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Germ Line Transmission and Expression of a Corrected HPRT Gene Produced by Gene Targeting in Embryonic Stem Cells

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Summary

The deletion mutation in the HPRT-deficient mouse embryonic stem (ES) cell line E14TG2a has been corrected by gene targeting. The presence of plasmid sequences in the correcting vector DNA did not affect the frequency of correction. We have characterized three different HPRT gene structures in correctants. Cells from one corrected clone have been introduced into mouse blastocysts, and germ line transmission of the ES cell-derived corrected gene has been achieved. The corrected gene has the same pattern of expression as the wild-type gene, with the characteristic elevated level of expression in brain tissue. Hence, we have demonstrated the feasibility of introducing targeted modifications into the mouse germ line by homologous recombination in ES cells.

Introduction

A strategy enabling precise modifications to be made to the mammalian genome would be of benefit both to biological and medical research. It would become possible to manipulate the expression of genes by targeting changes to their control sequences. This would be of value to the study of gene expression. It could also be of potential commercial value if used, for example, in livestock animals to increase output, or produce novel materials. In addition, genes could be inactivated to create animal models for human genetic diseases or to study the action of developmental genes, while the ability to correct mutant genes has implications for gene therapy. An advantage of being able to target modifications is that genes are manipulated in their natural chromosomal environment, whereas the use of conventional methods for introducing DNA sequences into the germ line (Jaenisch, 1988) allows no control over the chromosomal site of integration or the number of copies introduced. At the very least, this complicates the interpretation of gene expression studies and may result in insertion, either into sites that are inappropriate for expression or into essential genes, with deleterious consequences.

Several recent advances have made gene targeting in the mammalian genome possible. Here we report the generation of mice that show germ line transmission of a targeted gene modification. This has been achieved using homologous recombination in mouse embryonic stem (ES) cells.

Mouse ES cells can be cultured in vitro and still retain their pluripotency (Evans and Kaufman, 1981; Martin, 1981). These cells, when reintroduced into mouse blastocysts, can contribute to the germ line of the resultant chimeras (Bradley et al., 1984). ES cells deficient for the enzyme hypoxanthine–guanine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase; EC 2.4.2.8) have been used to produce mice that transmit the same enzyme deficiency through their germ line (Hooper et al., 1987; Kuehn et al., 1987). The pathology of this deficiency is interesting because in humans the result is the severe neurological disorder Lesch–Nyhan syndrome (Lesch and Nyhan, 1964; reviewed in Stout and Caskey, 1988). Consequently, it is noteworthy that, in normal individuals, HPRT expression is elevated in brain relative to other tissues. Hooper et al. (1987) selected for spontaneous HPRT-deficient ES cells in culture. Kuehn et al. (1987) used multiple retroviral infection of cultured ES cells to promote insertional mutation, and subsequently selected for HPRT-deficient clones. Neither procedure abolished the ability of the mutant ES cells to contribute to germ line chimerism.

Gene targeting, mediated by recombination between introduced vectors and homologous chromosomal sequences, has been employed in ES cells both to inactivate gene function (Thomas and Capecchi, 1987) and to correct the activity of a mutant gene (Doetschman et al., 1987). In both cases the target gene was HPRT, which has the advantage that it is possible to select either for, or against, activity while the ES cells are in culture. Additional advantages of the system are that the gene is X-linked and is therefore hemizygous in male cells, and the structure of the gene is well characterized (Melton et al., 1984). In their gene inactivation experiments, Thomas and Capecchi (1987) used vectors containing a modified neomycin resistance (neo^R) gene inserted into the eighth exon of the HPRT gene. The neo^R gene has the dual function of disrupting the HPRT coding sequence and providing an additional selectable marker. Homologous recombination between such a vector and the chromosomal HPRT gene results in the inactivation of the chromosomal gene. Thomas and Capecchi (1987) described targeting vectors as either sequence replacement or sequence insertion vectors, depending on their mechanism of integration, and found that both types worked with similar efficiencies.

We have previously described the targeted correction of an HPRT-deficient ES cell line, E14TG2a, using homologous recombination (Doetschman et al., 1987). E14TG2a was used by Hooper et al. (1987) to produce HPRT-deficient mice. The characterization of the mutation in E14TG2a is presented here. We describe the use of sequence insertion vectors to correct the HPRT deficiency

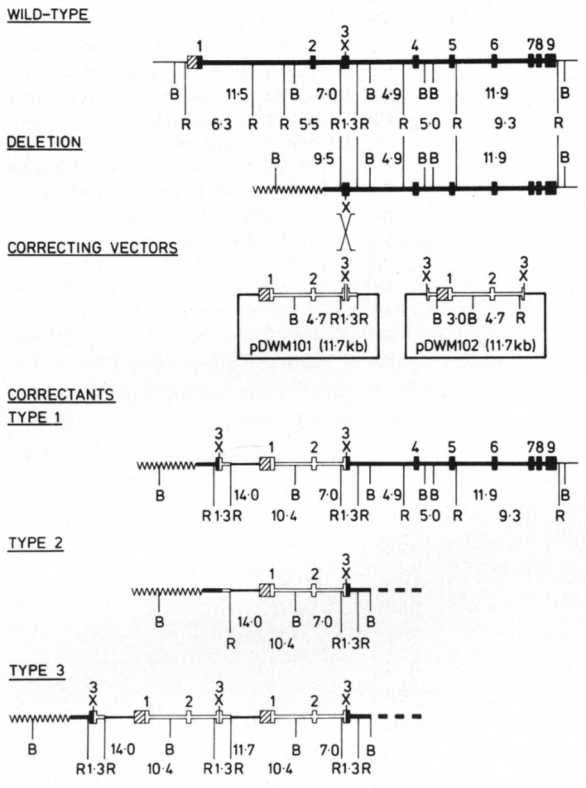

Figure 1. Targeted Correction of the Deletion in E14TG2a Cells and Correctant Structures

