Gene correction in hematopoietic progenitor cells by homologous recombination

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Homologous recombination (gene targeting) has many desirable features for gene therapy, because it can precisely correct mutant genes and restore their normal expression, and random nonhomologous integration of DNA is infrequent in cells in which homologous recombination has occurred. There are, however, no reports of attempts to use homologous recombination to correct mutant genes in normal hematopoietic stem cells (HSCs), which are prime cells for therapy of a variety of hematological and other conditions, presumably because of their low abundance and uncertainty that homologous recombination can occur at a usable frequency in these cells. The experiments reported here encourage optimism in this respect by demonstrating targeted correction of a defective hypoxanthine phosphoribosyltransferase gene in hematopoietic progenitor cells that can form colonies in methylcellulose culture. These clonogenic cells are in the same lineage as HSCs but are more abundant and more mature and so less pluripotent. Corrected colonies were identified by their survival in selective medium after electroporation of correcting DNA into unfractionated mouse bone marrow cells and were confirmed by reverse transcription–PCR and sequencing. The observed frequency ($4.4 \pm 3.3 \times 10^{-5}$ per treated clonogenic cell) is the same as in embryonic stem cells ($2.3 \pm 0.4 \times 10^{-5}$) with the same DNA and mutation. These data suggest that gene targeting to correct mutant genes eventually will prove feasible in HSCs capable of long-term bone marrow reconstitution.

The ideal form of gene therapy would correct a mutant gene directly without causing changes elsewhere in the genome (1). Many of the problems associated with gene therapy would thereby be greatly reduced or eliminated, including lack of adequate expression, extinction of expression, and the mutagenesis associated with integrating the correcting sequences into random sites in the genome. Homologous recombination has the necessary prerequisites for use in this context, because it is capable of precisely correcting mutant genes (2), and random nonhomologous integration of targeting DNA into the genome is infrequent in cells in which homologous recombination has occurred (3). However, we find no reported attempts of using homologous recombination to correct mutant genes in normal hematopoietic stem cells (HSCs; ref. 4), which are prime cells for therapy of a variety of hematological and other conditions (5). The likely reasons appear to be the low abundance of these cells combined with uncertainty that homologous recombination can occur in them at a usable frequency. The experiments we report here were designed to test the possibility of using homologous recombination to correct a mutant gene in hematopoietic progenitor cells that can form colonies in tissue culture. These colony-forming cells (CFCs) are in the same lineage as HSCs but are more abundant and more mature, and therefore less pluripotent. We demonstrate that the hypoxanthine phosphoribosyltransferase (HPRT) gene, which is mutated in humans with Lesch-Nyhan disease (6, 7), can be corrected by homologous recombination in CFCs at frequencies equivalent to those seen in embryonic stem (ES) cells, encouraging optimism that homologous recombination to correct mutant genes in pluripotent stem cells capable of long-term hematopoietic repopulation eventually will prove feasible.

Materials and Methods

Hprt$^-$ Bone Marrow (BM) Cells and ES Cells. Hprt$^-$ BM cells were isolated from C57BL/6J-*Hprt*$^{b\text{-}m3}$ mice descended from E14TG2a ES cells (8) and were obtained from The Jackson Laboratory. ES cells (HM-1), isolated from mice derived from E14TG2a ES cells, were from David W. Melton (University of Edinburgh, Edinburgh, U.K.; ref. 9).

Reconstruction Experiments. Assays were in 24-well plates with mixtures of Hprt$^+$ cells/Hprt$^-$ cells: $5 \times 10^4/5 \times 10^5$; $5 \times 10^3/5 \times 10^5$; and $5 \times 10^3/5 \times 10^6$, and in 35-mm dishes when the mixtures were $5 \times 10^3/5 \times 10^7$ and $5 \times 10^2/5 \times 10^7$. The mixtures were plated in standard methylcellulose medium (MethoCult GF M3434; StemCell Technologies, Vancouver) containing the following recombinant cytokines: mouse stem cell factor (50 ng/ml)/mouse IL-3 (10 ng/ml)/human IL-6 (10 ng/ml)/human erythropoietin (3 units/ml)/bovine pancreatic insulin (10 μg/ml). Selection was with HAT (120 μM hypoxanthine/0.4 μM aminopterin/20 μM thymidine). Colonies were counted under dark-field illumination at day 14.

