MOLECULAR AND CELLULAR BIOLOGY, Feb. 1994, p. 1009–1016
0270-7306/94/$04.00+0

Vol. 14, No. 2

Use of Double-Replacement Gene Targeting To Replace the Murine α-Lactalbumin Gene with Its Human Counterpart in Embryonic Stem Cells and Mice

ALEXANDER STACEY,[1]* ANGELIKA SCHNIEKE,[1] JIM McWHIR,[2] JULIAN COOPER,[1] ALAN COLMAN,[1] AND DAVID W. MELTON[2]

Pharmaceutical Proteins Ltd., Roslin, Midlothian EH25 9PP,[1] and Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR,[2] Scotland

Received 13 August 1993/Returned for modification 13 October 1993/Accepted 10 November 1993

The mouse α-lactalbumin gene has been replaced with the human gene by two consecutive rounds of gene targeting in hypoxanthine phosphoribosyltransferase (HPRT)-deficient feeder-independent murine embryonic stem (ES) cells. One mouse α-lactalbumin allele was first replaced by an HPRT minigene which was in turn replaced by human α-lactalbumin. The end result is a clean exchange of defined DNA fragments with no other DNA remaining at the target locus. Targeted ES cells at each stage remained capable of contributing efficiently to the germ line of chimeric animals. Double replacement using HPRT-deficient ES cells and the HPRT selection system is therefore a powerful and flexible method of targeting specific alterations to animal genes. A typical strategy for future use would be to generate a null mutation which could then be used to produce multiple second-step alterations at the same locus.

Gene targeting in murine embryonic stem (ES) cells has had an enormous impact on biology over the past 3 to 4 years because of its ability to produce mice with specific genetic alterations. Although the first mice produced by this method contained a corrected hypoxanthine phosphoribosyltransferase (HPRT) gene (32), most subsequent reports describe loss of function mutations or "knockouts" generated by the integration of a selectable marker into genes of interest (reviewed in references 4, 13, 19, and 37).

Gene replacement is potentially a more powerful technique than gene knockout. Possible applications include fine analysis of regulation of gene expression by mutagenesis of control elements, analysis of protein structure and function by alteration of amino acid sequence, generation of animal models of human diseases (e.g., cystic fibrosis), and in our case precise placement of heterologous genes in transgenic mice.

Our interest in gene replacement is as a means of manipulating milk composition for pharmaceutical and therapeutic applications. We have used a simple and straightforward strategy to replace the mouse α-lactalbumin gene with its human equivalent. The method, termed double replacement, which was first suggested by Reid et al. (24), uses the HPRT-selectable marker in HPRT-deficient ES cells. The target region is first replaced with an HPRT minigene by using a standard replacement vector. This is then replaced with the desired DNA fragment in a second round of targeting. This approach exploits the ability to select first for and then against HPRT expression in a feeder-independent HPRT-deficient ES cell line, HM-1 (18), which contributes to the germ line at high frequency. The system is simple and efficient to operate, and targeted cells retain their ability to colonize the germ line throughout. This method should be widely applicable for the production of mice with a variety of subtle gene alterations.

* Corresponding author. Phone: 031-440-4777. Fax: 031-440-4888.

MATERIALS AND METHODS

DNA cloning and constructs. The mouse α-lactalbumin gene was isolated from a library of strain 129 mouse genomic DNA (Stratagene) by hybridization with a mouse α-lactalbumin probe generated by PCR. Similarly, the human gene was isolated from a human genomic library (Stratagene) by hybridization with a human α-lactalbumin PCR fragment. Two overlapping λ clones were isolated and characterized for each gene. A restriction map of the cloned mouse α-lactalbumin locus is shown in Fig. 1. The identity of cloned genomic fragments was verified by comparison of partial DNA sequences (data not shown) with the published human genomic (9) and mouse cDNA (35) α-lactalbumin sequences.

MALHT. The MALHT (mouse alpha-lactalbumin, HPRT, TK) targeting vector shown in Fig. 1 was constructed from five DNA fragments: (i) a 4.3-kb mouse α-lactalbumin 5′ flanking region (derived from *Xba*I and *Bam*HI sites 4.9 to 0.57 kb upstream of the transcription initiation site) subcloned into pUC18 and excised with *Sal*I and *Bam*HI; (ii) a 2.7-kb fragment comprising the mouse phosphoglycerate kinase (PGK) promoter linked to an HPRT minigene, subcloned as an *Eco*RI fragment from PGK/pDWM1 (26) into pPoly3 (16), and used as a *Bam*HI-*Xba*I fragment; (iii) a 0.85-kb *Xba*I-*Bam*HI mouse α-lactalbumin 3′ flanking fragment (from 0.147 to 1 kb downstream of the translation stop site); (iv) a 3.0-kb fragment consisting of the PGK promoter linked to the herpes simplex virus thymidine kinase (TK) gene (26) excised with *Bam*HI and *Hin*dIII from Bluescript KS$^-$ (Stratagene); and (v) plasmid vector pSL1180 (Pharmacia) cut with *Sal*I and *Hin*dIII.