The structures of the wild-type HPRT gene, the E14TG2a deletion, the two correcting vectors, and the three correctant types are shown schematically. Closed boxes, endogenous exons; thick closed lines, endogenous introns; cross-hatched boxes, promoter regions; thin lines, HPRT flanking sequences; jagged line, distant flanking sequence brought into proximity as a result of the deletion in E14TG2a; open boxes, vector-derived exons; open thick lines, vector-derived introns; closed intermediate thickness lines, plasmid sequence. The number of each exon is shown directly above it. Selected restriction sites are shown: R, EcoRI; B, BamHI; X, XhoI. The sizes (in kb) of all EcoRI and BamHI restriction fragments containing exon elements are shown between the restriction sites. The thick closed broken line in the type 2 and 3 correctants denotes that beyond exon 3, they have the same structure as type 1 correctants. With the exception of the plasmid in the correcting vectors and the exon elements themselves, the same scale is used throughout.

in E14TG2a, and the identification of three types of corrected clone. Cells from one corrected clone have been injected into mouse blastocysts, and have successfully contributed to the germ line of a chimeric mouse. We have crossed this germ line chimera with HPRT-deficient mice in order to analyze expression from the corrected gene. The corrected gene shows levels of expression equivalent to the normal gene, with the same characteristic elevation in brain.

Results

Characterization of the E14TG2a Mutation

The structure of the wild-type mouse HPRT gene is shown schematically in Figure 1. The coding sequence of the gene is divided into nine exons that span approximately 33 kb of the X chromosome (Melton et al., 1984). We have used Southern blot hybridization to characterize the mutation in the male ES cell line E14TG2a, which is deficient for HPRT enzyme activity. The restriction enzyme sites used in this analysis are shown in Figure 1. Southern hybridization of DNA from the wild-type ES cell line E14 and from E14TG2a, using a full-length mouse HPRT cDNA probe, is shown in Figure 2A. The sizes of the bands are indicated, and the exon elements they contain can be seen in Figure 1. In the EcoRI-digested E14TG2a lane the 6.3 kb band, which contains exon 1, and the 5.5 kb band, which contains exon 2, are both absent. In the BamHI digest of E14TG2a the doublet band, consisting of the 11.5 kb fragment, which contains exon 1, and the 11.9 kb fragment, which contains exons 5 to 9, is reduced in intensity relative to the wild-type band. The 7.0 kb BamHI fragment, which contains exons 2 and 3 of the wild-type gene, is replaced by a 9.5 kb band containing exon 3. From these results we infer that the mutation in E14TG2a is a deletion spanning exons 1 and 2 of the HPRT gene. Using a PvuII digest (data not shown) we have mapped the 3′ end of the deletion to between 1.0 kb and 2.9 kb upstream of exon 3. We have used probes from the region flanking the HPRT gene to show that the 5′ end of the deletion is at least 10 kb upstream of the gene (data not shown).

Since the wild-type allele present in strain 129 mice is *Hprt*^{b}, in accordance with the rules of the International Committee for Standardized Genetic Nomenclature for Mice (Mouse News Letter *72*, 2–27, 1985) we designate the mutant allele present in E14TG2a as *hprt*$^{b\text{-}m3}$.

Correction of the E14TG2a Mutation by Gene Targeting

To correct the mutation in E14TG2a we constructed the sequence insertion vector pDWM101 (see Figure 1 and Experimental Procedures for details of construction). pDWM101 contains mouse exons 1, 2, and 3 and the mouse HPRT promoter. The exon 3 region provides homology with the target locus. The extent of homology between the targeting vector and the genome is between 2.3 kb and 4.2 kb, depending on the precise 3′ endpoint of the deletion. pDWM101 contains 650 bp of upstream flanking sequence, which is sufficient for HPRT expression in cultured cells (Melton et al., 1986). The anticipated outcome of correction has the same structure as the wild-type gene, except that the first intron is reduced from 10.8 kb to 4.1 kb (see Figure 1). The targeted locus has a restriction map distinct from both the wild-type gene and the deletion mutant, and thus enables correctants to be identified by Southern analysis. We have called the predicted structure type one (see Figure 1). In the EcoRI restriction map a novel 10.4 kb fragment is generated, containing exons 1 and 2, and the 1.3 kb band, containing exon 3, is duplicated. In the BamHI restriction map a 14.0 kb fragment, containing exon 1 and the duplicated exon 3, is generated and the 9.5 kb band, containing exon 3, is replaced by the wild-type 7.0 kb band which contains exons 2 and 3. The 7.0 kb and the 14.0 kb bands are the products of one BamHI site in the vector and one BamHI site in the ge-

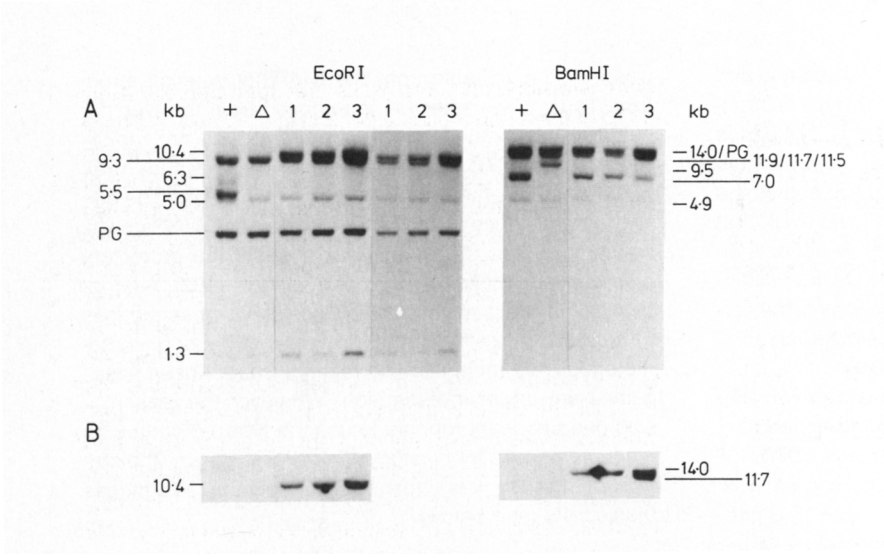

Figure 2. Southern Analysis of E14TG2a and Correctant DNA

Genomic DNA (10 μg) was restricted with EcoRI or BamHI, electrophoresed, transferred, and hybridized. Probes: In (A), a full-length HPRT cDNA probe was used. In (B), the same transfer was reprobed with pUC8. DNA: +, E14; Δ, E14TG2a; 1, a type 1 correctant; 2, type 2-1; 3, type 3-1. Both a long and a short exposure of EcoRI-digested correctant DNA are included in (A). The sizes (in kb) of hybridizing bands are shown adjacent to each panel. The fragments containing the HPRT pseudogene sequences are indicated by PG. The exon elements present in each band can be determined by consulting Figure 1.

nome. While the 7.0 kb band is also present in the wild-type gene, the 14.0 kb band is diagnostic for targeted correctants.