Electroporation. Electroporations were performed with a cuvette having a gap size of 4 mm and an area of 160 mm^2 (BTX, San Diego) with a 1-s pulse from a 250 μF capacitator charged to 300 V. BM and ES cells were suspended at a density of $0.3–1.5 \times 10^8$ cells/ml in conventional ES-cell medium (10, 11), containing DMEM with 15% heat-inactivated FBS and 10 μM 2-mercaptoethanol. Linearized correcting DNA was 1.6–5 nM. After electroporation at room temperature, the cell suspensions were held at room temperature for 5–10 min before plating.

Correcting DNA Plasmid. The MP8neo plasmid used for preparing correcting DNA has a copy of the pMC1neo gene (not used in these experiments) between the 5′ homologous region and the promoter region of the MP8 plasmid described (ref. 3; Fig. 1*C*). The MP8neo plasmid DNA was linearized with *Bam*HI before use.

Selection of HAT-Resistant Colonies. BM cells were flushed from the femur and tibia by using DMEM with 10% heat-inactivated FBS and made into a single-cell suspension by repeated passage through an 18-gauge needle; $5–10 \times 10^7$ BM cells from individual male mice were electroporated in 0.5–0.8 ml of medium and plated in 35-mm dishes (1 ml per dish) after mixing with 10 ml

Abbreviations: BM, bone marrow; CFC, colony-forming cell; ES, embryonic stem; HAT, hypoxanthine/aminopterin/thymidine; HPRT, hypoxanthine phosphoribosyltransferase; HSC, hematopoietic stem cell; RT-PCR, reverse transcription–PCR.

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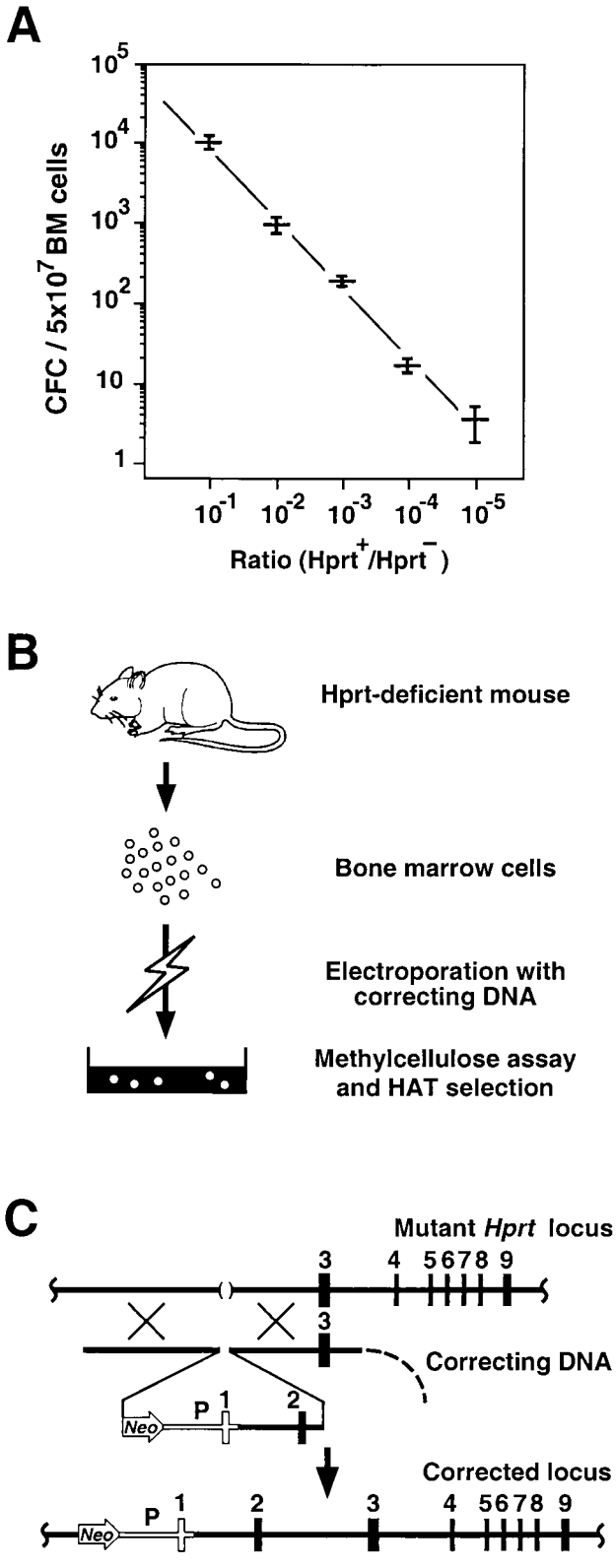

