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Rapid detection of homologous recombinants in nontransformed human cells

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ABSTRACT **Gene targeting is a technique by which a preselected site in the genome of a living cell can be modified by inserting, deleting, or exchanging DNA sequences. The application of this technology to cells with a limited life-span, such as nontransformed human somatic cells, requires the development of simplified and efficient procedures to allow the isolation of correctly modified cells from the much larger pool of random integrants. The current study describes the development of a widely applicable strategy for detecting homologous recombinants in human cells by using an ELISA-based screen. When this system is used accurately targeted clones can be detected with high efficiency as soon as 14 days following transfection. Data are presented demonstrating the utility of this detection system in isolating targeted recombinants at the β_2-microglobulin locus in both human retinal pigmented epithelial cells and human keratinocytes.**

The use of living cells and tissues in the treatment of human disease has been limited by the strong tendency for graft recipients to reject cells derived from an unmatched donor (1). This rejection is mediated by the recipient's immune system, and the primary targets of the response are the major histocompatibility (MHC) antigen complexes expressed on the surface of the transplanted (allogeneic) cells. These antigens are encoded by a cluster of highly polymorphic genes located on chromosome 6 in humans (2, 3). Elimination of these antigens from the surfaces of allogeneic cells could provide a route to reducing the rejection response. Permanent removal of a cell's MHC antigens requires the modification of the genes encoding these antigens. Currently, gene targeting is the only technique by which a preselected site in the genome of a living cell can be modified by precisely inserting, deleting, or exchanging DNA sequences (4–6).

Recent studies utilizing genetically engineered mice have demonstrated that removal of class I MHC antigens from the surface of pancreatic β-islet cells greatly enhances their ability to survive long-term after transplantation across both MHC and minor antigen barriers (7, 8). In these experiments gene targeting was used to inactivate the β_2-microglobulin (β_2m) gene from a line of mouse embryonic stem (ES) cells (9, 10). These cells were used to generate a new strain of mice which was β_2m-deficient (β_2m$^-$). Since the β_2m protein is required for the proper assembly of all MHC class I antigen complexes, cells derived from these mice are severely deficient in their cell-surface expression of class I MHC antigens (11, 12). When pancreatic β-islets were transplanted from β_2m$^-$ mouse donors into allogeneic recipients, the majority of grafts showed long-term survival. In contrast, grafts derived from MHC-matched, wild-type donors were promptly rejected by all allogeneic recipients (7). Similar yet less dramatic results were seen when mixed populations of cell types derived from either the liver or the kidney of β_2m$^-$ mice were used in comparable allogeneic transplantation studies (13, 14).

Application of these findings to humans requires the modification of somatic cells. In contrast to mouse ES cell lines, nontransformed human cells have a finite life-span *in vitro* (15). This makes their modification by gene targeting especially challenging, given the low efficiency of the targeting process (10^{-5} to 10^{-8} recombinants per input cell) (6). This procedure is further complicated by the fact that in mammalian cells, transfected DNA is integrated at random sites 100- to 1000-fold more frequently than at the homologous site (6).

The present study describes the successful targeting of the β_2m locus in two different nontransformed, human somatic cell types. A system was devised for the rapid identification of numerous independently targeted clones. The strategy for detecting successful targeting events was based on the concept that gene targeting can be used to create recombinant proteins, as well as recombinant genes. A recombinant locus was designed to create a unique fusion protein which would be secreted by correctly targeted cells and detected by using a simple ELISA-based assay. In this gene trapping system, the 5′ end of the recombinant locus is derived from the targeting vector, while the 3′ end of the recombinant locus is derived from the target gene. This design is the converse of the epitope-tagging and promoter trapping systems described by Jasin *et al.* (16, 17), which utilize the promoter region of the target gene to drive transcription of a novel recombinant locus. In the gene trapping system, since the entire 5′ end of the recombinant expression unit is determined experimentally, both the expression level and ultimate transport fate of the recombinant protein can be directed.

