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Site-Directed Point Mutations in Embryonic Stem Cells: a Gene-Targeting Tag-and-Exchange Strategy

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Sequential gene targeting was used to introduce point mutations into one α2 isoform Na,K-ATPase homolog in mouse embryonic stem (ES) cells. In the first round of targeted replacement, the gene was tagged with selectable markers by insertion of a Neo^r/HSV-tk gene cassette, and this event was selected for by gain of neomycin (G418) resistance. In the second targeted replacement event, the tagged genomic sequence was exchanged with a vector consisting of homologous genomic sequences carrying five site-directed nucleotide substitutions. Embryonic stem cell clones modified by exchange with the mutation vector were selected for loss of the HSV-tk gene by resistance to ganciclovir. Candidate clones were further screened and identified by polymerase chain reaction and Southern blot analysis. By this strategy, the endogenous α2 isoform Na,K-ATPase gene was altered to encode two other amino acids so that the enzyme is resistant to inhibition by cardiac glycosides while maintaining its transmembrane ion-pumping function. Since the initial tagging event and the subsequent mutation-exchange event are independent of one another, a tagged cell line can be used to generate a variety of mutant lines by exchange with various mutation vectors at the tagged locus. This method should be useful for testing specific mutations introduced into the genomes of tissue culture cells and animals and for developing animal models encompassing the mutational variability of known genetic disorders.

The concerted application of targeted gene modification and production of animals derived from embryonic stem (ES) cells has established a powerful method for studying gene function in the context of the whole animal (for reviews, see references 6 and 13). In this scheme, modification of a particular locus is performed first by targeted homologous recombination (21) in cultured ES cells. In turn, genetically modified ES cells are used to generate mice by injection into blastocysts and allowing the chimeric blastocyst to mature to term following transfer into a pseudopregnant foster mother (7). This combination of techniques has been used to develop many lines of mice carrying null alleles at specific loci as a means of testing the general physiological roles of particular gene products. Such experiments provide an alternative method of studying planned mutations in known genes, rather than relying on fortuitous identification of random mutations. This approach has already led to new insights on gene function. In many cases the phenotypes of animals harboring gene-targeted null mutations have confirmed predictions which were based on knowledge of the gene product (14, 23, 28). However, in several instances, surprising phenotypic results have implied new functions and expanded the biological understanding of previously well-characterized proteins (20, 22).

This relatively new tool of mammalian genetics presents the potential for testing the physiological significance of subtle functional mutations and for developing animal models of genetic disorders. To date, however, animals derived from gene-targeted ES cells have been designed exclusively to study the effect of null or "knockout" mutations. In order to examine the effect of more modest mutations which modify rather than eliminate the gene product, reliable methods for introducing such mutations into the genome, with fidelity, must be devised. In order to avoid artifacts due to extraneous genetic elements introduced during gene targeting, such as selectable markers, introduction of test mutations must be subtle in terms of perturbing the context of the gene. Since introduction of subtle mutations at most loci is not directly selectable, indirect selection for such recombination events is required.

Several strategies for introduction of minor mutations into nonselectable genes in ES cells have been explored. Microinjection of a mutation-targeting vector into ES cells resulted in targeted mutation of the *Hox* 1.1 gene at a frequency high enough to preclude the use of selectable markers (29). While the rate of targeted recombination was high, no reports of subsequent repetition of these experiments or of animals derived from the mutant ES cells have been published. The use of coelectroporation of a mutation vector and a selectable marker vector resulted in identification of targeted mutations of the hypoxanthine phosphoribosyltransferase (HPRT) gene (3). This method allows targeted mutation of loci which may be unable to express selectable marker genes. However, the rate of simultaneous homologous recombination and cotransfection with a selectable marker has been shown to be low (3, 17), and selection of cotransfected cells often results in multimeric insertions at the site of homologous recombination (3). A strategy for subtle mutation based on gene-targeted insertion (O-type recombination) and subsequent excision by intrachromosomal recombination, referred to as hit-and-run or in-out, has recently been reported (9, 25). Here we report the novel introduction of subtle point mutations into the α2 Na,K-ATPase gene of mouse ES cells by a sequential gene-targeted replacement (omega-type recombination) strategy which we call tag and exchange.

The target gene encodes one of the three isoforms of the catalytic (alpha) subunit of the ATP-dependent sodium-potassium pump. The Na,K-ATPase maintains the opposed sodium and potassium gradients across the plasma membranes of higher eukaryotes and also serves as the pharma-

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cological receptor for the cardiac glycoside (digitalis) class of drugs which act through inhibition of this enzyme. Point mutations introduced into the α2 Na,K-ATPase gene encode two amino acid changes which reduce the affinity of the enzyme for these drug ligands by 10^2- to 10^3-fold without interrupting the ion-pumping function of this integral membrane protein.

MATERIALS AND METHODS

Reagents. Enzymes used for cloning and analysis of DNA were purchased from Bethesda Research Laboratories, New England Biolabs, Pharmacia, Promega, Stratagene, and United States Biochemical. G418 (Geneticin) was purchased from Bethesda Research Laboratories, mitomycin was purchased from Boehringer Mannheim, and ganciclovir (GANC) sodium (Cytovene, manufactured for Syntex) was purchased locally. Oligonucleotides were synthesized on an Applied Biosystems 394 DNA synthesizer.

Cell culture, transfection, and selection of drug-resistant clones. D3 mouse embryonic stem cells were maintained by coculture on a feeder layer of mouse embryonic fibroblasts (which were arrested from proliferation by mitomycin treatment) in Dulbecco's modified Eagle's medium, 15% fetal bovine serum (Cellect-Gold; Flow Laboratories), and 10^{-4} M β-mercaptoethanol (Sigma). Mouse embryonic fibroblasts were prepared from 16-day postcoital embryos, which were transgenic for the neomycin resistance gene, according to the 4°C trypsinization method (2). For transfection, ES cells were dispersed by trypsin digestion for 2 to 3 min at 37°C, washed, resuspended in ES cell culture medium at 2×10^7 cells per ml, and electroporated with 3.5 nM vector DNA at 200 μF and 400 V/cm. All targeting vectors were excised as inserts from their parent plasmid and gel purified prior to transfection. Electroporated ES cells were seeded onto neomycin-resistant embryonic fibroblasts at 5×10^5 cells per 100-mm dish. These electroporation conditions resulted in 10 to 25% ES cell viability.

Neomycin (G418)-resistant ES cell clones were selected by exposure to 400 μg of G418 per ml starting 24 h after electroporation. The concentration of G418 was 400 μg/ml for the first 24 h of selection and was reduced to 300 μg/ml for the remainder of the selection period. During selection, cells were fed every other day until resistant colonies were picked on days 13 and 14 following electroporation. $G418^r$ homologous recombinants were maintained under G418 selection (300 μg/ml) prior to transfection with the mutation vector.

