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Correction of murine mucopolysaccharidosis VII by a human β-glucuronidase transgene

(glycosaminoglycan/Sly syndrome/lysosomal enzyme/transgenic mice)

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ABSTRACT **We recently described a murine model for mucopolysaccharidosis VII in mice that have an inherited deficiency of β-glucuronidase (β-D-glucuronoside glucuronosohydrolase, EC 3.2.1.31). Affected mice, of genotype *gus*^{mps}/*gus*^{mps}, present clinical manifestations similar to those of humans with mucopolysaccharidosis VII (Sly syndrome) and are shown here to have secondary elevations of other lysosomal enzymes. The mucopolysaccharidosis VII phenotype in both species includes dwarfism, skeletal deformities, and premature death. Lysosome storage is visualized within enlarged vesicles and correlates biochemically with accumulation of undegraded and partially degraded glycosaminoglycans. In this report we describe the consequences of introducing the human β-glucuronidase gene, *GUSB*, into *gus*^{mps}/*gus*^{mps} mice that produce virtually no murine β-glucuronidase. Transgenic mice homozygous for the mucopolysaccharidosis VII mutation expressed high levels of human β-glucuronidase activity in all tissues examined and were phenotypically normal. Biochemically, both the intralysosomal storage of glycosaminoglycans and the secondary elevation of other acid hydrolases were corrected. These findings demonstrate that the *GUSB* transgene is expressed in *gus*^{mps}/*gus*^{mps} mice and that human β-glucuronidase corrects the murine mucopolysaccharidosis storage disease.**

Mucopolysaccharidoses are a subgroup of lysosomal storage diseases that result from deficiencies of specific lysosomal enzymes involved in the stepwise degradation of glycosaminoglycans (for review, see ref. 1). They are characterized by progressive intralysosomal accumulation of undegraded glycosaminoglycans that leads eventually to cellular and organ dysfunction. Studies of cells from patients with these disorders have provided important insights into the mechanisms of targeting acid hydrolases to lysosomes by the mannose 6-phosphate-dependent pathways (2, 3).

Mucopolysaccharidosis type VII (MPSVII or Sly syndrome) results from deficiency of β-glucuronidase activity (4). β-Glucuronidase (β-D-glucuronoside glucuronosohydrolase, EC 3.2.1.31) is a tetrameric glycoprotein acid hydrolase localized predominantly in lysosomes of virtually all mammalian cells (for review, see ref. 5). It acts in lysosomes as an exoglycosidase to remove β-glucuronic acid residues from the nonreducing termini of glycosaminoglycans. We recently characterized the murine equivalent of human MPSVII in mice that have an inherited, nearly complete deficiency of β-glucuronidase (6, 7). The MPSVII mice have many features in common with humans with MPSVII, including a chronic and progressive course characterized by dwarfism, skeletal deformities, and premature death (1, 4, 6, 7). The MPSVII mice show excessive storage of undegraded glycosaminoglycans within lysosomes, and their leukocytes exhibit the characteristic inclusions described in humans with MPSVII (4).

We recently isolated the human gene for β-glucuronidase from a genomic cosmid library (8); this provided us with the opportunity to study the expression of the human gene in transgenic mice homozygous for the *gus*^{mps} mutation.

METHODS

Construction of Transgenic Mice. The human β-glucuronidase cosmid clone, pHGUS, was digested with *Eco*RV to generate a 28-kilobase (kb) fragment containing the entire structural gene with 1.6 kb of 5′-flanking sequence and 3.8 kb of 3′-flanking sequence. This *Eco*RV fragment was purified by sedimentation in a 5–20% (wt/wt) sucrose gradient. The 38-ml gradient was centrifuged at 26,000 rpm in a Beckman SW 28 rotor for 20 hr at 4°C. After precipitation in ethanol, the DNA fragment was resuspended in 10 mM Tris·HCl, pH 7.5/0.1 mM EDTA at a final concentration of 20 μg/ml. Male pronuclei of both F_1 and F_2 zygotes were microinjected as described (9). The F_1 zygotes were derived from C57BL/6J × LT/Sv crosses, and the F_2 zygotes were derived from an intercross of C57BL/6J × SJL/J F_1 hybrids. Of 29 animals born from the injected zygotes, four animals contained the human transgene, as determined by Southern blot analysis of tail DNA.

