Selective disruption of genes expressed in totipotent embryonal stem cells

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Two retrovirus promoter trap vectors (U3His and U3Neo) have been used to disrupt genes expressed in totipotent murine embryonal stem (ES) cells. Selection in L-histidinol or G418 produced clones in which the coding sequences for histidinol-dehydrogenase or neomycin-phosphotransferase were fused to sequences in or near the 5' exons of expressed genes, including one in the developmentally regulated *REX-1* gene. Five of seven histidinol-resistant clones and three of three G418-resistant clones generated germ-line chimeras. A total of four disrupted genes have been passed to the germ line, of which two resulted in embryonic lethalties when bred to homozygosity. The ability to screen large numbers of recombinant ES cell clones for significant mutations, both in vitro and in vivo, circumvents genetic limitations imposed by the size and long generation time of mice and will facilitate a functional analysis of the mouse genome.

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The long reproduction cycles and large genomes of mammals generally preclude the types of genetic analysis possible with simpler organisms. Although genes responsible for organismal phenotypes can be isolated, the process is slow, requires relatively detailed physical maps, and is limited to a small number of mutant alleles (Reith and Bernstein 1991).

Alternative approaches may circumvent some of these limitations. Particularly promising has been the use of embryonal stem (ES) cell lines to construct strains of mice in which genes have been mutationally disrupted. ES cells, derived from the inner cell mass of mouse blastocysts, can be cultured for extended periods while maintaining a totipotent differentiation potential (Evans and Kaufman 1981; Martin 1981; Doetschman et al. 1985). Early embryos injected with ES cells are able to develop as chimeras and transmit genes from the ES cells to their transgenic offspring.

Although it is possible to target genes by homologous recombination (for review, see Capecchi 1989; Kessel and Gruss 1990), it is also desirable to isolate previously unknown genes responsible for recessive phenotypes, because genes required for biological function generally cannot be predicted in advance. One approach has used exogenous DNA or retroviruses as insertional mutagens

(Schnieke et al. 1983; King et al. 1985; Stewart et al. 1985; Sanes et al. 1986; Soriano et al. 1987; Kratochwil et al. 1989; Spence et al. 1989; Weiher et al. 1990), in which genes associated with recessive phenotypes can be cloned from sequences near the sites of integration. Unfortunately, the probability that a recessive phenotype will result from DNA or provirus integration is low, 10% and 5%, respectively (Gridley et al. 1987; Jaenisch 1988).

To increase the efficiency of insertional mutagenesis, several types of vectors have been developed that select for integrations in expressed genes. One strategy involves inserting a selectable marker gene downstream of a splice acceptor site. Integration of the vector, delivered either by DNA transfer or retrovirus infection, into introns of expressed genes allows expression of the marker gene (Brenner et al. 1989; Gossler et al. 1989; Friedrich and Soriano 1991). A second strategy involves inserting a selectable marker gene into U3 (von Melchner and Ruley 1989; von Melchner et al. 1990; Reddy et al. 1991). When the viruses are passaged, the elongated U3 regions form part of the long terminal repeats (LTRs) that flank the integrated provirus. As a result, coding sequences in the 5' LTR are placed only 30 nucleotides from the flanking cellular DNA. Selection for U3 gene expression invariably generates cell clones in which the gene in the 5' LTR is expressed on transcripts initiating in the flanking cellular DNA. Cellular promoters that activate U3 gene expression are typically expressed prior to integration; thus, the viruses function as promoter traps (von Melchner et al. 1990).

In this study we demonstrate that the U3His and

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U3Neo promoter trap vectors can be used to selectively disrupt genes expressed in totipotent ES cells. Of 10 ES cell lines tested that expressed cell-virus fusion genes, 8 generated germ-line chimeras, including one involving the disruption of a known developmentally regulated gene. The results demonstrate that in principle, large numbers of recombinant clones recovered after promoter trap selection can be screened both in vitro and in vivo for genetically significant mutations, thus facilitating a functional analysis of the mouse genome.