MATERIALS AND METHODS

Genomic Cloning. A human placental genomic library (Clontech) was screened with a probe amplified from the pooled library by PCR using primers derived from the published sequence of exons 3 and 4 of the human β_2m gene (18) (primer sequences: HB1, 5′-ATCGAGACATGTAAGCAGCATCATGGAG; HB2, 5′-CAGAATTTGAATTCACTCAATCCAAATGCGGCATCTTCAAAC). Hybridizing clones were isolated from the library and a full-length clone was identified and characterized. The 16.5-kb insert was recovered with *Xho* I ends and subcloned in a pUC plasmid containing a *Not* I site on the 5′ side of the gene to create pLV5.

cDNA Cloning. Total RNA was prepared from RPE cells and was reverse-transcribed, using random hexamer primers. The first-strand cDNA was then amplified by PCR using primers derived from the published sequence of the human β_2m cDNA (19) (primer sequences: no. 748, 5′-CTTAATGGGCCCTCGGCGGGCATTCCTGAAGCTGACAGC; no. 746, 5′-CTGATAGGGCCCTCGCGGCATCTTCA-

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Abbreviations: MHC, major histocompatibility complex; β_2m, β_2-microglobulin; CMV, cytomegalovirus.

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AACCTCCATGATGCTGC). The product of this reaction was then digested with *Apa* I and inserted into the vector pIK.5.1 to generate pIK.β_2m.

Recombinant DNA Constructions. pIK.CD4 is a cytomegalovirus (CMV)-based expression vector containing the full-length human CD4 cDNA. The vector pIK.CD4/β_2m was derived from pIK.CD4 by removing the CD4 sequences except for the signal sequence and first two immunoglobulin domains (up to the *Nhe* I site) and insertion of a PCR product consisting of the full-length mature coding region of the β_2m cDNA from pIK.β_2m. The vectors pIK.5.1 and pIK.CD4 were generous gifts from Mitchell Finer and Isabel King (Cell Genesys).

The targeting vector SW137 contains a 7.6-kb *Not* I–*Mlu* I upstream homology sequence from pLV5, a neomycin-resistance expression unit amplified by PCR from pMC1Neo (6) as a *Hin*dIII cassette, a 1.6-kb *Xba* I–*Sma* I fragment from pLV5; a herpesvirus thymidine kinase gene contained on a 2-kb *Bam*HI fragment inserted into a *Bgl* II site, a 1.7-kb *Hin*dIII–*Nhe* I fragment from pIK.CD4 comprising a CMV promoter and enhancer, a spliced region, and a partial human CD4 cDNA sequence encoding the signal sequence and the first two immunoglobulin domains of CD4; and a 1-kb *Nhe* I–*Apa* I fragment amplified by PCR from a derivative of pLV5 (primer sequences: no. 1214, 5′-GTTTATTGCTAGCTATC-CAGCGTGAGTCTCTCCTACCCT; no. 1202, 5′-GCCAG-GTACTTAGAAAGTGCTCAAGATG), consisting of the final 10 bp of exon 1 of the β_2m gene fused in-frame to the CD4 coding region, the exon 1 splice donor sequence, and part of the first intron. Finally, a 2.5-kb *Apa* I–*Cla* I fragment from a derivative of pLV5 comprising additional sequences from the first intron was used to complete the 3′ homology. The targeting vector SW148 is identical, except that it lacks 3.7 kb of upstream homolog sequence (up to the second *Sca* I site from the 5′ end of the vector).

Cell Culture. Human retinal pigmented epithelial (RPE) cells were cultured in Dulbecco's modified Eagle's medium/F12 medium (DMEM/F12) containing 15% fetal bovine serum. Human keratinocytes were cultured in DMEM/F12 containing 10% fetal bovine serum, bovine pituitary extract (Hammond Cell Tech, Alameda, CA) at 100 μg/ml, hydrocortisone at 0.5 μg/ml, and recombinant human epidermal growth factor (GIBCO/BRL) at 100 pg/ml. All media contained penicillin at 100 units/ml and streptomycin at 100 μg/ml. Keratinocytes were cultured with γ-irradiated (4300 rads; 1 rad = 0.01 Gy) G418-resistant mouse embryo fibroblasts that had been immortalized by transfection with a simian virus 40 tumor (T)-antigen expression vector.