MPSVII mice were bred and maintained at The Jackson Laboratory. The human β-glucuronidase gene (*GUSB*) was placed onto the MPSVII mouse genetic background by mating male mice heterozygous for the human transgene (Tg*GUSB*/+) with female mice homozygous for the *gus*^{mps} mutation. Offspring with the human transgene (Tg*GUSB*/+, *gus*^{mps}/+) were then mated to offspring without the human transgene to generate mice homozygous for the *gus*^{mps} mutation and heterozygous for the human transgene (Tg*GUSB*/+, *gus*^{mps}/*gus*^{mps}). Mice homozygous for the human transgene were generated by additional brother–sister matings.

Nucleic Acid Techniques. All nucleic acid and recombinant DNA techniques were done according to procedures compiled by Maniatis *et al.* (10).

Lysosomal Enzyme Assays. Lysosomal enzymes were assayed fluorometrically by using 4-methylumbelliferyl substrates, as described (11, 12). Tissues were dissected and either stored at −80°C or homogenized immediately in 2–5 vol of homogenization buffer (25 mM Tris·HCl, pH 7.2/140

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Abbreviations: MPSVII, mucopolysaccharidosis VII (Sly syndrome); *GUSB*, human β-glucuronidase gene; *gus*^{mps}, heritable β-glucuronidase deficiency in mice; Tg*GUSB*, human β-glucuronidase transgene.

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mM NaCl) by Brinkmann Polytron homogenization for 30 sec at 4°C. Homogenates were then sonicated for 10 sec on ice and clarified by centrifugation at 12,000 × *g* for 30 min. Supernatants were assayed for lysosomal enzyme activities. Proteins were determined according to Lowry *et al.* (13).

Nondenaturing PAGE Gels. Human and mouse β-glucuronidase were resolved by nonreducing PAGE activity gels (14). Approximately 2000 units of β-glucuronidase was deglycosylated by overnight digestion with 0.25 unit of endoglycosidase F (grade II, Boehringer Mannheim), according to the supplier's directions, with the exception that no detergents were used. The removal of carbohydrates was necessary to reduce the heterogeneity in the human β-glucuronidase mobility. There was no loss of β-glucuronidase activity after endoglycosidase F treatment. The deglycosylated β-glucuronidase samples were resolved on a nondenaturing discontinuous PAGE gel with a 3% stacking gel and a 5% separating gel (15). After electrophoresis, the gel was incubated in 0.2 M acetate buffer (pH 4.8) for 30 min. β-Glucuronidase activity was visualized after staining for activity with naphthol-AS-BI β-D-glucuronide (Sigma) and Fast Garnet GBC salt (Sigma) (14).

Isolation and Characterization of Glycosaminoglycans. Kidney, spleen, and liver were weighed and homogenized in 25 ml of chloroform/methanol (2:1, vol/vol) in a Polytron homogenizer. The homogenized tissue was collected by filtration on a glass fiber filter and was extracted twice more with additional chloroform/methanol. The delipidated tissue was lyophilized and weighed. The dried residue was then dispersed in distilled water by sonication and placed in a boiling water bath for 30 min (16). Dispersed samples were cooled to room temperature and digested with papain (35 units per 100 mg of tissue) in 100 mM acetate buffer, pH 5.0, at 60°C for 24 hr. The proteolytic digests were then clarified by centrifugation at 12,000 × *g* for 30 min at 4°C. Liberated glycosaminoglycans in the supernatant were precipitated with cetylpyridinium chloride at a final concentration of 1%. After 3 hr at room temperature, the precipitate was collected by filtration through a cellulose ester membrane filter (Millipore; HV, 0.45-μm pore size) (17). The cetylpyridinium chloride precipitates were dissolved in 200 μl of water and collected by precipitation with 2.5 vol of 95% (vol/vol) ethanol. This precipitate was dissolved in 200 μl of distilled deionized water. Glycosaminoglycan content was estimated by measuring the total hexuronic acid content of the isolated glycosaminoglycans by the carbazole-borate method (18).