To promote recombination, the vector was linearized at the unique XhoI site in exon 3 prior to being introduced into E14TG2a cells by electroporation. The conditions chosen were those that resulted in minimal cell death in control plating experiments. Correctants were selected in HAT medium. Cells only become HAT^R if the correct targeting event takes place between pDWM101 and the defective HPRT gene. As a positive control E14TG2a cells were electroporated with pDWM100, a fully functional HPRT minigene derivative of pDWM101, which can rescue HPRT-deficient cells without the requirement for homologous recombination.

In the first experiment, which used late-passage stem cells, eight HAT^R colonies were generated from 4.6×10^7 cells electroporated (see Table 1). The ratio of HAT^R colonies obtained from cells electroporated with pDWM101 to HAT^R colonies obtained from cells transformed with pDWM100 was 0.007 (see Table 1, targeting index). This frequency gives an indication of the number of targeting events relative to the number of random integrations, although it does not take into account integrations of pDWM100 into sites that inhibit expression. Southern hybridization showed that seven of the eight HAT^R clones had the structure predicted for type 1 correctants (see Figure 2). In the EcoRI digest the new 10.4 kb band is visible, and the intensity of the 1.3 kb band is doubled with respect to the equivalent band in the E14TG2a lane. The intensity of the pseudogene band (Isamat et al., 1988) in the EcoRI digests allows comparison of the relative loading of each lane, thus acting as an internal control. In the BamHI digest the disappearance of the 9.5 kb fragment, characteristic of the deletion, and the regeneration of the 7.0 kb wild-type band can be seen. The presence of the 14.0 kb fragment, which comigrates with the pseudogene fragment, is indicated by the increased intensity of the band at that molecular weight.

One HAT^R clone had the structure we have called type 3 (see Figure 1). This clone has two correcting vectors integrated in tandem. The effect on the restriction map is that, in an EcoRI digest, the 10.4 kb fragment is duplicated and there are three copies of the 1.3 kb fragment compared with two copies in type 1 correctants and one copy in the deletion mutant. This is evident in the relative intensities of these bands in Figure 2A. In a BamHI digest the 14.0 kb and 7.0 kb fragments, seen in type 1 correctants, are present but there is an additional 11.7 kb, vector-sized fragment. This can be seen more clearly in Figure 2B, which shows a pUC8 reprobe of the transfer in Figure 2A. The pUC8 sequence is present within the 10.4 kb EcoRI band, and consequently the intensity of this band is doubled in type 3 DNA relative to type 1. In a BamHI digest

Table 1. Summary of Correction Experiments

Experiment	Correcting Vector	Number of HAT^R Colonies	Frequency of HPRT Correctants[a]	Positive Control[b]	Targeting Index[c]	Correctant Types 1	2	3
1	pDWM101	8	1.75×10^{-7}	2.5×10^{-5}	0.007	7	–	1
2	pDWM101	2	4.0×10^{-8}	6.4×10^{-6}	0.006	1	1	–
3	pDWM101	12	2.2×10^{-7}	6.3×10^{-6}	0.035	12	–	–
	pDWM102	8	1.5×10^{-7}	6.3×10^{-6}	0.024	8	–	–

[a] Number of HAT^R colonies generated per cell electroporated with correcting vector DNA.
[b] Number of HAT^R colonies generated per cell electroporated with pDWM100 DNA.
[c] Frequency of HAT^R colonies generated per cell electroporated with correcting vector DNA relative to HAT^R colonies generated per cell electroporated with pDWM100 DNA (a/b).

the pUC8 fragment is present in both the 14.0 kb band and the 11.7 kb band. This is diagnostic for the type 3 structure. The equal intensities of the 14.0 kb and 11.7 kb bands in the type 3 lane show that there are two copies of the vector integrated. If there were more than two copies the intensity of the 11.7 kb band would be at least twice that of the 14.0 kb band. There was no evidence either from these correctants, or from any of the others we have examined subsequently, for any random integrations of the correcting vector, which would have been detected as additional bands hybridizing to the pUC8 probe.

To generate a corrected ES cell line suitable for reintroduction into mouse blastocysts, a second experiment was performed using low-passage E14TG2a cells. pDWM101 was used as the targeting vector; electroporation of 5×10^7 cells produced two HAT^R clones (see Table 1). Southern analysis was used to determine the structure of the HPRT gene in these correctants. One clone had a structure indistinguishable from the seven type 1 correctants generated in the first experiment, and so was called type 1-8. The second HAT^R clone had a subtly different pattern, which we have called type 2 (see Figures 1 and 2). In this clone, the exon 3–containing 1.3 kb EcoRI band has the same intensity as in the E14TG2a lane, indicating that exon 3 is not duplicated. In addition, the intensity of the 14.0 kb BamHI band is lower than in type 1 correctants, although the size of the band is not visibly different at this resolution. An XhoI digest for this clone shows that the XhoI site in the upstream copy of exon 3 is absent (data not shown). The presence of the 10.4 kb EcoRI band indicates that the EcoRI site immediately upstream of the plasmid sequence is present. From this we infer that this clone, correctant type 2-1, has the targeting vector inserted correctly, but a small deletion has occurred to remove the upstream copy of exon 3. Therefore in this correctant, unlike type 1 correctants, there is no exon 3 duplication. This does not affect the HPRT-positive status of the clone because the deleted exon is not part of the functional gene.