HMAL. The HMAL (human, mouse alpha-lactalbumin) targeting vector shown in Fig. 2 was constructed from four DNA fragments: (i) a 4.3-kb *Xba*I-*Bam*HI mouse α-lactalbumin 5′ flanking fragment (4.9 to 0.57 kb upstream of the transcription initiation site); (ii) a 2.97-kb human α-lactalbumin fragment (derived from an *Xho*I site 0.77 kb upstream of the transcription initiation site and an *Eco*RI site 0.136 kb downstream of the translation stop site) subcloned into Bluescript KS$^-$ (the *Xho*I site was converted into a *Bcl*I site,

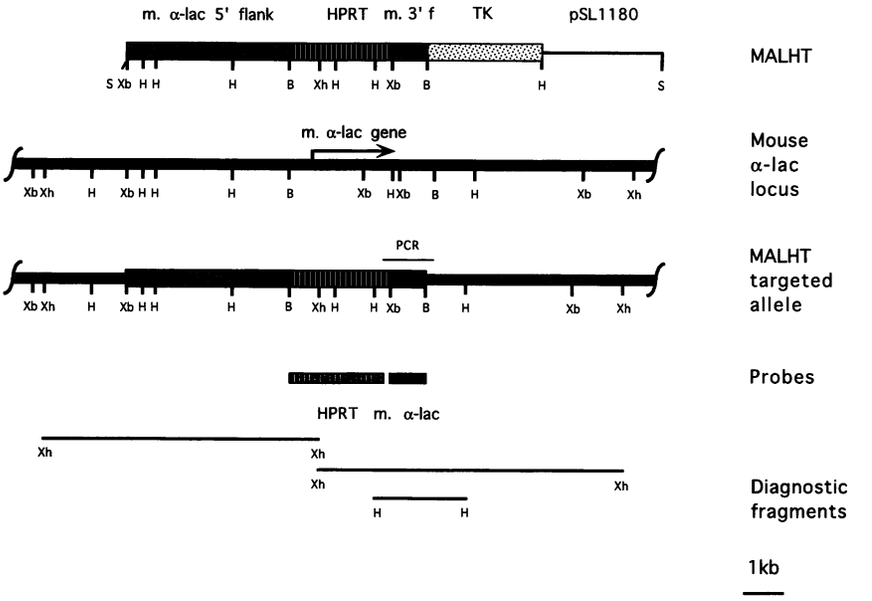

FIG. 1. Restriction maps of the linearized MALHT (mouse alpha-lactalbumin, HPRT, TK) vector, mouse α-lactalbumin locus, predicted structure of the targeted allele, and diagnostic fragments used for Southern analysis. The MALHT map shows restriction sites used in its construction and those used in Southern analysis. The lower two maps show only those sites used in Southern analysis. Striped bar, HPRT; dotted bar, TK; broad shaded bar, mouse (m) α-lactalbumin present in MALHT; narrow shaded bar, endogenous mouse α-lactalbumin; solid line, plasmid vector. The mouse α-lactalbumin transcribed region is indicated by the single arrow. The fragment amplified in the PCR assay is indicated by a line over the structure of the targeted allele. The probes used are indicated beneath the map of the targeted allele. Diagnostic restriction fragments correspond to those visualized in Fig. 3. The restriction enzyme sites shown, *Bam*HI (B), *Hin*dIII (H), *Sal*I (S), *Xba*I (Xb), and *Xho*I (Xh), are those used in the final construction of MALHT and diagnostic digests of targeted clones.

and a *Bcl*I-*Eco*RI fragment was used in the final construct); (iii) a 4.8-kb mouse α-lactalbumin 3′ flanking fragment (derived from *Xba*I sites 0.147 and 4.95 kb downstream of the translation stop site) subcloned into pUC18 and excised with *Eco*RI and *Sal*I; and (iv) Bluescript KS$^-$ (Stratagene) plasmid cut with *Xba*I and *Sal*I.

ES cell culture and electroporation. The ES cell line used throughout was HM-1 (18). Growth of HM-1 and targeted derivatives was as described previously (26).

(i) MALHT targeting. For MALHT targeting, 1.75×10^7 HM-1 cells (passage 11) were electroporated with 150 μg of *Sal*I linearized MALHT DNA as described elsewhere (32). Following electroporation, cells were plated at either 0.5 or 1.25×10^6 cells per 10-cm-diameter dish. Hypoxanthine-aminopterin-thymidine (HAT) (30) selection was applied after 30 h, and 0.5 μg of ganciclovir (Syntex Research Co.) per ml was added after 4 days. HAT selection was maintained throughout the growth of each cell line. Ganciclovir selection was maintained until colonies were picked.

(ii) HMAL targeting. Cells of the MALHT-targeted line M2 (see Results) were grown in nonselective medium for 7 days before the second round of targeting. HAT selection was removed, replaced with hypoxanthine-thymidine (lacking aminopterin) for 3 days, and then changed to ordinary medium. M2 cells (2×10^7) (passage 15) were electroporated with 200 μg of *Xho*I-linearized HMAL as described above. Following electroporation, cells were separated into 10 pools and grown for 6 days to allow HPRT activity in targeted cells to decay. Samples of each pool were plated at a density of 1.5×10^6 cells per 10-cm-diameter dish in medium containing 10 μM 6-thioguanine (6-TG; Sigma). Mock targeting was carried out at the same time as HMAL targeting and used the same cells, plating density, and selection conditions as for HMAL targeting. The electroporation step was omitted, and cells were plated directly into 6-TG selective medium as described above.

PCR and Southern analysis. Approximately one-third of the cells from each colony picked were used to prepare DNA (21) for PCR. Standard PCR conditions were used. Cycle parameters were 35 cycles of 45 s at 94°C, 2 min at 60°C, and 2 min at 72°C. Primers used were as described below.

(i) MALHT targeting. A 1.3-kb fragment from correctly targeted clones was amplified with primer H (AGCCTAC CCTCTGGTAGATTGTC), which hybridizes to the 3′ end of the HPRT gene at positions 960 to 983 of the mouse cDNA sequence (14), and primer M3 (GTGTCGAAGCCAGGATT AGCCTGG), which hybridizes to a region of the mouse α-lactalbumin 3′ flank not present in MALHT (unpublished sequence data). The region amplified is indicated in Fig. 1.