Electron Micrographic Analysis. Tissue preparation and electron microscopy were performed as described (7).

RESULTS

Construction of Transgenic Mice and Expression of the Human β-Glucuronidase Transgene in MPSVII Mutant Mice. The entire human β-glucuronidase gene was isolated from a cosmid library and shown to express the human enzyme in transfected rat XC cells (8). The cosmid clone was digested with *Eco*RV to isolate the entire β-glucuronidase structural gene with 1.6 kb of upstream sequences and ≈3.8 kb of sequences 3′ of the last exon. Transgenic mice were produced by microinjection of this 28-kb *Eco*RV fragment into the male pronucleus of zygotes.

Southern blot analysis of tail DNA from the 29 animals obtained from injected zygotes showed that four contained the *GUSB* transgene. Two of these founder animals expressed the human enzyme at high levels in all tissues examined, but only one (founder 4) of these two contained the transgene in the germ line and produced heterozygous transgenic offspring. Transgenic male progeny of founder 4 were mated to gus^{mps}/gus^{mps} females, and the pups were raised by foster mothers. Animals carrying the transgene were identified by analyzing homogenates of tail tissue with standard cellulose acetate electrophoretic methods and staining procedures to detect human enzyme activity (19). Transgenic offspring (Tg*GUSB*/+, gus^{mps}/+) were then mated to nontransgenic offspring (+/+, gus^{mps}/+) obtained from the same parents. The progeny from this mating were screened for the presence of human and mouse β-glucuronidase activity in homogenates of tail tissue using both cellulose acetate electrophoresis and a fluorometric enzyme assay. Of 211 progeny obtained, 95 (45%) appeared phenotypically normal and produced both mouse and human enzyme, 87 (41%) appeared normal and produced only mouse enzyme, 12 (5.7%) appeared normal and produced only human enzyme, and 17 (8.1%) were dwarfed and had no detectable β-glucuronidase activity. It is interesting to note that only 29 (14%) of the animals appeared to be gus^{mps}/gus^{mps} because the expected frequency is 25% if all mutant animals lived until weaning. A deficiency in the predicted number of mutant mice has been reported, but the deficiency seen in our study occurred even when the human transgene was present and the mice otherwise appeared phenotypically normal (6).

Fig. 1 shows an example of a native gel analyzing kidney tissue of transgenic mutant (Tg*GUSB*/Tg*GUSB*, gus^{mps}/gus^{mps}) mice expressing only *GUSB* and kidney tissue of transgenic normal mice expressing both human and murine enzymes. The latter produced mixed tetramers that were distinguishable on nonreducing native PAGE gels stained for β-glucuronidase enzyme activity (Fig. 1).

Because transgenic MPSVII mice produced only human enzyme, we determined the level of *GUSB* enzymatic activity in each tissue by using a fluorometric assay. Fig. 2 presents the β-glucuronidase levels in different tissues of transgenic MPSVII mice homozygous for the *GUSB* transgene (Tg*GUSB*/Tg*GUSB*, gus^{mps}/gus^{mps}). All tissues examined from transgenic animals showed high levels of β-glucuronidase activity compared to levels found in normal mice. Transgenic animals heterozygous for the *GUSB* transgene had ≈10-fold

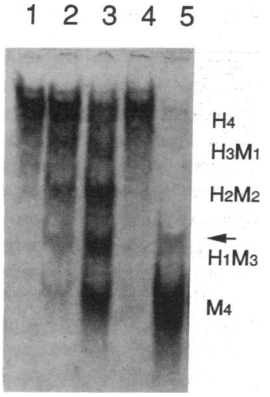

FIG. 1. Expression of human, mouse, and human/mouse β-glucuronidase heterotetramers in transgenic mice. Kidneys dissected from normal and transgenic mice were homogenized, and the homogenates were treated with endoglycosidase F to remove carbohydrate side chains before electrophoresis in a nondenaturing PAGE gel. The gel was stained for β-glucuronidase activity by using naphthol-AS-BI β-D-glucuronide and Fast Garnet GBC. Mixed tetramers were found in mice expressing both human and mouse β-glucuronidase. Lanes: 1, purified recombinant human β-glucuronidase; 2, transgenic mouse homozygous for the human transgene and heterozygous for the mouse gus^{mps} mutation; 3, transgenic mouse homozygous for the human transgene and homozygous for the normal mouse gene; 4, transgenic mutant mouse homozygous for the human transgene and homozygous for the gus^{mps} mutation; 5, normal mouse. H_4, H_3M_1, H_2M_2, H_1M_3, and M_4 indicate mobilities of human (H_4), mouse (M_4), and human/mouse heterotetramers (H_3M_1, H_2M_2, and H_1M_3). Arrow, precursor (microsomal) mouse β-glucuronidase (20).