Ganciclovir-resistant ES cell clones were selected by exposure to 2.5 μM Cytovene for 4 days, at which time colonies were picked and transferred with a disposable-tip pipetter to individual wells of 24-well tissue culture dishes preseeded with embryonic fibroblasts.

In order to minimize the time in culture for isolated ES cell clones, each line was expanded by a single passage and an aliquot was frozen for storage. Two days after transfer to individual wells, ES cells were dispersed with trypsin and allowed to resettle in the same well. Two to three days later, each well was roughly confluent and the cells were trypsinized and resuspended in 250 μl of freezing medium (culture medium plus 10% dimethyl sulfoxide). One drop (about 10 μl) of this cell suspension was used to seed each of two sets of 24-well tissue culture dishes: one set for G418 selection and one set for maintaining the lines and for preparation of genomic DNA. The remaining (230 μl) of cell suspension was frozen for storage in 500-μl microcentrifuge tubes (Marsh Biomedical). Using this scheme, we froze all selected $GANC^r$ cell lines within 14 days of electroporation and maintained the lines in culture for screening and identification of homologous recombinants.

Neo^r/HSV-tk expression cassettes. All constructs were prepared in the pBluescript II SK (+) cloning vector (Stratagene). For both cassettes the Neo^r gene was first subcloned directly from pMC1Neo (Stratagene) into pBluescript II SK (+) as an *Xho*I-*Sal*I fragment, generating pBSSK-Neo. The thymidine kinase gene of herpes simplex virus (HSV-tk) driven by the pMC1Neo (*Xho*I-*Eco*RI) promoter (a gift from Jay Degen) was then subcloned into the *Cla*I site of pBSSK-Neo by using *Cla*I linkers. The head-to-tail and tail-to-tail constructs were isolated from the same ligation reactions.

Preparation and analysis of genomic DNA. Small-scale preparations of genomic DNA were prepared from ES cell clones grown to confluence on 24-well plates by the method of Laird et al. (12), with the exception that cell lysates were transferred to microcentrifuge tubes for isopropanol precipitation. Large-scale genomic DNA isolation from confluent ES cell cultures on P-100 plates was done by standard proteinase K digestion and phenol-chloroform extraction followed by RNase treatment. ES cell clones from the first round of gene targeting were screened for homologous recombination by polymerase chain reaction (PCR) with a primer specific to the 3′ end of the HSV-tk gene (of the sequence 5′-CTGAGCAGACAGACCCATGC-3′) and a primer specific to intron 10 of the α2 isoform Na,K-ATPase gene (of the sequence 5′-CACGATTGCAGAGACCAGCG-3′).

$GANC^r$ $G418^s$ ES cell clones were screened for the presence of site-directed mutations in genomic DNA by PCR amplification and diagnostic restriction digestion of a 270-bp sequence encompassing the nucleotide substitutions of the mutation vector. This 270-bp fragment was amplified with a forward primer of the sequence 5′-AGCTCAGGACATTC TGG-3′ based on the exon 4 sequence and a reverse primer of the sequence 5′-TGAGCTCCTGACTGGGTGTG-3′ based on the intron 4 sequence. The genomic PCR products were extracted once with chloroform; one half of the product was then digested for 2 h with *Bst*BI restriction endonuclease, and the other half was used as a nondigested control. Control and *Bst*BI-digested PCR products were separated on 3% agarose gels and visualized by ethidium bromide staining. In this assay, the 270-bp PCR product of the site-directed mutants is uniquely cleaved by *Bst*BI, generating diagnostic 176- and 94-bp digestion fragments, while the PCR product of the wild-type allele remains at 270 bp.

To check for delivery of each nucleotide substitution in targeted mutations of homologous recombinants, the 270-bp PCR product (described above) was cloned and sequenced. This 270-bp fragment was cloned by blunt-end ligation into the *Eco*RV site of pBluescript II SK (Stratagene) after blunting of the PCR product with T4 DNA polymerase. Sequence of the cloned PCR product was determined by double-strand sequencing with Sequenase (United States Biochemicals).

Genomic Southern blot analysis was performed according to the standard protocol. Briefly, 10 μg of genomic DNA was digested overnight in a 300-μl reaction volume, ethanol precipitated, and electrophoreticaly separated on a 0.8% agarose gel. DNA was transferred to Magna N/T membrane (MSI) by capillary action with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After transfer, DNA was fixed to the membrane by baking and prehybridized in standard buffer. Southern blots were hybridized with either

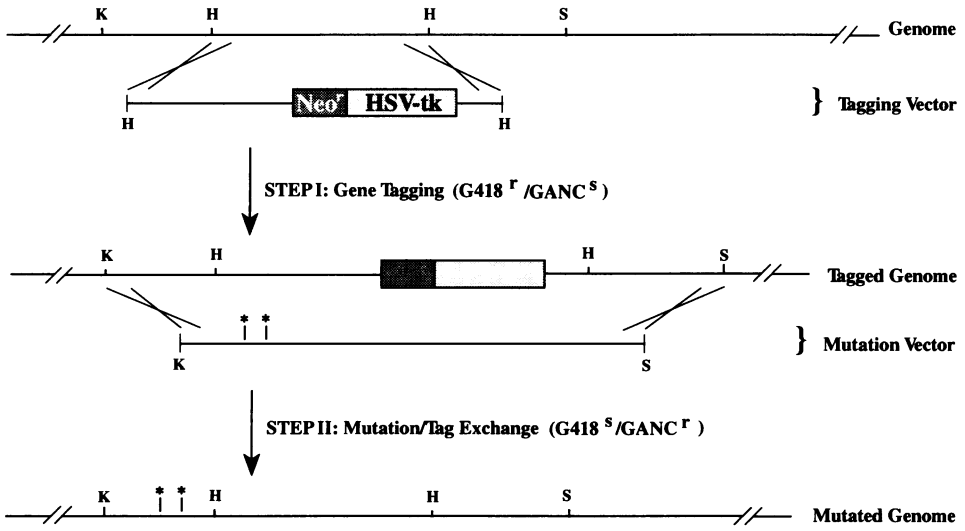

FIG. 1. Schematic diagram illustrating the gene tag-and-exchange strategy. In this illustration, the target site is the region between the *Hin*dIII (H) sites. The tagging vector is composed of a Neo^r/HSV-tk selectable marker cassette flanked by roughly 3 and 1 kb (5′ and 3′, respectively) of genomic DNA homologous to the target sequence. Step I represents the homologous recombination and subsequent tagging of the target gene with the tagging vector. The mutation vector is composed of a larger fragment of genomic DNA homologous to the target sequence and contains sequence mutations (**). Step II represents homologous recombination between the tagged locus and the mutation vector resulting in a mutated genomic sequence in a marker-free context. The *Kpn*I (K) and *Sma*I (S) site are included to provide landmarks.

random hexamer-labeled probe in standard hybridization buffer (50% formamide, 6× SSC, 5× Denhardt's solution, 10% dextran sulfate, 100 μg of denatured salmon sperm DNA per ml, 0.1% sodium dodecyl sulfate [SDS]) or with an end-labeled oligonucleotide probe in oligonucleotide hybridization buffer (5× SSC, 1% SDS, 5× Denhardt's solution, 100 μg of denatured salmon sperm DNA per ml).