(ii) HMAL targeting. A 742-bp fragment between positions −761 and −18 of the human α-lactalbumin gene (9) was amplified with primers Hu5 (GAGCTCCTGGGCTCAAGT GATCG, positions −761 to −739) and Hu6 (TTTGGCTAC CCCCAAGAACC, positions −18 to −37). The region amplified is indicated in Fig. 2.

Southern analysis was carried out as described elsewhere (32).

Mice. The mouse strains used for the identification of chimeras and germ line transmission of ES cell markers were as described previously (26). Blastocyst injection and embryo transfer were carried out by standard techniques (11).

RESULTS

We have replaced in two steps a 2.72-kb fragment containing the complete mouse α-lactalbumin transcribed region

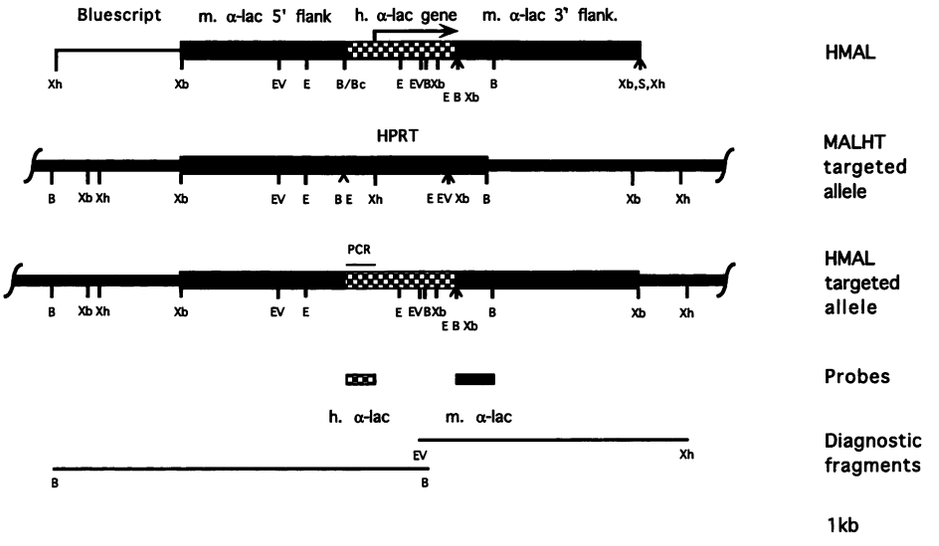

FIG. 2. Restriction maps of the linearized HMAL (human, mouse alpha-lactalbumin) vector, MALHT-targeted allele, predicted structure of the HMAL-targeted allele, and diagnostic fragments used for Southern analysis. The HMAL map shows restriction sites used in its construction and those used in Southern analysis. The lower two maps show only those sites used in Southern analysis. Striped bar, HPRT; checkered bar, human (h) α-lactalbumin; broad shaded bar, mouse (m) α-lactalbumin present in MALHT or HMAL; narrow shaded bar, endogenous mouse α-lactalbumin; solid line, plasmid vector. The fragment amplified in the PCR assay is indicated by a line over the structure of the targeted allele. Probes used are indicated beneath the map of the HMAL-targeted allele. Diagnostic restriction fragments correspond to those visualized in Fig. 4. The restriction enzyme sites shown, *Bam*HI (B), *Bam*HI-*Bcl*I fusion (B/Bc), *Eco*RI (E), *Eco*RV (EV), *Sal*I (S), *Xba*I (Xb), and *Xho*I (Xh), are those used in the final construction of HMAL and diagnostic digests of targeted clones.

and 0.57 kb of promoter with a 2.972-kb fragment containing the human gene and 0.772 kb of promoter. The 3′ boundaries of the exchanged regions were almost identical, 147 bp downstream of the mouse translation stop site and 136 bp downstream of the human translation stop site.

First replacement step. The targeting vector MALHT (Fig. 1) is a replacement vector which uses positive and negative markers to select for homologous recombinants (20). It consists of the HPRT positive selectable marker gene PGK/pDWM1 (26) between 4.3 kb of 5′ and 0.85 kb of 3′ mouse α-lactalbumin flanking sequences, flanked at the 3′ end with the herpes simplex virus TK counterselectable gene. All mouse α-lactalbumin fragments used were derived from strain 129 DNA to ensure sequence identity between MALHT and the target locus in HM-1 cells, since this has been shown to improve the frequency of homologous recombination (31). Transcription of both HPRT and TK genes was directed by the mouse PGK promoter (28). The PGK-HPRT and PGK-TK cassettes used have been demonstrated to work efficiently in ES cells (26).

Targeting of HM-1 cells with MALHT resulted in 343 HAT-resistant clones per 10^6 cells plated or 40 HAT^r clones per microgram of MALHT DNA. Comparison of colony numbers obtained after selection with HAT only and HAT plus ganciclovir double selection revealed that approximately 20% of the clones were HAT^r Gan^r. Therefore, ganciclovir selection provided a fivefold enrichment of potentially targeted cells. Ninety-six HAT^r Gan^r colonies were analyzed by PCR for the juxtaposition of HPRT and mouse α-lactalbumin 3′ flanking sequences predicted for a correct targeting event (Fig. 1). The diagnostic 1.3-kb fragment was detected in two independently derived cell lines, M2 and M52.

Southern analysis was used to investigate the DNA structure at the target locus in each clone. The predicted structure of the targeted α-lactalbumin allele following HPRT replacement of mouse α-lactalbumin is shown in Fig. 1. Analysis of M2 and M52 cells showed them both to have the predicted structure. Figure 3 shows M2 and HM-1 genomic DNA digested with two diagnostic restriction enzymes, *Hin*dIII and *Xho*I, and probed with an HPRT cDNA clone pHPT5 (14) and the 0.85-kb mouse α-lactalbumin 3′ fragment present in MALHT. The positions of the probes are shown in Fig. 1.