Results

Construction of the U3Neo promoter trap vector

The U3Neo vector was derived from pGgTKNeo-U3His(-) (von Melchner and Ruley 1989) by replacing the *HisD* gene with the neomycin-phosphotransferase (*neo*)-coding sequence derived from Tn5 and deleting the TkNeo from the body of the virus (Fig. 2, below). Cell lines producing ecotropic U3Neo viruses were generated by transfecting 20 µg of pGgU3Neo(en-) into Ψ2 helper cells. Viruses produced from cloned producer lines were titered on NIH-3T3 cells by selecting in G418. Because the U3Neo vector lacked a constitutively expressed drug resistance marker, titers were derived by multiplying the number of neomycin resistance (Neo^r) colonies with the average frequency of integrations that enable U3 gene activation of other promoter trap vectors (von Melchner and Ruley 1989; von Melchner et al. 1990; Reddy et al. 1991).

Selection for U3His and U3Neo gene fusions in cultured ES cells

ES cells are susceptible to retrovirus infection and can be used to introduce integrated proviruses to transgenic offspring (Evans et al. 1985; van der Putten et al. 1985; Robertson et al. 1986; Stewart et al. 1985). However, the proviral LTRs are transcriptionally inactive; therefore, drug resistance genes expressed from the LTR are transduced into ES cells 10⁴–10⁶ times less efficiently than into murine fibroblasts. Moreover, the polyadenylation signal in the LTR and binding sites for *trans*-acting repressors interfere with the activation of proviral genes by transcriptional elements in the flanking cellular DNA (Sorge et al. 1984; van der Putten et al. 1985; Barklis et al. 1986; Feuer et al. 1989; Tsukiyama et al. 1989; Loh et al. 1990; Akgün et al. 1991). Consequently, the proviruses in rare resistant clones contain deletions or have integrated in regions near strong transcriptional promoters where LTR sequences are removed by RNA splicing (Barklis et al. 1986; Peckham et al. 1989).

Nevertheless, we assumed that the U3His and U3Neo vectors would usurp cellular promoters in ES cells as efficiently as in NIH-3T3 cells, because integration fuses the histidinol-dehydrogenase (*his*) or *neo* genes directly to the cellular DNA, outside of the viral sequences that otherwise interfere with activation (von Melchner et al. 1990). This assumption proved correct with the U3His

virus, as similar numbers of histidinol-resistant (His^r) (40/10⁶ ES-D3 and 122/NIH-3T3 cells, respectively) colonies arose in both cell types, following infection with 10⁶ Neo^r transducing viruses. However, although similar numbers of Neo^r colonies were obtained after infecting ES-D3 cells with U3Neo viruses, transduction of Neo^r to NIH-3T3 cells was 100-fold more efficient. This is because transcripts initiating in the 5' LTR and extending through the 3' *neo* gene are expressed in 3T3, but not in ES, cells (see below).

The response of ES cells to selection in 3.5 mM L-histidinol was complex (Fig. 1). Many cells appeared to differentiate, forming an adherent fibroblast-like monolayer. In cultures infected with the U3His virus, two types of His^r colonies developed after 14 days of selection. The first type resembled adherent embryoid bodies containing peripheral endodermal cells separated from a central core of undifferentiated cells (Robertson 1987). In some cases, cells in the interior of the colony died, leaving a ring of differentiated cells. The second type (10–15% of all colonies) resembled rapidly proliferating undifferentiated stem cells. Only colonies of this type were expanded for further analysis. In contrast, G418 selection was more efficient. Thus, after 6 days in G418, only rapidly proliferating undifferentiated colonies survived.

Southern hybridization analysis of His^r and Neo^r clones indicated that in all cases *his* or *neo* sequences had duplicated as part of the LTR (data not shown). Most His^r clones contained one or two proviruses, consistent with the estimated multiplicity of infection (m.o.i.) (data not shown). However, each Neo^r line contained one provirus per cell indicating that the actual m.o.i. was <1. Rearrangements of proviral sequences were observed in 5 of 15 His^r and 1 of 10 Neo^r lines. In the two His^r clones examined further, the body of the provirus was deleted as a result of homologous recombination between the elongated LTRs. This is a higher rate of deletion than has been observed for U3His in NIH-3T3 cells and may reflect selection against a transcriptional silencer located in the tRNA primer-binding site (Feuer et al. 1989; Loh et al. 1990; Petersen et al. 1991).

