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Genetics

Positive genetic selection for gene disruption in mammalian cells by homologous recombination

(gene targeting in mammalian cells/genetic selection of gene targeting/homologous recombination in mammalian cells)

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ABSTRACT **Efficient modification of genes in mammalian cells by homologous recombination has not been possible because of the high frequency of nonhomologous recombination. An efficient method for targeted gene disruption has been developed. Cells with substitution of exogenous sequences into a chromosomal locus were enriched, by a factor of 100, using a positive genetic selection that specifically selects for homologous recombination at the targeted site. The selection is based on the conditional expression of a dominant selectable marker by virtue of in-frame gene fusion with the target gene. The dominant selectable marker was derived by modification of the *Escherichia coli neo* gene so that it retains significant activity in mammalian cells after in-frame fusion with heterologous coding sequences. In the example presented here, homologous recombinants were efficiently recovered from a pool in which the targeted gene was disrupted in 1 per 10,000 cells incorporating exogenous DNA.**

The ability to disrupt or modify chromosomal genes by targeted homologous recombination with exogenous DNA would fundamentally advance the molecular and genetic analysis of many processes in mammalian cells. The primary obstacle to gene targeting is the occurrence of nonhomologous recombination, whose frequency is typically several orders of magnitude higher than homologous recombination (1, 2). Frequencies of homologous versus nonhomologous recombination ranging from 1/100,000 (1, 3) to 1/1000 (4, 5), and even as high as 1/100 (6–8) and 1/10 (9) have been reported. High targeting frequencies are probably special cases due to, e.g., locus-to-locus variation, cell type differences, methods of introducing DNA, etc. It is probable that targeting frequencies in the 1/10,000 to 1/1000 range will be the rule with most loci. Several procedures have been found that enhance the frequency of homologous recombination: (*i*) linearization of the input DNA within the region of homology (10–12); (*ii*) maximization of the extent of homology between input and chromosomal sequences (3, 5); (*iii*) modification of the 3′ hydroxyls of the transfected substrate DNA by the addition of dideoxynucleotides (13).

Unfortunately, the disruption of most chromosomal genes cannot be directly selected. Recovery of homologous recombinants at such loci has been primarily approached by the utilization of physical screening procedures that allow the identification of rare homologous events in large pools of cells (4, 35). This can be a very time consuming and tedious endeavor. It should be possible to develop general genetic selections to facilitate the exclusive recovery of homologous targeting events. Two formal alternatives are possible: (*i*) positive selection for recombination at the homologous locus; and (*ii*) negative selection against recombination at nonhomologous loci. This communication explores the first possibility, and reports the development of a selection based on the conditional expression of a dominant selectable marker by homologous recombination at the target locus. The selection provides a 100-fold enrichment by reducing the number of recovered nonhomologous events and is generally applicable for the disruption or modification of any gene.

MATERIALS AND METHODS

Standard genetic nomenclature (14) as now used for *Escherichia coli* (15) has been adopted. The MT-1.4 cell line was constructed as follows. The *neo* gene was deleted from plasmid pZipPyMT (16). The resultant plasmid (pZipPyMT-*dlneo*; Fig. 1) was transfected into the packaging cell line ψ-2 (17). NIH 3T3 cells were infected with a transient lysate and plated for a focus assay (16). Foci were isolated, replated by limiting dilution, and subcloned. Transfection of DNA was either as described (18) or by electroporation at 1040 μF and 175 V (19). All recombinant DNA constructions were by standard procedures. The complete DNA sequences of all plasmids have been compiled in the digital VAX VMS operating system and are available upon request. DNA sequences flanking the site of provirus insertion were cloned from cell line MT-1.4 in a λ phage vector. Nonrepetitive sequences were used as probes for analysis of homologous recombinants. All phage libraries were made with the vector λ ZAP according to the supplier's instructions (StrataGene). Southern blot (18) and Western blot (20) analyses were as described. The anti-Neo and anti-Pmt rabbit antisera were the kind gifts of Lucy Shapiro (Columbia) and Tom Roberts (Harvard), respectively. DNA was sequenced by the dideoxynucleotide termination method (21) from supercoiled templates. Universal M13 primers and primers homologous to various portions of the *pmt* gene were used.

