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# Neonatal gene transfer leads to widespread correction of pathology in a murine model of lysosomal storage disease

**(adeno-associated virus/mucopolysaccharidosis type VII/animal models/metabolic disease/gene therapy)**

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**ABSTRACT** **For many inborn errors of metabolism, early treatment is critical to prevent long-term developmental sequelae. We have used a gene-therapy approach to demonstrate this concept in a murine model of mucopolysaccharidosis type VII (MPS VII). Newborn MPS VII mice received a single intravenous injection with $5.4 \times 10^6$ infectious units of recombinant adeno-associated virus encoding the human β-glucuronidase (GUSB) cDNA. Therapeutic levels of GUSB expression were achieved by 1 week of age in liver, heart, lung, spleen, kidney, brain, and retina. GUSB expression persisted in most organs for the 16-week duration of the study at levels sufficient to either reduce or prevent completely lysosomal storage. Of particular significance, neurons, microglia, and meninges of the central nervous system were virtually cleared of disease. In addition, neonatal treatment of MPS VII mice provided access to the central nervous system via an intravenous route, avoiding a more invasive procedure later in life. These data suggest that gene transfer mediated by adeno-associated virus can achieve therapeutically relevant levels of enzyme very early in life and that the rapid growth and differentiation of tissues does not limit long-term expression.**

Mucopolysaccharidosis type VII (MPS VII) is a lysosomal storage disease caused by the lack of β-glucuronidase (GUSB) activity (1). This defect results in a progressive accumulation of undegraded glycosaminoglycans (GAGs) in lysosomes, leading to lysosomal distention in multiple tissues. The clinical features of MPS VII include skeletal deformities, shortened life span, hearing and vision defects, and mental retardation. Reconstitution of GUSB activity can reverse lysosomal disease both *in vitro* and *in vivo*. This enzyme may be supplied from either an intracellular source (as seen after transduction of affected cells) or taken up from extracellular sources via the mannose-6-phosphate receptor (cross-correction; ref. 2). The murine MPS VII model closely resembles the human disease and has been used to evaluate a number of potential therapies for this condition. Reversal of established lysosomal disease in many tissues can be accomplished in adult animals by either enzyme replacement therapy (3) or bone marrow transplantation (4). It is clear from enzyme replacement therapy and bone marrow transplantation studies that neonatal treatment provides a more complete correction of functional defects, such as skeletal dysplasia (5) and deficiencies in hearing and behavior (6). Unfortunately, the transient nature of enzyme replacement therapy and the lack of suitable donors for bone marrow transplantation limits the clinical application of these therapies. An ideal treatment would be one that can be administered early in life, has low morbidity, and leads to long-term reconstitution of GUSB activity.

Adeno-associated virus (AAV) is a human parvovirus that is being developed as a gene therapy vector (7). Persistent *in vivo* expression from AAV vectors has been shown in many tissues, including retina, muscle, liver, and brain (8–14). This ability of AAV to transduce multiple organs, coupled with the nonpathogenic nature of the wild-type virus, makes AAV a potentially useful vector for the neonatal treatment of lysosomal storage diseases such as MPS VII. Previously, we have shown that, although neonatal intramuscular injection of AAV results in high levels of GUSB expression at the site of injection, the level and kinetics of secretion were insufficient to prevent the accumulation of lysosomal storage in distant tissues (15). However, results from that study suggested that an intravenous route of administration in neonates might provide more widespread delivery of therapeutic amounts of GUSB.

We show here that intravenous AAV-mediated gene therapy in neonatal MPS VII mice results in high-level GUSB expression in multiple tissues at a developmental stage where there is minimal evidence of disease. GUSB activity persisted for the duration of the study and was sufficient to reduce lysosomal distention dramatically in many cell types, including Kupffer cells of the liver, cardiac stromal fibroblasts, retinal pigment epithelial cells, meninges, and central nervous system (CNS) neurons. These data suggest that neonatal AAV-mediated gene transfer may represent an effective and noninvasive method of achieving widespread persistent therapeutic expression of a relevant enzyme for the treatment of lysosomal storage disease.

## METHODS

**Construction of AAVβGEnh Expression Cassette and Recombinant Virus.** The AAV vector AAVβGEnh has been described (15) and consists of the cytomegalovirus enhancer, chicken β-actin promoter, human GUSB cDNA, and rabbit β-globin and simian virus 40 polyadenylation signals. The initial intron of the chicken β-actin gene is included to increase protein expression. Viral stocks were prepared from homogenates of 293 cells after cotransfection of AAVβGEnh and the helper plasmid pIM45 (16) and superinfection with adenovirus type 5 at a multiplicity of infection of 2. Purified virus was isolated from cell lysates over two continuous isopycnic cesium gradients. The final product was concentrated and incubated at 56°C for 45 min to inactivate residual adenovirus. Infectious units were determined by an expression-based assay on a GUSB-deficient cell line. Replication-competent adenovirus

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Abbreviations: AAV, adeno-associated virus; CNS, central nervous system; GAG, glycosaminoglycan; GUSB, β-glucuronidase; MPS VII, mucopolysaccharidosis type VII.

