Introduction of a point mutation into the mouse genome by homologous recombination in embryonic stem cells using a replacement type vector with a selectable marker

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ABSTRACT

The introduction of small mutations instead of null alleles into the mouse genome has broad applications to the study of protein structure-function relationships and the creation of animal models of human genetic diseases. To test a simple mutational strategy we designed a targeting vector for the mouse pro-opiomelanocortin (POMC) gene containing a single nucleotide insertion that converts the initial tyrosine codon of β -endorphin 1-31 to a premature translational termination codon and introduces a unique *Hpa*I endonuclease restriction site. The targeting vector also contains a *neo* cassette immediately 3' to the last POMC exon and a herpes simplex virus thymidine kinase cassette to allow positive and negative selection. Homologous recombination occurred at a frequency of 1/30 clones of electroporated embryonic stem cells selected in G418 and gancyclovir. 10/11 clones identified initially by a polymerase chain reaction (PCR) strategy had the predicted structure without evidence of concatemer formation by Southern blot analysis. We used a combination of *Hpa*I digestion of PCR amplified fragments and direct nucleotide sequencing to further confirm that the point mutation was retained in 9/10 clones. The POMC gene was transcriptionally silent in embryonic stem cells and the targeted allele was not activated by the downstream phosphoglycerate kinase-1 promoter that transcribed the *neo* gene. Under the electroporation conditions used, we have demonstrated that a point mutation can be introduced with high efficiency and precision into the POMC gene using a replacement type vector containing a retained selectable marker without affecting expression of the allele in the embryonic stem cells. A similar strategy may be useful for a wide range of genes.

INTRODUCTION

Gene targeting in murine embryonic stem (ES) cells by homologous recombination and the subsequent production of transgenic mice carrying specific gene mutations from the transfected totipotent ES cells has been a recently exploited technique to create mouse models of human genetic diseases (1–2) and to explore the role of specific proteins in mammalian development (3–6). Virtually all studies reported to date have used gene targeting vectors containing deletions of essential protein coding regions resulting in null alleles. Although useful for many purposes, null alleles may result in a lethal phenotype precluding a complete analysis of gene function. Furthermore, many recessive genetic diseases are caused by point mutations that result in hypofunctional or dysfunctional protein products rather than by complete loss of function mutations. Consequently, several strategies have been described to introduce small mutations into the genome. Two groups have described methods to introduce subtle mutations based on insertional type vectors containing selectable markers (7–8). The necessity of spontaneous intrachromosomal recombination to resolve the targeted gene locus with a relatively high rate of gene conversions to wild type decreases the final frequency of recombination and the resulting increased passage number of the selected cells may reduce the chances of later germ line transmission of the desired mutations. Coelectroporation of a targeting vector and an independent selectable marker involves a more complicated primary screening procedure than standard Southern hybridization analysis or polymerase chain reaction of the initially obtained clones (9). An alternative method involving direct screening without the use of a selectable marker has not been widely used and is technically demanding (10).

A class of proteins whose function has not been studied using targeted mutagenesis is the polypeptide hormone family. Neuropeptides are typically processed from larger prohormones by a series of specific posttranslational endoproteolytic cleavage steps to yield the biologically active peptides. Pro-

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opiomelanocortin (POMC) is a neuropeptide precursor that generates a large family of peptides including adrenocorticotrophic hormone (ACTH), melanocyte stimulating hormones (MSH), and β -endorphin, each possessing distinct biological activities (11–12). An analysis of the phenotype resulting from a null allele for the POMC gene would be complicated by the diversity of peptides generated from the single precursor. POMC is therefore an excellent model for the introduction of subtle mutations whose aim is to selectively alter the peptide profile resulting from prohormone processing. In this report we describe the design of a novel replacement type targeting vector including selectable markers that introduces a point mutation into the translated portion of the mouse POMC gene that will result in the selective lack of β -endorphin production from a carboxyl-terminally truncated precursor. The targeting vector recombined as predicted and the point mutation was retained with high efficiency in ES cells. An identical strategy should be applicable to the mutation of neuropeptide precursors at dibasic processing sites precluding the release of specific peptides from otherwise normal prohormones.

