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A dominant positive and negative selectable gene for use in mammalian cells

(gene fusion/herpes simplex virus thymidine kinase/bacterial neomycin phosphotransferase)

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ABSTRACT **We have constructed three different fusion genes containing the herpes simplex virus thymidine kinase (HSV tk) and the bacterial neomycin phosphotransferase (neo) genes. All three fusion genes utilize the HSV tk promoter but differ at the junction of their components. We have determined if the fusion genes are bifunctional by introducing them into mammalian cells and testing for function of the individual components. One of the fusion genes, TNFUS 69, produced a bicistronic message and a fusion protein that has TK and NEO protein functions. This and other fusion genes of a similar nature could serve as dominant positive and negative selectable markers in mammalian cells.**

Gene transfer into somatic mammalian cells as well as into fertilized mouse embryos has become an extremely valuable tool for study of gene structure–function relationships. Gene transfer can be accomplished by several different methods that include microinjection, calcium phosphate coprecipitation, and electroporation. With the exception of direct microinjection of DNA into the nuclei of recipient cells, these methods require the presence of a selectable gene in the input DNA to permit isolation of cells that have stably incorporated the exogenously introduced DNA. Early gene transfer experiments utilized cells mutant at the thymidine kinase (*tk*) locus, and a cloned herpes simplex virus (HSV) tk gene was used as a directly selectable marker in transfecting these cells (1). Subsequent experiments allowed introduction of nonselectable DNA sequences by ligating them to the selectable *tk* or by cotransfection (2). Although other selectable genes, such as the hypoxanthine phosphoribosyltransferase (*hprt*), have been introduced into appropriate mutant mammalian cells, the need for the target cell to be mutant has limited the types of cells that could be used as recipients. The development of dominant selectable systems has removed this limitation. The first such system used was the xanthine guanosine phosphoribosyltransferase gene (*gpt*) from *Escherichia coli* (Eco-gpt; ref. 3). The *gpt* expression could be selected for in hprt$^-$ or in wild-type cells. Other dominant selectable genes have been developed, among which the neomycin phosphotransferase (*npt*II or *neo*) is perhaps the most widely used (4).

The ability to apply positive and negative selection systems during the introduction of DNA into cells has proven to be an extremely valuable genetic tool (5). The most widely used genes that allow positive and negative selection schemes are *hprt*, *tk*, and adenine phosphoribosyltransferase (*aprt*). By using appropriate mutants as recipients, transformed cells that have acquired the gene can be selected positively. Cells that have expression of these genes can subsequently be selected in a negative manner. Unfortunately, no single gene is available that can be selected for and against in wild-type cells. The availability of such genes would be valuable.

To achieve this goal, we have constructed and tested fusion genes between the bacterial *neo* and HSV *tk* genes. The *neo* serves as a positive selectable marker and the *tk* serves as a negative selectable marker. We here report experiments showing that this type of bifunctional gene can be used as a dominant positive and negative selectable marker in mammalian cells.

MATERIALS AND METHODS

Cells. Mouse LMTK$^-$ cells (6) were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (FCS). Mouse embryonic stem (ES) cells were maintained on irradiated feeder layers of STO cells in DMEM/10% FCS and 2% 2-mercaptoethanol.

Transfections. LMTK$^-$ cells were transfected by the calcium phosphate coprecipitation method (7). For each transfection, 5–7 × 10^5 cells were plated in 60-mm culture dishes and incubated overnight. A calcium phosphate precipitate with the appropriate DNA was added to each plate. No carrier DNA was used. After 4 hr, the cells were treated for 2 min with 10% dimethyl sulfoxide in 138 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/6 mM dextrose/20 mM Hepes, pH 6.92. Mouse ES cells were transfected by electroporation using a Bio-Rad gene pulser at 500 μF and 250 V. The transfected cells were incubated overnight in DMEM containing 10% FCS, after which they were transferred to 100-mm culture dishes and subjected to selection with G418 at 200 μg/ml (Geneticin, GIBCO) or hypoxanthine (15 μg/ml), aminopterin (0.2 mg/ml), and thymidine (5 μg/ml) (HAT medium; ref. 8). Resistant colonies were counted after 14 days of selection. Individual colonies were isolated and expanded for further analysis.