Preparation and analysis of DNA clones. A mouse strain 129 sv genomic DNA library of *Sau*3AI partial-digest fragments (18 to 21 kb) was prepared in the Lambda Dash II vector (Stratagene). Screening this library with a radiolabeled rat cDNA probe specific for the α2 isoform Na,K-ATPase gene resulted in isolation of 14 clones containing fragments of the α2 isoform Na,K-ATPase gene. Two of these clones, λ41-2 and λ78-1, which overlap, represent the entire α2 isoform gene and were characterized by mapping the recognition sites for several restriction enzymes by indirect end labeling of partial digests. The general exon organization of the gene was then mapped by Southern blot analysis with serial fragments of the sheep α1 Na,K-ATPase cDNA. Genomic inserts were positively identified as α2 isoform Na,K-ATPase gene fragments by partial sequencing of exons 4 and 8 to 10.

Targeting and control vectors, described in Results, were prepared with subcloned restriction fragments from the genomic insert of clone λ78-1. The mutation vector was prepared with an 8.6-kb *Kpn*I-*Sma*I genomic fragment of the λ78-1 clone. Site-directed mutations of coding sequence within exon 4 were introduced by oligonucleotide-directed mutagenesis by the method of Kunkel (11). Briefly, a 1.1-kb *Bam*HI fragment of the 8.6-kb *Kpn*I-*Sma*I subclone was transferred to M13mp18 to generate M13mp18-α21.1 and a uracil-rich template of M13mp18-α21.1 was prepared from BW313 cells. A mutagenesis oligonucleotide with the sequence 5′-TACGGTATCCGGGCCGCCATGGAGGACGA GCCTTCGAATGATGATGTGAGCC-3′ was phosphorylated and used to prime DNA synthesis from the U-rich template. Inserts of M13mp18-α21.1 clones containing the mutated sequence were sequenced in their entirety, and the mutant 1.1-kb *Bam*HI fragment was transferred back into the pmα2-8.6 kb K-S subclone, generating the mutation construct pmα2-8.6RD.

RESULTS

Tag-and-exchange strategy and testing the positive and negative selection cassette. The strategy for introduction of point mutations into the α2 isoform Na,K-ATPase gene in a marker-free context was to sequentially replace the gene sequences by homologous recombination. The general scheme used, shown in Fig. 1, was to first tag the gene of interest with selectable markers and then replace the tagged sequence with a sequence carrying a cluster of site-directed point mutations. This method requires two selectable markers, a positive marker for identification of recombinants in the first targeting step and a negative marker for selection of recombinants from the second targeting step in which the tagged sequence is lost by exchange with the mutation vector. In the experiments described here, we used the neomycin resistance gene (Neo^r) and the HSV-tk gene, which confers sensitivity to ganciclovir, as positive and negative selection markers, respectively. These marker genes were fused to prepare a positive and negative selection cassette in which the genes are driven by identical promoters.

Two positive and negative selection constructs were prepared by fusing the Neo^r and HSV-tk genes and inserting the pair into a polylinker, providing a cassette which could be easily subcloned. Each gene is driven independently by the HSV-tk promoter fused to polyomavirus enhancer repeats, which together were subcloned as a 177-bp *Xho*I-*Eco*RI fragment from pMC1Neo (Stratagene). In planning the construction of these selection cassettes, all four possible orientations of the two genes were considered. Two orientations were chosen for construction and empirically tested: one with the genes head to tail with the Neo^r gene 5′ of the

HSV-tk gene, and the other with the genes oriented tail to tail.

The selection properties of the head-to-tail and tail-to-tail selection cassettes were examined in order to determine which provided G418 resistance accompanied by the lowest reversion to GANC^r. To measure reversion from a GANC^s to a GANC^r phenotype, ES cells were first electroporated independently with either the head-to-tail or tail-to-tail marker cassette, and G418^r recombinants were selected in 300 μg of G418 per ml. For each construct, cells from a plate of 300 to 400 G418^r colonies were examined for their plating efficiency (reversion to GANC^r) in 2.5 μM GANC and 300 μg of G418 per ml. The results of these empirical measurements demonstrated that the head-to-tail configuration provides a lower rate of reversion than the tail-to-tail, 0.03 versus 0.4% plating efficiency, respectively, in ganciclovir. This translates to an estimated background of 300 colonies for a typical gene-targeting electroporation experiment in which 10^7 cells are electroporated under conditions resulting in 90% cell death. The head-to-tail cassette was therefore used as the selectable tag for construction of the gene-tagging vector. While these measurements were useful for choosing which positive and negative marker cassette to use in vector construction, it should be emphasized that the results indicate reversion at random sites of integration and may be subject to locus-specific rates of HSV-tk inactivation.

Targeting constructs. Both targeting constructs were prepared with cloned genomic sequences from the same mouse strain (129) as that used in preparation of the D3 line of blastocyst-derived ES cells (5). This precaution was taken to maximize the degree of sequence homology between the targeting vector and the target gene, thereby avoiding potential reduction of targeting efficiency due to genomic sequence polymorphisms between vector and target DNA. This concern is supported by recent comparative studies of targeting frequency between homologous and heterologous vector and target combinations in ES cells (4, 18, 26). The gene-tagging vector (mα2-4Ntk) illustrated in Fig. 2 was constructed by inserting the 3-kb Neo^r/HSV-tk cassette at the *Bal*I site of exon 8 in a 4-kb genomic *Hin*dIII fragment of the α2 isoform Na,K-ATPase gene. Genomic sequences flanking the selection cassette are 2.9 kb 5′ and 1.1 kb 3′ of the positive and negative selection cassette, and the marker genes are in the same transcriptional orientation as the α2 isoform Na,K-ATPase gene.