MATERIALS AND METHODS

Construction of the targeting vector

The plasmid pHAL (gift of M. Uhler), carrying a 10.2 kb *Eco* RI fragment containing the entire POMC gene isolated from a Balb/c mouse genomic library (13), was double digested with *Asp* 718 and *Hind* III and a 2.9 kb fragment was subcloned in pSelect (Promega, WI). A single base insertion was introduced by site-directed mutagenesis converting the codon for the amino-terminal tyrosine of β -endorphin into a termination codon (TAC to TAA). The same mutation creates a *Hpa* I restriction endonuclease site (GTTAC to GTTAAC). A blunt-ended neomycin (*neo*) resistance gene cassette was released from pPGKneobpA (14) and ligated into a *Pml* I site located 273 bp downstream of the single base insertion. A 6.3 kb *Eco* RI/*Asp* 718 fragment encompassing 5' flanking sequences and exon 1 and 2 of the POMC gene was added using Bluescript KS (+/–) (Stratagene, CA) as a cloning vector. A herpes simplex virus thymidine kinase (*tk*) gene was placed further upstream in the opposite transcriptional orientation with respect to the POMC gene to permit negative selection. Both the *neo* and the *tk* cassettes (gifts of P. Soriano) are under the transcriptional control of the phosphoglycerate kinase-1 (PGK) promoter. The resulting replacement targeting vector, designated pPOMCX*4, contains a long 5' arm of 8 kb and a short 3' arm of 1.2 kb homologous with the endogenous POMC gene (Fig. 1B).

Electroporation and selection of ES cells

AB1 (provided by A. Bradley) and D3 (provided by T. Doetschman) embryonic stem (ES) cells were grown on γ -irradiated G418 resistant SNL 76/7 feeder cells (provided by A. Bradley) (4) on gelatinized plates. ES cells were cultured as previously described (15). Confluent 10 cm plates (approximately 2×10^7 cells) were trypsinized and disaggregated by repetitive pipetting and resuspended in 0.8 ml Hepes buffered saline (21 mM Hepes pH 7.0, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 6 mM glucose). Electroporations were performed in the presence of 25 μg of *Not* I digested linearized pPOMCX*4 at 280 V and 330 μF at room temperature using a cell porator (Bethesda Research Laboratories, MD). After 10 min, ES cells

were divided into 3 equal aliquots and plated on 10 cm plates containing feeder cells. Following 24 to 36 h the medium was changed and contained G418 (300 $\mu\text{g}/\text{ml}$; Gibco-BRL, MD). Two plates were also treated with gancyclovir (2 μM ; Syntex, CA). Media were changed every 48 h and after 8 to 10 days double resistant colonies were collected under a dissecting microscope with a fine Pasteur pipette and expanded as described elsewhere (16).

Identification of homologous recombinant clones by PCR and Southern hybridization