To determine whether transcripts in His^r and Neo^r clones initiate within the flanking cellular DNA, total RNA was analyzed by an RNase protection assay (von Melchner and Ruley 1989). As shown in Figure 2, all clones expressed transcripts through the left (5') LTR, indicating that U3 gene expression was activated by flanking cellular promoters. Unlike NIH-3T3 cells, transcripts extended only occasionally through the right (3') LTR (e.g., clone 1B6; Fig. 2) because the LTR and thymidine kinase (*tk*) promoter are transcriptionally inactive in ES cells (Pellicer et al. 1980; Jahner et al. 1982; Stewart et al. 1982; Gautsch and Wilson 1983; Niwa et al. 1983; Stewart et al. 1985).

Sequences adjacent to U3His and U3Neo proviruses hybridize to single-copy DNA and contain transcribed exons

DNA was extracted from His^r and Neo^r cell lines that

Figure 1. ES cell cultures after 14 days in 3.5 mM L-histidinol at 100 $\times$ magnification. (A) Undifferentiated colony; (B) differentiated cells surrounding the area of detached cells; (C) simple embryoid bodies.

contained a single provirus (Table 1). Sequences upstream of the proviruses were amplified by inverse polymerase chain reaction (PCR) (von Melchner et al. 1990) and cloned. Each of the flanking sequences hybridized to a single restriction fragment present in ES cell DNA and thus were derived from single-copy cellular DNA (Table 1). As expected, an additional hybridizing fragment was observed in DNA from the appropriate His^r or Neo^r line, corresponding to the allele occupied by the provirus (data not shown).

Five flanking sequence probes (4A1, 4A3, 4A7, 1A4, and 1B4) hybridized to cellular transcripts in uninfected cells (Table 1; Fig. 3). This indicates that promoters were active prior to integration and were not activated as a result of integration. In the appropriate provirus-containing cells, each probe detected an additional RNA species that also hybridized to *his* (4A1, 4A3, and 4A7) or *neo* (1A4 and 1B4), as expected of fusion transcripts expressed by the allele occupied by the provirus (Fig. 3B).

his and *neo* transcripts in each clone varied in size, reflecting differences in the amount of appended cellular RNA (Fig. 3B). Cell-derived sequences were short, presumably because efficient translation would select for integrations in which the U3 gene provides the first initiation codon in the fusion transcript and the untranslated leader of the average mammalian transcript is only ~75 nucleotides (Kozak 1980; Mulligan and Berg 1981; Peabody et al. 1986; Kaufman et al. 1987; Kozak 1987b). For this reason some upstream sequences may have been too short to detect corresponding transcripts (Table 1). For example, PCR products cloned from the 1A4 cell line contained only 38 nucleotides of flanking sequence and initially did not detect cellular sequences in either Southern or Northern blots. However, satisfactory probes were prepared (Fig. 3, and data not shown) after labeling a flanking sequence oligonucleotide with terminal transferase (Johnson 1991).

Disruption of the developmentally regulated REX-1 gene

Computer analysis of upstream flanking sequences revealed that a 59-nucleotide region flanking one provirus (4A1) was identical to 5'-noncoding sequences within the cDNA of a previously characterized gene, *REX-1* (Fig. 4) (Hosler et al. 1989). *REX-1* is highly expressed in embryonal carcinoma cells and is repressed during differentiation. The *REX-1* protein contains four zinc finger motifs and a highly acidic domain, suggesting a possible function as a transcription factor.