RESULTS

General Selection for Homologous Recombination. A targeting module was designed such that the expression of a dominant selectable marker, *neo* (neomycin phosphotransferase), was made conditional upon integration by homologous recombination, which enabled its transcription and in-frame translation. To provide selection for in-frame translation, the fusion of the coding sequences of *neo* to the target gene is required. However, in the majority of cases the fusion of protein sequences to the N terminus of the Neo protein results in loss of activity (22, 23). Therefore, the *neo* gene was redesigned by the inclusion of a "bridge" peptide, encoded by the amino-terminal portion of the *cam* (chloramphenicol acetyltransferase) gene (22, 24), such that potentially disruptive influences could be removed from the immediate vicinity

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of the Neo enzyme. This should allow the *cam–neo* gene to function as a general marker for the selection of in-frame gene fusions. To demonstrate the utility of gene targeting by this principle, we chose to disrupt a transforming oncogene, the polyoma middle T antigen gene (*pmt*), present as a stable haploid locus in the genome of NIH 3T3 cells (cell line MT-1.4). Disruption of the *pmt* gene in the MT-1.4 cell line should result in a reversion to the parental nontransformed phenotype and thus provide a visual morphological screen for homologous recombination. The targeting module consisted of the in-frame fusion of the *pmt* gene to the *cam–neo* marker. The *pmt–cam–neo* gene encoded Neo activity, since its expression in mammalian cells (pSV2*pmt–cam–neo*; Fig. 1) conferred full resistance to G418 (Table 1). A single crossover targeting module (pCO1*pmt–cam–neo*; Fig. 1) was made by removing the promoter and the translational start (ATG) sequences from pSV2*pmt–cam–neo*. A double crossover targeting module (pCO2*pmt–cam–neo*; Fig. 1) was made by including a segment with homology to the 3′ end of the chromosomal *pmt* locus in pCO1*pmt–cam–neo*. Homologous recombination of either module should cause (*i*) activation of the *pmt–cam–neo* gene resulting in G418 resistance (the chromosomal *pmt* locus providing both transcriptional and translational signals); and (*ii*) inactivation of the *pmt* gene, resulting in a reversion to the nontransformed cellular morphology.

Enrichment Factor Provided by the Selection. A key experiment was the quantification of the recovery of G418-resistant colonies, determined as the efficiency of plating of the targeting modules with respect to pSV2*pmt–cam–neo* or other controls (pSV2*neo*). This provided a measure of the enrichment factor inherent in the genetic selection for homologous recombination based on in-frame fusions to the *cam–neo* marker. The ratio of G418-resistant colonies was ≈0.01 (Table 1). The experiment was repeated a number of times with (*i*) supercoiled and linearized plasmids; (*ii*) a variety of cell lines (NIH 3T3, MT-1.4, mouse L cells, monkey BSC-40 cells); (*iii*) the Moloney murine leukemia virus long terminal repeat driving the triple fusion gene (pLTR*pmt–cam–neo*); and (*iv*) transfection by electroporation as well as calcium phosphate coprecipitation. The ratio varied between 0.05 and 0.005, with the 0.01 value (100-fold enrichment) being an average.