From each electroporation, 150 double resistant ES cell colonies were grown until confluence on 24-well plates. DNA was isolated as described previously (17) and resuspended in 0.15 ml of a solution containing 10 mM Tris–HCl and 0.1 mM EDTA, pH 7.5. PCR conditions were established on cells that had previously been transfected with a mock targeting construct. For the first screening round, pools were obtained combining DNA from 10 individual colonies. A second screening round was performed using DNA samples from positive pools differently combined in pools of 6 individual colonies. Finally the colonies present in the positive pools were individually analyzed. Typically PCR was carried out in a 50 μl final volume with 1 μl of each sample of DNA and the following mixture: 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM MgCl_2 , 0.001% (w/v) gelatin, 200 μM of each dNTP, 1 μM of each primer and 1.25 U of Amplitaq DNA Polymerase, (Perkin Elmer Cetus, CA). Temperature cycles was as follows: 94°C, 1 min; 60°C, 1 min; 72°C, 1 min. Forty amplification cycles were used for the first and second screening and 35 for the third round. The following oligonucleotides were used to identify homologous recombination events: Primer 1 (Fig. 1C): PGKneobpA sense (5'GAGG-ATTGGGAAGACAATAGCA3') and primer 2: 3' end of the POMC genomic clone outside of the targeting vector antisense (5'GACATGTTTCATCTCTATACATAC3'). PCR products were resolved in a 0.8% agarose gel and those showing a 1.2 kb band were treated as putative positives. 10 μg of genomic DNA from individual clones were digested with 20 U *Eco* RI at 37°C overnight, ethanol precipitated, run on a 0.8% agarose gel, electrophoretically transferred to Nytran (Schleicher & Schuell, OH) and hybridized with a ^{32}P -labeled random primed (Boehringer-Mannheim, IN) probe using a 0.9 kb *Hind* III/*Eco* RI fragment located 3' to the short arm of the targeting vector (probe A, Fig. 1C). After developing the autoradiograms, the blots were stripped and rehybridized with a 0.8 kb *Pst* I/*Xba* I *neo* probe. Hybridizations were performed overnight at 42°C in the following conditions: 50% formamide, 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.2), 5 $\times$ Denhardt's, 0.5% SDS, 25 mM sodium phosphate buffer pH 7.6, 100 mg/ml sheared and denatured salmon sperm DNA and 2.5 mM EDTA. Washes were performed at a final stringency of 0.1 $\times$ SSC, 0.1% SDS at 65°C. To confirm the retention of the single base insertion, PCR was performed with each homologous recombinant clone using the same conditions as described above but with primers 3: POMC exon 3 sense (5'GGCAAGCGCTCC-TAC3') and 4: PGK promoter antisense (5'CTAAA-GCGCATGCTCCAGAC3'). The 576 bp products were digested with *Hpa* I and resolved on a 1.5% agarose gel. Direct sequencing by the dideoxy chain termination method (Sequenase 2.0; USB, OH) of the PCR products was performed to assure that unwanted mutations had not occurred in the POMC coding sequences.

Analysis of POMC gene expression in homologous recombinant clones

Two homologous recombinant clones and wild type D3 cells were grown to confluence on 10 cm gelatinized plates in the presence of 1000 U/ml murine leukemia inhibitory factor (Esgro, Gibco-BRL, MD) and total RNA was prepared using the proteinase K/NP-40 lysis method described elsewhere (18). Similarly, total RNA was isolated from three pooled C57Bl/6 mouse pituitaries. 20 μ g of total RNA from each sample were loaded into a formaldehyde gel (18), run at 3 V/cm for 4 h, electrophoretically transferred to Nytran and hybridized with the *neo* probe as described above. After developing the autoradiogram, the blot was stripped and rehybridized with a 32 P-labeled random primed probe using a 0.5 kb *Nco* I/*Pml* I fragment present in the third exon of the mouse POMC gene. Hybridization and washing conditions were the same as described for Southern blots.

Figure 1. Schematic representation of a point mutation introduced in the POMC gene by homologous recombination. **A:** Restriction map of the wild type POMC locus in the region homologous to pPOMCX*4. Black boxes denote exons. **B:** Restriction map of the linearized targeting construct pPOMCX*4 after *Not* I digestion. A *neo* cassette was inserted after the natural polyadenylation signal of the POMC gene and a tk cassette was added at the 5' end. The plasmid contains 8.1 kb of homology 5' and 1.2 kb 3' of the *neo* insertion. The sequence in the box (GTTAAC) denotes the *Hpa* I restriction site created after the insertion of a single A. Arrows indicate transcriptional orientation of the *neo* and tk promoters. **C:** Predicted structure of the mutated allele after homologous recombination. Half arrows indicate the approximate positions of primers 1 and 2 used for PCR amplification in colony screening and primers 3 and 4 used to confirm the retention of the point mutation. Probes A and B were used in Southern blots to identify homologous recombination events. Abbreviations: E, *Eco* RI; H, *Hind* III; B, *Bam* HI; S, *Sma* I.