The 4A1 and *REX-1* sequences diverged at the boundary between the third exon and adjacent intron (L. Gudas, pers. comm.). The 4A1 flanking sequence detected a single 1.8-kb RNA expressed in D3 cells, (the *REX-1* transcript) and an additional 2.1-kb transcript in 4A1 cells (Fig. 3A). The larger transcript also hybridized to *his* and corresponded to a fusion transcript in which 275 nucleotides of cellular RNA was appended to U3His, as predicted from the structure of the U3His-*REX-1* fusion gene (Fig. 3B). These results support the notion that

Figure 2. Ribonuclease protection analysis of provirus transcripts. Structure of U3His (A) and U3Neo (B) proviruses. (His-D) histidinol-dehydrogenase-coding sequences; (–enh) deletion in U3 removing the viral enhancer; (tk) HSV-2 thymidine kinase promoter; (Neo) neomycin phosphotransferase-coding sequences; (U3RU5) regions of the viral LTR. Fragments protected at the 5' [RT (readthrough transcripts)], and 3' ends of the provirus are indicated below each provirus. Lanes in C are designated as follows: (Probe) Native probe; (tRNA) tRNA control; (P93T3) His^r NIH-3T3 clone; (4A1–6A3) His^r ES cell clones. Lanes in D are designated as follows: (Probe) Native probe; (D3-3) His^r ES cell clone; (1A4, 1B4, 1B6) Neo^r ES cell clones. Size markers at left were estimated from the relative mobilities of radiolabeled 1-kb ladder (BRL) and pBR322 *Msp*I restriction fragments.

U3 gene expression is activated by integrations within or near untranslated 5' exons.

The body of the 4A1 provirus was deleted by homologous recombination between the LTRs (Fig. 4). Sequences downstream of the provirus appeared conserved because hybridization to *REX-1* cDNA probes failed to detect gross sequence rearrangements or deletions downstream of the provirus integration site. Furthermore, the size of the restriction fragment occupied by the provirus was exactly the size expected for a single U3His LTR (data not shown).

Germ-line transmission of genes disrupted by U3His or U3Neo proviruses

C57BL6 blastocysts were injected with His^r and Neo^r ES cells and transferred to pseudopregnant CD1 foster mothers (Hogan et al. 1986). When the resulting male chimeras were mated, a high incidence of germ-line transmission (five of seven His^r and three of three Neo^r lines tested) was observed (Table 1), indicating that infection and selection in histidinol or G418 did not affect stem cell totipotency. However, except for 4A1 and 4A4,

chimeras derived from His^r lines produced only a few Agouti offspring, and only one U3His fusion gene was passed into the germ line. In contrast, all U3Neo fusion genes were transmitted to transgenic offspring (Table 1).

Of the four disrupted genes introduced into the germ line, three were autosomal and two (4A4 and 1B6) resulted in embryonic lethality when bred to homozygosity (Table 2). The embryos appeared to die after implantation because approximately one-fourth of the fetuses were in the process of being reabsorbed by day 8–9 postcoitum. One cell line (1B4) generated offspring homozygous for the disrupted allele; however, no obvious abnormality was observed. The fourth disrupted gene (1A4) was X-linked. However, both males and females homozygous for the disrupted gene were apparently normal (Table 2).

Coincidentally, the 4A4 and 1B6 inserts that generated lethal phenotypes did not hybridize to cellular transcripts (Table 1), whereas cellular transcripts were detected with sequences flanking the 1A4 and 1B4 inserts (Fig. 3). Thus, the mutagenic potential of the viruses seems extremely high, as judged by both molecular and phenotypic criteria.

Table 1. Summary of results obtained with ES cell lines and cloned flanking sequences

ES cell line	U3 gene	Provirus flanking DNA (nucleotides) ^a	Hybridization to cellular transcripts	Germ-line transmission	Fusion-gene inheritance
4A1	<i>his</i>	315 (M)	+	+	— (0/20)
4A3 ^b	<i>his</i>	615 (M)	+	+	— (0/1)
4A4	<i>his</i>	300 (H)	—	+	— (0/1)
4A7	<i>his</i>	185 (M)	+	+	— (0/2)
5A4	<i>his</i>	90 (H)	—	+	— (0/6)
6A3	<i>his</i>	410 (H)	—	—	—
1A4	<i>neo</i>	38 (H)	+	+	— (0/2)
1B4	<i>neo</i>	275 (H)	+	+	— (0/6)
1B6	<i>neo</i>	245 (M)	—	+	— (0/6)
					+

Genomic DNAs from His^r Neo^r cell lines were digested with *Mse*I (M) or *Hinf*I (H) and ligated at concentrations of 5 µg/ml to obtain circular molecules. After cleavage with *Pvu*II, 1 µg of DNA from each sample was used for PCR as described in Materials and methods. Numbers in the last column indicate the frequency of fusion gene inheritance and the number of Agouti offspring tested.