A positive control construct (mα2-1.8Ntk) illustrated in Fig. 2 was also prepared for optimizing PCR conditions used to screen for homologous recombination in the first round of gene targeting. The PCR assay for homologous recombination between the tagging vector and the target α2 isoform Na,K-ATPase locus requires a set of primers which only participate in amplification of a DNA template unique to the tagged α2 isoform Na,K-ATPase gene. This primer set includes a forward primer specific for the HSV-tk gene of the tagging vector and a reverse primer specific for the genomic target sequence immediately flanking the 3′ end of the region of homology between vector and target. The positive control construct (Fig. 2) was prepared by inserting the selection cassette into the *Bal*I site of exon 8 in a 1.8-kb *Eco*RI fragment of α2 isoform Na,K-ATPase gene. This construct mimics the product of homologous recombination between the tagging vector and the chromosomal target in that it contains a template for PCR which is identical to the correctly tagged α2 locus. It was used to optimize and check for successful PCR.

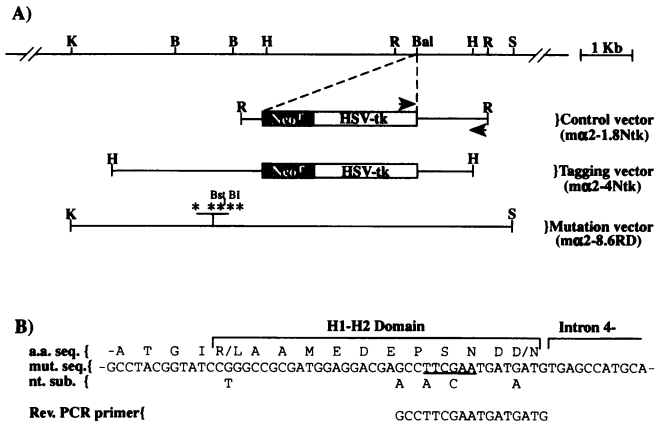

FIG. 2. Gene-targeting vectors. (A) Schematic diagram of the genomic restriction map of the α2 isoform Na,K-ATPase gene. The restriction sites represented include *Bal*I (Bal), *Bam*HI (B), *Eco*RI (R), *Kpn*I (K), and *Sma*I (S). The control vector (mα2-1.8Ntk) was constructed by insertion of the head-to-tail Neo^r/HSV-tk marker cassette into the *Bal*I site of the 1.8-kb *Eco*RI fragment of the α2 isoform Na,K-ATPase gene. The horizontal arrows represent the forward (leftmost) and reverse (rightmost) primers used to screen for homologous recombinants by PCR. Note that the reverse primer is unique to the genome and to the control construct. The tagging vector (mα2-4Ntk) was constructed by insertion of the selection cassette into the *Bal*I site of a 4-kb *Hin*dIII genomic fragment of the gene. The mutation vector (mα2-8.6RD) is composed of an 8.6-kb *Kpn*I-*Sma*I genomic fragment of the α2 isoform Na,K-ATPase gene and carries five point mutations (* ****) and a unique *Bst*BI recognition site in exon 4. (B) Details of the nucleotide substitutions in exon 4 of the mutation vector. The amino acid sequence (a.a. seq.) encoded by the mutation shows the substitutions (L111R and N122D) at the border residues of the extracellular H1-H2 domain. The mutation sequence (mut. seq.) shows the *Bst*BI recognition sequence (underlined) resulting from A→T and C→G substitutions. The wild-type nucleotides which were substituted (nt. sub.) are in the line below the mutation sequence. A reverse primer (Rev. PCR primer) designed for mutation-specific PCR amplification is also shown.

The mutation vector (mα2-8.6RD) illustrated in Fig. 2 consists of an 8.6-kb genomic *Kpn*I-*Sma*I fragment of the α2 gene carrying five nucleotide substitutions in exon 4. The objectives of these planned point mutations were twofold. First and foremost are the codon changes resulting in two amino acid substitutions which modify the cardiac glycoside receptor function of the mouse α2 isoform Na,K-ATPase. Second, this combination of mutations provides several means of screening and identifying cell clones which have been mutated by exchange at the tagged locus. The point mutations were first introduced by M13-mediated site-directed mutagenesis (11) into a 1.1-kb *Bam*HI fragment, which was then switched with its wild-type counterpart to complete the mutation vector. The nucleotide and amino acid substitutions encoded by the mutation vector are shown in Fig. 2B. All five nucleotide substitutions are within the coding region for the first extracellular domain of the enzyme. The amino acid substitutions of the border residues, L111R and N122D, of this extracellular domain were previously demonstrated to confer a 10^3- to 10^4-fold resistance to enzyme inhibition by cardiac glycosides by decreasing the affinity of the enzyme for this steroid ligand (15). To provide a means of screening and identifying correctly mutated ES cell clones, three additional nucleotide substitutions were introduced at wobble positions. These mutations introduce a

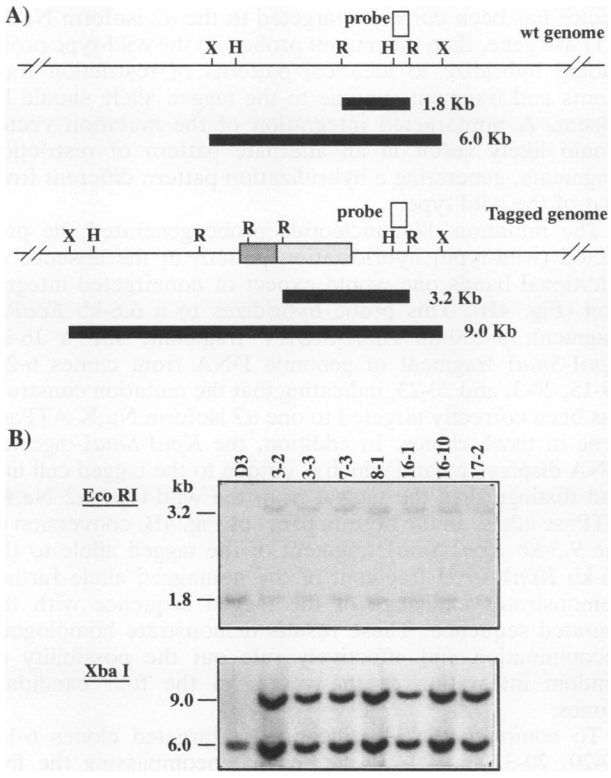

FIG. 3. Analysis of the tagging event. (A) Restriction maps of the wild-type (wt) and tagged alleles used to predict the diagnostic restriction fragment lengths of *Eco*RI (R)- and *Xba*I (X)-digested genomic DNA. The probe (empty block) is a 308-bp *Hin*dIII-*Eco*RI fragment which is external to the genomic sequence of the tagging vector. The predicted *Eco*RI and *Xba*I fragments for the wild-type and tagged α2 isoform Na,K-ATPase alleles which hybridize to the 308-bp probe are drawn as solid bars below the restriction maps. (B) Southern blot analysis of *Xba*I- and *Eco*RI-digested genomic DNA of D3 ES cells and seven G418^r cell lines tagged by homologous recombination with the tagging vector.

unique restriction endonuclease recognition site and provide a mutation-specific primer sequence for PCR without altering the amino acid coding sequence. A *Bst*BI recognition site, unique to the mutated sequence, was introduced by the substitutions A→T in the proline 118 codon and C→G in the serine 119 codon. The wobble mutation A→G in the glutamate 117 codon adds a fourth nucleotide difference to a 14-nucleotide stretch in the mutated sequence of exon 4. This additional substitution was included to increase specificity for mutation-specific PCR with a primer which overlaps the divergent 14-nucleotide stretch.