Fig. 1. Reconstruction experiment and strategy for gene targeting in BM cells. (*A*) Reconstruction experiment. Mixtures were made with decreasing numbers of wild-type Hprt$^+$ BM cells added to an excess of mutant Hprt$^-$ BM cells in ratios ranging from 1/10 to 1/100,000 to simulate the correction of the mutant Hprt$^-$ cells at frequencies ranging from 10^{-1} to 10^{-5}. Colonies were counted at day 14 in a HAT-containing methylcellulose medium (see *Materials and Methods*), and the number of CFCs (CFCs per 5×10^7 BM cells) was plotted against the ratio of Hprt$^+$ to Hprt$^-$ cells. The center and error bars indicate average numbers and their standard deviations. (*B*) Strategy to detect gene targeting in hematopoietic CFCs. BM cells are prepared from an Hprt-deficient male mouse, electroporated in the presence of correcting DNA, and plated in methylcellulose medium. Two days later, HAT-selection medium is added (see *Materials and Methods*). (*C*) Gene targeting at the *Hprt* locus. The structures of the mutant *Hprt* locus in C57BL/6J-*Hprt*$^{b\text{-}m3}$ mice (*Top line*), correcting DNA (*Middle line*), and corrected targeted locus (*Bottom line*) are shown. The correcting DNA is 12 kb in length and includes two 3-kb regions of homology with the target gene. Black boxes represent exons; heavy horizontal lines represent mouse sequences; open boxes and lines indicate that in the correcting DNA the *HPRT* promoter (P) and its first exon are derived from the human *HPRT* gene. *Neo* indicates a neomycin-resistance gene (not used in these experiments). The dashed light line indicates plasmid sequences. The parentheses show a 55-kb deletion in the mutant gene.

of methylcellulose culture medium (MethoCult GF M3434; StemCell Technologies). Cell numbers did not exceed 1×10^7/ml. One milliliter of twice final concentration HAT ($2\times$ = 240 μM hypoxanthine/0.8 μM aminopterin/40 μM thymidine) was added along the wall to each dish of cells 2 days after electroporation. These conditions support the growth of HAT-resistant clonogenic progenitor cells. The input numbers of CFCs were determined by culturing 5×10^4 BM cells in 1 ml of the same methylcellulose medium without HAT. Colonies were counted under dark-field illumination at day 14. The procedure used for gene targeting in ES cells was essentially as described (10) except that HAT selection was imposed 2 days after electroporation.

Reverse Transcription–PCR (RT-PCR) Analyses and Sequencing of Transcripts. HAT-resistant colonies were picked under a dissecting microscope and washed with 200 μl of cold PBS. Preparation of RNA and cDNA synthesis using an oligo(dT) primer were with a Cells-to-cDNA kit supplied by Ambion (Austin, TX). PCR conditions were: 94°C for 2 min, then 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min. The primers were 5′-TCCTCCTCCTGAGCAGTCAG-3′ (for human exon 1), and 5′-ATCTCCACCAATAACTTTTATGTCCC-3′ (for mouse exon 4). The expected PCR product was 402 bp. The quality of the tested RNA was confirmed by PCR with primers specific for the murine glyceraldehyde-3-phosphate dehydrogenase gene, 5′-GTTCCAGTATGACTCCACTCACG-3′ and 5′-AGATCCACGACGGACACATTG-3′, which gives a 597-bp fragment. PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide. The 402-bp PCR product was isolated and purified by QIAquick (Qiagen, Chatsworth, CA) and sequenced by using an automatic DNA sequencer (Applied Biosystems).