RESULTS AND DISCUSSION

Targeting a single base insertion to the POMC gene by homologous recombination

We used a 10.2 kb *Eco* RI/*Eco* RI fragment isolated from a Balb/c mouse genomic DNA library (13) to target the POMC locus. This fragment contains the complete transcriptional unit of the POMC gene (Fig. 1A). With the ultimate aim of creating mice capable of producing normal ACTH and MSH peptides but unable to produce functional β -endorphin, we constructed the replacement targeting vector pPOMCX*4 (Fig. 1B) which carries two independent mutations. First, there is a single base insertion in the first codon of β -endorphin that converts the amino-terminal tyrosine into a premature translational termination codon (TAC to TAA). Because the coding region for β -endorphin is located 3' with respect to ACTH and MSH sequences, the predicted carboxyl-terminally truncated prohormone is likely to be processed to all possible POMC peptides with the exception of β -endorphin and its derivatives. Our strategy makes the assumption that the truncated prohormone will be correctly targeted to secretory granules, however the location of the signal(s) for intracellular trafficking within POMC are not known. The second mutation is the insertion of a *neo* expression cassette PGKneobpA (14) that is used to select transformed cells. *Neo* was placed downstream of the natural polyadenylation sequence of the POMC gene and 1.2 kb from the 3' end of the construct. This positioning permitted the identification of potential homologous recombinants using PCR with a primer complementary to the sense strand of the *neo* cassette (primer 1, Fig. 1C) and another one complementary to the antisense strand of a region of the POMC locus not present in the targeting construct (primer 2). A herpes simplex virus thymidine kinase expression cassette (PGKTK) was added at the 5' end of the long arm of the construct to select against random integration events. pPOMCX*4 was used to electroporate 2×10^7 AB1 or D3 ES cells and the transformants were selected in G418 and gancyclovir. The enrichment obtained with gancyclovir was 3.9 fold in AB1 cells and 1.6 fold in D3 cells (Table 1).

To detect homologous recombinant clones, we used PCR primers specific for a junction fragment at the 3' end of the construct (Fig. 1C). In order to avoid the presence of feeder cells during the preparation of genomic DNA, ES cells were grown during the last passage in the presence of 1000 U/ml murine leukemia inhibitory factor. PCR positive clones were confirmed by Southern blot analysis of genomic DNA digested with *Eco* RI and hybridized to probe A, a flanking probe in the 3' vicinity of the targeting vector (Fig. 1C). Ten of 11 positive clones identified initially by PCR showed the expected restriction pattern: a 10.2 kb band corresponding to the normal allele as well as the

Table 1. Introducing a point mutation and a selectable marker in the POMC locus

ES Cells	No. of Cells Electroporated	No. of Colonies G418 ^r	No. of Colonies G418 ^r /GANC ^r	Enrichment	No. of Recombinants	Frequency of Recombination	Retention of the Point Mutation
AB1	2×10^7	3,400	880	3.9	5/140	1/28	4/4
D3	2×10^7	720	460	1.6	6/144	1/24	5/6
Total	4×10^7	4,120	1,340	2.8*	11/284 [†]	1/26	9/10 [†]

* Represents the average obtained between both cell lines. GANC, gancyclovir.

[†] Eleven clones were identified by a PCR strategy. Ten of these had the correct structure based on subsequent Southern blot analysis.

Figure 2. Southern blot analysis of genomic DNA from recombinant embryonic stem (ES) cell clones. Ten micrograms of DNA from wild type (WT) AB1 cells or ES cell clones identified as recombinants by an initial PCR screen were digested with *Eco* RI and sequentially hybridized with a 32 P-labeled random primed probe using a 0.9 kb *Hind* III/*Eco* RI fragment (probe A, Figure 1C) and with a 32 P-labeled random primed probe using a 0.8 kb fragment of *neo*. The 10.2 kb band corresponds to the wild type allele while the 11.8 kb band coincides with the predicted homologous recombination event. Autoradiograms were exposed for 3 to 4 days.