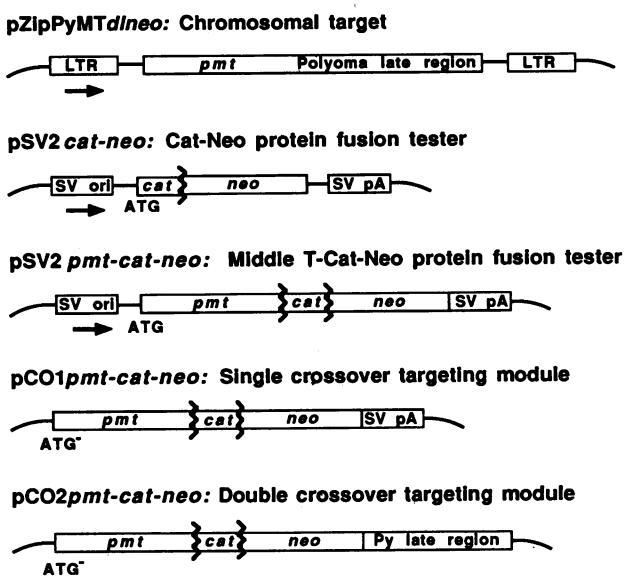

FIG. 1. Schematic representation of plasmid constructs. Bold arrows indicate direction of transcription. Serrated line indicates in-frame gene fusion. The *cam–neo* gene encoded as follows: amino acid residue (aa) 1–73, *cam* (aa 1–73 of native *cam*); aa 74–81, polylinker (22); aa 82–341, *neo* (aa 5–264 of native *neo*). The *pmt–cam–neo* gene encoded as follows: aa 1–381, *pmt* (aa 1–381 of native *pmt*); aa 382–385, linker (pUC13 plasmid); aa 386–707, *cam–neo* (aa 20–341 of *cam–neo*, above). LTR, long terminal repeat of Moloney murine leukemia virus; SV ori, origin of replication and early promoter of simian virus 40 (SV40); SVpA, early polyadenylylation site of SV40.

Table 1. Quantification of the enrichment factor

Plasmid	Experiment A	Experiment B	Experiment C	Experiment D
Control	4.5×10^{-3}	ND	1.5×10^{-1}	ND
pSV2/S	ND	2.7×10^{-3}	1.3×10^{-1}	ND
pLTR/S	ND	ND	1.8×10^{-1}	1.7×10^{-1}
pCO1/S	1.2×10^{-4}	6.0×10^{-5}	1.0×10^{-3}	2.0×10^{-3}
pCO1/L	9.8×10^{-5}	6.9×10^{-5}	ND	5.5×10^{-3}
pCO2/S	1.0×10^{-4}	3.0×10^{-5}	ND	3.6×10^{-3}
pCO2/L	6.0×10^{-5}	1.1×10^{-4}	ND	7.6×10^{-3}

All plasmids contained the *pmt–cam–neo* fusion gene, except the control, which was supercoiled pSV2*neo*. The form of the DNA used is indicated as either S (supercoil) or L (linear). pCO1 was linearized with *Esp* I; pCO2 was linearized with *Acc* I and *Eco*RV. Experiments A and B were performed by calcium phosphate coprecipitation into cell line MT-1.4; the data are given as the number of G418-resistant colonies per transfected cell. Experiments C and D were performed by electroporation into cell lines NIH 3T3 and MT-1.4, respectively; the data are given as the number of G418-resistant colonies per surviving cell. ND, not determined.

The majority of G418-resistant colonies from transfections of the MT-1.4 cell line with the targeting modules had the transformed morphology. Southern blot hybridization analysis of several clonal isolates revealed that (*i*) the *pmt* locus remained unchanged; and (*ii*) the introduced *pmt–cam–neo* module was apparently integrated at various unrelated locations (data not shown). Cell lines containing single copy as well as multicopy insertions were recovered. These isolates represent nonhomologous insertions of the targeting module into the genome such that the *pmt–cam–neo* gene became activated in a position-dependent manner. The frequency of nontransformed colonies, as scored by microscopic observation, was $\approx 2 \times 10^{-3}$ and could be as high as 5×10^{-3} in individual transfections (Table 2). That the majority of these nontransformed isolates did not contain targeting events was immediately apparent, since the frequency of such colonies remained unchanged in control transfections with, e.g., pSV2*neo*. Plating of the MT-1.4 cell line at limiting dilution revealed colonies with a nontransformed morphology at a frequency of $\approx 5 \times 10^{-4}$. The MT-1.4 cell line had been subcloned twice, and great care was always taken to avoid