Recombination between MALHT and the target locus on the 5′ side of the HPRT minigene is indicated by the presence of a 7.3-kb *Xho*I fragment, from a site within the HPRT minigene and a site in the α-lactalbumin 5′ flank not included in MALHT, which hybridizes to the HPRT probe (Fig. 3, lane A). Recombination on the 3′ side of the HPRT minigene is indicated by two diagnostic fragments: (i) a 10-kb *Xho*I fragment derived from the same *Xho*I site within the HPRT gene and a site in the 3′ flank, not present in MALHT, which hybridizes to the mouse α-lactalbumin probe (Fig. 3, lane E), and (ii) a 2.4-kb fragment from a *Hin*dIII site within the HPRT minigene and a site in the 3′ flank not present in MALHT detectable with either HPRT or mouse α-lactalbumin probes (Fig. 3, lanes C and G).

The mouse α-lactalbumin probe also detected the targeted and untargeted α-lactalbumin alleles, which could be distinguished as equimolar 2.4- and 2-kb *Hin*dIII fragments (Fig. 3, lane G) and as 10- and 17-kb *Xho*I fragments (Fig. 3, lane E).

Generation of mice carrying the MALHT-targeted α-lactalbumin allele. M2 and M52 cells were microinjected into BALB/c blastocysts which were allowed to develop in foster mothers. Eight chimeras were generated, and the first three were test mated against BALB/c mice. One chimera from each ES clone transmitted the ES coat color markers to offspring (Table 1). It may be noted that chimera B was

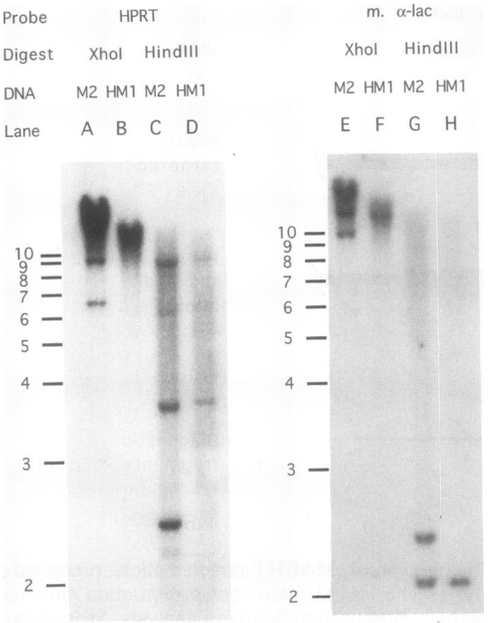

FIG. 3. Southern analysis of gene-targeted clones after the first replacement. Cell lines used were HM-1, the parental line, and M2, a MALHT-targeted line positive in PCR assay. m. α-lac, mouse α-lactalbumin. The source of DNA in each lane, the restriction digests, and the probes used are all indicated above the lane markers. Molecular size markers at the left of each autoradiograph are given in kilobases.

female. In this and other experiments, we have observed that the (male) HM-1 ES cell line contributes efficiently to the germ line of chimeras of both sexes. Southern analysis of genomic DNA prepared from tail biopsies from the 28 germ line pups analyzed showed that 16 carried the targeted allele (Table 1). These animals are being bred to homozygosity to reveal the phenotype of α-lactalbumin deficiency.

Thus, both cell lines resulting from the first round of targeting had retained totipotency and were therefore suitable for the second round.

Test for residual HPRT$^-$ cells. As the second targeting step involved selection for loss of HPRT function, it was important to determine whether any HPRT$^-$ HM-1 cells had survived HAT selection by metabolic cooperation with the HPRT$^+$-targeted cells (12).

The importance of this test was illustrated by a study on another series of targeted clones. We have previously corrected HPRT deficiency in the HPRT$^-$ ES cell line E14TG2a by gene targeting and selection for HPRT$^+$ cells in HAT medium (32). These experiments generated approximately five colonies per 5×10^6 cells plated onto each 10-cm-diameter dish. The proportion of HPRT$^-$ cells surviving amongst HPRT$^+$ E14TG2a targeted cells was determined individually for 22 clones. HAT selection was removed from each clone for 10 days, and then identical numbers of cells were plated in nonselective medium and in 6-TG medium. Comparison of plating efficiencies in 6-TG standardized against nonselective medium gave a measure of the purity of each clone. Nineteen clones showed purity of >99.9%, two clones showed 90 to 99%, and one clone showed only 79% purity. That the 6-TG-resistant cells in the correctant populations were the result of survival of original HPRT$^-$ cells rather than a product of secondary recombination at an unstable corrected locus was suggested by the observation that all subclones examined from a number of independent correctants were >99.9% pure. These results indicate the difficulty of isolating pure gene-targeted ES clones even from dishes with small numbers of colonies. This problem presumably arises because of the particularly intimate contact ES cells make with their neighbors. In recognition of this, when targeting with MALHT we took the obvious precaution of plating cells at very low density for HAT selection (see Materials and Methods).

TABLE 1. Generation of mice from targeted ES clones

Clone	Chimera	No. of pups		
		Total	Germ line[a]	Targeted allele[b]
M2	A (male)	64	53	14/26
M52	B (female)	10	2	2/2
M2	C (female)	12	0	
F6	D (male)	51	31	14/31
I2	E (female)	8	1	NT[c]

[a] Number of pups displaying ES coat color markers.
[b] Number of pups bearing the targeted allele/total pups analyzed.
[c] NT, not tested.