Frequency of 6-Thioguanine Resistant Cells in Correctant Populations

To assay the frequency of $HPRT^-$ cells in the two correctant populations (type 1-8 and type 2-1) generated in the second experiment, cells were grown in the absence of selection for 2 weeks, and then their plating efficiency in medium containing 6-thioguanine (6-TG), relative to their plating efficiency in nonselective medium, was measured. The relative plating efficiency for type 2-1 was 0.03%, which is approximately the same as the figure obtained for wild-type ES cells. However, the relative plating efficiency for type 1-8 was 21.0%; that is, after 2 weeks of growth in nonselective medium, 21.0% of the cell population in the type 1-8 culture was HPRT deficient.

To analyze further the presence of $6\text{-}TG^R$ cells in the type 1-8 population, we used Southern hybridization to examine DNA from cells grown under different selection conditions. The structure of the gene in DNA prepared from the type 1-8 culture grown nonselectively for 24 days was indistinguishable from the E14TG2a deletion structure. Thus, when selection is removed from the type 1-8 culture, cells containing the corrected gene are rapidly replaced by $6\text{-}TG^R$ cells which are $6\text{-}TG^R$ because their HPRT gene has the E14TG2a structure. This could be due to the persistence in the HAT^R culture of E14TG2a cells, surviving, since the original isolation of the correctant clone, by metabolic cooperation (Hooper, 1982). Alternatively, $6\text{-}TG^R$ cells might arise by loss of vector sequences from corrected cells, due to a reversal of the original homologous recombination (correcting) event. These possibilities are under investigation. Whatever the reason, it was deemed inappropriate to use the type 1-8 culture as a source of cells for blastocyst injections. Hence, the correctant type 2-1 was chosen for introduction into mouse blastocysts (see below).

The Effect of Plasmid Sequences on Targeting Efficiency

The use of the targeting vector pDWM101 results in the integration of plasmid sequences into the genome. To determine whether the incorporation of plasmid sequences was detrimental to the efficiency of gene targeting, a third experiment was performed (see Table 1). In this experiment E14TG2a cells were electroporated with either pDWM101 or pDWM102 DNA (see Figure 1 and Experimental Procedures for details of construction). pDWM102 contains precisely the same HPRT gene-derived sequences as pDWM101, but they are rearranged so that when pDWM102 DNA is restricted with XhoI the pUC8 sequences are excised. Consequently, correctants generated with this vector do not have pUC8 sequences integrated into the genome. The predicted, type 1, restriction map for pDWM102 correctants differs from that for pDWM101 correctants in the following ways: In the EcoRI restriction map the 10.4 kb and the duplicated 1.3 kb fragments seen in pDWM101 correctants are replaced by a fragment of 9.0 kb, which is equal to their combined size minus the size of pUC8 (2.7 kb), due to the absence of the EcoRI site immediately upstream of the promoter (see Figure 1). In a BamHI digest the loss of pUC8 and the gain of a BamHI site upstream of the promoter results in the replacement of the 14.0 kb fragment by a 3.0 kb internal fragment and an 8.3 kb fragment extending into the upstream flanking sequence. A comparison of the Southern hybridization patterns for BamHI-digested DNA from type 1 correctants, generated with pDWM101 and pDWM102, is shown in Figure 3.

In the third experiment, 12 HAT^R colonies were generated from cells electroporated with pDWM101 and eight correctants were generated from cells electroporated with pDWM102 (see Table 1). The frequency of gene targeting relative to random integrations, calculated as for previous experiments, was 0.035 for pDWM101 and 0.024 for pDWM102. Thus we conclude that the presence of plasmid sequences in the targeting vector does not affect the frequency of gene targeting. All correctants generated in this experiment had the type 1 gene structure.

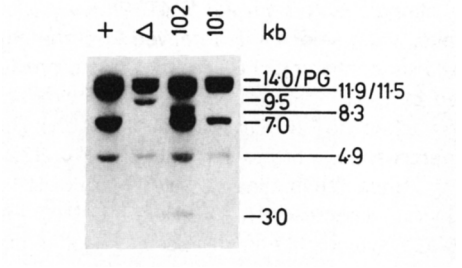

Figure 3. Comparison of Type 1 Correctants Produced by Correcting Vectors pDWM101 and pDWM102

Genomic DNA (10 μg) was restricted with BamHI and probed with an HPRT cDNA probe. DNA: +, E14; △, E14TG2a; 102, a pDWM102 type 1 correctant; 101, a pDWM101 type 1 correctant. The sizes (in kb) of the hybridizing bands are shown. The pattern is explained in the text.

Production of Chimeric Mice

Cells from the correctant clone type 2-1 were injected into F2 (C57BL/6/Ola × CBA/Ca/Ola) blastocysts. The latter are homozygous for the wild-type alleles at the *c* and *p* loci (*C/C, P/P*) and segregating for the alleles *A* (agouti) and *a* (nonagouti), whereas the 129/Ola-derived type 2-1 cells are homozygous for the alleles c^{ch} (chinchilla) and *p* (pink-eyed dilution), which both lighten the coat color, and for A^w (white-bellied agouti). Hence, chimeric mice are identifiable by the presence of light coloring in an otherwise dark coat. Twenty-six mice were born from 93 blastocysts injected. Fifteen chimeric mice were obtained, 12 of which were male. Male chimeras were tested for germ line transmission of the c^{ch} and *p* markers, derived from ES cells, by crossing with strain 129/Ola females. Offspring which, like strain 129/Ola, are light yellow with pink eyes are diagnostic of germ line transmission (see Table 2). One out of eight males tested showed germ line transmission to 12 of 16 offspring (see Figure 4). Although type 2-1 cells have a modal chromosome number of 40 and those metaphase spreads with 40 chromosomes have no detectable abnormalities in G-banding, only eight out of 20 metaphases examined had the modal number. Nevertheless, when the male germ line chimera was mated to an F1 (C57BL/6/Ola × CBA/Ca/Ola) female, the resulting 11 fertilized eggs, studied at the one-cell stage, all had 40 chromosomes (data not shown).

