 2,000 G418-resistant colonies were examined, 6-TG-resistant colonies were detected (Table 1). This indicated that coelectroporation could be used to disrupt the HPRT locus at a usable frequency. The 6-TG^r/G418^r ratio was approximately 1/500, which is similar to the ratio obtained by electroporation of the control plasmid (RV6.8Neo) which contained the *neo* gene within the targeting construct (Fig. 1 and Table 1). Thus, coelectroporation targets at a frequency similar to that of conventional positive selection replacement

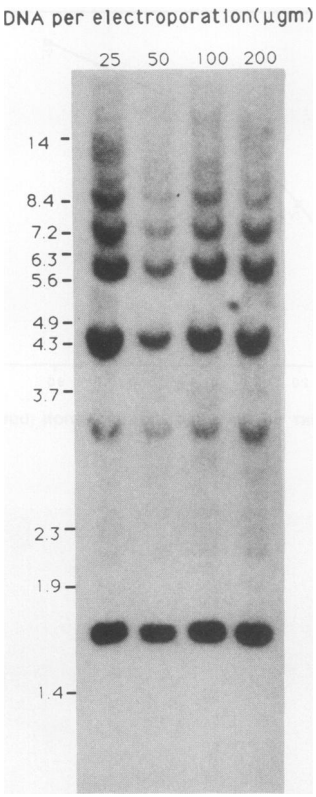

FIG. 3. Integration of concatemers after electroporation. Genomic DNAs from all G418^r colonies from each of four electroporations with differing amounts of DNA were digested with *Eco*RI, an enzyme that cuts once at the 5′ end of the *neo* fragment, and probed with *neo* such that each integration should give a unique-size band. μgm, micrograms.

vectors. This was surprising, as we expected that some targeted clones would not be G418 resistant and thus be lost in the coelectroporation experiments. The integration of concatemers would explain this result if those concatemers included both the targeting and the selectable constructs. Varying the molar ratio of selectable to targeting vector between 1:1 and 1:10 gave similar 6-TG^r/G418^r frequencies (1/520 versus 1/868) and thus did not improve chances of detecting a targeted event while decreasing the targeting frequency per cell electroporated.

Nature of the targeting event after coelectroporation. The purpose of this study was to determine the feasibility of introducing small mutations into selected genes by coelectroporation. Thus it was important to characterize the structure of the genomic DNA in the targeted clones. We analyzed 36 clones targeted with RV6.8XP. In all cases, 200 μg of DNA was used per electroporation, with a 1:1 molar ratio of targeting to selectable marker. RV6.8XP replaces an *Xho*I site with a *Pvu*I site when accurate targeting has occurred. A *Hin*dIII digest of untransfected ES cells hybridized with an HPRT probe from exon 4 gives a band of 7.5 kb. If the *Xho*I site is present, as it is in the wild type, then a *Hin*dIII-*Xho*I digest reduces the size of the band to 6.9 kb (Fig. 1), whereas the successfully mutated HPRT should not be restricted with *Xho*I and thus should have only a 7.5-kb band. A Southern blot analysis of 36 6-TG^r clones showed that 11 clones had only a 7.5-kb band, indicating a loss of the *Xho*I site, and that 25 clones had both a 7.5- and a 6.9-kb band (Fig. 4). The presence of both bands is probably due to a partial *Xho*I digest, as this restriction enzyme is methylation sensitive. The alternate possibility of two HPRT genes, one of which is mutated and one of which is a wild type, is unlikely, as these clones are TG^r, indicating the absence of a functional HPRT gene. The 6.9-kb band was taken to indicate the presence of an *Xho*I site and thus the small mutation had not been successfully introduced into exon 3 of the HPRT locus. By this reasoning, 11 6-TG^r clones remained as candidates for the desired mutational recombination event. We examined the HPRT locus for any other evidence of disruption in these 11 clones since we wished to introduce only the small mutation. An *Nco*I digest of wild-type genomic DNA gives a 23-kb fragment when probed with a fragment not contained in the targeting vector (Fig. 1). Field inversion gel electrophoresis allows separation of fragments of this size such that a gain or loss of 1 kb can be detected. There are no *Nco*I sites in the targeting vectors but there is a single site in the *neo* gene (Fig. 1). Thus, alteration in the size of the *Nco*I fragment would indicate a disruption of the locus. The results of this analysis are presented in Table 2. Southern analysis of clones lacking the *Xho*I site is shown in Fig. 5. For clones 21, 22, 25, and 32, the *Nco*I fragment was normal in size. To confirm that no other DNA had cointegrated into the HPRT locus at the time of recombination, we probed a filter first with HPRT and then with pKS and *neo* (Fig. 6). Clone 21 showed *neo*/pKS hybridization of the HPRT band and thus has integrated either pKS or *neo* into the HPRT locus. Of the original 36 clones, 3 met the criteria for having undergone a replacement event which introduced a small mutation with no other disruption of HPRT.