Table 2. Quantification of gene disruption

Exp.	Number of recovered colonies: Total $G418^R$	Nontransformed morphology: Total	Targeted	Other
1	≈3000	8	1	7
2	≈2000	4	1	3
3	686	5	0	5
4	421	4	0	4
5	533	4	3	1

Exps. 1–4 were performed by electroporation of the MT-1.4 cell line with plasmid pCO2*pmt–cam–neo* linearized with *Acc* I and *Eco*RV. Exp. 5 was performed with the *Acc* I/*Eco*RV fragment containing the targeting module purified by preparative gel electrophoresis. The concentration of DNA in the electroporation cuvette was 200 μg/ml in Exps. 1–3 and 100 μg/ml in Exps. 4 and 5. Cell line FR-1.20 was obtained from Exp. 1, cell line FR-1.42 was from Exp. 2, and cell lines FR-2.37, FR-2.46, and FR-2.47 were from Exp. 5. $G418^R$, G418 resistance.

contamination with NIH 3T3 cells. Direct analysis by Southern blot hybridization revealed a complete loss of the *pmt* locus in each individual cell line and reversion to the parental NIH 3T3 pattern. The loss of the *pmt* locus could be the result of either a deletion event that included flanking chromosomal sequences or, more likely, a mitotic chromosome nondisjunction event. This reversion was apparently stimulated by some component of the transfection regimen, such as the presence of exogenous DNA, the application of the electric field, the G418 selection, etc.

Recovery and Analysis of Targeted Events. The utility of the double crossover targeting module was tested as follows: (*i*) plasmid pCO2*pmt–cam–neo* was cut with *Acc* I and *Eco*RV to generate recombinogenic ends in regions homologous to the target (Fig. 2); (*ii*) the restricted plasmid was introduced into the MT-1.4 cell line by electroporation; (*iii*) G418-resistant colonies were selected; (*iv*) all colonies were examined microscopically to identify clones with nontransformed morphology; (*v*) nontransformed colonies (as well as a few representative transformed colonies as controls) were cloned and expanded into cell lines; (*vi*) the structure of the *pmt* locus was examined by Southern blot hybridization. The above experiment has been repeated several times, and a number of independent recombinants have been identified (Table 2).

Southern blot hybridization analysis was performed with four probes: nonrepetitive chromosomal sequences 5′ and 3′ to the *pmt* locus, as well as *pmt* and *neo* sequences (Fig. 3). In this way, alterations at the *pmt* locus, as well as linkage with transfected sequences, could be unambiguously demonstrated. Several types of events were observed. The first was precise double crossover (cell lines FR-1.42, FR-2.46, FR-2.47). The second was precise recombination at the 5′ side (which was demanded by the G418 selection) accompanied by illegitimate events in the rest of the targeting module (FR-1.20, FR-2.37). One event of this type was best explained by a partial duplication of the targeting module prior to homologous crossover on both 5′ and 3′ sides (FR-2.37). Another event was best explained as a homologous crossover on the 5′ side accompanied by an illegitimate rearrangement on the 3′ side (FR-1.20). In addition, the above types of events were sometimes found against a backdrop of multiple nonhomologous recombinations throughout the genome (FR-1.20, FR-1.46), while at other times no nonhomologous events were seen (FR-2.37, FR-2.46, FR-2.47).

DNA Sequence Analysis. Homologous recombination between transfected and chromosomal sequences can result in point mutations around the region of crossover (25), although some have failed to observe this phenomenon (8). The recombinant locus from the cell line FR-1.42 was therefore cloned into a bacteriophage vector and subjected to DNA sequence analysis (it was of particular interest to determine the fidelity of homologous recombination in a cell line that had undergone multiple concomitant nonhomologous events). A contiguous sequence of 1579 base pairs (bp) was obtained, spanning the entire *cam* and *pmt* gene fragments and beyond in the 5′ direction, unambiguously demonstrating the linkage of the transfected and chromosomal sequences. The crossover must have occurred in the 947 bp between the *Acc* I site that formed the 5′ boundary of the transfected DNA and the 3′ boundary of the region of homology (*pmt–cam* junction). The sequence of this entire region, as well as the 459 bp 5′ of it and the 173 bp 3′ of it, contained no changes whatsoever from the published sequence.