Germ Line Transmission and Expression of the Corrected Gene

To study the expression of the corrected gene in mice, the male chimera showing germ line transmission of cell line–derived markers was crossed to homozygous HPRT deficient ($hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$) females, bred from the series of chimeras produced with E14TG2a (see Experimental Procedures). The gene pool of these females is derived entirely from strain 129/Ola and from the outbred strain MF1. The latter is a strain of mice whose precise genetic status has not been characterized, but coat colors observed in the progeny of crosses are consistent with their being homozygous for the albino allele (*c/c*) and for the nonagouti allele (*a/a*). White homozygous $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$ females were chosen for mating to chimeras; if the genotype deduced above for MF1 is correct, these homozygotes must either be of genotype *c/c* or carry the gene combination c^{ch}/c^{ch}, *p/p*, *a/a* (Silvers, 1979). In both cases, host blastocyst–derived offspring are agouti or black, while cell line–derived offspring are lighter than agouti (Table 2). The cell line–derived marker was transmitted to nine of 12 progeny (see Figure 4). Cell line–derived female offspring inherit one X chromosome carrying the corrected gene, which we designate $Hprt^{b\text{-}m4}$, derived from their father, and the other X chromosome, carrying deletion $hprt^{b\text{-}m3}$, from their mother. Two such females were screened for the corrected gene by Southern hybridization of tail DNA (see Figure 5).

EcoRI digests of DNA from these two mice show the 10.4 kb band diagnostic for the corrected gene. The relative intensity of this band is reduced in this hybridization, with respect to the corrected cell line DNA, because the ES cell line is male and therefore contains just one corrected HPRT allele per cell, whereas in the female mice only one of the two alleles is corrected. The absence of a wild-type HPRT allele in these mice is indicated by the lack of the 5.5 kb and 6.3 kb bands. DNA from a heterozygous $hprt^{b\text{-}m3}/Hprt^{b}$ mouse is shown in Figure 5, for comparison. In BamHI-digested DNA from mice carrying the corrected gene, heterozygosity is indicated by the presence of both the 9.5 kb band, containing exon 3 from the deletion, and the 7.0 kb band, containing exons 2 and 3 from the corrected allele. These two bands are also present in DNA from heterozygous $hprt^{b\text{-}m3}/Hprt^{b}$ mice, but

Table 2. Expected Phenotypes of Progeny Mice from Test Crosses of Chimeras

Host Blastocyst F2 (C57BL/6/Ola × CBA/Ca/Ola)	Stem Cells (Strain 129/Ola)	Tester Female[a]	Coat Color	
			Host Progeny	Stem Cell Progeny
C/C *P/P*	c^{ch}/c^{ch} *p/p* A^w/A^w	(1) c^{ch}/c^{ch} *p/p* A^w/A^w	agouti	light yellow[b]
		(2) *c/c*	agouti or black	light chinchilla
		(3) c^{ch}/c^{ch} *p/p* *a/a*	agouti or black	light yellow[b]

[a] Tester females: (1), strain 129/Ola; (2) and (3), differing possible genotypes of white homozygous $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$ females (see text).
[b] These animals are predicted to have pink eyes, the remaining four classes dark eyes.

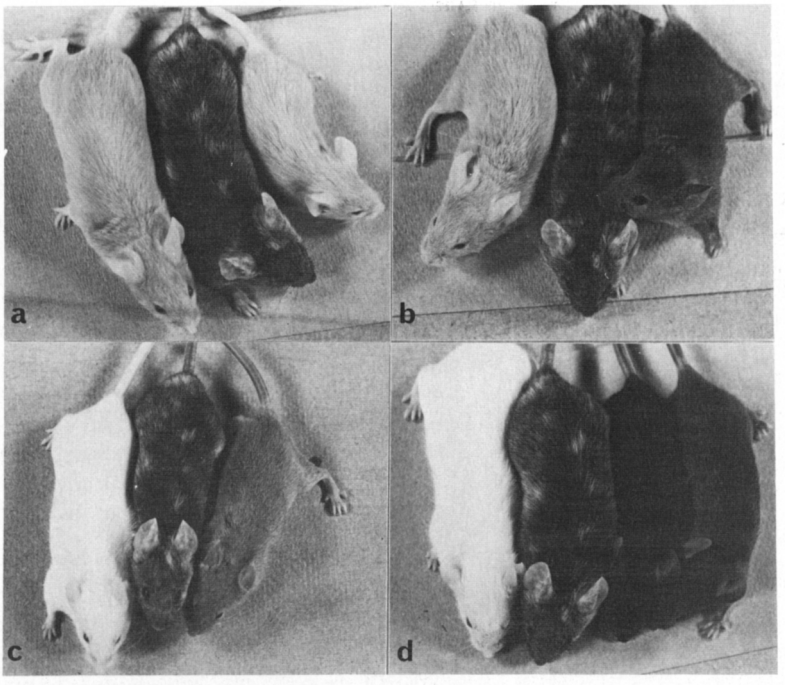

Figure 4. Germ Line Transmission of Coat Color Markers from Type 2-1 Chimeras

Mice are listed from left to right in each case. (a) 129/Ola female; male type 2-1 germ line chimera; cell line–derived light yellow offspring. (b) First two animals as in (a); host blastocyst–derived agouti offspring. (c) White *hprt*$^{b\text{-}m3}$/*hprt*$^{b\text{-}m3}$ female; male type 2-1 germ line chimera; cell line–derived light chinchilla offspring. (d) First two animals as in (c); host blastocyst–derived black offspring; host blastocyst–derived agouti offspring.

the *hprt*$^{b\text{-}m3}$/*Hprt*$^{b\text{-}m4}$ mice are distinguishable by the EcoRI digests.