Electroporations. Both RPE cells and keratinocytes were electroporated in DMEM/F12 at 8×10^6 cells per ml, using 0.5 ml of the cell suspension in 0.4-cm cuvettes with a Bio-Rad Gene Pulser set at 270 V and 960 μF. The cells were then plated in nonselective media in cell wells. After one day, the medium was supplemented with G418 at 400 μg/ml (active concentration), and thereafter the medium was changed twice weekly. After 7–14 days, G418-resistant clones were apparent (1–10 clones per well), and a sample of the supernatant from each well was tested by ELISA.

ELISA. Nunc Immuno MaxiSorp plates were coated with a rabbit anti-human β_2m IgG [Dako; 1:450 dilution in 170 mM NaCl/3 mM KCl/10 mM phosphate buffer, pH 7.2 (PBS)] overnight at 4°C. Subsequent steps were performed at room temperature. Plates were washed three to five times with PBS/0.1% Tween 20 after each step. Nonspecific binding sites were blocked by incubating the wells with blocking buffer (5 g of ovalbumin per liter in PBS) for 2 hr. Samples (neat or diluted in blocking buffer) were applied and incubated for 1 hr. After washing, a monoclonal anti-CD4 antibody (OKT4A, Ortho Diagnostics; diluted 1:200 in blocking buffer) was added to the wells and incubated for 1 hr. After washing, the wells were incubated for 1 hr with an alkaline phosphatase-conjugated rabbit anti-mouse IgG2a antibody (Organon Teknika–Cappel; diluted 1:200 in blocking buffer). After washing, the chromogenic substrate *p*-nitrophenyl phosphate (Sigma; 1 g/liter in 10 mM diethanolamine/0.5 mM $MgCl_2$) was added, the reaction was developed for 30–60 min, and the A_{405} was read on a plate reader. A reading of 4 times background was considered positive.

Immunoprecipitations. Cells in 35-mm cell wells were incubated with [^{35}S]methionine and [^{35}S]cysteine [Tran^{35}S-label, ICN; 265 μCi (1 μCi = 37 kBq) per well in methionine- and cysteine-deficient medium] for 5 hr. The conditioned medium was collected, the cells were lysed in lysis buffer (50 mM Tris·HCl, pH 8.0/150 mM NaCl/1% Nonidet P-40/0.1 g of phenylmethylsulfonyl fluoride per liter) on ice for 30 min, and the debris was removed by centrifugation. SDS was added to 10 g/liter and the samples were boiled to denature the proteins, then diluted 5-fold in incubation buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% Nonidet P-40/1 mM EDTA/gelatin at 2.5 g/liter/NaN_3 at 0.2 g/liter). After preclearing with fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem), the samples were incubated with either a rabbit anti-human β_2m IgG preparation (Dako) or a rabbit anti-human CD4 IgG preparation (American Biotechnologies, Cambridge, MA) overnight at 4°C, then precipitated with Pansorbin. After washing three times in incubation buffer and once in 10 mM Tris·HCl, pH 7.5/0.1% Nonidet P-40, the Pansorbin-bound proteins were released by boiling in SDS sample buffer, then electrophoresed on 4–20% gradient polyacrylamide gels (NOVEX, San Diego) alongside molecular weight standards. The gels were fixed, soaked in sodium salicylate, dried, and exposed to x-ray film.

Southern Analysis. High molecular weight genomic DNA was isolated from each cell clone. A 5-μg sample of each DNA was digested with either *Hpa* I or *Eco*RV and electrophoresed on a 0.8% agarose gel. The gel was then subjected to a Southern blotting procedure (20). The blot was hybridized with a random-primed preparation of probe 2R (see Fig. 1*C*), washed stringently, and exposed to x-ray film.