Preparation of Cell Extracts. Cell monolayers were washed with phosphate-buffered saline, harvested, and centrifuged at 2000 × *g*. The cell pellet was suspended in cell lysis buffer (0.1 M Tris, pH 6.8/0.5% Triton X-100/1 mM phenylmethylsulfonyl fluoride) at 10^8 cells per ml. Cells were sonicated three times, 10 sec each time, and cell lysis was monitored by phase-contrast microscopy. Lysed cells were centrifuged for 2 min to sediment cell membranes, nuclei, and cell debris, and the supernatant was removed for protein analysis. Pro-

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Abbreviations: HSV, herpes simplex virus; neo, neomycin phosphotransferase; tk, thymidine kinase; SV40, simian virus 40; ^{R}, resistant; ES, embryonic stem; HAT, hypoxanthine/aminopterin/thymidine; nt, nucleotide(s).

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tein concentration was estimated by the Bio-Rad protein assay.

Gel Electrophoresis and Western Blot Analysis. Duplicates of each sample were analyzed by SDS/polyacrylamide gel electrophoresis, using 80–100 μg of total protein per lane according to the procedure of Laemmli (9). Electrophoretic transfer of proteins and antibody binding were carried out by a modification of the protocol of Towbin *et al.* (10). Proteins were transferred to nitrocellulose filter paper overnight at 200 mA in 25 mM Tris, pH 8.1/192 mM glycine made in 20% (vol/vol) methanol. After transfer, the nitrocellulose filter was cut in half and soaked in a solution of 3% bovine serum albumin (fraction V, Sigma) in saline (0.9% NaCl/10 mM Tris·HCl, pH 7.4) for 1 hr at room temperature. One half of the nitrocellulose filter was incubated with goat antiserum prepared against the TK protein of the HSV type I (a gift from T. Silhavy, Princeton University, Princeton, NJ). The secondary antibody was rabbit anti-goat IgG (a gift from W. Carey Hanly, University of Illinois, Chicago) diluted 1:1000 in the above buffer. The second half of the nitrocellulose filter was incubated with primary rabbit antiserum prepared against bacterial neo (anti-NEO antibody; a gift from L. Shapiro, Stanford University, Palo Alto, CA). After each incubation, filters were extensively washed in a solution of 0.05% Tween 20 prepared in Tris-buffered saline. Immunoreaction was visualized using goat anti-rabbit immunoglobulins coupled to horseradish peroxidase (Bio-Rad) and staining with horseradish peroxidase color development reagent according to manufacturer's specifications. The developed membrane shows purple bands against a white background. The sensitivity of detection by this procedure is 100 pg for a selected purified protein.

Extraction of RNA and Northern Blot Analysis. Total cellular RNA was isolated by the method of Soeiro and Darnell (11). Ten-microgram samples of RNA were dissolved in 50 mM boric acid/5 mM sodium borate/10 mM sodium sulfate/0.1 mM EDTA/50% formamide/6% formaldehyde, heated at 65°C for 5 min, and fractionated on 1.2% agarose gels containing 3% formaldehyde. The RNA was transferred to nitrocellulose according to the method of Thomas (12) and hybridized with nick-translated DNA probes. Hybridization was conducted at 45°C in 50% formamide/5× SSC (1× SSC = 0.15 M sodium chloride/15 mM sodium citrate)/50 mM sodium phosphate, pH 6.8/5× Denhardt's solution (50× Denhardt's = 5 g of Ficoll, 5 g of polyvinylpyrrolidone/5 g of bovine serum albumin and H_2O to 500 ml)/0.1% sodium dodecyl sulfate (SDS)/60 μg of denatured salmon sperm DNA per ml. The filters were washed at 55°C with 0.1× SSC/0.1% SDS. The filters were exposed to Kodak XAR film to generate autoradiographs.