In a mock targeting experiment, carried out in parallel with HMAL targeting, 4.5×10^7 M2 cells were plated into 6-TG medium at 1.5×10^6 cells per 10-cm-diameter dish. After 22 days in culture, no colonies were evident, indicating that residual HPRT$^-$ cells were present at a frequency less than 1 in 4.5×10^7. This experiment also demonstrated the stability of the HPRT minigene in M2 cells, since either physical loss by chromosome rearrangement or deletion or loss of function would have resulted in 6-TG-resistant survivors.

Second replacement step. The second targeting vector, HMAL, is shown in Fig. 2. It consists of the human α-lactalbumin gene flanked by 4.3 kb of 5′ and 4.8 kb of 3′ mouse α-lactalbumin sequences. The TK gene was considered unnecessary, because selection in the second step is for loss of HPRT function and random HMAL integrants are killed by 6-TG. When targeting with HMAL, the number of 6-TG^r clones per microgram of DNA was expected to be significantly lower than the number of HAT^r clones obtained after targeting with MALHT but the proportion of targeted clones greater.

6-TG-resistant colonies were expected to arise in three possible ways: (i) persistence of untargeted HM-1 cells; (ii) cells that had spontaneously lost HPRT function; and (iii) loss of the HPRT minigene by replacement with human α-lactalbumin, i.e., gene targeting. Results from the parallel mock targeting experiment described in the preceding section indicate that cells in the first two classes are effectively absent.

Six days after electroporation, one-third of the cells present from each of 10 pools were plated into 6-TG. Sixty-two resistant colonies were obtained, representing ~1 6-TG^r colony per 10^6 cells plated. Forty-eight colonies were isolated and assayed for the presence of the human α-lactalbumin gene by PCR amplification of the region shown in Fig. 2. Four cell lines, three of which were known to be independent isolates since they arose in different pools, contained the gene.

Figure 4 shows a Southern analysis of diagnostic *Bam*HI and *Eco*RV-*Xho*I digests of genomic DNA from two PCR-positive targeted lines (I2 and F6), two PCR-negative lines (G3 and F4), the MALHT-targeted line, M2, and the parental

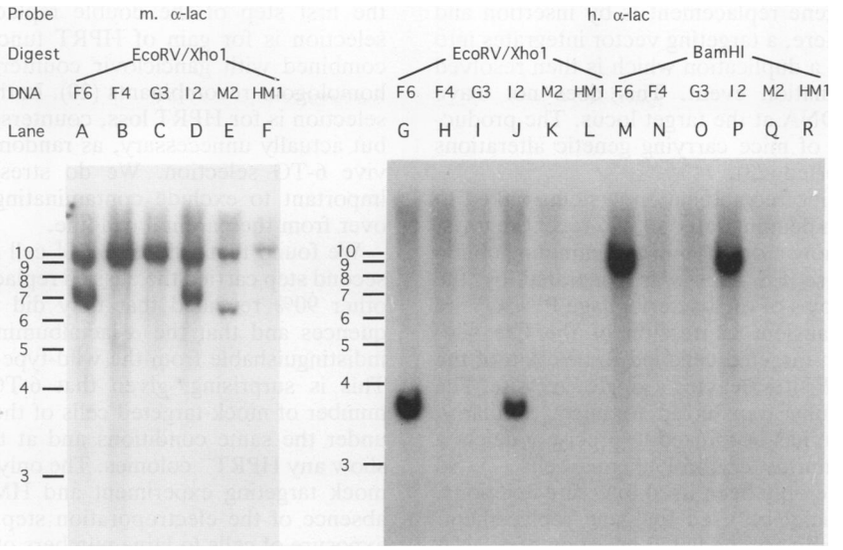

FIG. 4. Southern analysis of gene-targeted clones after the second replacement. Cell lines used were HM-1, the parental line, and M2, the MALHT-targeted line; I2 and F6, HMAL-targeted lines positive in PCR assay; and G3 and F4, lines negative in PCR assay. The source of DNA in each lane, the restriction digests, and the probes used are all indicated above the lane markers. Molecular size markers at the left of each autoradiograph are given in kilobases. m. α-lac, mouse α-lactalbumin; h. α-lac, human α-lactalbumin.

line, HM-1. Blots were hybridized with human and mouse α-lactalbumin probes. The position of DNA fragments used as probes is indicated in Fig. 2.

The human α-lactalbumin probe hybridizes only to DNA from the two PCR-positive targeted lines (I2 and F6). Recombination between HMAL and the target locus on the 5′ side of the human α-lactalbumin gene is indicated by the release of a 9.5-kb *Bam*HI fragment from a site in the human gene and a site in a region of the mouse 5′ flank not present in HMAL (Fig. 4, lanes M and P). The *Eco*RV-*Xho*I digest releases a 3.7-kb fragment from within HMAL (Fig. 4, lanes G and J).

The mouse α-lactalbumin probe recognizes a 7-kb *Eco*RV-*Xho*I fragment in clones I2 and F6, which indicates recombination on the 3′ side (Fig. 4, lanes A and D). The *Eco*RV site is within the human gene, and the *Xho*I site is in the mouse 3′ flank distal to that present in HMAL (Fig. 2). This probe also hybridizes to a 6-kb *Eco*RV-*Xho*I fragment in the M2 line, which is indicative of the presence of the HPRT minigene at the MALHT targeted locus (Fig. 4, lane E). A 10-kb *Eco*RV-*Xho*I fragment from the untargeted murine α-lactalbumin gene is present in all cell lines (Fig. 4, lanes A to F).

The cell lines G3 and F4 are representative of 10 PCR-negative cell lines analyzed. All digests carried out so far are consistent with the presence of wild-type murine α-lactalbumin only, i.e., identical to HM-1 (Fig. 4). Hybridization of PCR-negative cell lines to HPRT cDNA did not reveal the presence of HPRT minigene sequences (data not shown).