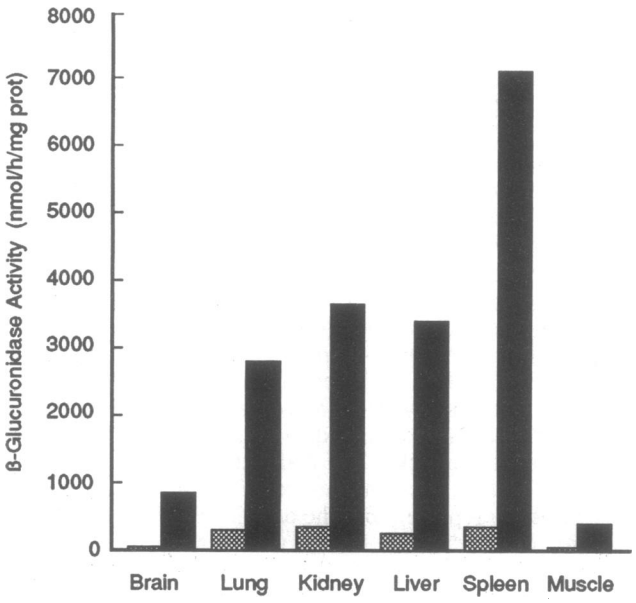

FIG. 2. β-Glucuronidase activity in transgenic and normal mouse tissue. Tissues dissected from normal mice (stippled bars) and transgenic mutant mice homozygous for the gus^{mps} mutation (black bars) were homogenized and assayed fluorometrically, as described. One unit of enzyme activity was defined as the activity that released 1 nmol of 4-methylumbelliferone per hr. Values are the average obtained from assays of four male mice of each genotype; mice ranged in age from 4 to 7 mo.

more activity than normal mice (data not shown). In mice homozygous for the transgene, the level was 20-fold higher (Fig. 2). It is not known how much of this difference in activity reflects differences in k_{cat} between mouse and human enzyme for the 4-methylumbelliferyl β-D-glucuronide substrate and how much the difference reflects actual differences in amount of protein present. In either case, the human promoter clearly functions in a way that produces a relative tissue distribution of enzyme activity similar to that seen for the mouse enzyme. Thus, brain and muscle are relatively low in activity when compared with other tissues.

Correction of the Secondary Elevation in Other Lysosomal Enzymes by the Human Transgene in MPSVII Mutant Mice. β-Glucuronidase-deficient MPSVII mice show a secondary elevation of many other lysosomal enzymes, including β-hexosaminidase, β-galactosidase, α-mannosidase, acid phosphatase, aryl sulfatase A, and α-fucosidase (data not shown). Similar results have been found in humans with MPSVII. Fig. 3 shows that the secondary elevation for α-galactosidase is eliminated in transgenic mice homozygous for the gus^{mps} mutation (Tg*GUSB*/Tg*GUSB*, gus^{mps}/gus^{mps}). The secondary elevation of the other acid hydrolases listed above was also corrected in mutant mice expressing the human transgene (data not shown).