RESULTS

Targeting in RPE Cells. Human RPE cells were selected for the initial studies due to their reported growth in culture (21), their availability from a tissue source (Northern California Transplant Bank), and their potential involvement in a variety of human retinopathies (22). RPE cells were isolated from human eye tissue as described (23). They were cultured as an uncloned population which demonstrated a 27-hr doubling time and contact-inhibited growth.

The targeting vector SW137 is shown in Fig. 1*A*. This vector consists of two regions of homology derived from the human β_2m locus surrounding a nonhomologous insert which is used to select/detect correctly targeted clones. The region of nonhomology contains a neomycin resistance marker to enable selection of cells which stably integrate the vector and the 5′ end of the recombinant expression unit, including a strong promoter/enhancer derived from the human CMV, a cDNA fragment encoding the signal sequence, and the first two immunoglobulin domains of the human CD4 protein joined in-frame to the splice donor sequence from the end of exon 1 of the β_2m gene. Following a precise targeting reaction, this structure will create a fusion gene, composed of an artificial exon 1 encoding CD4 epitopes and exons 2–4 of the native β_2m locus. The human β_2m locus is shown in Fig. 1*B* and the correctly targeted recombinant locus is shown in Fig. 1*C*. A second targeting vector, SW148, was generated, which carries a shorter 5′ homology region.

To develop an ELISA for the CD4-β_2 fusion protein, RPE cells (passage 6) were transfected with either the CD4 or the