Small mutations can be introduced into HPRT by coelectroporation. Confirmation of the desired mutation in clones 22, 25, and 32 requires demonstration that an *Xho*I site in the wild type had been replaced by a *Pvu*I site in the mutant clones. Southern analysis did not reveal the presence of a new *Pvu*I site, but this enzyme is methylation sensitive. We therefore used primers to amplify a 1.5-kb band by PCR, which included exon 3 of HPRT (Fig. 7A). It should be noted that one of the primers was not contained in the electropo-

TABLE 1. Targeting the HRPT locus by using coelectroporation

No. of electroporations	Amt of DNA per electroporation (μg)				Total no. of colonies		
	PGKNeobpA	RV6.8XP	RV6.8Neo	Molar ratio	G418^r	6-TG^r and G418^r	Ratio (6-TG^r/G418^r)
8	67	133		1:1	16,150	31	1/520
3	34	66		1:1	3,030	3	1/1,010
16	10	190		1:10	6,081	7	1/868
15	25				18,144	0	
20			25		26,792	51	1/525

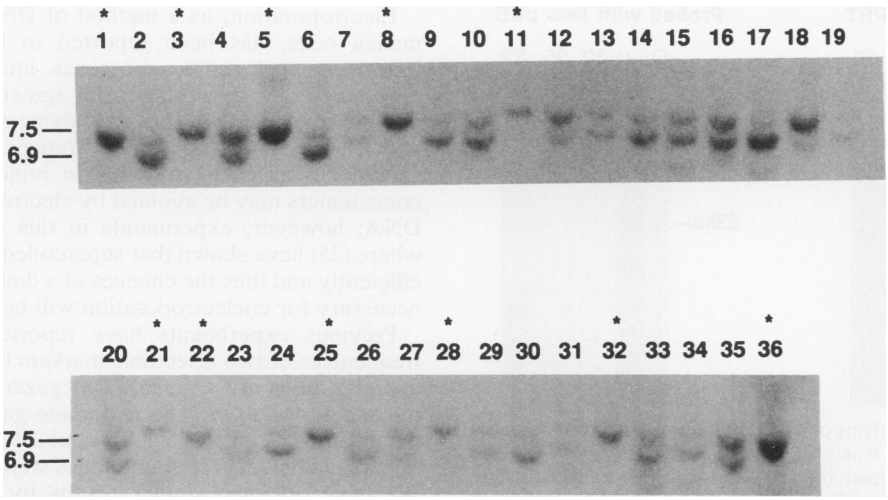

FIG. 4. Southern analysis of G418^r and 6-TG^r clones targeted by coelectroporation of RV6.8XP and PGKneobpA. The targeted locus and the probe used are diagrammed in Fig. 1B. (A) Genomic DNA from 36 clones was digested with *Hin*dIII and *Xho*I. The wild-type band is 6.9 kb, whereas the loss of an *Xho*I site gives a 7.5-kb band. Digests are partial and thus the presence of a 6.9-kb band is taken as evidence that the *Xho*I site is present. The 11 clones which appear to be missing the *Xho*I site and thus are potentially mutated are marked with asterisks.