A Northern analysis of HPRT mRNA from stem cells and mouse tissues is shown in Figure 6. The level of the 1500 nucleotide HPRT mRNA in the corrected cells is indistinguishable from the level in wild-type E14 cells. No HPRT transcripts are detected in RNA from E14TG2a cells. In normal mice the level of HPRT mRNA in brain tissue is elevated approximately 5-fold relative to liver tissue (see Figure 6). Because HPRT is X-linked, some cells in heterozygous mice will be functionally HPRT deficient, because of random inactivation of one X chromosome in each cell. Hence to provide a positive control for HPRT expression in the two heterozygotes containing one deleted and one corrected allele, RNA from two heterozygous *hprt*$^{b\text{-}m3}$/*Hprt*^{b} mice was included. The pattern of expression in mice heterozygous for the corrected allele is essentially the same as in mice heterozygous for the wild-type allele. All heterozygotes showed the characteristic low level of HPRT mRNA in liver and elevated levels in brain. Differences in the degree of mosaicism between HPRT-expressing cells and cells in which the wild-type or corrected HPRT gene is on the inactive X chromosome probably account for the slight variations observed in the absolute levels of expression between mice. Thus we conclude that the corrected gene functions in the same way

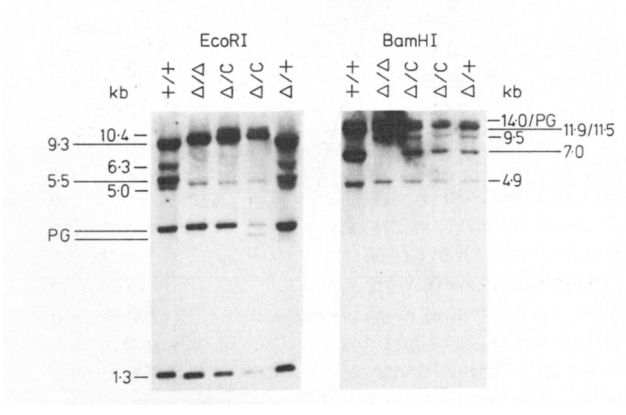

Figure 5. Southern Analysis to Show Germ Line Transmission of the Corrected HPRT Gene

Tail DNA prepared from female mice was digested with either EcoRI (left) or BamHI (right) and probed with the full-length HPRT cDNA. Lanes: +/+, homozygous *Hprt*^{b}/*Hprt*^{b}; Δ/Δ, homozygous *hprt*$^{b\text{-}m3}$/*hprt*$^{b\text{-}m3}$; Δ/C, heterozygous *hprt*$^{b\text{-}m3}$/*Hprt*$^{b\text{-}m4}$; Δ/+, heterozygous *hprt*$^{b\text{-}m3}$/*Hprt*^{b}. The size (in kb) of each hybridizing band is shown. The fragments containing the HPRT pseudogene are labeled PG. One of the heterozygous *hprt*$^{b\text{-}m3}$/*Hprt*$^{b\text{-}m4}$ mice shows a previously characterized pseudogene polymorphism (Isamat et al., 1988).

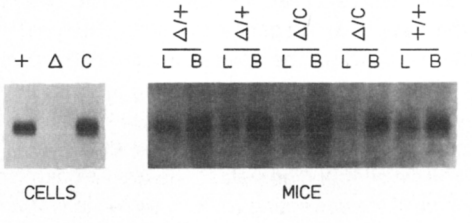

Figure 6. Northern Analysis Showing Expression from the Corrected Gene in ES Cells and Mouse Tissues

Total RNA (30 μg) was prepared from ES cells or mouse tissues, electrophoresed on formaldehyde–agarose gels, transferred, and probed with full-length HPRT cDNA. Cell RNA: +, E14; Δ, E14TG2a; C, correctant type 2-1 grown nonselectively. Tissue RNA: Liver (L) and brain (B) RNAs are shown from two heterozygous *hprt*$^{b\text{-}m3}$/*Hprt*^{b} mice, Δ/+; two heterozygous *hprt*$^{b\text{-}m3}$/*Hprt*$^{b\text{-}m4}$ mice, Δ/C; and one homozygous *Hprt*^{b}/*Hprt*^{b} mouse, +/+. The size of the HPRT mRNA is approximately 1500 nucleotides.

as the wild-type gene in mice. That is, the function of the mutant gene in E14TG2a has been fully corrected by gene targeting.

Discussion

In this study we have demonstrated the feasibility of making targeted modifications to the mouse genome by homologous recombination in ES cells. We have used targeting vectors of the type defined as insertion vectors (Thomas and Capecchi, 1987) to correct an HPRT deficiency in the ES cell line E14TG2a, which has a deletion of the first two exons of the HPRT structural gene. Insertion of the targeting vector pDWM101 into the genome by homologous recombination corrects the deletion mutation. Three electroporation experiments generated a total of 30 HAT^R colonies. To give an indication of the number of gene targeting events relative to random integrations, we have calculated the ratio of the number of HAT^R colonies per cell electroporated with pDWM101 to the number of HAT^R colonies per cell electroporated with pDWM100, a fully functional minigene derivative of pDWM101, and denoted this the targeting index (see Table 1). All electroporations were carried out under identical conditions. The 4-fold variation in targeting index observed between experiments could not be correlated with the state of the stem cell cultures at the time of electroporation. The highest targeting index we observed was 0.035, which is more than 10-fold higher than the frequency reported by Thomas and Capecchi (1987). Neither calculation allows for random integrations into regions of the genome that prevent expression of the introduced gene. However, a relatively high frequency of targeting events is implied by our observations, and those of Thomas and Capecchi (1987) and Doetschman et al. (1987), that no random integrations, in addition to the targeting event, have been found in any homologous recombinants analyzed so far. This suggests that it should prove possible to make targeted genome alterations without the risk of deleterious consequences arising from random integrations.

Southern analysis of the HPRT gene in correctants shows that the majority (28/30) have the predicted (type 1) gene structure. However, we have identified two correctants that have different gene structures. The type 2 structure has a deletion of the upstream copy of the exon 3 region, which may have occurred during, or after, the integration event. This could be equivalent to the deletions reported by Doetschman et al. (1988) during their HPRT inactivation experiments. The type 3 structure has two copies of the correcting vector inserted in tandem. We have shown that there is no significant difference in the frequency of correction between a vector that does result in the integration of plasmid sequences, pDWM101, and one that does not, pDWM102. This is helpful, because generally it is easier to construct insertion vectors that do result in the integration of plasmid sequence. In both targeting vectors, pDWM101 and pDWM102, the extent of homology with the target locus is the same (between 2.3 kb and 4.2 kb). Others have shown that the greater the extent of homology, the higher the efficiency of gene targeting (Thomas and Capecchi, 1987).