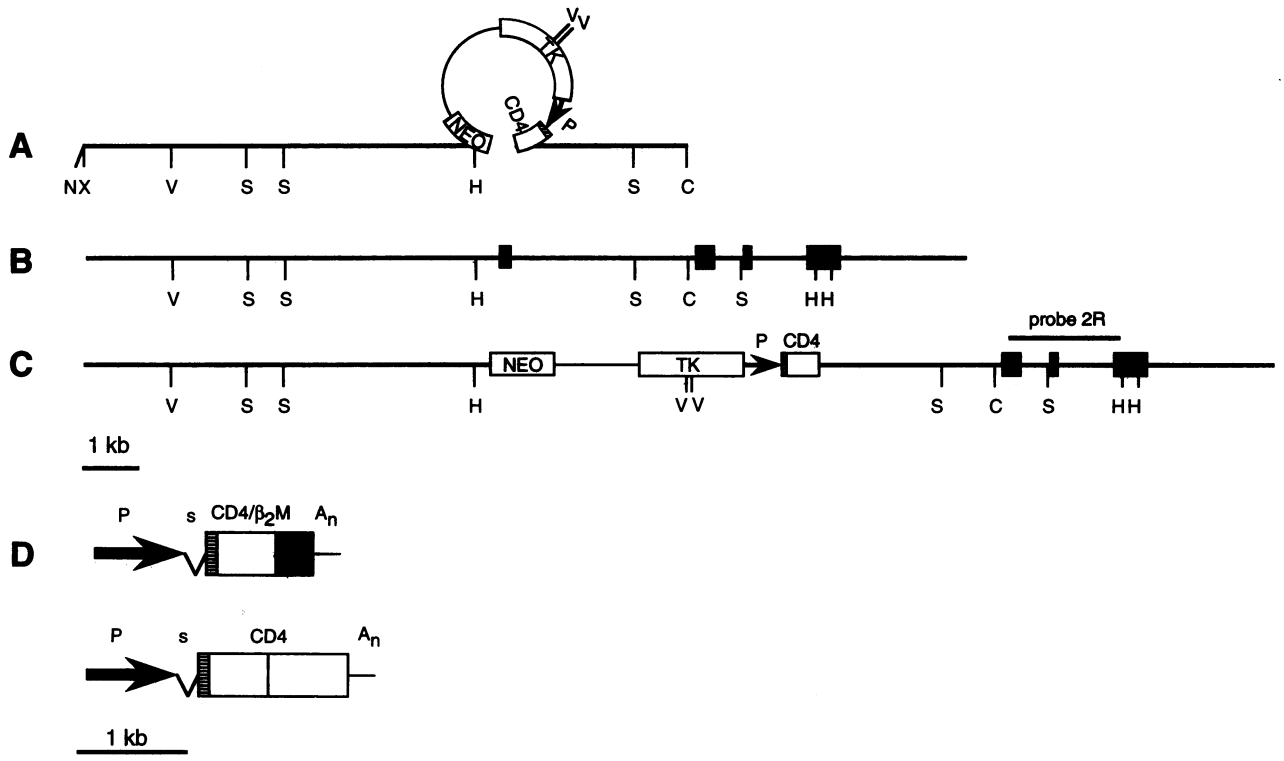

FIG. 1. (*A*) The β_2m targeting vector, SW137. Restriction sites: C, *Cla* I; H, *Hpa* I; N, *Not* I; S, *Sca* I; V, *Eco*RV; and X, *Xho* I. TK, thymidine kinase; P, CMV immediate-early promoter and enhancer; NEO, neomycin (G418) resistance. (*B*) The human β_2m gene. Black boxes denote exons. (*C*) The correctly targeted recombinant locus. The position of the probe, designated 2R, used for the Southern blot is shown. (*D*) Control vectors used to develop and validate the ELISA. P, CMV immediate-early promoter and enhancer; s, spliced sequence; striped boxes, the CD4 signal sequence; CD4/β_2m, a reconstructed cDNA sequence consisting of the first two immunoglobulin domains of CD4 fused to the full-length mature coding region of β_2m; CD4, the full-length CD4 cDNA coding region; A_n, simian virus 40 polyadenylylation signal.

CD4-β_2m expression vectors shown in Fig. 1*D*. One day after transfection, conditioned medium was harvested from the transfected cells and tested by ELISA. The assay was designed to bind proteins containing a β_2m-encoded epitope to the surface of a microtiter well and to detect bound proteins which possess a CD4-encoded epitope. The resulting assay was shown to be specific for the CD4-β_2m fusion protein, allowing detection of secreted protein from as few as 1000 expressing cells (S.R.W., unpublished data).

To generate recombinants, the targeting vector SW137 was linearized and introduced into RPE cells (passage 7–18) by electroporation. Colonies resistant to the antibiotic G418 were selected, and conditioned medium from pools of 3–10 colonies was screened, using the ELISA to detect the CD4–β_2m fusion protein expected from correctly targeted clones. Conditioned medium obtained from individual clones derived from each positive pool was rescreened, and recombinant clones were identified. The data from nine independent targeting experiments are summarized in the upper part of Table 1. Different forms of the linearized SW137 vector were tested on the same day in experiment 9 to directly compare their efficiency of generating recombinants. As can be seen from the data, day-to-day variation in the cells' propensity to undergo recombination had a greater impact on the absolute recombination efficiency observed in the experiments than did the homologous sequence(s) at the end(s) of the linearized vector. Several ELISA-positive clones were chosen for further analysis.

Characterization of the Targeted Clones. To directly confirm the expression of the CD4–β_2m fusion protein by the ELISA-positive clones, a small subculture of each selected clone was radiolabeled with [^{35}S]methionine plus [^{35}S]cysteine. After labeling, both the cells and their supernatants were harvested. The cell lysates and supernatants were immunoprecipitated with either CD4-specific or β_2m-specific antibodies, run on denaturing SDS/PAGE, and fluorographed. The results are shown in Fig. 2*A*. In the parental RPE cells, a 12-kDa protein band was observed in the β_2m immunoprecipitate, and no clear bands were observed in the CD4 immunoprecipitate. In contrast, the two ELISA-positive transfectants produce an additional 35-kDa protein with the β_2m-immunoprecipitate which was also present when they were immunoprecipitated with a CD4-specific

Table 1. Targeting results

Vector	Amount, μg	ELISA-positive clones	ELISA-positive efficiency × 10^7
	Targeting in RPE cells		
SW137/NotI	2.5	11	5.9
SW137/NotI	4	7	2.3
SW137/NotI	4	3	3.8
SW137/NotI	4	10	3.3
SW137/NotI	7.5	2	0.67
SW137/NotI	2	7	5.9
SW137/NotI	2	18	2.8
SW137/XhoI	2	150	28
SW137/NotI*	2	9	23
SW137/ClaI*	2	10	25
SW137/NotI + ClaI*	2	17	43
	Targeting in keratinocytes		
SW148/NotI + ClaI	2	2	1.8
SW148/NotI + ClaI	2	9	6.4
SW137/NotI + ClaI	2	7	8.8

In each experiment 4–64 × 10^6 cells were electroporated in samples of 2–4 × 10^6. The ELISA-positive efficiency is expressed per input cell.
*Samples run on the same day.