Tagging the α2 isoform Na,K-ATPase gene. In the first gene-targeting event, an α2 isoform Na,K-ATPase homolog was tagged by replacement with the tagging vector (mα2-4Ntk) in which exon 8 had been disrupted by insertion of the Neo^r/HSV-tk selection cassette. The tagging vector was prepared as a gel-purified *Hin*dIII fragment and transfected into ES cells by electroporation. Transfectants were subject to G418 selection, and 600 G418^r clones were screened for homologous recombinants by PCR as described in Materials and Methods. PCR-positive clones were isolated, expanded, and analyzed by genomic Southern blotting for correct gene-targeted recombination according to diagnostic restriction endonuclease patterns. Identification of ES cell clones correctly targeted with the gene-tagging construct is illustrated in Fig. 3. Southern blots of genomic DNA isolated from PCR-positive clones, digested with either *Eco*RI or *Xba*I, were probed with a 308-bp genomic *Hin*dIII-*Eco*RI fragment which lies 3′ and adjacent to the flanking genomic sequences of the targeting construct. For *Eco*RI digests, this probe hybridizes to a 1.8-kb fragment of the wild-type α2 isoform Na,K-ATPase allele and a 3.2-kb fragment of a correctly targeted allele. For *Xba*I digests, the wild-type allele yields a 6-kb hybrid and the targeted allele yields a 9-kb hybrid with this probe. By these criteria, we isolated 15 ES cell lines which were tagged at the α2 isoform Na,K-ATPase locus with an overall targeting efficiency of 1:40 homologous recombinants to G418^r colonies (Table 1).

Exchange with the mutation vector. An ES cell line (16-10) with a tagged α2 isoform Na,K-ATPase gene, as described above, was expanded and maintained under G418 selection. This cell line was then transfected by electroporation with the mutation vector (mα2-8.6RD) which was prepared as a gel-purified *Kpn*I-*Sma*I fragment. Transfected cells were selected for resistance to 2.5 μM ganciclovir beginning at day 0, 1, 2, 3, 4, or 5 following electroporation. This variable lag period prior to selection was designed to test for the optimum recovery period required for decay and dilution of HSV-tk activity, since recombinants which have lost the HSV-tk gene will remain sensitive to ganciclovir as long as active enzyme exists in the cell. Selection was complete after 3 days, and resistant colonies were isolated and transferred with a disposable-tip pipetter to 24-well plates for expansion. The electroporation of 10^7 cells yielded an estimated total of 1,000 colonies which survived the GANC selection for loss of HSV-tk activity, and 500 of these clones were isolated for further analysis.

Upon expansion of the isolated GANC^r clones, a portion of the cells was frozen for storage and the remaining cells were passaged in duplicate to new 24-well culture plates. One set of plates was used for preparation of genomic DNA, and the other set was used to analyze the clones for the loss of the Neo^r gene by G418 selection. Clones carrying the exchange-mediated mutation should have lost the entire Neo^r/HSV-tk cassette and therefore would be G418^s

TABLE 1. Comparative targeting frequency data for tag and exchange at the α2 Na,K-ATPase locus

Event	No. of cells		Total no. of colonies	No. of clones picked and screened	No. of targeted cell lines	Frequency	
	Electroporated (10^7)	Surviving electroporation (10^6)				Per cell	Per resistant colony
Tagging	1	1	600 (G418^r)	600	15	1.5×10^{-5}	1:40
Exchange	1	2.5	1,000 (GANC^r)	216 (days 0–3), 288 (days 4–5)	0 (days 0–3), 4 (days 4–5)	5.5×10^{-6} (days 4–5)	1:54 (days 4–5)

TABLE 2. Selection[a] data for tag and exchange at the α2 Na,K-ATPase locus

Day of GANC selection (postelectroporation)	No. of GANC^r colonies		No. of GANC^r G418^s colonies	Targeted exchange
	Per plate	Picked and screened		
0	17	17	2	0
1	32	26	6	0
2	29	29	1	0
3	34	144	9	0
4	31	144	17	3
5	32	144	13	1
Subtotal (days 4 and 5)	31	288	30	4
Total		504	48	4

[a] Selection for exchange by homologous recombination.

GANC^r. To test for the loss of the Neo^r gene, one set of the isolated clones was selected in 400 μg of G418 per ml for 3 weeks. Of the 504 GANC^r clones, only 48 were also G418^s, indicating that at least 90% of the GANC^r clones had not lost the selection cassette and therefore could not be complete products of homologous recombination with the mutation vector. The GANC and G418 selection data are provided in Table 2. The 48 Neo^r$^-$/HSV-tk$^-$ clones were subsequently examined for the presence of the mutation vector by PCR, and then homologous recombinants were verified by genomic Southern blot analysis.

Testing for the presence of the mutation vector in GANC^r G418^s clones provided an independent screen for clonal products of targeted exchange. Because the probability of random recombination with the vector and concomitant loss of the selectable markers in the same cell is exceedingly low, clones which have gained the vector and lost the markers are most likely homologous recombinants (see Discussion). The GANC^r G418^s clones were examined for the presence of the mutation vector by PCR amplification of a 270-bp fragment encompassing the point mutations and testing for the mutation-specific *Bst*BI cleavage of this 270-bp PCR product (data not shown). This assay revealed the presence of the mutated sequence in four clones (6-20, 19-15, 20-3, and 20-23) which were then analyzed for homologous recombination as described below.