Results

To establish conditions able to detect homologous recombination events in a clonogenic assay, we first carried out reconstruction experiments with mixtures of wild-type and mutant BM cells. BM cells were prepared from male mice having a partial deletion of the X-linked *Hprt* gene; these Hprt$^-$ cells are killed by HAT-containing medium. We also prepared BM cells from wild-type mice having a functional *Hprt* gene; these Hprt$^+$ cells survive in HAT-containing medium. Mixtures were made with decreasing numbers of wild-type Hprt$^+$ BM cells added to an excess of Hprt$^-$ BM cells in ratios ranging from 1/10 to 1/100,000, thereby simulating the correction of the Hprt$^-$ BM cells at frequencies ranging from 10^{-1} to 10^{-5}. The mixtures were plated in a standard methylcellulose medium for murine clonogenic hematopoietic progenitor cells, but in the presence of HAT. The numbers of colonies, and so of CFCs, counted at day 14, were normalized to a constant number of treated cells, and plotted against the input ratio of Hprt$^+$ to Hprt$^-$ cells (Fig. 1*A*). The data show that the progressively decreasing numbers of Hprt$^+$

Table 1. Correction of an HPRT$^-$ mutation by homologous recombination

HPRT$^-$ cell type	Cell no., $\times 10^{-7}$	Input CFC, $\times 10^{-5}$	HAT^r colonies, no.	Correction frequency, $\times 10^5$
BM (*Hprt$^{b\text{-}m3}$*)	7.3	1.5*	9	6.0
BM (*Hprt$^{b\text{-}m3}$*)	12	2.4*	10	4.2
BM (*Hprt$^{b\text{-}m3}$*)	8.5	0.3†	0	NA
BM (*Hprt$^{b\text{-}m3}$*)	12	4.8†	8	1.7
BM (*Hprt$^{b\text{-}m3}$*)	10	2.5†	3	1.2
BM (*Hprt$^{b\text{-}m3}$*)	10	1.2†	11	9.2
				4.4 ± 3.3‡
ES (HM-1)	2.0	NA	420	2.1
ES (HM-1)	1.5	NA	300	2.0
ES (HM-1)	2.0	NA	564	2.8
				2.3 ± 0.4‡

CFC, colony-forming cells; HAT^r, HAT resistant; and NA, not applicable.
*Assuming 200 CFC/10^5 BM cells, as calculated from the data in Fig. 1*A*.
†Number determined directly by a clonogenic assay without HAT selection.
‡Means ± standard deviation.

(HAT-resistant) colonies are readily detected in the presence of an increasingly vast excess of Hprt$^-$ BM (HAT-sensitive) cells. No signs of deviation from linearity were observed even when the proportion of Hprt$^+$ colonies was only 1 in 100,000. The presence of a large number of dying cells (HAT kills dividing Hprt$^-$ cells in about 3 days) reduces the sizes of the individual colonies, but the linearity of the plot in Fig. 1*A* shows that this does not affect the colony count.

Our procedure for assaying homologous recombination in hematopoietic colony-forming progenitors is illustrated in Fig. 1 *B* and *C*. BM cells are isolated from descendants of mice originally generated by Hooper *et al.* (8) from an ES cell that is Hprt$^-$ as a consequence of a spontaneous 55-kb deletion (12) that includes the promoter and exons 1 and 2 of the *Hprt* gene. The correcting DNA, which has ≈6 kb of its sequence homologous to the target locus, supplies the missing promoter and exons after homologous recombination (Fig. 1*C*). We introduce correcting DNA into the Hprt$^-$ BM cells by electroporation, the treated cells are plated in methylcellulose, an equal volume of 2× HAT medium is added after 2 days, and the number of HAT-resistant (Hprt$^+$) colonies are counted 12 days later. Because the mutation in the Hprt$^-$ BM cells is a deletion, which never reverts to Hprt$^+$ spontaneously, no background of false-positive HAT-resistant cells occurs. The colony assay consequently counts the number of CFCs that have been corrected by homologous recombination. Dividing this number by the total number of CFCs in the BM sample at the time of electroporation gives the frequency of gene correction.