Figure 3. Analysis of the retention of the point mutation. DNA samples from D3 ES cell homologous recombinant clones were used as templates to PCR amplify a 576 bp band using primers 3 and 4 (shown in Figure 1C). These bands were further digested with *Hpa* I and separated on a 1.5% agarose gel. The size of the predicted bands are 320 bp and 256 bp. An undigested aliquot of the PCR product obtained with DNA from clone 76 was run on the left lane as a control.

predicted 11.8 kb band created following homologous recombination (Fig. 2). When the same blot was stripped and rehybridized with a *neo* probe, only the 11.8 kb band was observed suggesting that none of the targeted clones carried an additional integrated insertion of the construct or contained any gross structural rearrangement of the locus (Fig. 2). Homologous recombination at the 5' extreme of the construct was confirmed also by Southern blot analysis of genomic DNA digested with *Bam* HI and *Sma* I and hybridized to probe B (data not shown). Table 1 summarizes these experiments and shows a relatively high frequency of recombination at the POMC locus, 1/28 and 1/24 for AB1 and D3 cells respectively.

Retention of the single base insertion in the homologous recombinant clones

Although the mechanisms of homologous recombination in mammalian cells are not yet completely understood, some evidence suggests that the process is similar to that found in

Figure 4. Northern blot analysis of POMC and PGK promoter transcriptional activities in embryonic stem cells. Total RNA from wild type D3 ES cells (WT), from homologous recombinant D3 clones 14 and 96 and from normal mouse pituitaries (Pit.) was obtained and 20 μ g were loaded per lane. The Northern blot was first hybridized with a 32 P-labeled random primed probe using a 0.8 kb fragment of *neo*. The autoradiogram was exposed for 4 d. After stripping the blot was rehybridized with a 32 P-labeled random primed probe using a 0.5 kb *Nco* I/*Pml* I fragment of the third exon of the mouse POMC gene and the autoradiogram was exposed for 5 h.

bacterial and fungal systems: double reciprocal crossover events followed by the resolution of Holliday structures or by gene conversion (19). A typical replacement vector contains regions of homology with the endogenous locus at both sides of the mutated sequence and the vector is thought to pair via shared homology with its chromosomal counterpart.

Because the targeting vector pPOMCX*4 carries two different mutations, it was necessary to confirm the existence of the single base insertion responsible for knocking out β -endorphin production in those clones selected for homologous recombination by G418 and gancyclovir resistance. Using genomic DNA from those clones and primers 3 and 4 (Fig. 1C) we PCR amplified a 576 bp product that contains the first codon of β -endorphin and the newly created *Hpa* I restriction endonuclease site. *Hpa* I digestion should generate two fragments of 320 bp and 256 bp. All the recombinant clones in AB1 ES cells analyzed (data not shown) as well as five out of six D3 recombinant clones carried the single base mutation. Figure 3 shows the restriction pattern of the PCR products amplified with primers 3 and 4 after digestion with *Hpa* I. Clones 14, 15, 53, 76 and 96 had the predicted 320 and 256 bp bands while the uncut band of clone 131 suggests that the *Hpa* I site was absent. Direct sequencing of the 576 bp PCR products confirmed the predicted mutation in all the clones that were cut with *Hpa* I. However, clone 131 had the same sequence as the wild type POMC gene (data not shown). The presence of a recombinant that carries only one of the mutations could be due to either a cross-over event on the long arm between both mutations or an initial cross-over event upstream of the point mutation, with restoration of the wild type sequence by branch migration. The high retention of the point mutation in 9 out of 10 recombinant events may be due to the relatively short distance between the insertional point mutation and the PGK*neo* cassette (273 bp). Further experiments are necessary to distinguish between these possibilities.