RESULTS

Nature of the Test Plasmids. The HSV *tk* gene was derived from pHSV106 (13) and the *neo* gene was derived from pSV2neo (4). Using plasmid pSV010 (4) as a vector, an initial plasmid was made that contained the *tk* gene of the HSV type I (HSV I *tk*) together with its own promoter and all of the necessary 5′ regulatory sequences (14); a promoterless copy of the bacterial aminoglycoside phosphotransferase gene *neo* (15) was inserted downstream of the *tk* gene. This initial plasmid also contained the simian virus 40 (SV40) replication origin and the SV40 early promoter regions in reverse orientation with respect to the *tk* gene; its structure is illustrated in the top line of Fig. 1. Several modifications of the initial plasmid were made in the *tk* and *neo* genes resulting in three different constructs designated TNT, TNSSS 31, and TNFUS 69. The regions corresponding to the end of the *tk* coding sequences and beginning of the *neo* in the initial plasmid and in each of these three constructs are also shown in Fig. 1. In the TNT (*tk–neo* terminator) plasmid, the region corresponding to last 7 amino acids of the TK protein was replaced by a region coding for 6 new amino acids. The end of the TK protein and the initiation codon for the NEO protein are separated by 12 nucleotides (nt). Thus, the *tk* and *neo* genes in this plasmid have their own translation initiation and termination codons. In the TNFUS 69 (*tk–neo* fusion) plasmid, the region corresponding to the carboxyl-terminal 6 amino acids as well as the termination codon of the *tk* gene was replaced by a small segment of DNA coding for 3 amino acids. This sequence was fused in frame to the *neo* coding region starting from the translation initiation codon. Thus, TNFUS 69 contains only one translation termination codon, at the end of the *tk–neo* fusion gene. In the TNSSS 31 (*tk–neo* stop-stepback-start) plasmid, the *tk–neo* junctional region was further modified so that the last 6 amino acids of the *tk* gene were replaced by a DNA segment coding for 20 new amino acids. In addition, the junction between the coding

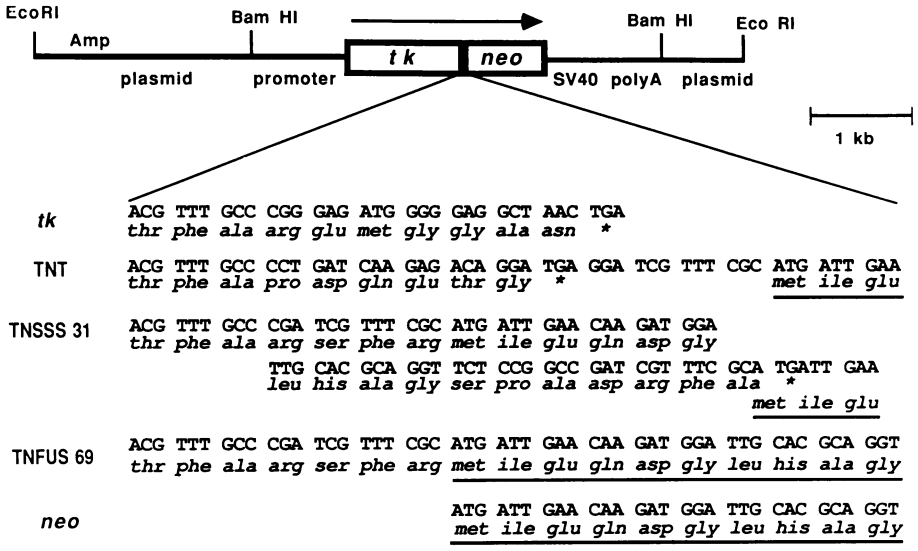

FIG. 1. Junction region between the *tk* and *neo* genes in the TNT, TNSSS 31, and TNFUS 69 plasmids. The top line indicates the basic structure of the three fusion plasmids (see text for details). *tk*, The DNA sequence of the 3′ end of the *tk* gene and the amino acids it codes for; *neo*, the 5′ end of the *neo* gene and the amino acids it codes for. The DNA sequence and the corresponding amino acid sequences of the region at the junction of *tk* and *neo* in the three fusion plasmids are shown. kb, Kilobase.

sequences for the *tk* and *neo* genes is such that 4 nt code for the termination codon of the *tk* and for the initiation codon of the *neo*. These 4 nt are ATGA; the TGA triplet in this sequence serves as the termination codon for the *tk*, and the ATG triplet serves as the initiation codon for *neo*. Thus, the *neo* sequence is in the −1 reading frame with respect to the *tk* sequence.

Fusion Genes Are Functional in Mammalian Cells. Each of the three modified plasmids was introduced into mouse LMTK$^-$ cells to test for the expression of *tk* and *neo* genes. Two plasmids, pHSV106 and pSV2neo, were used as controls. Half of the transfected cell population was selected in HAT medium, and the other half was subjected to selection with G418. Results from these experiments are shown in Table 1. The data show that the *tk* gene in TNT did not function and that the *neo* gene in this plasmid functioned poorly. The carboxyl-terminal end of the TK protein is known to be necessary for its function (16); our data suggest that replacement of the last seven amino acids of the TK protein produces a nonfunctional protein. Transfection of cells with TNSSS 31 plasmid yielded HAT^R and G418^R colonies (Table 1), but the TNFUS 69 plasmid yielded the highest frequencies of HAT^R and G418^R colonies (Table 1).

