Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6567–6571, July 1993
Genetics

A single-base-pair deletion in the β-glucuronidase gene accounts for the phenotype of murine mucopolysaccharidosis type VII

(animal models/restriction fragment length variation detection/translation termination/mRNA metabolism)

MARK S. SANDS AND EDWARD H. BIRKENMEIER*

The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609

Communicated by William S. Sly, April 14, 1993 (received for review January 14, 1993)

ABSTRACT **Murine mucopolysaccharidosis type VII is a heritable disease caused by a spontaneous mutation, *gus^{mps}*, closely linked to the β-glucuronidase structural gene on chromosome 5. Mice homozygous for the mutation have a >200-fold decrease in β-glucuronidase mRNA levels and virtually no enzyme activity detectable by a sensitive fluorometric assay. Approximately 20 kb of genomic DNA containing the β-glucuronidase gene *Gus* and >2 kb of 5′ and 3′ flanking sequences were cloned from both a *gus^{mps}/gus^{mps}* mouse and a +/+ mouse of the progenitor strain. Restriction enzyme digests containing DNA fragments 20–400 bp in length were generated from each of the two *Gus* alleles and then compared by using nondenaturing polyacrylamide DNA-sequencing gels. This method rapidly identified a large number of restriction sites and was sensitive enough to detect a restriction fragment length variation resulting from a 1-bp deletion in the *gus^{mps}* allele. DNA-sequence analysis of the mutant genomic fragment showed that the 1-bp deletion created a frameshift mutation within exon 10. Insertion of the deleted nucleotide by oligonucleotide site-directed mutagenesis restored function to the corrected mutant gene when transfected into *gus^{mps}/gus^{mps}* fibroblasts. We concluded that the frameshift mutation, which introduces a premature stop codon at codon 497 in exon 10, accounts for the molecular, biochemical, and pathological abnormalities associated with the *gus^{mps}* phenotype.**

Mutant mice lacking β-glucuronidase (β-D-glucuronoside glucuronosohydrolase, EC 3.2.1.31) activity develop the lysosomal storage disease murine mucopolysaccharidosis type VII (MPS-VII) (1). Mice homozygous for the MPS-VII mutation *gus^{mps}* have a dramatically shortened life-span and share many clinical and pathological characteristics with humans having MPS-VII or Sly syndrome (1–3). The MPS-VII mouse provides an excellent animal model to study the pathophysiology of the corresponding human disease and to examine the therapeutic efficacy of bone marrow transplantation, i.v. enzyme infusion, and somatic-cell gene therapy (1–4).

The murine β-glucuronidase locus *Gus* is a well characterized genetic locus in the mouse and is composed of several elements referred to as *Gus-s*, *Gus-r*, *Gus-t*, and *Gus-u* (5). *Gus-s* is the protein-coding region, *Gus-r* modulates androgen induction, *Gus-t* controls differential and developmental expression, and *Gus-u* regulates aspects of β-glucuronidase expression common to all cells in an organism (5). The autosomal recessive *gus^{mps}* mutation maps to within 3.7 centimorgans of *Gus-s* (1). Even though Southern blot analysis of *Gus-s* in *gus^{mps}/gus^{mps}* and +/+ mice failed to identify differences in the structure or methylation pattern of the two *Gus-s* alleles, β-glucuronidase mRNA levels were decreased by ≈200-fold in *gus^{mps}/gus^{mps}* mice (1). Interestingly, the relative level of kidney gus^{mps} mRNA responded normally to androgen induction by increasing ≈11-fold (1). These results suggested that the regulatory genes controlling β-glucuronidase expression are normal in *gus^{mps}/gus^{mps}* mice and that the genetic defect may reside adjacent to or within the structural gene, affecting either basal levels of promoter activity or mRNA metabolism.

To more precisely define the defect we isolated β-glucuronidase genomic clones from a *gus^{mps}/gus^{mps}* mouse and from a +/+ mouse of the progenitor strain. High-resolution PAGE was used to search 20 kb of both genomic DNAs for the presence of restriction fragment length variations (RFLVs) not identified by routine Southern blot analysis. A single-base-pair deletion detected within exon 10 of the mutant gene changed the predicted amino acid sequence starting at codon 490. Correction of this mutation in the *gus^{mps}* allele restored expression of β-glucuronidase activity in MPS-VII fibroblasts transfected *in vitro*. We concluded that the frameshift mutation, which introduces a premature translation termination codon in exon 10, is sufficient to explain the microscopic pathology, biochemical defects, and molecular abnormalities associated with the murine MPS-VII phenotype.