Correction of Levels of Glycosaminoglycan Storage by the Human Transgene. The elevated lysosomal enzymes found in MPSVII mice are presumably a consequence of the accumulation of undegraded glycosaminoglycans. To determine whether the correction in lysosomal enzyme levels found in the transgenic mutant mice correlated with normal glycosaminoglycan levels, glycosaminoglycans were isolated by cetylpyridinium chloride precipitation of tissue digests obtained after proteolytic digestion of the dried, delipidated tissues. Precipitated glycosaminoglycans were assayed for hexuronic acid content, and the total hexuronic acid per mg of dried tissue weight was used as a measure of accumulated storage material. Fig. 4 shows the elevated levels of hexuronic acid in tissues isolated from MPSVII mice. The transgenic mutant mice carrying *GUSB* (Tg*GUSB*/Tg*GUSB*,

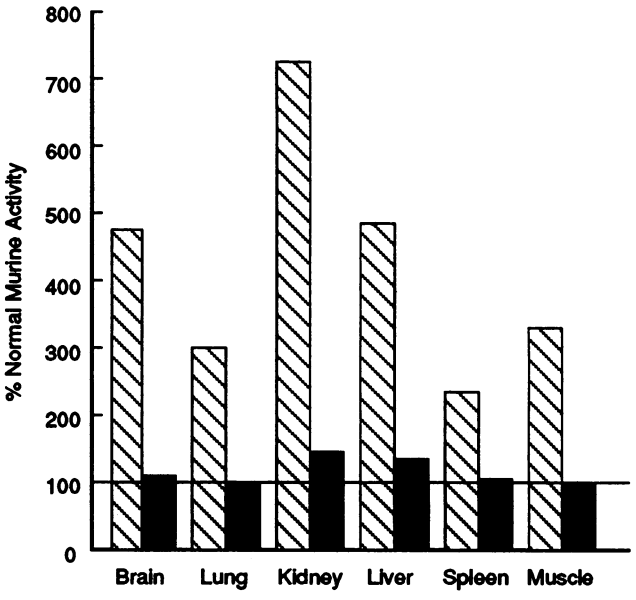

FIG. 3. Correction of secondary elevation of α-galactosidase activity in transgenic mice. Tissues were dissected and homogenized as for Fig. 2. Homogenates from mutant gus^{mps} mice without the human transgene (hatched bars) and mutant mice with the human transgene (black bars) were assayed fluorometrically by using 4-methylumbelliferyl α-D-galactoside. Activity is expressed as percentage of normal mouse α-galactosidase activity. Activities (nmol/hr per mg of protein) for normal mouse α-galactosidase in respective tissues were as follows: brain, 17; lung, 24; kidney, 18; liver, 19; spleen, 57; and muscle, 4. One unit of enzyme activity was defined as the activity that released 1 nmol of 4-methylumbelliferone per hr. Values are the average obtained from assays of four male mice of each genotype; mice ranged in age from 4 to 7 mo.

gus^{mps}/gus^{mps}) had normal levels of hexuronic acid, indicating that expression of *GUSB* alone was sufficient to correct the glycosaminoglycan storage normally present in mutant gus^{mps}/gus^{mps} mice.

Correction of Pathological Findings in Tissues of Transgenic Mice. We had shown (6, 7) that morphological analysis of tissues from MPSVII mice reveals lysosomes distended by fine granular and fibrillar storage material in many tissues including spleen and kidney. To determine whether these changes were corrected in mice carrying the human trans-

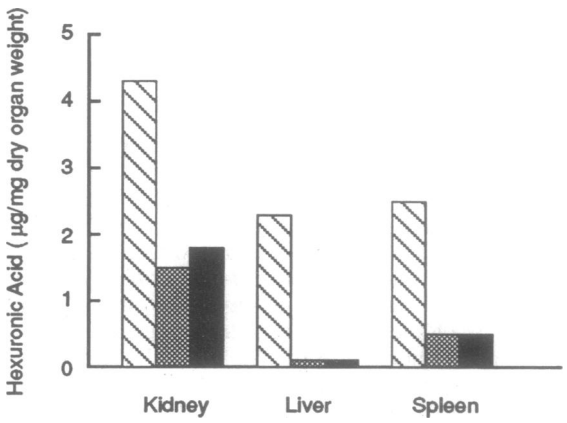

FIG. 4. Glycosaminoglycan levels in tissues of MPSVII, normal, and transgenic mice. Glycosaminoglycans were isolated by cetylpyridinium chloride precipitation after proteolytic digestion of delipidated tissues and assayed for total hexuronic acid content (18). Values are expressed as μg of hexuronic acid per mg of dry delipidated tissue. Mutant gus^{mps}/gus^{mps} mice without the human transgene (hatched bars), normal mice (stippled bars), and transgenic mutant mice homozygous for the gus^{mps} mutation (black bars).