Generation of mice carrying the second-step HMAL-targeted α-lactalbumin allele. As in the first replacement step, chimeric animals were generated by microinjection of targeted ES cells into BALB/c blastocysts. A total of five chimeras were obtained by using cell lines I2 and F6. Test matings against BALB/c mice revealed the presence of the ES genotype in over half the offspring of male chimera D and a single offspring from female chimera E (Table 1). This confirmed that clones F6 and I2 had both retained totipotency through two rounds of targeting. The remaining three chimeras were not analyzed. Fourteen of 31 germ line pups analyzed from chimera D carried the targeted allele. These animals are being bred to homozygosity to study the consequences of replacing the mouse α-lactalbumin gene with its human counterpart.

DISCUSSION

We describe here the first instance in which gene targeting has been used to produce mice in which a whole gene has been replaced by that of another species at the original locus. Human-mouse α-lactalbumin gene replacement was carried out in two stages: replacement of the mouse gene by the HPRT minigene, followed by replacement of the HPRT minigene by the human gene.

Gene replacement by gain and loss of the HPRT gene was first proposed in 1990 by Reid et al. (24) but has not been demonstrated until now. This has been largely due to the lack of an ES culture system suited to HPRT selection regimes. Until recently, it was thought that ES cells had to be grown on layers of feeder cells to maintain totipotency (4). However, HPRT selection of feeder-dependent ES cells is complicated by intercellular exchange of metabolites (12). The isolation of a feeder-independent HPRT-deficient totipotent ES cell line, HM-1 (18), has now removed this obstacle.

Other methods of gene replacement have been reported. Recently, an alternative double replacement method termed "tag and exchange" has been described (1). This method uses a combination of neo-positive and TK-negative selectable markers to achieve two-step gene replacement in wild-type, feeder-dependent ES cells. Gene replacement can also be achieved in a single step by cotransfer of DNA linked to a positive selectable marker (neo) in a replacement vector (6). Unfortunately, this results in deposition of the marker gene close to the gene replacement, which may be undesirable in many circumstances. Transfer of gene replacements into the mouse germ line by either of these methods has yet to be demonstrated.

A third method of gene replacement is by insertion and excision (10, 15, 33). Here, a targeting vector integrates into a target locus, forming a duplication which is then resolved by a second recombination event. This does not leave undesired exogenous DNA at the target locus. The production by this technique of mice carrying genetic alterations has recently been reported (23).

The use of site-specific recombination systems linked to gene targeting has been demonstrated in two recent reports. Gu et al. (8) describe how a deletion in the immunoglobulin switch region in mouse ES cells was generated by the introduction of two copies of the bacteriophage P1 *loxP* site by gene targeting. Transient expression of the Cre site-specific recombinase in targeted cells led to deletion of the region between the *loxP* sites, leaving a single *loxP* site. The deletion was successfully transferred to mice. Similarly, yeast FLP recombinase has been used to precisely delete a selectable marker in murine erythroleukemia cells (7). So far, these methods have only been used to create deletions, but they could potentially be used for gene replacement. This could be by recombinase-mediated insertion of a DNA fragment at a single *loxP* site (2) left after a deletion step. Alternatively, site-specific recombination could be used to delete a positive-negative-selectable cassette used to cotransfer DNA containing genetic alterations.

Conversion of vector sequences to chromosomal sequences at the target locus during gene targeting experiments has been observed and ascribed either to DNA mismatch repair (29) or exolytic extension of double-strand breaks during homologous recombination (34). The latter mechanism is thought to be responsible for loss of end-proximal markers from insertion vectors (5, 15, 34). Interestingly, replacement vectors are less susceptible to this effect (5). This is a potentially serious disadvantage of the insertion-excision technique. Clearly, desired sequences can be placed away from the ends of a linearized insertion vector in the first step. However, the intrachromosomal or sister chromatid recombination responsible for resolution of the duplication in the second step is uncontrolled and can initiate at any point and possibly result in sequence conversion. Recombination between stably integrated plasmids on the same chromosome has been shown to result in frequent gene conversion events (3, 17). In contrast, alteration or loss of engineered mutations is unlikely to occur with our method of double replacement, because homologous wild-type sequences at the target allele are completely removed in the first step, before mutant sequences are introduced. Furthermore, recombination events are designed to occur in flanking sequences relatively distant from DNA bearing engineered alterations.

It has been claimed that insertion events are more frequent than replacements (10); however, this does not seem to apply when vector and target locus are isogenic (5). Our results of targeting with MALHT showed two replacements in 96 HAT^r Gan^r colonies, which indicates a frequency of approximately 1 in 250 of the total (non-ganciclovir-enriched) $HPRT^+$ transformants, certainly enough for practical purposes.

Any advantage that insertion vectors may provide in increased frequency of homologous recombination over replacement vectors is, in our opinion, outweighed by the convenience of double drug selection as an initial screen for putative homologous recombinants. The use of insertion vectors relies solely on screening (e.g., by PCR) colonies resistant to a single drug for homologous recombinants. This can entail the processing of large numbers of samples (27). In the first step of the double replacement method, where selection is for gain of HPRT function, HAT selection is combined with ganciclovir counterselection to enrich for homologous recombinants (20). In the second round, where selection is for HPRT loss, counterselection is also possible but actually unnecessary, as random integrants do not survive 6-TG selection. We do stress, however, that it is important to exclude contaminating $HPRT^-$ cells carried over from the parental cell line.