MATERIALS AND METHODS

β-Glucuronidase Genomic Cloning. Genomic DNA was isolated from the spleen of a wild-type B6.C-*H-2^{bml}*/By mouse and from the spleen of a *gus^{mps}/gus^{mps}* mouse obtained from the B6.C-*H-2^{bml}*/ByBir-*gus^{mps}*/+ mutant strain (1). The DNAs were partially digested with *Mbo* I, and fragments ranging in size from 9–20 kb were electroeluted from a 0.4% agarose gel. The size-selected fragments were ligated to commercially available λ-DASH arms digested with *Bam*HI and packaged, as described by the manufacturer (Stratagene). Approximately 1×10^6 plaques from both the wild-type and mutant libraries were screened by standard techniques (6) by using a random-primed radiolabeled *Pst* I–*Sca* I restriction fragment from the rat β-glucuronidase cDNA, pGUS-1, as a probe (7). Wild-type and mutant clones containing 3–5 kb of 5′ flanking sequence and extending beyond a unique *Sal* I site within intron 9 (8) were isolated. The 3′ portion of both genes was cloned initially as a 9-kb *Eco*RI fragment in λ-DASH and were then subcloned in pUC19 by using standard techniques (9). A 330-bp *Sal* I–*Eco*RI fragment derived from the wild-type and mutant gene was cloned onto the 5′ end of the wild-type and mutant 9-kb *Eco*RI fragment, respectively. Full-length wild-type and mutant genes were reconstructed by cloning the 5′ and 3′ portions into λ-DASH using the common *Sal* I site.

High-Resolution Restriction Fragment Analysis. To uniformly label the clones, 2 μg of λ DNA containing either the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MPS-VII, mucopolysaccharidosis type VII; RFLV, restriction fragment length variation.

*To whom reprint requests should be addressed.

wild-type or mutant β-glucuronidase gene were incubated with 7.5 units of *Escherichia coli* DNA polymerase I (BRL) and 16.7 pmol (50 μCi) of [^{32}P]dCTP (Amersham; 3000 Ci/mmol; 1 Ci = 37 GBq) in 50 mM Tris, pH 7.5/5 mM $MgCl_2$/1 mM dithiothreitol/0.5 mM each of dATP, dTTP, and dGTP for 1 hr at 37°C. The uniformly radiolabeled DNA was phenol-chloroform extracted, ethanol precipitated, and digested with *Eco*RI. The 2.2-, 3-, 6-, and 9-kb *Eco*RI restriction fragments encompassing the entire β-glucuronidase gene including 2–4 kb of 5′ and 3′ flanking sequences were separated on a 1% agarose gel in 50 mM Tris/50 mM boric acid/1 mM EDTA buffer (TBE). The gel was briefly exposed to x-ray film, the fragments were excised, and the DNA was electroeluted in 0.2× TBE. The individual radiolabeled *Eco*RI fragments were then digested to completion with restriction enzymes that frequently cleave the β-glucuronidase gene. Approximately 2 × 10^4 cpm (Cerenkov) from each digestion were electrophoresed on 0.4-mm-thick, 8% polyacrylamide sequencing-type gels run in TBE buffer under nondenaturing conditions. The gels were then dried and exposed to Kodak X-Omat AR film.

Sequencing, PCR, and Mutagenesis. A 186-bp *Taq* I–*Bam*HI restriction fragment encompassing the RFLV was cloned into M13mp18 and sequenced as described (10). The PCR was done, as described (11), with genomic DNA as template and the following two primers: 5′-GGATCCTGTGTCATTTGCATGTG-3′ and 5′-AACGTTTACACAGATAACATCCACG-3′ from intron 9 and exon 10, respectively. The PCR products from the mutant and wild-type genes were 113 and 114 bp in length, respectively. Oligonucleotide site-directed mutagenesis was done on a 650-bp *Eco*RI–*Bam*HI restriction fragment from the mutant gene, as described (12). The sequence of the mutagenic primer was 5′-CATCCACGTACGGGGCCTAAGACAAGAGCG-3′. Sequence analysis confirmed the insertion of the deleted nucleotide and the integrity of the remaining sequences.