Cells from the correctant clone type 2–1, in which the corrected gene is essentially as stable as a wild-type gene, were introduced into mouse blastocysts. The frequency of chimeras per animals born was comparable to the value reported previously for the parental (E14TG2a) stem cells (Hooper et al., 1987). We believe that the lower frequency of blastocysts surviving to term in the experiments described here reflects difficulties associated with establishing the technique in our hands, rather than a reduction in the ability of targeted stem cells to contribute to the developing embryo. One chimeric male, from eight tested, was shown to transmit the corrected gene through the germ line. This is a lower proportion than for E14TG2a-derived male chimeras, of which 19 out of 34 showed germ line transmission (Hooper et al., 1987). The reduced ratio may be a consequence of the lower fraction of diploid cells in type 2–1. In total, the chimera described here transmitted corrected cell line–derived markers to 21 of 28 offspring. Chimeras produced with E14TG2a have shown transmission of stem cell–derived markers to 100% of offspring and to as few as 1% of offspring (Hooper et al., 1987). The ES cells used here for blastocyst injections were subjected to the mildest electroporation conditions possible, and their time in culture was kept to a minimum. This may have contributed to the successful production of germ line chimerism from the corrected stem cells.

By crossing to HPRT-deficient females, we have produced female mice in which the corrected gene is the only functional HPRT gene. This enabled us to study expression of the corrected gene in the absence of a wild-type gene. We chose to analyze expression in these females because males with a corrected gene would not be available until the next generation. Mosaicism in the female mice, caused by random X inactivation, did not complicate the interpretation of the expression data. HPRT is a member of the housekeeping class of genes, which are generally expressed constitutively at low levels in all cell types. However, a characteristic of HPRT expression is the elevation of mRNA levels in brain relative to liver and other tissues. The deletion in E14TG2a extends at least 10 kb upstream of the HPRT gene. Correction with pDWM101 replaces just 650 bp of 5′ flanking sequence, which we show is sufficient to control HPRT expression with the characteristic liver and brain mRNA levels. Hence, the mechanism by which expression is elevated in brain can have no requirement for sequences located more than 650 bp upstream of the transcription initiation point. In cultured cells 50 bp of 5′ sequence is sufficient for a wild-type level of expression from an HPRT minigene (Melton et al., 1986). Although the minigene experiments were subject to random integration and variable copy number, the corrected gene in mice is present in only one copy per cell, in its natural chromosomal environment. By targeting changes both to the promoter and the 3′ untranslated region of HPRT, we hope to determine the mechanism by which expression is elevated in brain tissue.

The brain-specific elevation of HPRT expression is particularly noteworthy because a deficiency of HPRT in hu-

mans is the molecular basis of Lesch–Nyhan syndrome, which is characterized by learning difficulties, spasticity, choreoathetosis, and compulsive self-injurious behavior. Mice deficient for HPRT do not demonstrate the behavioral patterns characteristic of Lesch–Nyhan syndrome. This suggests that mice, unlike humans, can tolerate the HPRT deficiency, perhaps by utilizing other pathways to maintain the levels of purines and neurotransmitters in the brain. However, the levels of striatal dopamine are reduced by approximately 20% in HPRT-deficient mice relative to their normal littermates, which compares to a 70% to 90% reduction in Lesch–Nyhan patients (Finger et al., 1988). Hence, we would not expect to observe altered behavior in mice with a corrected HPRT gene, although we would predict that the levels of striatal dopamine would be restored to the wild-type level.

Although in these experiments the detection of targeting events is facilitated by the selection systems available for HPRT activity, strategies for targeting into nonselectable genes, using vectors containing selectable markers (Jasin and Berg, 1988; Mansour et al., 1988; Doetschman et al., 1988), or the detection of targeted clones by the polymerase chain reaction (Kim and Smithies, 1988), are being developed. Consequently, it should soon be possible to target modifications to any chosen gene in the mouse genome.

Experimental Procedures

Production of Homozygous *hprt*$^{b\text{-}m3}$/*hprt*$^{b\text{-}m3}$ Female Mice

Mice of inbred strains 129/Ola, C57BL/6/Ola, and CBA/Ca/Ola and outbred strain MF1 were obtained from Olac 1976 Ltd., Bicester, Oxon. A male E14TG2a chimera showing 100% transmission of the c^{ch} allele (Hooper et al., 1987) was mated to a wild-type MF1 female. Female offspring, heterozygous for the null *hprt*$^{b\text{-}m3}$ allele (generation 1), were mated to MF1 males, and male offspring (generation 2) carrying the null allele were identified by enzyme assay on blood samples. These HPRT-deficient males were mated to MF1 females, and embryos were transferred to foster mothers to improve their health status. Heterozygous female offspring (generation 3) were mated to strain 129/Ola males. HPRT-deficient male offspring were identified by enzyme assay on blood samples, and heterozygous female offspring were identified by Southern blot analysis of DNA obtained by tail biopsy (generation 4). HPRT-deficient males from generation 4 were mated to heterozygous females from either generation 3 or 4. Homozygous *hprt*$^{b\text{-}m3}$/*hprt*$^{b\text{-}m3}$ females were identified by enzyme assay on blood samples, and their status was confirmed by tail blots. Segregation of coat color markers in these animals made it possible to choose white homozygotes for mating to chimeras produced with corrected stem cells.

Embryonic Stem Cells

The wild-type male ES cell line E14 was derived from strain 129/Ola blastocysts by trypsinization of immunosurgically isolated inner-cell masses. The dissociated cells were cultured on STO fibroblast feeder layers in Buffalo rat liver cell (BRL)-conditioned medium (A. H. Handyside, G. T. O'Neill, M. Jones, and M. L. Hooper, submitted). The HPRT-deficient E14TG2a cell line was isolated as a spontaneous 6-TG^{R} derivative of E14 (Hooper et al., 1987). For all the experiments reported here ES cells were grown in BRL-conditioned medium without a feeder layer. The medium used was Glasgow modified Eagle's medium (Flow Laboratories) supplemented with 1× nonessential amino acids and 1 mM sodium pyruvate (Flow Laboratories), and 5% (vol/vol) of both fetal calf serum and newborn calf serum (Sera-Lab, Sussex). Batches of serum were chosen for their ability to give good ES cell plating efficiencies (typically 10%–30%) without affecting the ability of ES cells to differentiate in vitro. Cells were grown in the absence of antibiotics in 37°C incubators with 5% CO_2 in air.