^aSouthern blot analysis revealed that all flanking sequences hybridized to a single restriction fragment in ES cell DNA

^bA sibling of clone 4A3 was isolated from the same culture dish and failed to contribute to the germ line.

Finally none of 20 Agouti offspring analyzed inherited the U3His-*REX-1* fusion gene (Table 1). The reasons for this are not known but several possibilities merit consideration. First, the 4A1 cell line may have been contaminated by other ES cells which contributed to the germline. However, this seems unlikely, because each of

the 10 subclones analyzed carried the U3His-*REX-1* fusion gene. Second, since *REX-1* is actively transcribed in developing spermatocyte (Rogers et al. 1991), loss of *REX-1* functions may adversely affect post-meiotic sperm. Alternatively, as with herpes simplex virus (HSV) thymidine kinase (Wilkie et al. 1991), expression of his-

Figure 3. Northern blot analysis of cellular transcripts and cell-virus fusion genes. RNAs (20 µg) from uninfected and virus-infected D3 cell lines were hybridized to ³²P-labeled probes derived from flanking cellular sequences (A) and *his* or *neo* sequences (B). Transcripts from unoccupied (U) and occupied (O) alleles are indicated by arrows. In each case, fusion transcripts (O) comigrated with transcripts in B that hybridized to the appropriate *his* or *neo* probe. Fusion transcripts 1A4 and 1B4 (O) were similar in size to the cellular transcript (U). Mobilities of RNAs are indicated.

tidinol-dehydrogenase in may affect sperm fertility. Finally, transmission of the fusion gene may have been prevented by other chromosomal abnormalities linked to the provirus.

Discussion

Two retrovirus promoter trap vectors have been used to induce mutations in totipotent ES cells. Retrovirus vectors containing *hisD* or *neo* sequences inserted into the U3 region exhibited a normal ability to be passaged to D3-ES cells, generating proviruses flanked by the U3 genes. Selection for *his* or *neo* expression produced cell clones in which the U3 genes in the 5'LTR were expressed on transcripts initiating in the flanking cellular DNA. Most cell lines generated germ-line chimeras, indicating that selection in histidinol or G418 does not adversely affect ES cell totipotency. However, fusion genes obtained following G418 selection were passed to the germ line much more efficiently than U3His fusion genes. Histidinol may induce differentiation of ES cells,

as has been observed with other cell types (Pilz et al. 1987; Nordenberg et al. 1989). Nevertheless, *his* provides an alternative selectable marker in ES cells.

Sequences adjacent to U3His and U3Neo proviruses hybridized to single-copy DNA and frequently contained transcribed exons. One activating integration occurred in the third untranslated exon of the *REX-1* gene. These observations are consistent with the notion that promoter trap vectors select for integrations into 5' regions of genes transcribed by RNA polymerase II. The fact that promoters transcribed by RNA polymerases I and III do not activate U3 gene expression was not unexpected because the resulting fusion transcripts lack 5' caps and are not processed or translated efficiently (Banerjee 1980; Sisodia et al. 1987). In addition, polymerase III transcripts may terminate in short thymidine stretches present in the U3 genes (Bogenhagen et al. 1981).