Transfections. Fibroblasts (1.7 × 10^6) derived from a gus^{mps}/gus^{mps} mouse were cotransfected with 5 μg of wild-type, mutant, or corrected mutant β-glucuronidase genes and 2.5 μg of a neomycin resistance gene driven by the simian virus 40 promoter. The cells were electroporated at 250 μF and 450 V (Gene Pulser, Bio-Rad) and plated at 2.5 × 10^5 cells per 100-mm dish. Stable transformants were selected in medium containing G418 at 200 μg/ml (Gentecin, GIBCO). Ten days after transfection, cells expressing β-glucuronidase activity were detected by a histochemical stain using naphthol AS-BI β-D-glucuronide (Sigma) and pararosaniline hydrochloride (13).

RESULTS AND DISCUSSION

β-Glucuronidase Genes and High-Resolution RFLV Mapping. Genomic DNA encoding the 5′ and 3′ halves of the β-glucuronidase structural gene from mutant and wild-type mice was cloned in λ. Full-length clones were reconstructed by using the unique *Sal* I site common to overlapping regions of the partial clones. In this way, >20 kb of contiguous genomic DNA containing the entire structural gene and at least 2 kb of 5′ and 3′ flanking sequences, respectively, were isolated from both wild-type and mutant DNA (Fig. 1).

To rapidly compare 20 kb of genomic sequences representing the wild-type and mutant genes, we used *E. coli* DNA polymerase I to uniformly radiolabel the cloned DNA by nick translation. λ and plasmid DNA acquire sufficient nicks during purification to serve as excellent templates for the exonuclease and polymerase activities of DNA polymerase I without the addition of DNase. In this way, large fragments of uniformly radiolabeled DNA can be obtained with a specific activity of 10^5–10^6 cpm/μg. Nanogram quantities of radiolabeled DNA were digested with frequently cutting restriction enzymes, and the fragments were resolved on DNA-sequencing-type gels under nondenaturing conditions. By comparing restriction fragments from the wild-type and mutant genes in adjacent lanes, small differences in mobilities of homologous fragments were detected. For example, a slight alteration in the relative migration of a 215-bp *Dde* I