BRL-conditioned medium was made as follows: BRL cells were grown to confluence in 75 cm^2 tissue culture flasks. The medium was discarded, and 15 ml of fresh medium was added. This was collected after 2 days and replaced with another 15 ml of fresh medium. In total, 45 ml of conditioned medium, representing 6 days of collection, was obtained from each flask. The conditioned medium was filtered (pore size, 0.2 μm) and stored at −20°C.

ES cells were grown on gelatin-coated tissue culture flasks in 60% BRL-conditioned medium, 40% fresh medium, containing 0.1 mM β-mercaptoethanol. Typically, the medium was changed daily and cultures were split every 3–4 days. Under these conditions the cell number doubled every 24 hr and the majority of cells in the culture had the typical stem cell morphology with little spontaneous in vitro differentiation. We considered that the likelihood of obtaining germ line transmission from corrected stem cells would be enhanced by the use of early-passage cells and by minimizing their time spent in culture. For this reason correctant clones generated in the first electroporation experiment from late-passage stem cells were not used for blastocyst injections. For the second experiment, E14TG2a cells (passage 25 since the isolation of the E14 line) were grown up and the targeting procedures and analysis were carried out as rapidly as possible. The cumulative passage number of correctant type 2-1 cells at the time of blastocyst injection had risen to passage 36.

Vectors

The correcting vector used in these experiments, pDWM101, is similar in structure to the vector described by Doetschman et al. (1987) except that, in pDWM101, all correcting sequences are derived from the mouse HPRT gene (see Figure 1). pDWM101 was constructed by first cloning a 4.7 kb BamHI–EcoRI restriction fragment, containing exon 2, and derived from the mouse HPRT genomic clone, λHPT1 (Melton et al., 1984), into BamHI- and EcoRI-cut pUC8 (Vieira and Messing, 1982). A 3.0 kb BglII–BamHI fragment containing the HPRT promoter and exon 1, derived from λHPT32, was then inserted into the BamHI site, and a 1.35 kb EcoRI fragment, containing exon 3, isolated from λHPT1, was cloned into the EcoRI site. The resulting 11.7 kb plasmid, pDWM101, has the same organization as the equivalent part of the HPRT gene itself except that the first intron is reduced from 10.8 kb in the gene to 4.1 kb in the vector. The correcting vector can only rescue HPRT-deficient stem cells by homologous recombination with the defective gene. As a positive control for these correction experiments, an HPRT minigene derivative of pDWM101 was constructed by replacing the 1.25 kb XhoI–EcoRI fragment from pDWM101 with the 1.8 kb XhoI–EcoRI fragment from the minigene pDWM1 (Melton et al., 1986), which contains the remainder of the coding sequence and the 3′ untranslated region. This minigene derivative, pDWM100, is fully functional and can rescue HPRT-deficient stem cells without the requirement for homologous recombination. A rearranged version of the correcting vector, pDWM102, in which an XhoI digest is used to excise the pUC8 sequences prior to recombination, was also constructed: A 7.8 kb SalI (pUC8 polylinker site to the 5′ side of correcting sequence)–XhoI (site within exon 3) fragment from pDWM101 was first cloned into a pUC8 derivative, where an XhoI linker had been inserted into the SmaI site within the polylinker. This plasmid was then linearized at a SalI site immediately upstream of the HPRT promoter region, and a 1.25 kb fragment, containing the remainder of exon 3 and the following intervening sequence, from pDWM101, was inserted to generate the 11.7 kb plasmid, pDWM102, with the structure shown in Figure 1.

Electroporation and Selection for Correctants

Correcting vector DNA was introduced into ES cells by electroporation (Bio-Rad Gene Pulser). Approximately 5 × 10^6 cells were resuspended in 0.8 ml of HEPES-phosphate buffered saline (pH 7.05) (Graham and van der Eb, 1973) with 20 μg of XhoI-linearized vector DNA. The cells were given a single pulse (800 V, path length 0.4 cm, 3 μF), then allowed to stand at room temperature for 10 min before plating in nonselective medium. HAT selection (Littlefield, 1964) was imposed after 24 hr.

Southern and Northern Hybridizations

High molecular weight genomic DNA was prepared by the method of Pellicer et al. (1978). Total RNA was prepared from cultured cells, or mouse tissues, as described previously (Melton et al., 1981). GeneScreen

Plus (New England Nuclear Products) was used as the hybridization membrane. The conditions for transfer, hybridization, washing, and rehybridization were those recommended by the supplier. For Southern hybridizations the probe was either the purified insert from the mouse HPRT cDNA recombinant pHPT5 (Konecki et al., 1982) or plasmid pUC8. For Northern hybridizations the probe was the purified insert from pHPT4 (Konecki et al., 1982). Probes were labeled by the random priming method (Feinberg and Vogelstein, 1983).

Blastocyst Injections and Production of Chimeric Mice

Cells for microinjection were washed, trypsinized and resuspended in 10 mM HEPES-buffered Glasgow modified Eagle's medium containing 10% (vol/vol) newborn calf serum. F2 (C57BL/6/Ola × CBA/Ca/Ola) blastocysts were collected on the day of injection by flushing uterine horns 3.5 days postcoitum with Glasgow modified Eagle's medium containing 10% (vol/vol) newborn calf serum. Approximately 12–15 cells were injected into each blastocyst, using a Leitz micromanipulator and needles prepared with a Campden Instruments puller and microforge. Injected blastocysts were allowed to re-expand for 1–2 hr following manipulation and were then implanted into the uterine horns of pseudopregnant C57BL/6/Ola mice, mated 2.5 days previously to vasectomized F1 (C57BL/6/Ola × CBA/Ca/Ola) males.

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