Western Blot Analysis. The *pmt–cam–neo* fusion gene was active as an efficient selectable marker. The presence of the expected fusion polypeptide *in vivo* was demonstrated by Western blot analysis using antibodies directed either against the polyomavirus middle T antigen, or against the *E. coli* Neo protein (Fig. 4). Pools of cells stably transfected with the plasmids pSV2*neo*, pSV2*cam–neo*, and pSV2*pmt–cam–neo* displayed bands of mobilities consistent with the predicted values when probed with anti-Neo antiserum. When the same samples were probed with anti-Pmt antiserum, only the *pmt–cam–neo* gene product was visualized. Most importantly, both antisera displayed a band of identical mobility. Nontransfected NIH 3T3 cells did not react with either antiserum, and MT-1.4 cells produced a Pmt band of the expected mobility when probed with anti-Pmt antiserum. The recombinant cell line FR-2.46 displayed the expected full length Pmt–cam–Neo fusion protein when probed with either antiserum. All other recombinant cell lines also contained full-length fusion proteins (data not shown).

Frequency of Targeted Homologous Events. These experiments demonstrate that correct homologous targeting events can be obtained simply by the indirect selection for G418 resistance. The electroporations in experiments 1–4 (Table 2) were performed with total restriction digests of pCO2*pmt–cam–neo*. The frequency in experiments 1 and 2 was ≈1/2500. The selection plates in experiments 1 and 2 were seeded at a high density to allow the examination of a large number of G418-resistant colonies. Unfortunately, the resultant crowding of the colonies with transformed morphology greatly obscured the slower growing nontransformed colonies, and thus the given frequency is very likely an underestimate. Subsequently, the plates in experiments 3, 4,

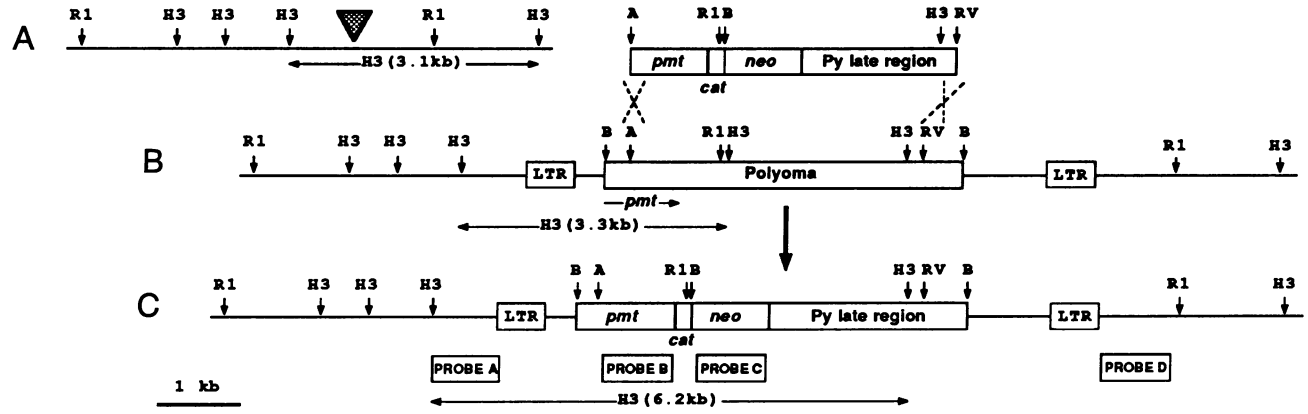

FIG. 2. Schematic representation of the disruption of the *pmt* locus. (*A*) The locus in NIH 3T3 cells. Shaded triangle indicates the site of insertion of the *pmt* retrovirus vector. (*B*) Parental *pmt* locus in the MT-1.4 cell line, with the targeting module drawn above. Dashed line indicates the regions of possible crossover. (*C*) Disrupted *pmt* locus in a recombinant cell line. Probes indicated below the diagram were used in Southern blot hybridization analysis (Fig. 3): probe A, 5′ chromosomal flank; probe B, *pmt*; probe C, *neo*; probe D, 3′ chromosomal flank. Note scale bar in lower left corner (kb, kilobases).