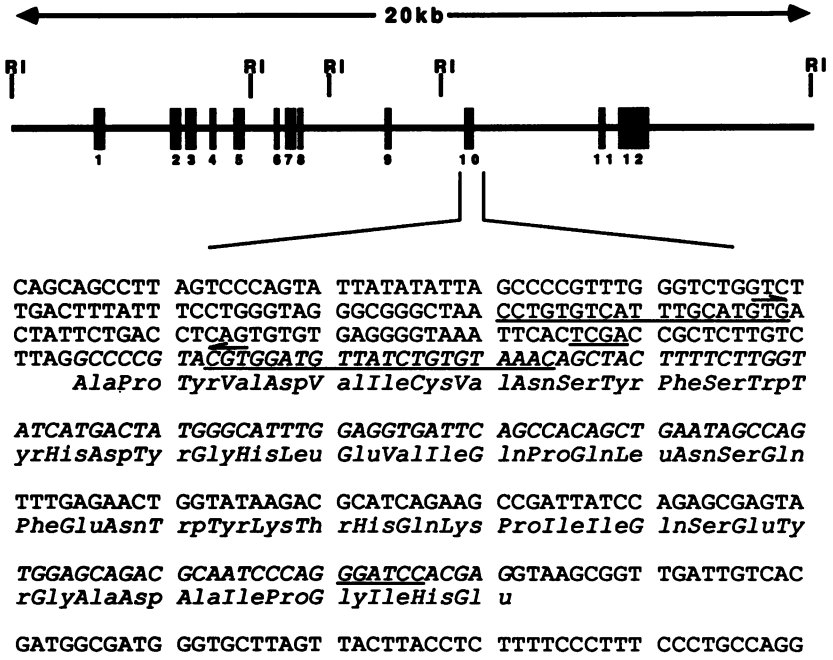

FIG. 1. Structure of murine β-glucuronidase gene. The diagram represents 20 kb of genomic sequences encompassing the murine β-glucuronidase gene and flanking sequences. The horizontal line represents the flanking and intron sequences. The solid rectangles numbered 1–12 represent the exons. R1 indicates the positions of *Eco*RI restriction sites. The nucleotide and deduced amino acid sequences adjacent to and within exon 10 are shown below the gene. Underlined nucleotide sequences TCGA and GGATCC identify *Taq* I and *Bam*HI restriction sites, respectively. Forward and reverse PCR primers corresponding to the two additional underlined nucleotide sequences were synthesized, the 3′ ends of which are indicated by short overlined arrows. The DNA sequence shown is from the murine *Gus-s*^{a} allele (8).

restriction fragment was observed in one of the mutant lanes (Fig. 2*A*). This fragment was derived from the radiolabeled 9-kb *Eco*RI fragment encompassing the 3′ portion of the β-glucuronidase gene (Fig. 1). Higher resolution of the RFLV was obtained when the *Dde* I fragment was further digested with *Bst*NI to generate a smaller 167-bp fragment (Fig. 2*A*). The difference between the wild-type and mutant alleles was confirmed by using PCR to selectively amplify a segment of genomic DNA containing the RFLV (Fig. 1) and analyzing the PCR products on a nondenaturing sequencing-type gel stained with ethidium bromide (Fig. 2*B*).

A Single-Base-Pair Deletion in Exon 10. The 186-bp *Taq* I–*Bam*HI restriction fragment shown in Fig. 1 contains the altered 167-bp *Dde* I–*Bst*NI fragment described above. DNA-sequence analysis of the *Taq* I–*Bam*HI fragment revealed that the only difference between mutant and normal DNA was a single-base-pair deletion in the mutant gene (Fig. 3*A*). As shown in Fig. 2*A*, this small deletion was initially detected

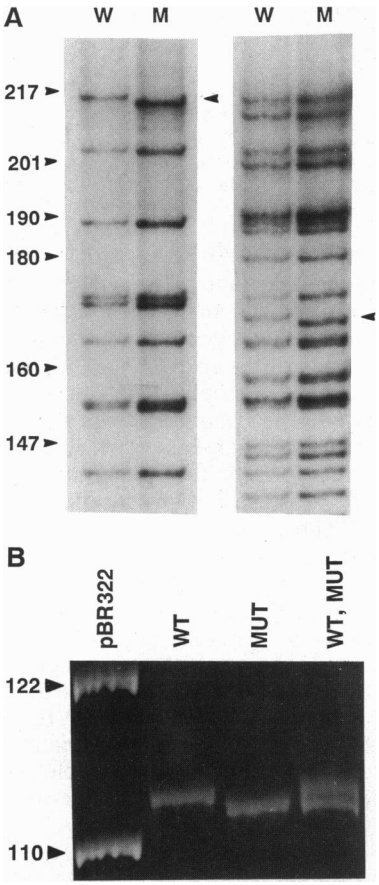

FIG. 2. High-resolution RFLV analysis of mutant and wild-type alleles. (*A*) Autoradiograph comparing relative mobilities of restriction fragments from wild-type (W) and mutant (M) β-glucuronidase genes resolved on a 0.4-mm-thick 8% polyacrylamide DNA-sequencing-type gel run under nondenaturing conditions. Fragments were generated by digesting a uniformly radiolabeled 9-kb *Eco*RI restriction fragment containing exons 10–12 (Fig. 1) with the restriction enzyme *Dde* I (left two lanes) or with a combination of *Dde* I and *Bst*NI (right two lanes). Arrowheads at right of lanes indicate the location of a faster-migrating fragment derived from the mutant gene. (*B*) Ethidium-stained 12% polyacrylamide gel illustrating the difference in mobility of wild-type (WT) and mutant (MUT) genomic DNA fragments amplified by using the two PCR primers indicated in Fig. 1. The fourth lane (WT, MUT) contains PCR products obtained by separately amplifying WT and MUT genomic DNA and then mixing together equal volumes of each completed reaction. For both *A* and *B*, arrowheads at left indicate mobility and size (bp) of restriction fragments from pBR322 digested with *Msp* I.