Since mouse ES cells are being extensively used for gene transfer and genetic manipulation, we tested the transfection efficiencies of TNFUS 69 and a modified version of TNFUS 69 containing a polyoma enhancer, designated TNFUS 69PY in these cells. The results are shown in Table 1. TNFUS 69 and TNFUS 69PY gene transfection efficiencies compare favorably with those obtained with pMClneopolA (17).

For fusion genes to be useful in positive and negative selection, cells carrying them must express TK and NEO functions simultaneously. We ascertained this feature by taking colonies obtained in each of the two selection systems (HAT and G418) with each of the TNSSS 31 and TNFUS 69 plasmids and testing them for cross resistance. We observed that cells isolated in HAT alone were capable of growing in G418 or in HAT plus G418, and all of the cells initially selected in G418 were capable of growth in HAT or in G418 plus HAT.

Fusion Genes Produce Fusion Transcripts. The structures of the fusion genes in TNT and TNSSS 31 are such that although a single transcript encoding the *tk* and *neo* should be produced, translation of the transcript leads to discrete TK and NEO proteins. In TNFUS 69, however, the single transcript generated by using the *tk* promoter is expected to yield a single fusion polypeptide. To test these expectations, we examined cells stably transfected with TNFUS 69 for a fusion transcript and for a fusion protein. The fusion gene should produce a single transcript that will hybridize to *tk*-specific and *neo*-specific DNA probes. To detect such a transcript, total RNA from four independent cell lines derived from the use of TNFUS 69 was fractionated on agarose gels and hybridized with *tk*- and *neo*-specific DNA probes. Results of an experiment of this type are presented in Fig. 2. Fig. 2*a*, lane A, contains RNA from a cell line transfected with a plasmid containing only the *tk* gene. This lane reveals a 1300-nt-long transcript that hybridizes to the *tk*-specific probe. Lanes B–E contain RNA isolated from the four independent TNFUS 69 transfectants. All of these cell lines contain a single 3200- to 3300-nt-long transcript that hybridizes to the *tk* probe. Results of hybridization of an identical filter to a *neo* probe are shown in Fig. 2*b*. The *neo* probe does not hybridize to the message produced by the cell line transfected with a *tk* (lane A), but it does hybridize to an 1840-nt transcript in cells transfected with a plasmid containing only a *neo* (lane F). The *neo* probe also hybridizes to a transcript in lanes B–E whose mobility is identical to that detected with the *tk* probe. Since the predicted size of the fusion transcript is 3240 nt, the observed size of 3200–3300 nt is consistent with the size expected for the *tk–neo* fusion transcript. These results thus indicate that the TNFUS 69 construct, when transfected into cells, produces a transcript that jointly carries *tk*- and *neo*-specific RNA sequences and is larger than either authentic *tk* or *neo* transcripts.

Table 1. Efficiency of transfection with *tk–neo* fusion plasmids

Plasmid	HAT^R		G418^R	
	Colonies/ μg*	Colonies per μg†	Colonies/ μg*	Colonies per μg†
pHSV106	750/10	75	—	—
TNT	0/8	0	1/8	0.125
TNSSS 31	14/4	3.5	23/4	5.75
TNFUS 69	240/28	8.57	1656/28	59.14
pSV2neo	—	—	800/10	80
pMClneopolA‡			955/25	38.2
TNFUS 69‡			1319/25	52.8
TNFUS 69PY‡			1256/25	50.2

R, Resistant.
*Total number of colonies/total amount of DNA used.
†Colonies obtained/μg of input DNA.
‡Transfections into a mouse ES cell line.