rated construct, thus ensuring that the amplified fragment came from the HPRT chromosomal locus. The 1.5-kb band amplified by PCR was gel purified and restricted with either *Xho*I or *Pvu*I. The wild-type allele should give a 1.35-kb and a 150-bp fragment when restricted with *Xho*I and should not be restricted with *Pvu*I. The successfully mutated allele in which the *Xho*I site has been replaced by a *Pvu*I site should not cut with *Xho*I but should give the same 1.35-kb and 150-bp fragments when digested with *Pvu*I. As shown in Fig. 7B, clones 21, 22, 25, and 32 are all restricted with *Pvu*I and not *Xho*I. To visualize the small fragment, the gel shown in Fig. 7B was analyzed by Southern transfer and probed with appropriate oligonucleotides. The correct-size 150-bp fragment is evident in all clones restricted with *Pvu*I but not with *Xho*I. Clone 21, which appears to have cointegrated either *neo* or pKS, also has the *Pvu*I mutation in exon 3. This could occur if a targeting vector *neo* concatemer integrated on the short arm such that the genomic locus has the mutation in the 3′ duplicated exon 3 and a *neo* gene at the 5′ end of the integration. The *Nco*I site in the *neo* gene would give approximately the correct-size fragment when probed with HPRT. We conclude that 3 of 36 clones tested or 8% of the 6-TG^r clones have had a small mutations introduced into exon 3 without disruption of the HPRT locus.

Thus, targeting by coelectroporation occurs at a frequency similar to that obtained with conventional replacement vectors which contain a positive selectable marker. Of the targeted events, 8% have a small mutation introduced in the absence of any other disruption as detected by Southern analysis.

TABLE 2. Southern analysis of 6-TG^r clones targeted by coelectroporation

Presence of *Xho*I site	No. of clones with *Nco*I fragments				
	Total	At size of:			
		<23 kb	23 kb (wild type)	>23 kb	ND[a]
*Xho*I site present (clone not mutated)	25	4	4	8	9
*Xho*I site absent (clone possibly mutated)	11	1	4	6	0

[a] ND, not determined.

DISCUSSION

We have used coelectroporation to introduce small mutations into the mouse HPRT gene. We have shown that a 4-bp insertion can be successfully introduced into exon 3 of HPRT in 1 of 12 of the targeted recombination events by using the RV6.8XP vector. In addition, coelectroporation targets the HPRT locus with an overall frequency similar to that of conventional positive selection vectors. Many genes that have been successfully targeted do so at frequencies of between 1 per 30 and 1 per 100 G418^r colonies (9, 13, 28). Thus, a nonselectable mutation could be introduced in a

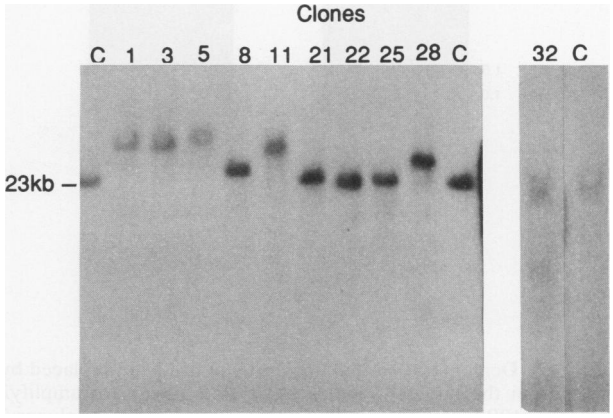

FIG. 5. Southern field inversion gel electrophoretic analysis of 6-TG^r clones lacking an *Xho*I site in exon 3. The potentially correctly mutated 6-TG^r clones (marked with asterisks in Fig. 4) were digested with *Nco*I and hybridized with an HPRT probe external to the targeting vector. The control lanes (C) are nonelectroporated ES cells which give a 23-kb band.