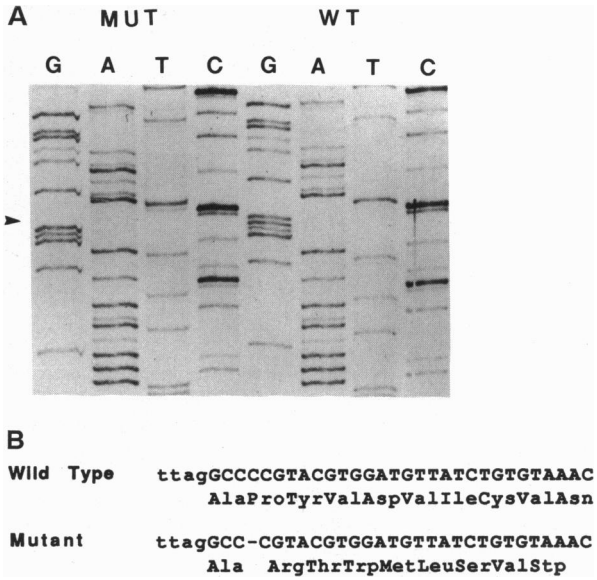

FIG. 3. Location of murine MPS-VII frameshift mutation. (*A*) Sequence of coding strand of exon 10 from mutant (MUT) and wild-type (WT) DNA contained within a *Taq* I–*Bam*HI restriction fragment (Fig. 1). The arrowhead indicates a single-base-deletion in the mutant gene. (*B*) Location of deleted nucleotide on noncoding strand. Lowercase letters indicate nucleotides within intron 9, and uppercase letters indicate protein-coding sequences within exon 10. The deleted nucleotide is indicated by a −, and the premature stop codon (Stp) is shown with the deduced amino acids beneath the nucleotide sequence.

using *Dde* I alone. We selected *Dde* I because it has 103 recognition sites within 14 kb of the *Gus-s*^{a}-allele, representing a total of 412 bp or ≈3% of the nucleotide sequence (8). Although the *gus*^{mps} mutation did not result in the loss or gain of a *Dde* I site, we could scan 20 kb of DNA on a single gel and detect a single-base-pair deletion within a *Dde* I fragment. Thus, small deletions or insertions that may not be detected by conventional Southern blotting of agarose gels may become apparent with this method. Because high-resolution RFLV mapping is easier and more reproducible than denaturing gradient-gel electrophoresis, it may be the method of choice when initially comparing the sequences of wild-type and mutant genes contained within relatively large fragments of genomic DNA.

Because the deletion generates a frameshift mutation within exon 10, the predicted amino acid sequence is altered beginning with codon 490, and a premature translation stop signal is introduced at codon 497 within the same exon (Fig. 3*B*). A truncated, presumably nonfunctional, polypeptide would result from the translation of this mRNA. β-Glucuronidase is composed of functional domains required for catalytic activity, tetramerization, intracellular trafficking, egasin binding, and glycosylation (5). It is unclear at present which functions might be affected by truncation and amino acid changes at the C-terminal portion of the protein because the locations of the various functional domains have not been clearly delineated.

Because β-glucuronidase mRNA levels are reduced >200-fold in MPS-VII mice (1), there is probably little or no truncated protein produced. Previous reports studying human β-globin, human triose-phosphate isomerase, human ornithine aminotransferase, and hamster dihydrofolate reductase have shown that premature translation-termination codons generated by nonsense or frameshift mutations often result in lowered steady-state levels of mRNA (14–17). Although the exact mechanisms responsible for altering the metabolism of mRNAs containing these mutations have not