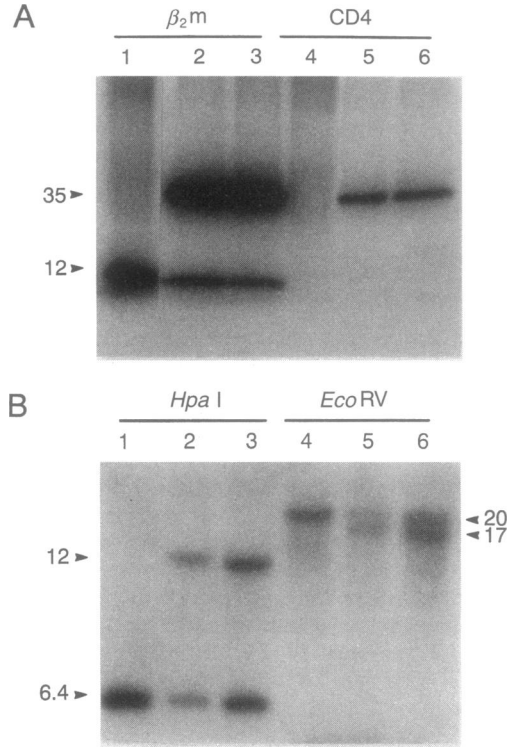

FIG. 2. (*A*) Immunoprecipitation of chimeric proteins from targeted cells. The samples were incubated with either a rabbit anti-human β_2m IgG preparation (lanes 1–3) or a rabbit anti-human CD4 IgG preparation (lanes 4–6). Shown are the immunoprecipitation results from the cell lysates; similar results were obtained with the conditioned media. Lanes 1 and 4, parental RPE cells; lanes 2 and 5, clone 24.1.3.2; lanes 3 and 6, clone 28.1.6.1. (*B*) Southern analysis of genomic DNA. High molecular weight genomic DNA isolated from parental RPE cells (lanes 1 and 4) and two targeted clones (lanes 2 and 5, clone 28.2.6.1; lanes 3 and 6, clone 33.1.4.6) was digested with *Hpa* I (lanes 1–3) or *Eco*RV (lanes 4–6), electrophoresed on an agarose gel, then subjected to a Southern blotting procedure. The blot was hybridized with the probe 2R (see Fig. 1*C*). The parental β_2m locus yields a 6.4-kb *Hpa* I band and a 20-kb *Eco*RV band with the 2R probe, while the recombined locus yields a 12-kb *Hpa* I band and a 17-kb *Eco*RV band with this probe.

antibody. In addition to the novel 35-kDa CD4–β_2m fusion protein expected of the correctly targeted clones, each clone also expressed the 12-kDa wild-type β_2m protein, indicating that the modification had affected only one of the two copies of the β_2m gene in the selected cells. Cells from additional clones were further expanded and genomic DNA was extracted for Southern analysis. Genomic DNA was digested with either *Hpa* I or *Eco*RV, fractionated on an agarose gel, blotted to a solid support, and hybridized with a probe which lies outside the 3′ region of homology (see Fig. 1*C*). Typical results are shown in Fig. 2*B*. With the parental RPE cells, a single hybridizing restriction fragment was observed for each digest (6.4-kb *Hpa* I fragment, 20-kb *Eco*RV fragment), whereas the ELISA-positive clones contained both the wild-type fragment and a new recombinant fragment of the size expected (12-kb *Hpa* I fragment, 17-kb *Eco*RV fragment) for an accurate targeting event. This demonstrated the recombinant nature of these cells, as well as validating the experimental approach.