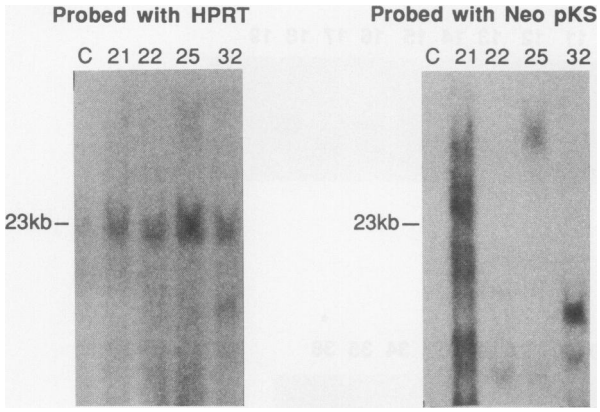

FIG. 6. Southern analysis of 6-TG^r potentially correctly mutated clones. An *Nco*I digest was probed with HPRT (left portion), and the same filter was stripped and reprobed with *neo* and pKS (right portion). The control lanes (C) are nonelectroporated ES cells which give a 23-kb band.

single step into such genes at frequencies of 1 per 360 to 1 per 1,200 G418^r colonies (1/12 the targeting frequencies). With careful design of PCR screening techniques, it should be possible to isolate these events. In addition, this is the only method, to date, available for targeting genes which suppress expression of a selectable marker after correct targeting.

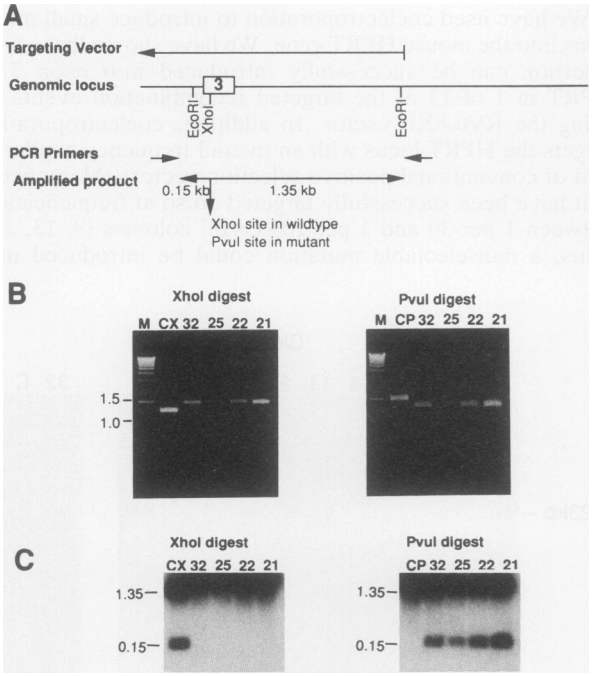

FIG. 7. Demonstration that an *Xho*I site has been replaced by a *Pvu*I site in the targeted clones. (A) PCR strategy for amplifying exon 3 of HPRT. (B) The 1.5-kb fragment amplified from clones 21, 22, 25, and 32 and control cells (C), purified, digested with *Xho*I or *Pvu*I, and separated by electrophoresis. M, size markers. (C) Southern analysis of the gel in panel B probed with a mixture of two oligonucleotides, one of which hybridizes to the large exon 3 fragment and the other of which hybridizes to the small exon 3 fragment.

Electroporation, as a method of DNA delivery to mammalian cells, has been reported to be a better way to introduce single copies of genes into chromosomes (1). However, our data show, in agreement with those of Toneguzzo et al. (26), that concatemers frequently integrate with electroporation and that strategies for targeting genes should be designed with this in mind. The formation of concatemers may be avoided by electroporating supercoiled DNA; however, experiments in this laboratory and elsewhere (25) have shown that supercoiled DNA integrates less efficiently and thus the chances of a double integration event necessary for coelectroporation will be exceedingly small.

Previous experiments have reported coelectroporation frequencies of two selectable markers between 23 and 100% at molar ratios of 1:1 (1, 25). Toneguzzo et al. (25) varied the molar ratio of a *gpt* gene and a *neo* gene and found that at molar ratios of 1:1 and 5:1, 100 and 23%, respectively, of the micophenolic acid-resistant clones were also G418 resistant. We have obtained similar results by measuring targeting events rather than random integrations. Thus, at high DNA concentrations the frequency of targeting to the HPRT locus is similar regardless of whether the selectable and targeting vectors are introduced on the same or different molecules. Altering the molar ratios of targeting to selectable constructs gives no advantage in the 6-TG^r/G418^r ratio but decreased the total number of targeted events per cell electroporated, presumably because of a decreased chance of integrating a *neo* gene.