gene, we compared light and electron micrographs of tissue sections obtained from three mutant mice with the human transgene (Tg*GUSB*/Tg*GUSB*, gus^{mps}/gus^{mps}) to those obtained from mutant mice without the transgene (gus^{mps}/gus^{mps}). Fig. 5 shows that mice expressing only the human enzyme have no evidence of lysosomal storage disease (Fig. 5 *b* and *d*). However, affected MPSVII mice without the transgene have the expected abnormalities (Fig. 5 *a* and *c*). Mutant mice carrying the human transgene also showed none of the other abnormalities seen in MPSVII mice—including dwarfism, gait disturbance, limited joint mobility, skeletal deformities, paucity of adipose tissue, and shortened lifespan (6, 7). In addition to appearing phenotypically normal visually, the transgenic gus^{mps}/gus^{mps} mice breed without difficulty, and the females raise their pups without complication.

DISCUSSION

Two copies of a mouse chromosome carrying a *GUSB* transgene express 10- to 20-fold more β-glucuronidase activity than is normally seen in mouse tissues assayed with 4-methylumbelliferyl β-D-glucuronide as substrate. This result clearly shows that 1.6 kb of 5′-upstream sequences are sufficient for high-level expression of the human gene product. Factors contributing to this high-level expression could be transgene copy number (estimated at two to four copies on Southern blot analysis), position effect (site of integration), absence of upstream cis-acting negative regulatory elements in the transgene, insensitivity to negative regulation by murine regulation factors, absence of translational control of expression (documented for murine β-glucuronidase) (21), and possibly slower turnover of the human enzyme in the murine cellular environment (the human enzyme is considerably more stable to heat inactivation) (12, 22). Although human gene expression was higher in all tissues examined, it is interesting that the relative tissue distribution of both human and mouse enzymes was similar in the transgenic mice.

The MPSVII mice exhibited secondary elevations of all five of the other acid hydrolases measured. It is not clear