Promoter trap mutagenesis provides a general method to study gene functions in mice. Several features of the approach are particularly powerful. First, retrovirus integration, unlike transferred DNA, preserves both viral

Table 2. Inheritance of U3His and U3Neo fusion genes

Clone	Number	Inheritance pattern			Phenotype
		heterozygotes	homozygotes (wild type)	homozygotes (fusion gene)	
4A4	21	17	4	0	embryonic death (9/32)
1A4 ^a	male (12) female (11)	male (0) female (3)	male (6) female (0)	male (6) female (8)	male (N.O.) ^a female (N.O.) ^a
1B4	10	2	6	2	N.O. ^a
1B6	52	20	32	0	embryonic death (11/45)

Heterozygous mice derived from the indicated cell lines were mated, and DNA from the offspring was analyzed by Southern blot hybridization. Restriction fragments corresponding to the normal and disrupted alleles were distinguished by hybridization flanking sequence probes. Numbers in parentheses indicate the number of embryos in the process of being reabsorbed per total number of embryos after 8–10 days postcoitum.

^aX-linked; (N.O.) none observed.

and cellular sequences so that the structure of the recombination product is predictable. Second, promoter trap selection favors integrations in or near 5' exons; consequently, 60% of upstream-flanking probes hybridized to transcripts expressed by both the occupied and unoccupied alleles. Thus, as with vectors activated by gene splicing (Brenner et al. 1989; Gossler et al. 1989; Friedrich and Soriano 1991), promoter trap viruses greatly simplify isolating cDNAs of the disrupted genes, as compared with vectors in which expression of a marker gene is activated by transcriptional enhancers (Hamada 1986; Taketo and Tanaka 1987; Gossler et al. 1989). Finally, selection for cells containing fusion genes significantly reduces the number of recombinants needed to screen for loss of gene function, as compared with cells in which viruses have integrated randomly. Two of four disrupted genes that have been passed to the germ line resulted in embryonic death when bred to homozygosity. In short, the mutagenic potential of promoter trap vectors seems extremely high, as judged either by molecular or biological criteria. The frequency of recessive mutations was similar to a recent study by Friedrich and Soriano in which 9 of 24 activating integrations caused recessive lethal mutations (Friedrich and Soriano 1991). Further analysis will be required to establish whether disrupted genes identified by molecular means generate subtle phenotypes as is frequently observed after specific genes have been targeted (Zijlstra et al. 1989; Zijlstra et al. 1990; Schorie et al. 1991; Soriano et al. 1991).

Two considerations suggest that most expressed genes can be targeted by promoter trap selection. First, the number of sites in the genome that can activate U3 gene expression, as judged either by the fraction of proviruses that express U3 genes (von Melchner and Ruley 1989; Reddy et al. 1991) or by the frequency with which haploid genes can be targeted following promoter trap selection (W. Chang, S.C. Hubbard, C. Friedel, and H.E. Ruley, in prep.), is similar to the total number of expressed genes (2×10^4 to 4×10^4), as judged by RNA renaturation kinetics (Lewin 1975). Second, genes displaced from their promoters as a result of virus integration are functionally inactivated (W. Chang, S.C. Hubbard, C. Friedel, and H.E. Ruley, in prep.). However, some genes may be easier to disrupt than others because integration may be biased for certain regions (Rohdewohld et al. 1987; Shih et al. 1988; Peckham et al. 1989; Scherdin et al. 1990), and regulated genes generally have longer untranslated leaders than widely expressed, housekeeping genes (Kozak 1987a).

These features of promoter trap mutagenesis make it feasible to screen a large collection of recombinant clones for disruptions of interesting genes before constructing strains of mice that harbor particular mutant alleles. For example, 5'-flanking regions can be isolated rapidly by inverse PCR and analyzed to determine whether the disrupted genes (1) have been cloned previously (e.g., *REX-1*), (2) map near important loci or chromosomal regions, and (3) are expressed in a tissue-specific manner. Although promoter trap mutagenesis is

presently limited to genes expressed at the time of virus integration, other vectors (e.g., U3lacZ) and improved methods to induce ES cell differentiation in vitro will simplify recovering mutations in developmentally regulated genes.

As physical maps of the mouse genome become more detailed and additional genes are characterized, collections of characterized ES cell clones will provide an increasingly important resource. For example, representation of a newly discovered gene in a library of ES cell integrants could eliminate the necessity of targeting the gene separately by homologous recombination. Finally, the efficiency with which disrupted loci can be introduced into the germ line makes it feasible to identify biologically significant genes directly, by assessing the phenotypes of mice homozygous for the disrupted alleles.