A limited number of strategies have been reported to introduce small mutations in the mammalian germ line. They include the 'it and run' (7) similar to the 'in-out' (8) method and coelectroporation of a targeting replacement vector together with a selectable marker in a different plasmid (9). The first strategy is essentially a double recombination. This procedure is very complicated because the positive clones obtained after the initial homologous recombination between an insertional targeting vector and the endogenous locus must be reselected for intrachromosomal recombination between the endogenous

sequences and the duplicated sequences provided by the vector. Coelectroporation, on the other hand, has the disadvantage of limiting the primary screening for targeted events in a large number of clones to the inefficient approach of direct sequencing of PCR products. Although the introduction of a restriction site allows screening of recombinants by Southern hybridization or endonuclease digestions of PCR products that encompass the mutation, most point mutations will not generate or disrupt a restriction site.

The methods described above, although using more complex or time consuming routines, do avoid the presence of a selectable marker with its promoter/enhancer in the genome of the mutated animal. Potentially, the promoter of the selection cassette could positively or negatively regulate expression of the targeted locus thereby complicating an interpretation of the phenotype resulting from a subtle mutation in the encoded protein sequences. In addition, the PGK promoter was reported to have limited bidirectional activity in transfected cell lines (20). For these reasons, we tested the capability of the PGK promoter to influence the expression of the POMC gene in ES cells targeted with pPOMCX*4. Total RNA was prepared from wild type and from two different homologous recombinants of D3 ES cells and from mouse pituitaries and used for Northern blots of *neo* and POMC transcripts. Figure 4 shows that a 1.3 kb band predicted for *neo* mRNA is only present in recombinant ES clones suggesting that the PGK promoter is active in these cells. A random primed probe using double-stranded POMC DNA as a template was used to detect potential POMC transcripts made in either orientation. After stripping and rehybridizing the Northern blot, only the lane containing RNA from mouse pituitaries showed the predicted 1.1 kb band corresponding to mouse POMC mRNA. Neither wild type nor the recombinant ES cells showed detectable levels of POMC gene expression even after 48 h of film exposure, suggesting that the active PGK promoter was unable to inappropriately activate the normally silent POMC gene in ES cells.

It is possible, therefore, that the retained PGK-*neo* cassette will have no effect on POMC gene expression in differentiated tissues of mice produced from the targeted embryonic stem cells. In at least one case where expression was assayed, a retained polyoma promoter/enhancer-*neo* selection cassette was actually silent *in vivo* and the disrupted insulin-like growth factor-2 allele was transcribed essentially in the normal developmentally regulated and tissue-specific pattern (21). On the other hand ectopic expression of a selectively mutated allele would not necessarily confound an interpretation of the phenotype. Several examples of transgenic mice with ectopic expression of a neuropeptide and no overt phenotype have been reported (22–25).

Our results demonstrate that it is possible to introduce precisely a point mutation into the mouse genome using a replacement type vector with a retained selectable marker. The absolute frequency of correctly targeted clones was at least 10-fold higher than reported with other more complicated strategies (7–9). POMC, like other neuropeptide precursors, has multiple proteolytic processing sites that are typically paired basic amino acid residues. Mutation of the Lys-Arg processing site flanking β -endorphin could therefore be an alternative approach to producing mice with a selective loss of the opiate peptide if the carboxyl-terminal truncated POMC prohormone encoded by the mutation described in this report is not properly targeted or processed. A similar mutational strategy could be used to target individual or multiple processing sites and selectively alter the resulting

peptide profile generated from prohormones to deduce the physiological functions of each peptide in mice. In the example reported, the point mutation was introduced into the terminal exon and the *neo* cassette was inserted into the 3' flanking region of the gene. Alternatively, it should be possible to insert the *neo* cassette into an intron to accommodate a small mutation in a central exon and maintain a short distance between the two mutations. The relative simplicity and efficiency of this strategy will likely make it applicable to a larger number of genes than the previously described methods.

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