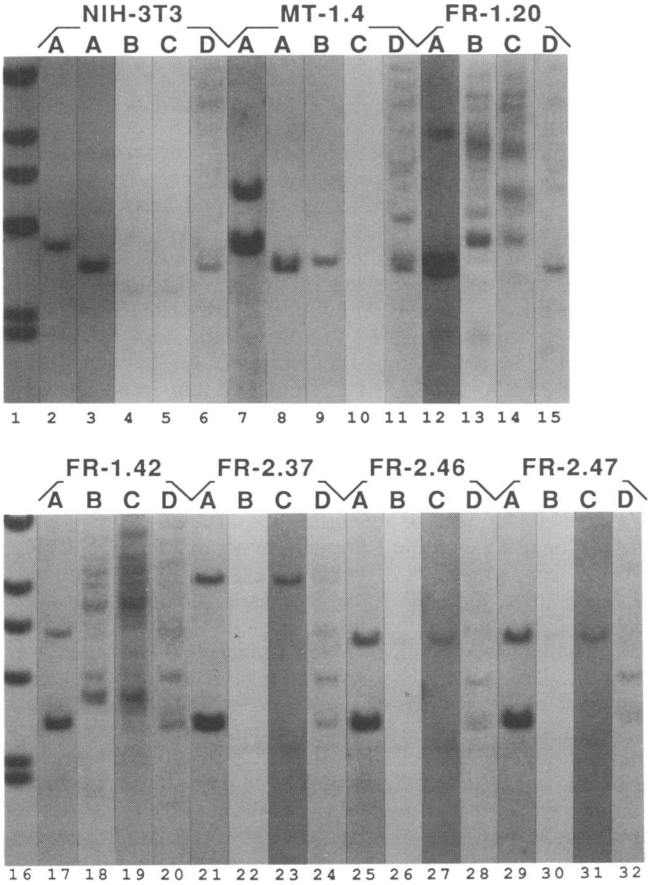

FIG. 3. Southern blot hybridization analysis of parental and recombinant cell lines. The probes were as follows: lanes A, 5′ chromosomal flank; lanes B, *pmt*; lanes C, *neo*; lanes D, 3′ chromosomal flank. Markers (lanes 1 and 16) were *Hin*dIII-digested phage λ DNA. All samples were digested with *Hin*dIII, except in lanes 2 and 7, which were digested with *Eco*RI. Refer to Fig. 2 for aid in interpretation. The 5′ flanking probe (probe A) showed the MT-1.4 locus to be haploid (*Eco*RI, lanes 2 and 7; *Hin*dIII, lanes 3 and 8); FR-1.42, FR-2.46, FR-2.47 to contain recombinant *Hin*dIII fragments of predicted size (lanes 17, 25, and 29); FR-1.20, FR-2.37 to contain *Hin*dIII fragments of larger than predicted size (lanes 12 and 21). Identical *Hin*dIII recombinant fragments were visualized with 5′ flank, *pmt* and *neo* probes (probes A, B, and C) in FR-2.37, FR-2.46, and FR-2.47, indicating linkage of transfected DNA to the chromosomal *pmt* locus (lanes 21–23, 25–27, and 29–31). *pmt* and *neo* probes (probes B and C) showed multiple nonhomologous insertion events in FR-1.20 and FR-1.42 (lanes 13, 14, 18, and 19). The 3′ flank probe (probe D) showed the identical pattern in FR-1.42, FR-2.37, FR-2.46, and FR-2.47 as in parental MT-1.42 (lanes 11, 20, 24, 28, and 32), indicating the 3′ crossover to be precise. By the same criterion, the 3′ crossover was probably nonhomologous in FR-1.20 (lane 15).