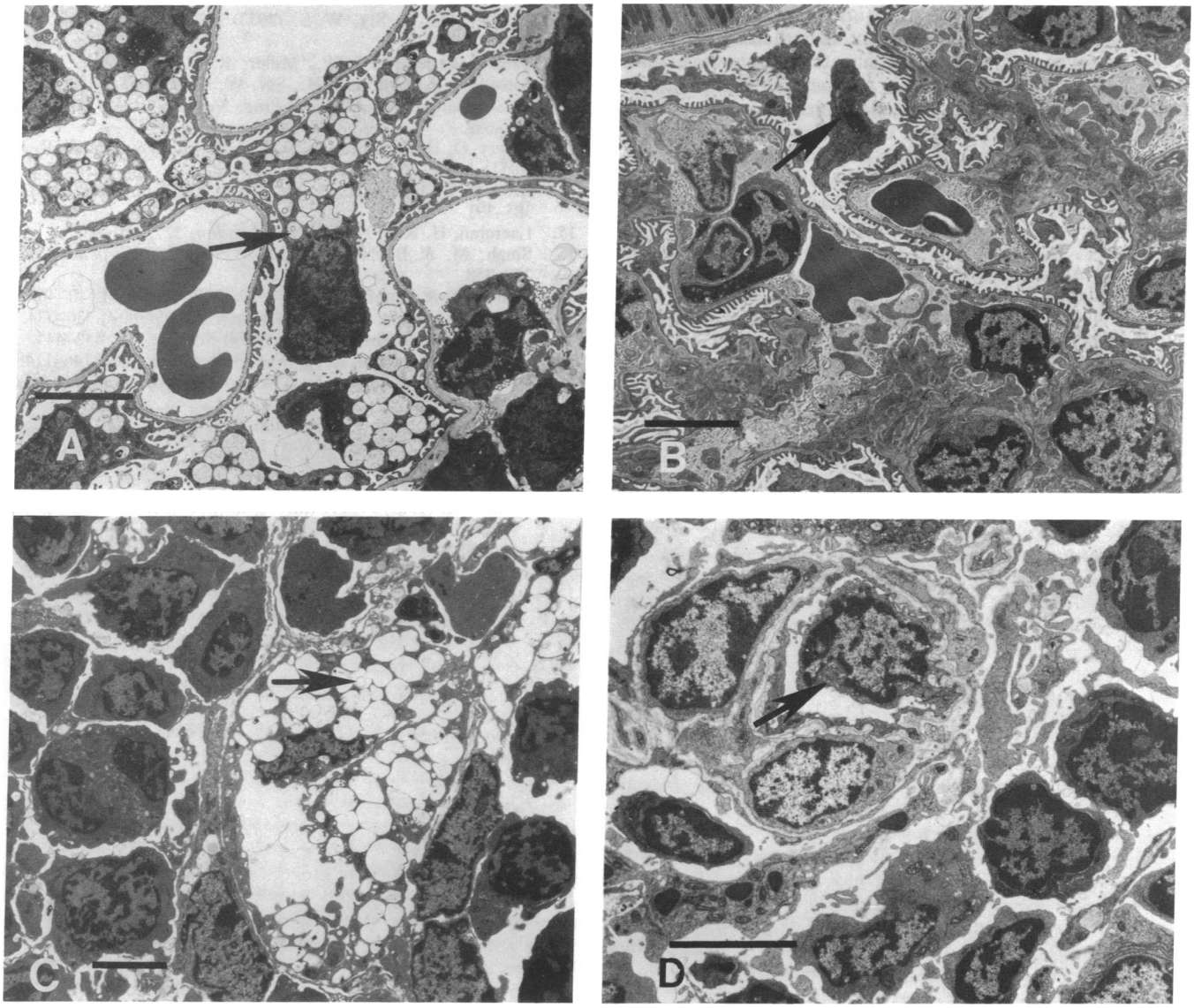

FIG. 5. Electron micrographic analysis of transgenic and MPSVII mice. Visceral epithelial cells in a glomerulus from a 3-mo-old mutant gus^{mps}/gus^{mps} male mouse (*A*) are distended by enlarged lysosomes (arrow) containing fine fibrillar material and small fragments of membranous debris. In contrast, a 2-mo-old male transgenic mutant gus^{mps}/gus^{mps} mouse (*B*) had no lysosomal distension in glomerular podocytes (arrow). A splenic vascular space from a newborn mutant pup (*C*) is lined by cells distorted by numerous expanded lysosomes. Much of the storage material was lost in processing, and only small membranous fragments (arrow) remain. A 2-mo-old male transgenic mutant gus^{mps}/gus^{mps} mouse (*D*) had no significant vacuolar storage in vascular lining cells (arrow). (*A*, ×3100; *B*, ×2850; *C*, ×2250; *D*, ×3850; bars = 5 μm; uranyl acetate-lead citrate.)

whether these elevations resulted from (*i*) increased synthesis of acid hydrolases in cells distended with storage material or from (*ii*) impaired degradation and turnover of these enzymes because the degradative enzymes are inhibited by stored glycosaminoglycans. In either case, the secondary elevations were fully corrected in the transgenic animals.

One interesting finding in the MPSVII mice, which has not been seen in humans with MPSVII, is the apparent deficiency of adipose tissue in normal sites of adipose storage (6, 7). The finding that this feature is fully corrected by expression of the human transgene suggests that this unexplained feature of murine MPSVII is, indeed, due to β-glucuronidase deficiency. Such a relationship between β-glucuronidase expression and adipose storage warrants further investigation.

There are several other interesting questions regarding regulation of expression of the human transgene and localization of its gene product. These include the following: (*i*) Is the human gene subject to androgen inducibility in the mouse, as is the normal murine gene (23)? (*ii*) Is the human gene subject to alternate splicing in murine tissues, as it is in a number of human tissues (8, 12)? (*iii*) Does the human gene product associate with the resident endothelial reticulum protein, egasyn, and have dual localization within the endothelial reticulum and lysosomes, as does the murine gene product (20)? (*iv*) Is the human gene subject to temporal and tissue-specific changes in level of expression during development as is the murine gene (24)? The transgenic mice, described here, that express only the human β-glucuronidase, provide an attractive model for addressing these questions.

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