Six experiments were carried out, each with between 7 and 12×10^7 unfractionated BM cells from a single adult male Hprt$^-$ mouse. The total number of nucleated Hprt$^-$ cells treated (cell no.) and the number of CFCs in this total (input CFC) are presented in Table 1. In the first two experiments, the input numbers of CFCs were calculated on the assumption that 10^{-5} BM cells contain 200 CFCs, a figure derived from the data in Fig. 1*A*, which were obtained with BM cells from mice of a similar age. In the remaining four experiments, the input numbers of CFCs were directly assayed by counting colonies formed in the absence of HAT selection and without electroporation. The frequency of gene correction ranged from 1.2 to 9.2 per 10^5 input CFCs, with a mean and standard deviation of $4.4 \pm 3.3 \times 10^{-5}$. This frequency is a conservative estimate, because no corrections were made for cells killed by the electroporation. One experiment failed to yield any HAT-resistant colonies, but this result is not unexpected statistically because the input number of CFCs in this BM sample was low (0.3×10^5).

The HAT-resistant colonies derived from Hprt$^-$ BM cells after gene correction were indistinguishable in gross appearance from the colonies derived from wild-type BM cells cultured in the presence of a comparable excess (>100×) of Hprt$^-$ BM cells (Fig. 2*A Left*). The types of cell present in the wild-type and corrected colonies, as judged from cytospin preparations (Fig. 2*A Right*), were likewise indistinguishable. Typically both included granulocytes and macrophages, indicating that they were derived from hematopoietic progenitors capable of yielding more than one type of fully differentiated cell.

To check whether the HAT-resistant colonies obtained in these experiments were truly the result of correction of their mutant *Hprt* genes by homologous recombination in the predicted manner, we devised a RT-PCR assay that amplifies a recombinant RNA fragment that is present only in cells in which the mutation has been corrected in the predicted manner. The assay uses one primer that hybridizes to sequences in exon 1 of the human *HPRT* gene, which is present in the correcting DNA but not in the mouse BM cells. The other primer hybridizes to exon 4 of the mouse *Hprt* gene, which is present in the target mouse BM cells but not in the correcting DNA. Thus, specific PCR amplification can occur only when a human/mouse chimeric mRNA is synthesized after the juxtaposition of human exon 1 and mouse exon 4 as a result of homologous recombination at the *Hprt* genomic locus. The size of the PCR product should be 402 bp. The ethidium bromide-stained gel illustrated in Fig. 2*B* shows that the recombinant RNA fragment is present in HAT-resistant colonies resulting from correction of the Hprt$^-$ mutation, as judged by the predicted 402-bp PCR product, but is absent in noncorrected Hprt$^-$ cells. The recombinant RNA is also absent in normal Hprt$^+$ control cells that synthesize *Hprt* mRNA having only mouse sequences (data not shown). Confirmation of the RT-PCR result was obtained by sequencing the chimeric 402-bp PCR product by using a primer corresponding to mouse exon 4 (Fig. 2*C*). The nucleotide sequence of exon 1 of the PCR product was human (the relevant positions are underlined); the nucleotide sequence of its exon 2 was mouse.

ES cells generally are recognized as efficient in homologous recombination. To serve as a comparative standard for the frequency of recombination in the BM CFCs, we therefore assayed the frequency of gene correction in HM-1 ES cells (9), which carry the same mutation as the Hprt$^-$ donor mice. The bottom three lines of Table 1 list the correction frequencies in HM-1 cells by using the same correcting DNA as in the BM experiments but with electroporation conditions optimized for ES cells. The average frequency of gene correction was $2.3 \pm 0.4 \times 10^{-5}$, which is not significantly different from that observed in the BM CFCs, $4.4 \pm 3.3 \times 10^{-5}$. We conclude that clonogenic hematopoietic progenitor cells are capable of mediating homologous recombination at a frequency comparable to that seen in ES cells.

Discussion

This demonstration of gene correction by homologous recombination in hematopoietic progenitor cells opens the way to correcting cells of this category with a view to gene therapy that provides short-term hematopoietic support (13). It also should encourage investigators to approach the task of achieving targeted gene correction in the more primitive HSCs that are capable of populating BM long term. We recognize that our currently demonstrated frequency of gene correction in hematopoietic CFCs is low. We also recognize that, although the exact numbers are debatable, HSCs represent a smaller fraction of BM cells than do the CFCs that we have corrected (which occur at a frequency of approximately two per 10^3 nucleated BM cells). Assays for HSCs are based on their capacity to provide long-term