and 5 were seeded at a low density. No targeting events were found in experiments 3 and 4 (0/686 and 0/421, respectively). The frequency in experiment 5 was 3/533. In this experiment, the fragment of pCO2*pmt–cam–neo* corresponding to the targeting module was purified by preparative gel electrophoresis before being used in electroporation. These observations suggest that transfection with a purified targeting module may enhance the recombination frequency. Since the cells were allowed to grow after electroporation for 48 hr in the absence of G418, during which time 1 or 2 doublings occurred, the true ratio of homologous to nonhomologous events was probably in the vicinity of 1/100. Multiplication of this net targeting value by the 100-fold enrichment factor inherent in the selection gives a value for the ratio of homologous to total nonhomologous events as ≈1/10,000. This value agrees well with reports in the literature, considering that the targeting module in pCO2*pmt–cam–neo* had relatively limited homology with the chromosomal locus (947 bp on the 5′ side and 2027 bp on the 3′ side).

DISCUSSION

The disruption of genes with unknown null phenotypes, a situation that is expected in the majority of applications of targeting technology, was approached by the development of a selectable marker whose expression can be made conditional upon recombination at the target locus. A selectable marker consisting only of an open reading frame without its translation initiation codon reduces the target for productive nonhomologous insertions to a minimum (in-frame insertions within exons of active genes transcribed by RNA polymerase II). The *E. coli neo* gene was redesigned by adding a sequence encoding a "bridge" peptide (the amino-terminal portion of the *E. coli cam* gene) to make it suitable for the selection of in-frame gene fusions. The target, the polyomavirus middle T antigen gene (*pmt*) was introduced as a stable haploid locus into the genome of NIH 3T3 cells; the characteristic transformed phenotype of the resultant cell line (MT-1.4) could then be reverted to the parental morphology by the disruption of the single *pmt* locus. The targeting module was a *pmt–cam–neo* fusion gene introduced by electroporation as a linear DNA fragment with homology at both ends to the chromosomal *pmt* locus. Transcription and translation start sequences were not present in the targeting module. It is important to note that the *pmt* locus was unselected, since the only selection during the gene disruption procedure was for resistance to G418.

The enrichment for the recovery of cells containing homologous recombination events was ≈100-fold. However, this is probably not the limit, since the *pmt* portion of the *pmt–cam–neo* gene contained numerous in-frame ATG codons. Therefore, nonhomologous activation of the fusion gene could be the result of translational restarts or fragmentation of the transfected DNA exposing internal ATGs. In the future, it is probable that the 100-fold enrichment could be improved by avoiding the presence of numerous in-frame start codons upstream of the *cam–neo* gene segment—e.g., by constructing fusions in small exons and using intron homology for targeting recombination. Southern blot hybridization analysis of the fate of the targeting module during productive illegitimate recombination revealed extensive fragmentation and rearrangements (data not shown). Numerous differences were seen when blots were sequentially probed with *pmt* and *neo* probes. The fragmentation of DNA during transfection will probably constitute an upper limit for possible enrichment in a positive selection, by acting to expose the selectable marker in any targeting module. The extensive fragmentation also implies that negative selection strategies (i.e., selections against recombination at nonhomologous loci) will probably yield only a limited enrichment, at least in the NIH 3T3 background. Whether other cell types may be less prone to rearrangements during electroporation remains to be established.