Targeting in Keratinocytes. To further generalize this procedure, similar manipulations were carried out on normal human keratinocytes isolated from neonatal human foreskins and grown in the presence of G418-resistant mouse embryo fibroblast feeder cells (24). Again transfections were performed with linearized preparations of either the SW137 or SW148 targeting vectors. G418-resistant colonies of the human keratinocytes were selected and screened for the expression of the CD4–β_2m fusion protein. The results from three independent experiments are presented in the lower part of Table 1. These studies demonstrate gene targeting in a second somatic cell type at frequencies very similar to those seen with the human RPE cells.

DISCUSSION

These results suggest that gene targeting is a process which can be utilized for the directed modification of multiple human cell types grown *in vitro*. These data also demonstrate the efficiency of the gene trapping system used to detect the recombinants generated in these studies. To date 22 ELISA-positive clones have been analyzed by Southern blotting, and 21 of these clones have been shown to be correctly targeted recombinants. One clone carried a small duplication on the 3′ side of the targeting site (S.R.W., unpublished data).

Given the efficiency of detecting targeted recombinants in this system, the next point of interest concerns the function of the recombinant locus. In many cases gene targeting is undertaken to produce a null mutation. In some cases, the addition of sequences of the amino terminus of a target protein can disrupt its structure enough to render the protein nonfunctional. The function of the secreted CD4–β_2m fusion protein can be assessed in the β_2m-deficient human tumor line HCT-15 (25). If the recombinant protein retains some residual function, the gene trap can be adjusted in several ways to allow production of null mutants. By choosing an appropriate promoter, it may be possible in some cases to alter a gene's expression pattern enough to create a functional null mutation. This may be especially relevant to cell cycle or developmentally regulated genes. A second route to mutagenesis in this system involves the inappropriate addition or deletion of the signal sequence at the 5′ end of the recombinant transcript. Removal of the signal sequence from the gene trap should allow the production of functional null mutants of secreted and cell surface proteins by forcing their retention within the targeted cell. Conversely, the addition of a signal sequence to intracellular protein targets should allow the production of functional null mutants by causing the targeted protein to be inappropriately transported outside the cell. Application of this latter approach to large, highly-structured proteins may result in inefficient secretion of fusion protein and poor cell viability. In these cases, a third route to mutagenesis may prove useful. This strategy involves the manipulation of the crossover point within the recombination reaction. This can be achieved by adding or deleting sequences at the 5′ end of the 3′ arm of homology. Using this scheme, it may be possible to remove essential epitopes from the amino terminus of a protein to destroy its function, while still retaining carboxyl-terminal epitopes of the target protein which could be used to detect the recombinant locus in a specific ELISA. This strategy should be most useful with larger genes which are encoded by multiple exons.

As expected, both the immunoprecipitation results and the Southern blot data demonstrated that each of the β_2m recombinant clones derived from one round of gene targeting was heterozygous at the β_2m locus, possessing one targeted allele and one wild-type allele. To create a β_2m-deficient, class I MHC-depleted cell from a null heterozygote, a second round of gene targeting is necessary. In contrast to the targeting of the first allele, where the targeted phenotype was created through the expression of a fusion protein, engineering of the second null allele is expected to automatically yield an identifiable phenotype. As is the case with homozygous β_2m-deficient human tumor cell lines (25), recombinant clones from a second round of gene targeting should be severely deficient in their cell surface expression of all three

of their class I MHC genes. This phenotype should be very clear after immunostaining with a pan anti-class I MHC antibody.

In general, heterozygous targeting events are difficult to detect in normal cells, but given a specific antibody directed against the gene product of the target locus and a genomic clone of the locus, the gene trapping system presented here can be adapted to allow the isolation of targeted recombinant cells for a wide variety of cell types and genetic loci. These experiments extend the scope of gene targeting to a variety of genetic systems beyond mouse ES cells and may permit the development of cell-based human therapeutics.

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