The genomic location of the mutant sequence in these four candidate clones was established by Southern blot analysis. To distinguish between random integration and homologous recombination, genomic Southern blots of the candidate clones were screened with mutant and wild-type oligonucleotide probes, as illustrated in Fig. 4. Wild-type and mutant versions of the 52-base oligonucleotide used to introduce the point mutations proved to be specific probes for this analysis. The conditions for specificity were determined with cloned sequences and then applied to genomic Southern blot analysis. Southern blots of *Eco*RV-, *Kpn*I-, and *Sma*I-digested genomic DNA were hybridized with the probe which can distinguish between the wild-type and mutated α2 sequences. These restriction digests were chosen because they represent a combination of infrequent recognition sites, providing a diagnostic set of predicted restriction fragment patterns. The predicted *Eco*RV fragment length is a result of one internal and one external recognition site. Since the mutation vector was transfected as a gel-purified *Kpn*I-*Sma*I fragment, the *Kpn*I and *Sma*I sites are disrupted and therefore are not transmitted with the vector. If the mutation vector has been correctly targeted to the α2 isoform Na,K-ATPase gene, then the mutant probe and the wild-type probe should hybridize to identical patterns of restriction fragments and fragments unique to the tagged allele should be absent. A nontargeted integration of the mutation vector would likely result in an alternate pattern of restriction fragments, generating a hybridization pattern different from that of the wild type.

The mutation oligonucleotide probe generated the predicted (wild-type) hybridization pattern in the absence of additional bands one would expect of nontargeted integration (Fig. 4B). This probe hybridizes to a 6.6-kb *Eco*RV fragment, a 5.0-kb *Kpn*I-*Eco*RV fragment, and a 16-kb *Kpn*I-*Sma*I fragment of genomic DNA from clones 6-20, 19-15, 20-3, and 20-23, indicating that the mutation construct has been correctly targeted to one α2 isoform Na,K-ATPase gene in these clones. In addition, the *Kpn*I-*Sma*I-digested DNA displays a band which is unique to the tagged cell line and distinguishes the tagged from the wild-type α2 Na,K-ATPase allele. In the bottom panel of Fig. 4B, conversion of the 9.3-kb *Kpn*I-*Sma*I fragment of the tagged allele to the 16-kb *Kpn*I-*Sma*I fragment of the nontagged allele further demonstrates exchange of the tagged sequence with the mutated sequence. These results demonstrate homologous recombination and effectively rule out the possibility of random integration of the vector in the four candidate clones.

To complete the identification of targeted clones 6-15, 19-20, 20-3, and 20-23, the region encompassing the five nucleotide substitutions was cloned from each cell line and sequenced. A 270-bp genomic fragment spanning the mutated region was amplified by PCR and cloned into a plasmid vector. PCR clones of the mutant allele were identified by *Bst*BI sensitivity and sequenced. These sequences (Fig. 4C) demonstrate delivery of all five point mutations to each of the modified cell lines.

All four correctly modified ES cell lines were from a population of 288 colonies which were allowed to grow for 4 or 5 days posttransfection before being selected in GANC. Of the 144 GANC^r colonies selected beginning on day 3 posttransfection, no correctly modified cells were identified. Therefore, 4 days is sufficient, if not the minimal required time, for dilution and decay of residual HSV-tk activity to allow for selection of gene-targeted exchange of the selection cassette. The selection results for this experiment are provided in Table 2.

Analysis of the GANC^r background population from the exchange step. The background population of GANC^r ES cell colonies selected from the targeted exchange step is derived from cells which have lost HSV-tk activity without acquiring the desired mutation by homologous recombination between the tagged locus and the mutation vector. Two subpopulations make up this background population: one (GANC^r G418^r) which has lost activity of HSV-tk only, and the other (GANC^r G418^s) which has lost activity of both HSV-tk and Neo^r. There are two basic explanations for this loss of marker activity; one is the physical loss of the marker gene(s), and the other is inactivation of marker gene expression. As a general assessment of the origin of the GANC^r background, the loss versus inactivation of the selectable markers was determined by assaying for the presence or absence of the Neo^r/HSV-tk selection cassette in GANC^r clones. In these genomic Southern blot assays of *Xba*I-digested DNA, a probe for the α2 Na,K-ATPase gene reveals a 9-kb fragment if the marker genes are present and a 6-kb fragment of the wild-type allele if the marker genes are