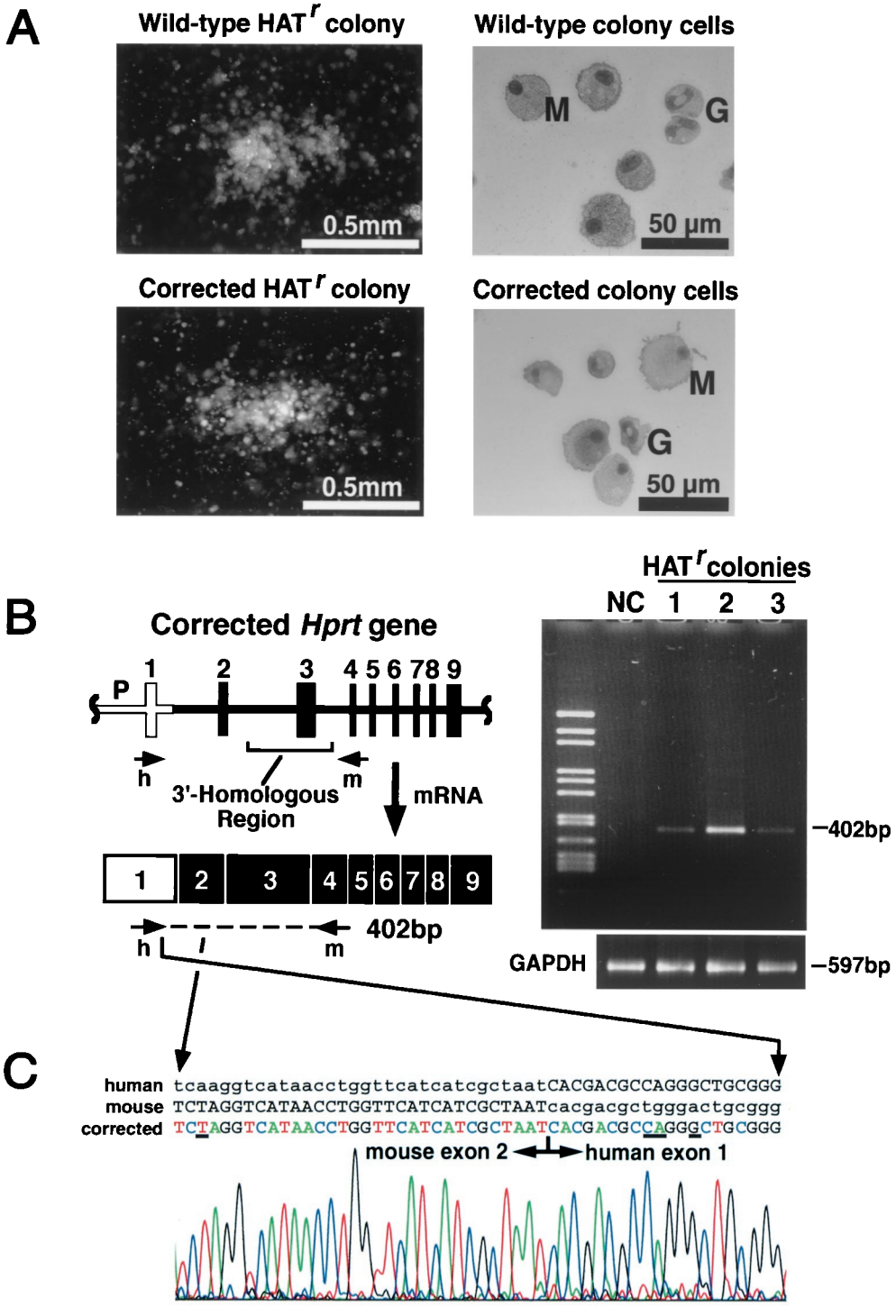

Fig. 2. Analyses of HAT^r colonies. (*A*) Typical HAT^r colonies (*Left*, dark field) and their component cells (*Right*, Wright–Giemsa-stained cytospin) from (*Upper*) wild-type BM cells mixed with Hprt$^-$ BM cells and (*Lower*) corrected Hprt$^-$ BM cells. M, macrophage; and G, granulocyte. (*B*) RT-PCR analyses to detect corrected *Hprt* transcripts. The diagram shows the corrected *Hprt* gene and the recombinant mRNA structure. The open box is human exon 1; the black boxes are mouse exons 2–9. The PCR analyses to test for gene targeting use a human-specific primer (h) from the first exon and a mouse primer (m) from the fourth exon. The photograph shows gel electrophoresis of the PCR products obtained when individual HAT^r colonies were analyzed by RT-PCR by using these primers. The 402-bp fragment was obtained with the HAT^r colonies (1, 2, and 3), which contain chimeric human-mouse transcripts from the corrected *Hprt* gene, but was not obtained with noncorrected (NC) cells. The same samples were tested with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers which amplify a 597-bp fragment. (*C*) Nucleotide sequence of RT-PCR product from a HAT^r colony. The printed nucleotide sequences are from human *HPRT* cDNA (*Top line*), mouse cDNA (*Middle line*), and the 402-bp PCR product sequenced with a primer corresponding to mouse exon 4 (*Bottom line*). The gel scan from the automatic DNA sequencer is shown. The first exon of the PCR product is identical to the human *HPRT* sequence, and the second exon is identical to the mouse *Hprt* sequence. Capital letters highlight the first exon of human and second exon of the mouse. Underlining shows the differences between human and mouse.

hematopoietic repopulation in recipient animals, and estimates of their numbers depend on the assay system used. Assays in which marrow cells are transplanted into lethally irradiated recipients (14) suggest a frequency of approximately one HSC per 10^5 nucleated BM cells, whereas those using lethally irradiated wild-type recipients also receiving short-term populating cells (15) or sublethally irradiated (16) and nonirradiated recipients (17) whose endogenous HSCs are genetically handicapped suggest a higher frequency (approximately one per 10^4 nucleated BM cells). These differences probably reflect the effects of radiation damage to the BM microenvironment (18), so that the higher estimate (one HSC per 10^4 marrow cells) is more applicable to the present discussion. In this case, if HSCs are corrected at the same frequency as CFCs (approximately one event per 2×10^4 cells), an average of 2×10^8 cells would be required for a single HSC correction event to be detected, which is approximately the number of marrow cells obtainable from four mice. This number of cells could not be injected into a single recipient mouse, and so some form of enrichment for stem cells would be desirable, such as the use of commercially available antibody-coated magnetic beads to discard the majority of unwanted cells but retain a subfraction that includes HSCs.