We cannot directly calculate the enrichment given by coelectroporation in comparison with electroporating a targeting vector alone, as we have previously determined that there is significant cross-killing by 6-TG at cell densities higher than 5×10^4 per 10-cm plate. As 10^7 cells are used per electroporation, the nonenriched targeting frequency is too low to measure accurately. Shulman et al. (20) found that coelectroporation of a construct designed to correct a 2-bp deletion in the immunoglobulin M heavy chain and a *neo* gene gave a minimum enrichment of sixfold, although continued growth in G418 appeared to lead to enrichment of nontargeted cells. Even sixfold seems lower than our data would predict. The numbers of targeted cells per electroporation, i.e., those which had alterations in the HPRT gene presumably due to a recombination event, were similar regardless of whether the selectable marker was introduced on the same or on a different DNA fragment. This suggests that few, if any, targeted events are being lost by G418 selection and that the enrichment could be calculated as the number of cells surviving electroporation divided by the number of G418^r colonies per electroporation. Assuming that one-half of the cells survive electroporation, this would give an enrichment of 500-fold. The reasons for the discrepancy between our results and those of Shulman et al. are not obvious. Given that concatemers are the most common form of integration event, it may be that a concatemer integrating at the immunoglobulin M heavy chain locus such that the mutation is corrected fails to express a cointegrated *neo* at a level sufficient to ensure survival in G418.

As in previous studies, the number of G418-resistant colonies increased as the DNA concentration increased to 100 μg/ml (35 pmol) and then remained constant (1, 26). This was not due to limiting culture conditions or nonspecific toxicity of the DNA. The failure of more cells to integrate a selectable marker at high DNA concentrations suggests that only a certain percentage of the total cell population is susceptible to stable DNA incorporation.

The vector RV6.8XP is a conventional replacement or

omega vector in that the vector sequences are colinear with the endogenous HPRT gene. In contrast to results of previous experiments with replacement vectors (23), the majority of the targeted events examined had alterations in the locus indicative of a recombination event within HPRT other than gene conversion or double crossover (i.e., replacement). This is to some extent an artifact of the selection system. We have shown that after electroporation, a single site of integration is most common and that electroporation with the *neo* gene alone does not result in 6-TG resistance. Thus, selecting for G418 and 6-TG resistance will result in the majority of clones having inserted a *neo* gene and a targeting vector into the HPRT locus (i.e., a concatemer). Frequent integration of multiple copies of a replacement vector into a targeted locus has been reported by other laboratories for experiments in which there was no bias in the selection method (5, 19). In our laboratory, we find that when all targeting events are scored, replacement-type vectors most frequently undergo insertion-type events (7). Most other replacement vectors have made use of the positive-negative selection method (9, 13, 24). In this system, random integration events are selected against by inclusion of a herpes simplex virus thymidine kinase gene outside the area of homology in the targeting vector. Ganciclovir selection eliminates clones containing an intact thymidine kinase gene and thus clones with multiple plasmid copy insertions either randomly inserted or targeted to the locus would not survive selection. It is interesting that even after using positive-negative selection, Thomas and Capecchi reported that disruption of the int-1 locus was due to a tandem integration of two copies of the targeting vector (24). This may have been due to the requirement for 2 *neo* genes to get expression sufficient to confer G418 resistance.

Coelectroporation is a suitable method for introducing nonselectable mutations into any target gene in ES cells. The only limitation is the targeting frequency of the candidate gene, which will vary from one region of the genome to the next. Coelectroporation provides a 500-fold enrichment for directed nonselectable mutations from the transfected population, and unlike the alternative methods, it is not technically demanding and does not rely on two successive recombination events and marker gene expression in the target site. Coelectroporated targeted cells will be sufficiently frequent for many genes that direct PCR screening is applicable. Because the transfection marker will be unlinked to the targeted gene, it can be eliminated from the genome once the cell line is established in the germ line to provide a clear interpretation of a potential phenotype in the resultant mice.

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