We found that 10% of $6\text{-}TG^r$ cell lines obtained from the second step carried the desired replacement. A sample of the other 90% revealed that they did not contain HPRT sequences and that the α-lactalbumin locus was apparently indistinguishable from the wild-type gene present in HM-1. This is surprising, given that 6-TG selection of a large number of mock-targeted cells of the same ES clone grown under the same conditions and at the same time failed to show any $HPRT^-$ colonies. The only difference between the mock targeting experiment and HMAL targeting was the absence of the electroporation step. It is conceivable that exposure of cells to large numbers of linear DNA molecules may trigger an SOS response involving induction of DNA repair enzymes and increased rates of gene conversion. It would be interesting to determine whether electroporation with unrelated DNA also results in such an apparently high rate of gene conversion or whether the effect is restricted to DNA homologous to the affected locus.

The frequencies of targeting events we obtained after each step were similar to those obtained by Askew et al. using neo-TK tag and exchange (1). Their analysis of nontargeted clones surviving drug selection after the second step also showed clones which had lost the neo-TK cassette at a frequency (83% of total drug-selected clones) similar to that we observed for the loss of the HPRT marker. This indicates that the effect is not restricted to a particular ES cell line or experimental protocol. Spontaneous production of homozygous cells from a heterozygous population has also been observed during culture of ES cells and is believed to arise as result of gene conversion or mitotic nondisjunction (22). Askew et al. also describe a second class of nontargeted clones in which the TK gene, while physically present, was inactive. Our findings were that the HPRT minigene does not undergo extinction of expression in this way.

Double replacement using the HPRT minigene is accomplished by two entirely independent targeting events. The target locus in cells from the first step can generally be expected to be stable, since no gene duplication is generated. These cell lines can be expanded and analyzed at will before a second step is commenced. This provides several important advantages, not the least of which is that totipotency can be tested by generating and breeding chimeras at the half-way stage. The brief test we carried out was sufficient to confirm that both cell lines obtained after MALHT targeting were totipotent.

When, as in our case, double replacement is used to target a functional gene, the first round of targeting will often inactivate the targeted allele. Recent reports illustrate that mice containing null mutations can be interesting in themselves. When used in combination with a second targeting step, they can also provide valuable controls for the phenotype of animals with more subtle genetic alterations.

The HPRT selection system is more suitable than neo-TK (1) for both testing totipotency and producing knockout mice because TK expression in male gonads results in sterility. This would clearly interfere with the generation and breeding of chimeric animals from first-step ES clones.

The most important application of double replacement that we foresee is the generation of multiple gene replacements at a single locus. This can be readily achieved by targeting first-step cells with many different second-step vectors. Thus, sets of animals which are isogenic apart from precisely defined alterations in a gene of interest can be generated. Such sets would be an extremely valuable resource for many aspects of biology.

Gene targeting experiments necessarily subject ES cells to long-term culture. Progressive loss of totipotency in populations of cultured ES cells has been described as a potential problem (36) and observed in some feeder-dependent ES cell lines (25). The results presented here show that a feeder-independent ES cell line can undergo two successive rounds of gene targeting, drug selection, and single-cell cloning and still contribute readily to the germ line of chimeric animals.

We believe that the power and simplicity of double replacement used in conjunction with HPRT selection will make it the method of choice for many future gene targeting experiments.

ACKNOWLEDGMENTS

This work has been supported in part by Wyeth-Ayerst Research, Philadelphia, Pa., and by United Kingdom Department of Trade and Industry SPUR grant 91/010.

We thank Ian Garner for constructive discussions and also Jim Selfridge, Ann Scott, and Angela Scott for technical assistance.