A TK–NEO Fusion Protein Is Made in TNFUS 69 Transfectants. Since the TNFUS 69 plasmid contains only one translational stop codon, at the end of the *neo* gene, it is expected that its transcript will be translated into one TK–NEO fusion protein. To test this prediction, proteins were extracted from a HAT^R, G418^R cell line obtained with the TNFUS 69 plasmid, separated by electrophoresis through polyacrylamide gel, and examined by Western blot analysis using antisera directed against either the HSV I TK protein or the NEO protein. Results of this analysis are shown in Fig. 3. Using anti-HSV I TK antiserum, a specific protein band of ≈66 kDa was detected (Fig. 3, lane A). The molecular mass of this protein corresponds to the expected size of the fusion protein, as deduced from the nucleotide sequences of the TNFUS 69 plasmid. Using antiserum against the NEO protein, a specific protein band of the same molecular mass was detected (Fig. 3, lane C). A protein band of this size was not detected with either antiserum in cell lysates from LMTK$^-$ cells that had been transfected (Fig. 3, lanes B and D). These results indicate that a single fusion TK–NEO protein is made from the TNFUS 69 plasmid.

The fact that cells transfected with TNFUS 69 can be selected efficiently in medium containing G418 is a clear indication that this gene can be used as a dominant selectable marker. The expression of HSV *tk* can be efficiently selected

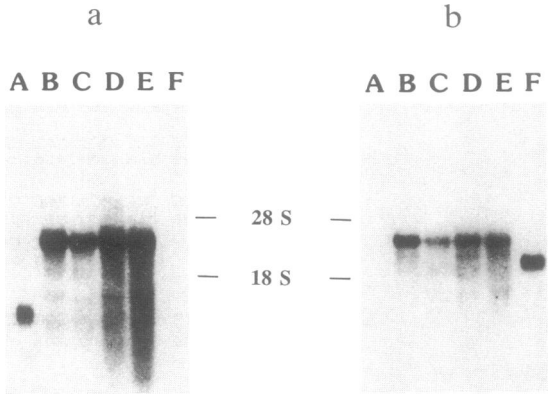

FIG. 2. Transcripts produced by TNFUS 69. Total RNA from four independent TNFUS 69 transfectants was electrophoresed and hybridized to *tk* (*a*) or *neo* (*b*) probes. Lanes A, RNA from a pHSV106 transfectant; lanes B–E, RNA from TNFUS 69 transfectants; lane F, RNA from a pSV2neo transfectant.

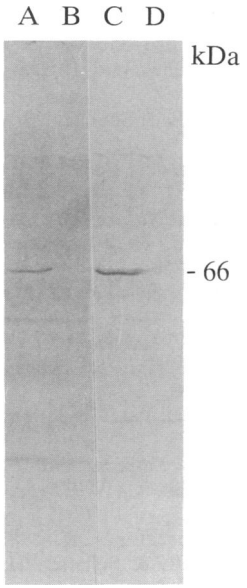

FIG. 3. Western blot analysis of the TK–NEO fusion protein. Total proteins were extracted either from HAT^R, G418^R cells obtained after transfection with the TNFUS 69 plasmid (lanes A and C) or from LMTK$^-$ cells that had not been transfected (lanes B and D). Proteins were electrophoresed through polyacrylamide gel, transferred to a nitrocellulose filter, and analyzed by Western blot. Samples in lanes A and B were incubated with antiserum prepared against the HSV I TK protein. Samples in lanes C and D were incubated with antiserum directed against the NEO protein. Antigen–antibody complexes were visualized with the use of goat anti-rabbit IgG coupled to horseradish peroxidase.

against by the use of acyclovir or ganciclovir (5). Our observation that the TNFUS transfectants are efficiently expressing the HSV *tk* gene product is an indication that the fusion gene can be selected against.

DISCUSSION

In an effort to generate genetic markers whose expression can be selected for and against in mammalian cells, we have tested three different constructs involving fusions between the HSV-*tk* and the bacterial *neo* genes. Of the three constructs tested, TNFUS 69 best fulfills the requirements for an effective positive and negative selectable gene. In this construct the fusion gene is expressed using the HSV *tk* promoter and has an in-frame fusion between the *tk* and *neo* gene sequences. We have shown that cells transfected with this fusion gene yield G418^R colonies at efficiencies that compare favorably with those obtained by the use of pSV2neo or pMClneopolA. The fusion gene yields a transcript of about 3200 nt that hybridizes to *tk*- as well as *neo*-specific probes. The size of the transcript corresponds very closely to the expected fusion transcript. We have also shown that cells made G418^R with TNFUS 69 are also expressing the HSV-*tk* gene by demonstrating that the cells can survive in HAT or in HAT plus G418. Further support for the view that the fusion gene is functioning as a single unit comes from the demonstration that antibodies against the authentic *neo* and HSV-*tk* gene products recognize a polypeptide that is of the same size expected from the fusion product.