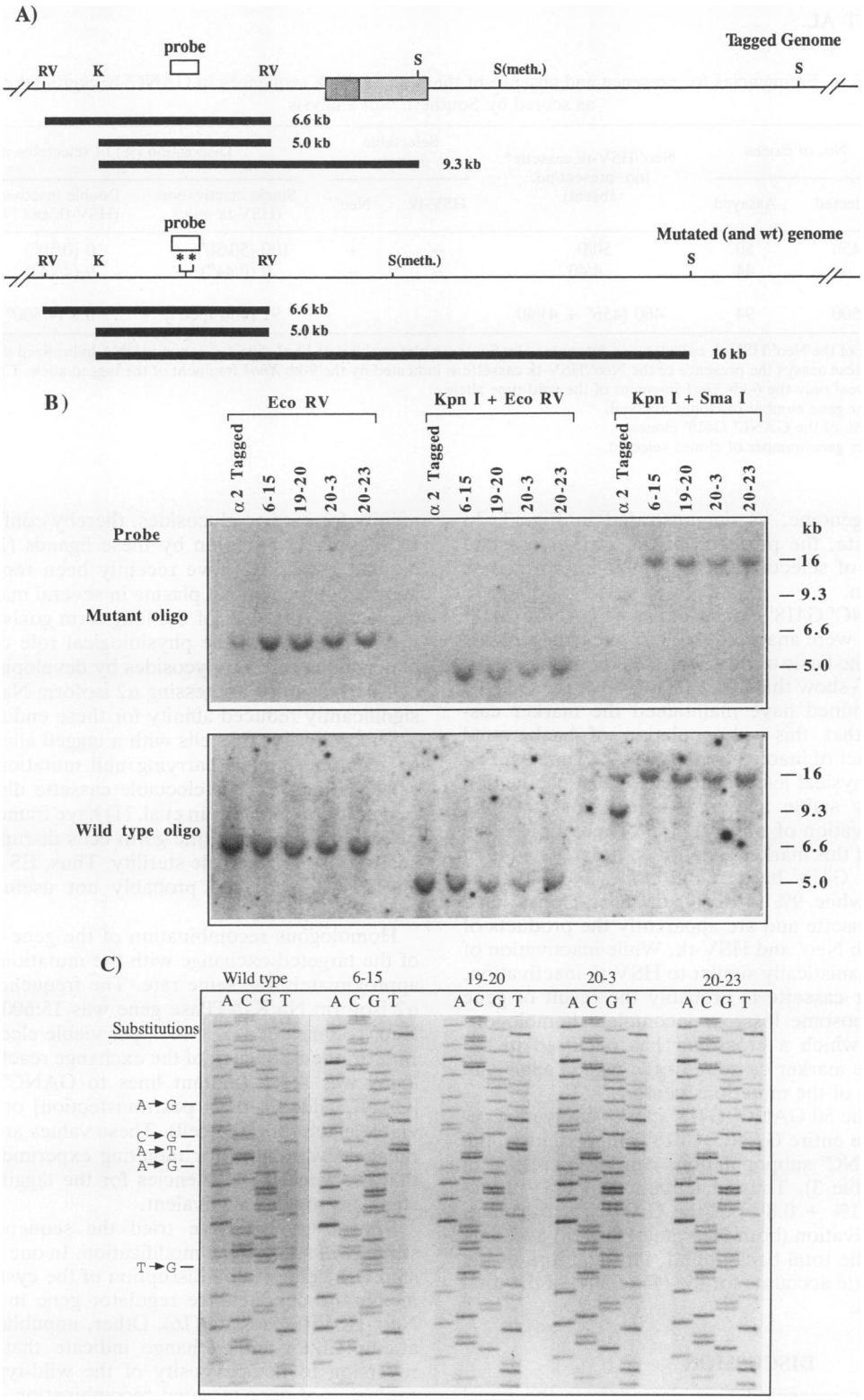

FIG. 4. Analysis of the exchange event. (A) Restriction maps of the tagged allele (top) and the mutated (exchanged) allele (bottom). The sites for cleavage by *Eco*RV (RV), *Kpn*I (K), and *Sma*I (S) were used to predict diagnostic restriction fragment lengths. A *Sma*I site [labeled S(meth.)], identified in genomic clones, is insensitive to *Sma*I digestion in any of the genomic analyses. The predicted fragment lengths for the tagged and mutated alleles are illustrated by solid bars below the maps. The wild-type (wt) and mutant probes (empty block) were 52-bp oligonucleotides encompassing the region of nucleotide substitutions and were used to distinguish between the wild-type and mutant sequences. (B) Southern blot analysis showing the tagged parent cell line (α2 tagged) and the four cell lines mutated by homologous recombination between the mutation vector and the tagged allele. The lower panel probed with the wild-type oligonucleotide shows the hybridization pattern of the wild-type and tagged alleles. The upper panel represents the same blot probed with the mutant oligonucleotide, demonstrating the hybridization pattern of the modified allele in each of the four modified cell lines. Note that since the mutant oligonucleotide (top panel) is not 100% free of cross-hybridization, the faint bands of cross-hybridization in the wild-type (α2 Tagged) lanes provide size markers with which to compare the hybridization patterns of the mutant sequence. (C) Sequence analysis showing all five nucleotide substitutions (indicated at the left) delivered to the modified α2 Na,K-ATPase gene.

TABLE 3. Frequencies for presence and absence of the Neo^r/HSV-tk sequences in GANC^r background clones as scored by Southern blot analysis

Phenotype	No. of clones		Neo^r/HSV-tk cassette[a] (no. present/no. absent)	Selectable activity		Disposition (%) of selectable marker genes		
	Selected	Assayed		HSV-tk	Neo^r	Single inactivation (HSV-tk only)	Double inactivation (HSV-tk and Neo^r)	Absent
GANC^r G418^r	456	50	50/0	−	+	100 (50/50[b])	0 (0/50[b])	0 (0/50[b])
GANC^r G418^s	44	44	4/40	−	−	0 (0/44[b])	9 (4/44[b])	91 (40/44[b])
Total	500	94	460 (456[c] + 4)/40			91 (456[c]/500[d])	0.8 (4/500[d])	8 (40/500[d])

[a] Presence or absence of the Neo^r/HSV-tk cassette was determined by Southern blot analysis of *Xho*I-digested genomic DNA hybridized to an α2 Na,K-ATPase probe as for Fig. 4. In these assays the presence of the Neo^r/HSV-tk cassette is indicated by the 9-kb *Xho*I fragment of the tagged allele. Clones which have lost the HSV-tk cassette reveal only the 6-kb *Xho*I fragment of the wild-type allele.
[b] Number with marker gene/number of clones assayed.
[c] Extrapolated as 100% of the GANC^r G418^r clones.
[d] Number with marker gene/number of clones selected.

absent from the genome, as demonstrated in Fig. 3. In analyzing these data, the presence of the marker cassette coupled with loss of selectable activity was interpreted as marker inactivation.

Fifty of the GANC^r G418^r clones and all 44 GANC^r G418^s background clones were analyzed. No gross rearrangements were detected on the basis of fragment sizes in this analysis. The data in Table 3 show that 100% (50 of 50) of the GANC^r G418^r clones examined have maintained the marker cassette, indicating that this subpopulation of background clones is the product of inactivation of HSV-tk gene expression rather than physical loss of the gene. This inactivation must be relatively stable and may reflect, for example, methylation inactivation of transcription or mutation in the coding sequence of this marker gene. In contrast, 91% (40 of 44) of the GANC^r G418^s background clones have lost the marker cassette, while 9% (4 of 44) of these clones have maintained this cassette and are apparently the products of inactivation of both Neo^r and HSV-tk. While inactivation of Neo^r may be mechanistically similar to HSV-tk inactivation, loss of the marker cassette is probably the result of gene conversion, chromosome loss, or incomplete homologous recombination in which a crossover has occurred in the region between the marker cassette of the tagged allele and the point mutation of the mutations vector.

Assuming that the 50 GANC^r G418^r clones assayed accurately represent the entire GANC^r G418^r subpopulation, the origin of both GANC^r subpopulations can be expressed in relative terms (Table 3). Thus, inactivation of HSV-tk accounts for 92% (91% + 0.8%) of the GANC^r background, while double inactivation (both Neo^r and HSV-tk) accounts for only 0.8% of the total background. Physical loss of the marker gene cassette accounts for 8% (44 of 504) of the total background clones.

DISCUSSION

The experiments presented here demonstrate the novel use of sequential gene-targeted replacement for the subtle introduction of nucleotide substitutions in one homolog of the α2 isoform Na,K-ATPase gene of murine ES cells. These mutations include two codon changes which result in amino acid substitutions and three functionally silent point mutations designed for identification of correctly modified cells. Na,K-ATPase is the receptor for cardiac glycosides which inhibit enzymatic activity of this receptor and are used in the treatment of congestive heart failure and certain cardiomyopathies. The amino acid substitutions introduced reduce affinity for cardiac glycosides, thereby conferring resistance to enzymatic inhibition by these ligands (15). Endogenous cardiac glycosides have recently been reported as normal steroid components of plasma in several mammals including humans (8, 19). One of the long-term goals of these experiments is to reveal the physiological role of these putative endogenous cardiac glycosides by developing a strain of ES cell-derived mice expressing α2 isoform Na,K-ATPase with significantly reduced affinity for these endogenous ligands.