REFERENCES

1. **Askew, G. R., T. Doetschman, and J. B. Lingrel.** 1993. Site-directed point mutations in embryonic stem cells: a gene-targeting tag-and-exchange strategy. Mol. Cell. Biol. **13**:4115–4124.
2. **Baubonis, W., and B. Sauer.** 1993. Genomic targeting with purified Cre recombinase. Nucleic Acids Res. **21**:2025–2029.
3. **Bollag, R. J., and R. M. Liskay.** 1988. Conservative intrachromosomal recombination between inverted repeats in mouse cells: association between reciprocal exchange and gene conversion. Genetics **119**:161–169.
4. **Bradley, A., P. Hasty, A. Davis, and R. Ramirez-Solis.** 1992. Modifying the mouse: design and desire. Biotechnology **10**:534–539.
5. **Deng, C., and M. R. Capecchi.** 1992. Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. Mol. Cell. Biol. **12**:3365–3371.
6. **Deng, C., K. T. Thomas, and M. R. Capecchi.** 1993. Location of crossovers during gene targeting with insertion and replacement vectors. Mol. Cell. Biol. **13**:2134–2140.
7. **Fiering, S., C. G. Kim, E. M. Epner, and M. Groudine.** 1993. An "in-out" strategy using gene targeting and FLP recombinase for the functional dissection of complex regulatory elements: analysis of the β-globin locus control region. Proc. Natl. Acad. Sci. USA **90**:8469–8473.
8. **Gu, H., Y.-R. Zou, and K. Rajewsky.** 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-*loxP*-mediated gene targeting. Cell **73**:1155–1164.
9. **Hall, L., D. C. Emery, M. S. Davies, D. Parker, and R. K. Craig.** 1987. Organization and sequence of the human α-lactalbumin gene. Biochem. J. **242**:735–742.
10. **Hasty, P., J. Rivera-Pérez, C. Chang, and A. Bradley.** 1991. Target frequency and integration pattern for integration and replacement vectors in embryonic stem cells. Mol. Cell. Biol. **11**:4509–4517.
11. **Hogan, B., F. Costantini, and E. Lacy.** 1986. Manipulating the mouse embryo. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Hooper, M. L., and C. Slack.** 1977. Metabolic cooperation in $HGPRT^-$ and $HGPRT^+$ embryonal carcinoma cells. Dev. Biol. **55**:271–284.
13. **Koller, B. H., and O. Smithies.** 1992. Altering genes in animals by gene targeting. Annu. Rev. Immunol. **10**:705–730.
14. **Konecki, D. S., J. Brennand, J. Fuscoe, C. T. Caskey, and A. C. Chinault.** 1982. Hypoxanthine phosphoribosyltransferase genes of mouse and Chinese hamster: construction and sequence analysis of cDNA recombinants. Nucleic Acids Res. **10**:6763–6775.
15. **Kumar, S., and J. P. Simons.** 1993. The effects of terminal heterologies on gene targeting by insertion vectors in embryonic stem cells. Nucleic Acids Res. **21**:1541–1548.
16. **Lathe, R., J. L. Vilotte, and A. J. Clark.** 1987. Plasmid and bacteriophage vectors for excision of intact inserts. Gene **57**: 193–201.
17. **Liskay, R. M., J. L. Stachelek, and A. Letsou.** 1984. Homologous recombination between repeated chromosomal sequences in mouse cells. Cold Spring Harbor Symp. Quant. Biol. **49**:183–189.
18. **Magin, T. M., J. McWhir, and D. W. Melton.** 1992. A new mouse embryonic stem cell line with good germ line contribution and gene targeting frequency. Nucleic Acids Res. **14**:3795.
19. **Magin, T. M., and D. W. Melton.** 1993. Gene targeting, p. 29–39. *In* R. N. Rosenberg, S. B. Prusiner, S. DiMauro, R. L. Barchi, and L. M. Kunkel (ed.), The molecular and genetic basis of neurological disease. Butterworth-Heinemann, Boston.
20. **Mansour, S. L., K. T. Thomas, and M. Capecchi.** 1988. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to nonselectable genes. Nature (London) **336**:348–352.
21. **McMahon, A. P., and A. Bradley.** 1990. The Wnt-1 (int-1) protooncogene is required for development of a large region of the mouse brain. Cell **62**:1073–1085.
22. **Mortenson, R. M., D. A. Conner, S. Chao, A. A. T. Geisterfer-Lowrance, and J. G. Seidman.** 1992. Production of homozygous mutant ES cells with a single targeting construct. Mol. Cell. Biol. **12**:2391–2395.
23. **Ramirez-Solis, R., H. Zheng, J. Whiting, R. Krumlauf, and A. Bradley.** 1993. Hoxb-4 (Hox-2.6) mutant mice show homeotic transformation of a cervical vertebra and defects in the closure of the sternal rudiments. Cell **73**:279–294.
24. **Reid, L. H., R. G. Gregg, O. Smithies, and B. H. Koller.** 1990. Regulatory elements in the introns of the human HPRT gene are necessary for its expression in embryonic stem cells. Proc. Natl. Acad. Sci. USA **87**:4299–4303.
25. **Schwartzberg, P. I., S. P. Goff, and E. J. Robertson.** 1989. Germ line transmission of a c-abl mutation produced by targeted disruption in ES cells. Science **246**:799–804.
26. **Selfridge, J., A. M. Pow, J. McWhir, T. M. Magin, and D. W. Melton.** 1992. Gene targeting using a mouse HPRT minigene/HPRT-deficient embryonic stem cell system: inactivation of the mouse ERCC-1 gene. Somatic Cell Mol. Gen. **18**:325–336.
27. **Shesely, E. G., H. S. Kim, W. R. Shehee, T. Papayannopoulou, O. Smithies, and B. W. Popovich.** 1991. Correction of a human beta S-globin gene by gene targeting. Proc. Natl. Acad. Sci. USA **88**:4294–4298.
28. **Soriano, P., C. Montgomery, R. Geske, and A. Bradley.** 1991. Targeted disruption of the c-src protooncogene leads to osteopetrosis in mice. Cell **64**:693–702.
29. **Steeg, C. M., J. Ellis, and A. Bernstein.** 1990. Introduction of specific point mutations into RNA polymerase II by gene targeting in mouse embryonic stem cells: evidence for a DNA mismatch repair mechanism. Proc. Natl. Acad. Sci. USA **87**: 4680–4684.
30. **Szybalska, E. H., and W. Szybalski.** 1962. Genetics of human cell lines. IV. DNA-mediated heritable transformation of a biochemical trait. Proc. Natl. Acad. Sci. USA **48**:2026–2034.
31. **te Riele, H., E. R. Maandag, and A. Berns.** 1992. Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. Proc. Natl. Acad. Sci. USA **89**:5128–5132.
32. **Thompson, S., A. R. Clarke, A. M. Pow, M. L. Hooper, and D. W. Melton.** 1989. Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. Cell **56**:313–321.

33. **Valancius, V., and O. Smithies.** 1991. Testing an "in-out" targeting procedure for making subtle genomic modifications in mouse embryonic stem cells. Mol. Cell. Biol. **11:**1402–1408.
34. **Valancius, V., and O. Smithies.** 1991. Double-strand gap repair in a mammalian gene targeting reaction. Mol. Cell. Biol. **11:** 4389–4397.
35. **Vilotte, J.-L., S. Soulier, and J.-C. Mercier.** 1992. Sequence of the murine α-lactalbumin-encoding cDNA: interspecies comparison of the coding frame and deduced protein. Gene **112:**251–255.
36. **Yagi, T., Y. Ikawa, K. Yoshida, Y. Shigetani, N. Takeda, I. Mabuchi, T. Yamamoto, and S. Aizawa.** 1990. Homologous recombination at c-fyn locus of mouse embryonic stem cells with use of diphtheria toxin A-fragment gene in negative selection. Proc. Natl. Acad. Sci. USA **87:**9918–9922.
37. **Zimmer, A.** 1992. Manipulating the genome by homologous recombination in embryonic stem cells. Annu. Rev. Neurosci. **15:**115–137.