The best targeting frequency achieved was ≈1/100 of total recovered G418-resistant colonies. Multiplication by the 100-fold enrichment factor provided by the selection results in a figure of 1/10,000 total nonhomologous events, which is in good agreement with reported values. The absolute frequency of homologous recombination seems to be strongly influenced by the extent of homology between the incoming DNA and the resident target locus (5). The extent of homology present in the *pmt–cam–neo* targeting module was quite limited: 2974 bp total, with 947 bp and 2027 bp on the 5′ and 3′ sides, respectively. It is therefore very likely that the net gene disruption frequency of 1/100 obtained to date could be

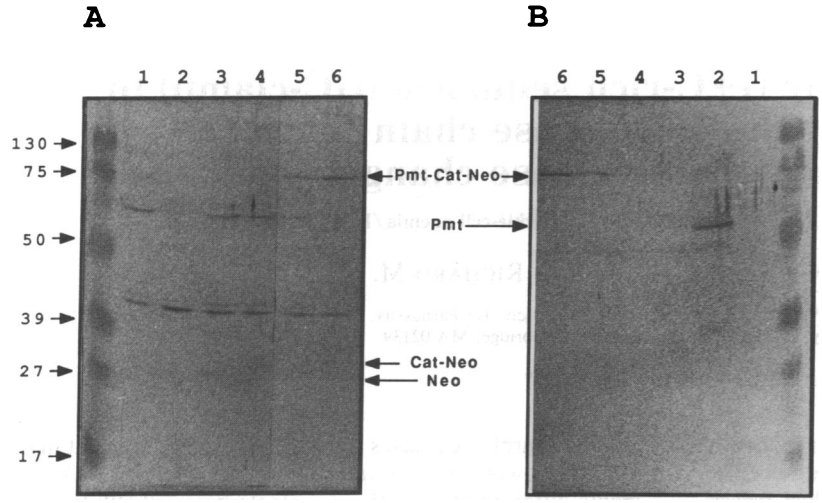

FIG. 4. Western blot analysis of cells expressing the *neo*, *cam–neo*, and *pmt–cam–neo* genes. Crude cell extracts were electrophoresed on two identical gels which were run in parallel. (*A*) Gel 1 probed with antiserum to the *E. coli* Neo protein; (*B*) gel 2 probed with antiserum to the polyomavirus middle T antigen. Lanes: 1, NIH 3T3; 2, MT-1.4; 3, NIH 3T3 pSV2*neo*; 4, NIH 3T3 pSV2*cam–neo*; 5, NIH 3T3 pSV2*pmt–cam–neo*; 6, FR-2.46. Computer-generated translation of the nucleotide sequences of the *pmt–cam–neo*, *cam–neo*, and *neo* genes produced polypeptides of 707, 341, and 264 amino acid residues, with molecular masses of 79,919, 37,896, and 29,047 Da, respectively. Prestained markers (Bio-Rad) were run in the outermost lanes.

substantially improved by a simple redesign of the targeting module to include larger homologous sequences. The DNA sequence of the entire 5′ region of crossover of one recombinant as well as significant amounts of neighboring sequences were determined (1579 bp total). No changes from the published sequence were found. The particular cell line also contained multiple nonhomologous insertions. The simplest explanation of these observations is that homologous and nonhomologous events can occur concomitantly but in all likelihood are independent events. In addition, the fidelity of homologous recombination at the DNA sequence level, even in a cell undergoing illegitimate events, appears to be very high.

It is to be expected that most null mutations produced by gene targeting will be recessive. In a diploid background, this necessitates mutation of the second copy of the gene, which could be approached by various means: classical mutagenesis (26), insertional mutagenesis (27), mitotic nondisjunction (28–30), gene conversion (31, 32), or even further gene targeting. In addition, since a homozygous null phenotype can often be lethal, a means of conditionally supplying the targeted gene product will have to be arranged. Gene targeting in totipotent embryonic stem cells (5, 33, 34) followed by rescue into the murine germ line presents a special case, since homozygosity of a single disruption event could be achieved by breeding the resultant transgenic animals. The determination of organismal phenotypes in transgenic animals is, in many ways, the ultimate experiment. Nevertheless, tissue culture systems have proven to be extremely useful models for numerous applications. The time consuming nature, as well as considerable expense, of transgenic constructions thus makes the development of efficient gene targeting systems in tissue culture of great importance.

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