There are several other genes whose expression can be selected for or against. The most well known of these are *hprt*, *tk*, and *aprt*. However, positive selection for these genes can be achieved only in cells that are mutant at the appropriate locus. On the other hand, positive selection for bacterial genes such as Eco-gpt and nptII (*neo*) inserted into mammalian expression vectors can be achieved, but no suitable negative selection systems for these genes are available. In contrast, the *tk–neo* fusion we describe here can be selected for or against in any cell type.

The use of a fusion gene for positive and negative selection schemes has significant advantages over the use of a plasmid that carries the two genes as separate cassettes. Transfection with a two-cassette plasmid followed by selection for one of the two genes does not require that the second gene be active. Thus, the gene that is not selected can accumulate mutations during the course of cell culture, which will lead to a high background when negative selection is applied. The *tk–neo* fusion gene that we have described here helps avoid this shortcoming. By designing the fusion protein structure so that the first selected activity (the dominant positive function) is located distally to the second selectable marker (the dominant negative function), problems due to mutations in the negative selectable gene are much reduced. Strategies similar to the ones described here may be useful in constructing additional fusion genes such as between the hygromycin-resistance gene and the HSV-*tk* gene. Several points need to be kept in mind in constructing such fusion genes. Most importantly, it should be noted that not all fusions will yield bifunctional proteins. Amino-terminal fusions with the *neo* gene have been studied by Reiss *et al.* (18). These investigators constructed several plasmids in which the amino-terminal region of the NEO protein, which was lacking the ATG translation initiation codon, was fused to foreign DNA sequences coding for 3–300 amino acids and looked for the expression of the fused proteins in *E. coli*. They found that all amino-terminal fusions examined conferred kanamycin resistance to bacteria (equivalent to G418 resistance in mammalian cells) but that the level of this resistance varied depending on the length of the added sequence. In general, short fusions induced high levels of resistance, whereas longer fusions showed only moderate to low kanamycin resistance. Furthermore, they found that most of the longer fusion proteins were not enzymatically active as such but had to be converted into active products by proteolytic cleavage. In addition, they found that not only the length but also the particular structure of the added sequence affected the enzymatic activity of the NEO moiety. Similarly, a number of different fusion constructs between polyoma middle tumor antigen and *neo* coding sequences had to be tested to find a suitable fusion gene that works in mouse cells (19). The results of our experiments with the TNFUS 69 plasmid indicate that amino-terminal fusions with the NEO protein can result in biologically active molecules in mammalian cells. In the TNFUS 69 plasmid, 480 amino acids of the TK protein are fused to the amino terminus of the NEO protein. Despite the length of the fusion, the resulting TK–NEO fusion protein has both enzymatic activities.

The fusion gene TNFUS 69 can be used for several purposes. For example, introduction of the fusion gene into each of the human chromosomes in different isolates derived from a normal diploid human cell line (by transfection and subsequent generation of somatic cell hybrids) could allow the selective retention and subsequent loss of individual human chromosomes.

Fusion genes can also play an important role in gene targeting by homologous recombination. Valencius and Smithies (20) and Hasty *et al.* (21) have described strategies to obtain subtle modifications in mouse genes by an in-out homologous recombination procedure. In one case (20), the vector contained a portion of the *hprt* gene able to correct a partial deletion of the *hprt* in the recipient *hprt*$^-$ ES cells. The vector also contained a 4-base-pair insertion. The "in" step was used to introduce the vector sequences into the *hprt* locus by gene targeting. In the second "out" step, homologous recombination (probably intrachromosomal) permitted loss of the input vector. Since the recipient cells used were

hprt$^-$, the in step was made possible by selecting for the *expression* of the *hprt* gene in HAT medium; the out step was achieved by selecting for the *loss* of *hprt* expression by use of 6-thioguanine-containing medium. The use of the *hprt* as the dominant and negative selectable marker requires that the target cells be *hprt*$^-$. The fusion gene that we have described here can be used for comparable experiments in which wild-type cells are the targets for the modification. In the second case (21), a similar in-and-out strategy involving a vector that contains separate positively and negatively selectable genes was utilized. The fusion gene that we describe here could be used for similar purposes.

While this manuscript was in its final stages of preparation, a report describing the use of a fusion gene between hygromycin phosphotransferase and HSV tk for positive and negative selections has been published (22).

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