Theoretically, ES cells with a tagged allele could be used to generate animals carrying null mutations of the tagged gene, as long as the selectable cassette disrupted a coding region. However, Braun et al. (1) have found that expression of HSV-tk in postmeiotic germ cells disrupts maturation of sperm, resulting in male sterility. Thus, ES cells tagged with an HSV-tk gene are probably not useful for generating knockout mice.

Homologous recombination of the gene-tagging step and of the targeted exchange with the mutation vector occur at approximately the same rate. The frequency of tagging the α2 isoform Na,K-ATPase gene was 15:600 (tagged lines to Neo^r colonies or 1.5×10^{-5} per viable electroporated cell). In turn, the frequency of the exchange reaction at the tagged locus was 4:288 (mutant lines to GANC^r G418^s colonies [GANC added 4 days posttransfection] or 5.5×10^{-6} per viable electroporated cell). These values are well within the range of variability for targeting experiments and indicate that the targeting efficiencies for the tagging and exchange steps are roughly equivalent.

Several groups have tried the sequential replacement strategy of subtle gene modification. In one account, the first step was achieved by disruption of the cystic fibrosis transmembrane conductance regulator gene in ES cells with a Neo^r/HSV-tk cassette (16). Other, unpublished accounts of attempted tag-and-exchange indicate that in many cases reversion to homozygosity of the wild-type allele, to the exclusion of gene-targeted recombination, is the major (or only) genetic event producing cells selected for the loss of marker gene activity. This may be due to poor targeting frequency, high background in the exchange selection, and/or elimination of positive clones by premature application of selection.

Design of the tagging and mutation vectors should provide for the most efficient targeting of both vectors and most efficient selection of correctly modified clones. The targeting vectors should include a length of homologous sequence, 4 to 10 kb, suitable to provide high-frequency gene targeting

(4) with the selectable marker cassette located near the position of the planned mutation. The vector arms should also be at least 1 kb to maximize precise recombination (24, 27). There are two important considerations in positioning the mutations in the mutation vector: distance from the vector ends and relative proximity of the selection cassette. First, as observed in the careful study of Jiang et al. (10), it is important to place the desired mutation at least 400 bp, ideally 1 to 2 kb, from the ends of the targeting vector to optimize delivery of the mutant sequence to the genome. Second, the mutation(s) should be close to or overlap the selectable markers in the tagged allele. This precaution should optimize correct modification by minimizing incomplete homologous recombination in which a crossover occurring between the mutant sequence and the selectable markers results in exchange with the marker cassette or the mutation sequence but not both.

The tag-and-exchange strategy presented here relies on omega-type homologous recombination. Two groups have recently reported the introduction of a subtle mutations by using O-type homologous recombination followed by intrachromosomal recombination. Hasty et al. (9) used the Neo^r and HSV-tk selection scheme to mutate the HPRT and *Hox-2.6* genes, whereas Valancius and Smithies (25) used the HPRT gene marked with a 4-base insertion to modify the HPRT locus in ES cells. While Hasty et al. reported that the reversion rate to GANC^r was 3.8×10^{-3} per cell generation, their recovery rate of successfully mutated clones was not provided. The frequency of the "out reaction" in the Valancius and Smithies studies was 8×10^{-7} per cell plated. In the work presented here, the overall rate of reversion to GANC^r was $10^3/10^7$ cells electroporated. Considering the 25% postelectroporation survival rate, the reversion to GANC^r becomes 4×10^{-4} per viable cell. The efficiency of targeted exchange with the tagged sequence is 5.5×10^{-6} per viable cell.

Analysis of the GANC^r population of ES cell clones from the exchange step suggests a more efficient selection scheme for the exchange step. In our experiments, three kinds of genetic events which lead to generation of GANC^r cells in the exchange step occurred: (i) background loss of the Neo^r/HSV-tk cassette, (ii) inactivation of the HSV-tk gene, and (iii) replacement of the selection cassette with the mutation construct by homologous recombination. The background frequency of physical loss of the marker genes was 8%, and the frequency of HSV-tk inactivation was 92%, while only 0.8% of the clones were inactivated for both HSV-tk and Neo^r. Since inactivation of a single marker gene accounted for roughly 90% of the background, while loss or inactivation of both marker genes was roughly 10%, eliminating single inactivation as a source of background would theoretically provide a 10-fold enrichment for homologous recombinants. If two negative markers were used, providing double selection, for the exchange step, single inactivation would not contribute to the background. Therefore, a selection scheme including two independent negative selection genes, for example HPRT and HSV-tk, would improve the efficiency of selection for targeted exchange.

In addition, we would like to emphasize the screening for the presence of to the mutation vector, for instance, by PCR, provides a useful enrichment step towards identifying homologous recombinants of the exchange reaction. Since targeted recombination must result in gain of the mutation vector (vector-positive) accompanied by loss of the selectable markers (GANC^r and G418^s), screening for presence or absence of the vector simply allows elimination of vector-negative clones from the population of potential homologous recombinants. Also, if nontargeted integration and marker loss are independent events, then because of the low frequencies for reversion to GANC^r G418^s (roughly 10^{-4} per viable cell) and for gain of the transfected vector (generally, 10^{-3} per viable cell), the frequency of both events occurring independently in the same cell is very low (roughly 10^{-7} per viable cell). This translates to less than one cell per transfection experiment, and consequently most if not all GANC^r G418^s vector$^+$ clones from a single transfection would be targeted recombinants. In the studies presented here, 4 of the 48 GANC^r G418^s clones had recombined with the mutation vector, and all 4 were homologous recombinants.

There are two advantages of targeted tag and exchange over the hit-and-run or in-out strategy. One advantage is the ability to use PCR analysis for presence of the mutation vector as a screen for homologous recombinants at the exchange step. In the insertion and excision scheme, this assay is precluded by the presence of the mutated sequence in the product of the insertion step. A second advantage is the potential of using the initial gene-tagged cell line as a substrate for replacement by different mutation constructs. Since the selection process of the second round of targeted recombinants is straightforward, production of cell lines carrying different mutations at the same locus would be routine once the cell line tagged at the gene of interest was prepared. In contrast, hit and run or in out requires that each mutation is separately targeted to the gene of interest, and then the targeted cell line is expanded and selected for the excision event. Therefore, tag and exchange may be especially useful in situations where multiple mutants are desired at a particular locus in a marker-free and otherwise wild-type context.

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