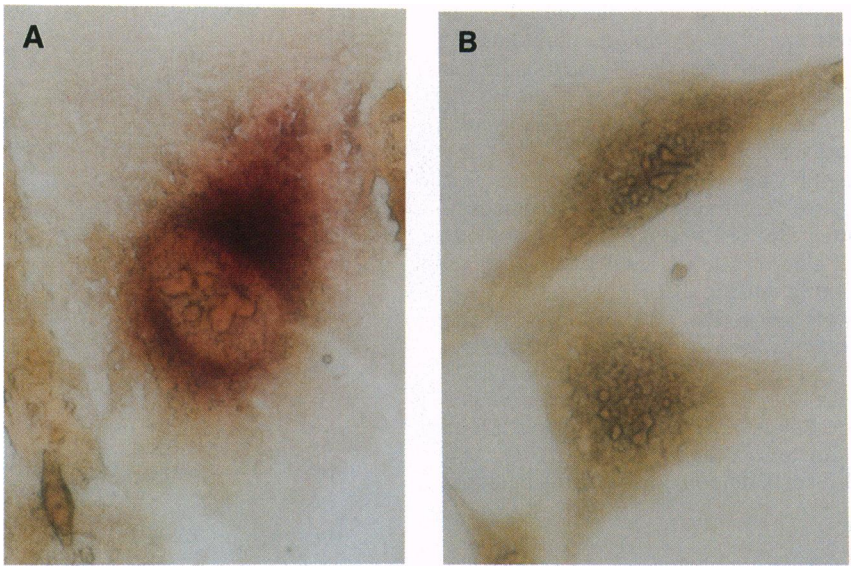

FIG. 4. Expression of corrected mutant β-glucuronidase gene. Fibroblasts from a gus^{mps}/gus^{mps} mouse were transfected with either the mutant or the corrected mutant β-glucuronidase gene. Structure of the 20-kb reconstructed genes extending between upstream and downstream *Eco*RI sites is diagrammed in Fig. 1. The amount of 5′ and 3′ flanking sequences were ≈2.5 kb and 3.6 kb, respectively. Transfected cells were stained histochemically, as described. Enzymatically active β-glucuronidase was detected by the formation of a red precipitate in a low percentage of cells transfected with the corrected mutant gene (*A*). None of the cells transfected with the mutant gene had detectable levels of activity (*B*).

been identified, it appears likely that intranuclear events, such as exon scanning or nuclear translocation/translation, are involved in most cases (14, 17). Interestingly, position of the mutation may be important, with premature-translation termination in upstream exons usually having a greater effect than in the final exon (15, 16). Because the frameshift mutation in the gus^{mps} allele introduces a premature translation stop codon in the 10th exon, it seems likely that the very low steady-state mRNA levels in MPS-VII mice are caused by mechanisms similar to those mentioned above.

It is also possible that the reduced level of β-glucuronidase mRNA in MPS-VII mice is a result of aberrant mRNA splicing. Although exon sequences sometimes have a role in splice-site selection (18), it seems unlikely that the single-base-pair deletion in exon 10 would be sufficient to alter splicing because a consensus of exon sequences, other than a guanine residue, has not been identified at the 3′ splice site (19). However, presence of the nonsense codon resulting from the frameshift mutation could affect splicing of gus^{mps} mRNA. A recent report showed that skipping of constitutive exons is induced by nonsense mutations (20). In addition, nonsense mutations inhibit splicing of autonomous parvovirus minute virus of mice RNA *in cis* when they interrupt the reading frame of either exon of the final spliced product (21). Whatever mechanism actually causes the reduced steady-state levels of β-glucuronidase mRNA in mutant mice, the MPS-VII mouse provides an interesting and well-characterized animal model useful for further studies on the relationship between nonsense-codon mutations and mRNA metabolism.

Correction of the gus^{mps} Frameshift Mutation. Oligonucleotide site-directed mutagenesis was used to insert the deleted nucleotide and restore the normal reading frame of the mutant β-glucuronidase gene. Mutant, corrected mutant, and wild-type genes were reconstructed and then transfected along with a neomycin-resistance gene into gus^{mps}/gus^{mps} fibroblasts. Ten days later, the comparable numbers of G418-resistant colonies present on all plates were stained histochemically for β-glucuronidase activity. Although none of the G418 resistant cells cotransfected with the mutant gene stained positive, enzyme activity was readily observed in ≈7.5% and 12% of the G418-resistant colonies cotransfected with the corrected mutant gene and the wild-type gene, respectively (Fig. 4). The intensity of staining was similar in cells transfected with either the wild-type or corrected mutant genes, and there was an average of 11 β-glucuronidase-positive colonies on each of four plates transfected with the corrected mutant gene (data not shown).