Materials and methods

Plasmids

pGgU3Neo^r(-) was derived from pGgU3TkNeoU3Hisen(-) (von Melchner and Ruley 1989) by replacing the *hisD* gene with a 900-nucleotides fragment containing the *neo*-coding sequence. The *neo* sequences were amplified by PCR from pGgU3TkNeoU3Hisen(-).

Viruses and cell cultures

ES-D3 cells (1×10^6) were infected with the U3His virus at a m.o.i. of 1 Neo^r CFU/cell (as assayed on NIH-3T3 cells), as described previously (von Melchner and Ruley 1989). ES cell clones surviving selection in 3.5 mM histidinol were isolated after 14–15 days and expanded on irradiated (3.2 Gy) mouse embryo fibroblast (MEF) feeder layers (prepared from 16-day-old embryos) in the absence of L-histidinol. The growth medium for ES cells was Dulbecco's modified Eagle medium (DMEM) (GIBCO) supplemented with 15% preselected and heat-inactivated fetal calf serum, 100 mM nonessential amino acids (GIBCO), 0.1 mM β -mercaptoethanol (Bio-Rad), 5 mg/ml of penicillin and streptomycin, and 10^3 U/ml of leukemia inhibitory factor (LIF) (Esgro[®]; Amrad, Australia).

Amplification and cloning of upstream sequences

Genomic DNAs from His^r and Neo^r cell lines were digested with *Mse*I(M) or *Hinf*I(H) and ligated at concentrations of 5 μ g/ml to obtain circular molecules. After cleavage with *Pvu*II, 1 μ g of DNA from each sample was used for PCR as described previously (von Melchner et al. 1990). Oligonucleotide primers complementary to *his* (5'-CCAGTCAATCAGGGTATTGA-3' and 5'-GTAAGCTTTAAACAGAAGTGACAGCGCTACGG-3' for *Mse*I circles; 5'-CCAGTCAATCAGGGTATTGA-3' and 5'-GTAAGCTTTGCAGGAAATCTTAACGTCGGCGG-3' for *Hinf*I circles) or *neo* (5'-CCATCTTGTTCATCATGC-GAAACGATCC-3' and 5'-GTAAGCTTGCTCCCGATTCC-CAGCGCATCGCCTT-3' for *Mse*I circles; 5'-CCATCTTGT-TCAATCATGCGAAACGATCC-3' and 5'-GTAAGCTTGC-GATGCCTGCTTGCCGAATATCATG-3' for *Hinf*I circles) sequences were used. Gel-purified PCR products were cleaved with *Nhe*I and *Hind*III and ligated to Bluescript(KS-) (Stratagene) plasmids digested with *Xba*I and *Hind*III. A 38-nucleotide synthetic oligonucleotide complementary to the 1A4 flanking

sequence was labeled by terminal transferase as described previously (Johnson 1991).

Nucleic acid hybridization analyses and ribonuclease protection assays

Nucleic acid hybridizations with labeled flanking sequences and ³²P-labeled probes complementary to the U3His provirus-coding strand were prepared as described previously (Reddy et al. 1991). Probes complementary to the U3Neo provirus-coding strand were prepared by using T3 RNA polymerase to transcribe an 820-nucleotide *Bam*HI–*Nae*I fragment of pGgU3Neoen(–) cloned into Bluescript(KS–). Hybridization of RNA samples to antisense RNA probes and RNase digests were performed as described previously (von Melchner and Ruley 1989). Protected fragments were resolved on denaturing 6% polyacrylamide/8.3 M urea gels and visualized by autoradiography.

Construction and analysis of germ-line chimeras

Germ-line contribution of His^r ES-D3 (129; Agouti/Agouti) cell lines was tested by injecting each of 15–20 C57BL6 blastocysts (3.5 days) with 15–20 cells. The blastocysts were implanted into the uterus of pseudopregnant CD1 recipients as described previously (Hogan et al. 1986), and chimeric males were mated with C57BL6 mice. Inheritance of the D3 Agouti coat color marker was observed in 3–100% of the offspring from 50% of the male chimeras.

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