Our optimism with regard to the feasibility of gene correction in HSCs is supported by the fact that electroporation is versatile and applicable to a wide variety of cell types (19). Moreover, the overall effectiveness of gene correction in HSCs is open to substantial improvements by modifying various parts of our experimental procedure. First, by doubling the length of the homologous sequences in the input DNA, the targeting frequency can be increased more than 10-fold (20). Second, the proportion of repopulating HSCs in the BM can be increased at least 10-fold by pretreatment with suitable cytokines (21). Third, additional genetic material can be introduced into the target locus at the time the HSCs are corrected to provide them with an advantage in engraftment and/or enhance their proliferation *in vivo*. For example, a transgene encoding a truncated erythropoietin receptor sensitive to exogenously administered erythropoietin is a demonstrated example of such a benign advantage sequence (22). A transgene encoding a chimeric protein that dimerizes and induces cell growth when exposed to a chemical inducer of dimerization is another example (23). Predicting the overall improvement in the effectiveness of HSC gene correction achievable by these strategies alone or in combination is difficult, but an improvement of more than 2 orders of magnitude is conceivable. Finally, it appears reasonable to expect that the long-sought goal of expanding HSCs *ex vivo* (24) eventually will be reached.

Whereas our optimism of achieving gene correction in HSCs at a usable frequency may be justified, there are still serious obstacles to consider. For example, therapeutic gene targeting in human subjects presents problems of genetic heterogeneity that are not observed in isogeneic experimental mice. Additionally, targeting at the *Hprt* locus occurs at a relatively high frequency and automatically provides a selectable marker for targeted cells; the frequency of targeting at therapeutically more important loci is likely to be less, and direct selection of corrected cells will not usually be possible. Our experiments are nonetheless encouraging because they clearly demonstrate that gene correction by homologous recombination can be achieved in clonogenic progenitor cells, which are in the same lineage as HSCs capable of long-term BM engraftment.

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