Although the gus^{mps} allele has been maintained by inbreeding $gus^{mps}/+$ mice for 16 yr (>40 generations), restoration of function *in vitro* was achieved by insertion of a single-base pair in exon 10. These results raise the interesting question of whether restoration of function *in vivo* would result in a normal phenotype. We predict that it would because expression of a human β-glucuronidase transgene corrected the metabolic defects in MPS-VII mice (22). Taken together, these results strongly suggest that the single-base-pair deletion in exon 10 accounts for the phenotype of MPS-VII mice. Now that the primary defect in MPS-VII mice has been defined at the molecular level, this animal model of Sly disease will continue to provide a valuable system in which to study the efficacy of gene- and enzyme-replacement therapies (4).

We thank Babette Gwynn for expert technical assistance; we also thank John W. Kyle for providing the cell lines used in the transfection studies. The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care. This research was supported by National Institutes of Health Grant DK41082 to E.H.B. and National Research Service Award Grants DK07449 and DK08546 to M.S.S.

1. Birkenmeier, E. H., Davisson, M. T., Beamer, W. G., Ganschow, R. E., Vogler, C. A., Gwynn, B., Lyford, K. A., Maltais, L. M. & Wawrzyniak, C. J. (1989) *J. Clin. Invest.* **83**, 1258–1266.
2. Sly, W. S., Quinton, B. A., McAlister, W. H. & Rimoin, D. L. (1973) *J. Pediatr.* **82**, 249–257.
3. Vogler, C., Birkenmeier, E. H., Sly, W. S., Levy, B., Pegors, C., Kyle, J. W. & Beamer, W. S. (1990) *Am. J. Pathol.* **136**, 207–217.
4. Wolfe, J. H., Sands, M. S., Barker, J. E., Gwynn, B., Rowe, L. B., Vogler, C. A. & Birkenmeier, E. H. (1992) *Nature (London)* **360**, 749–753.

5. Paigen, K. (1989) *Prog. Nucleic Acid Res. Mol. Biol.* **37**, 155–205.
6. Boguski, M. S., Birkenmeier, E. H., Elshourbagy, N. A., Taylor, J. M. & Gordon, J. I. (1986) *J. Biol. Chem.* **261**, 6398–6407.
7. Powell, P. P., Kyle, J. W., Miller, R. D., Pantano, J., Grubb, J. H. & Sly, W. S. (1988) *Biochem. J.* **250**, 547–555.
8. D'Amore, M. A., Gallagher, P. M., Korfhagen, T. R. & Ganschow, R. E. (1988) *Biochemistry* **27**, 7131–7140.
9. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
10. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
11. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239**, 487–491.
12. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488–492.
13. Wolfe, J. H., Schuchman, E. H., Stramm, L. E., Concaugh, E. A., Haskins, M. E., Aguirre, G. D., Patterson, D. F., Desnick, R. J. & Gilboa, E. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2877–2881.
14. Baserga, S. J. & Benz, E. J. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2935–2939.
15. Cheng, J., Fogel-Petrovic, M. & Maquat, L. E. (1990) *Mol. Cell. Biol.* **10**, 5215–5225.
16. Mashima, Y., Murakami, A., Weleber, R. G., Kennaway, N. G., Clarke, L., Shiono, T. & Inana, G. (1992) *Am. J. Hum. Genet.* **51**, 81–91.
17. Urlaub, G., Mitchell, P. J., Ciudad, C. J. & Chasin, L. A. (1989) *Mol. Cell. Biol.* **9**, 2868–2880.
18. Watakabe, A., Tanaka, K. & Shimura, Y. (1993) *Genes Dev.* **7**, 407–418.
19. Shapiro, M. B. & Senapathy, P. (1987) *Nucleic Acids Res.* **15**, 7155–7174.
20. Dietz, H. C., Valle, D., Francomano, C. A., Kendzior, R. J., Pyeritz, R. E. & Cutting, G. R. (1993) *Science* **259**, 680–683.
21. Naeger, L. K., Schoborg, R. V., Zhoa, Q., Tullis, G. E. & Pintel, D. J. (1992) *Genes Dev.* **6**, 1107–1119.
22. Kyle, J. W., Birkenmeier, E. H., Gwynn, B., Vogler, C., Hoppe, P., Hoffmann, J. W. & Sly, W. S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3914–3918.