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was absent as determined by cytopathic effect assay on 293 cells. The ratio of wild-type AAV to recombinant AAV was 0.18 as determined by infectious center analysis.

**Neonatal Injections.** Mutant mice were obtained from heterozygous matings of B6.C-*H-2*$^{bm1}$/ByBir-*gus*$^{mps}$/+ mice maintained by M.S.S. at Washington University. Identification of newborn mutants was accomplished by quantitative analysis of GUSB activity in toe clips on the day of birth. Each mouse received a single intravenous injection of 100 $\mu$l of viral suspension containing $5.4 \times 10^6$ infectious units of recombinant AAV via the superficial temporal vein (5) on day 2 of life, providing a dose of approximately $5 \times 10^9$ infectious units per kg.

**Quantitative Analysis of Lysosomal Enzyme Activities and Glycosaminoglycan Levels.** GUSB activities were measured on tissue homogenates by using fluorometric assays as described (3). For serum samples, reactions were incubated for 48 h at 37°C to increase the sensitivity of the assay. GUSB specific activity (1 unit = 1 nmol of 4-methylumbelliferyl $\beta$-D-glucuronide liberated per hour per milligram of total protein) was calculated and compared with GUSB specific activities from age-matched +/+ mice of the same strain and presented as a percentage of normal activity. Glycosaminoglycan levels were measured by the alcian blue method as described (17). Final GAG levels are expressed as micrograms of GAG per milligram of protein.

**Histochemical and Histopathological Analyses.** Histochemical analysis of GUSB activity was performed on 10-$\mu$m thick frozen sections as described by using naphthol-AS-BI $\beta$-D-glucuronide (ASBI) as the substrate (3). Sections were counterstained with 1% methyl green. Additional tissue samples were collected, fixed, and prepared for light and electron microscopy as described (4). Tissue samples were immersed in ice-cold 2% glutaraldehyde and 4% paraformaldehyde in PBS and then embedded in Spurr's resin. Sections of tissue 0.5 $\mu$m thick were stained with toluidine blue and evaluated for lysosomal storage.

**PCR and Southern Analysis of Tissues.** The locations of the primers and probes used in this study have been described (15). Primers specific for sequences in exon 6 (5′-CTGTGGCTGTCACCAAGAGC-3′) and exon 7 (5′-GGACACTCATCGATGACCAC-3′) of the human GUSB cDNA were used. These sequences are identical to the human cDNA (18) but have two mismatches within the murine exon 6 sequence (19). Expected products are a 240-bp fragment from the human cDNA and a 454-bp fragment from the endogenous murine gene. Blots were probed with a 527-bp *Cla*I fragment from the human GUSB cDNA. Tissue samples were resuspended in 50 $\mu$l of buffer containing 50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Triton X-100, 0.45% Tween 20, and 200 $\mu$g/ml Proteinase K and incubated at 56°C for 16 h. For each PCR reaction, 10 $\mu$l of this reaction was used.

## RESULTS

**Quantitative GUSB Expression After Neonatal Injection.** Newborn MPS VII mice were injected intravenously on day 2 of life with $5.4 \times 10^6$ infectious units of the recombinant vector AAV$\beta$GEnh. This vector has been described (15) and consists of the human GUSB cDNA driven by the chicken $\beta$-actin promoter and cytomegalovirus enhancer element. Significant levels of GUSB activity were detected in multiple organs at time points throughout the first 16 weeks of life (Fig. 1 *A* and *B*). In heart, lung, brain, and kidney, GUSB expression steadily increased for the first 4 weeks of the study, stabilizing at levels ranging from 1% to 2,000% of normal (Fig. 1*A*). These levels have been shown to be sufficient to reduce lysosomal storage in most tissues (3, 20). In contrast, GUSB activity in the liver initially approached normal levels but then decreased sharply over the course of the study, stabilizing at a final level of 1% of normal by week 16 (Fig. 1*B*). Relatively high levels of GUSB activity were observed in the spleen and serum between 1 and 4 weeks of age but then decreased dramatically by week 16 (Fig. 1*B*). Interestingly, the decrease in splenic and serum GUSB levels paralleled the hepatic activity.

**Distribution of Expression and Therapeutic Effect After Neonatal AAV Injection.** Histochemical and histopathological analyses of organs from treated mice showed widespread therapeutic response to neonatal AAV treatment. An example of this response can be seen in the heart, where GUSB activity was distributed throughout the cardiac muscle at week 16 (Fig. 2*A*). Histopathological lesions in cardiac-valve fibroblasts were decreased in AAV-treated MPS VII mice at week 16 (Fig. 2 *B* and *C*). Persistent GUSB expression was also noted in multiple areas of the CNS in AAV-treated mice. A limited survey at 2 weeks of age showed GUSB staining in the central gray matter,

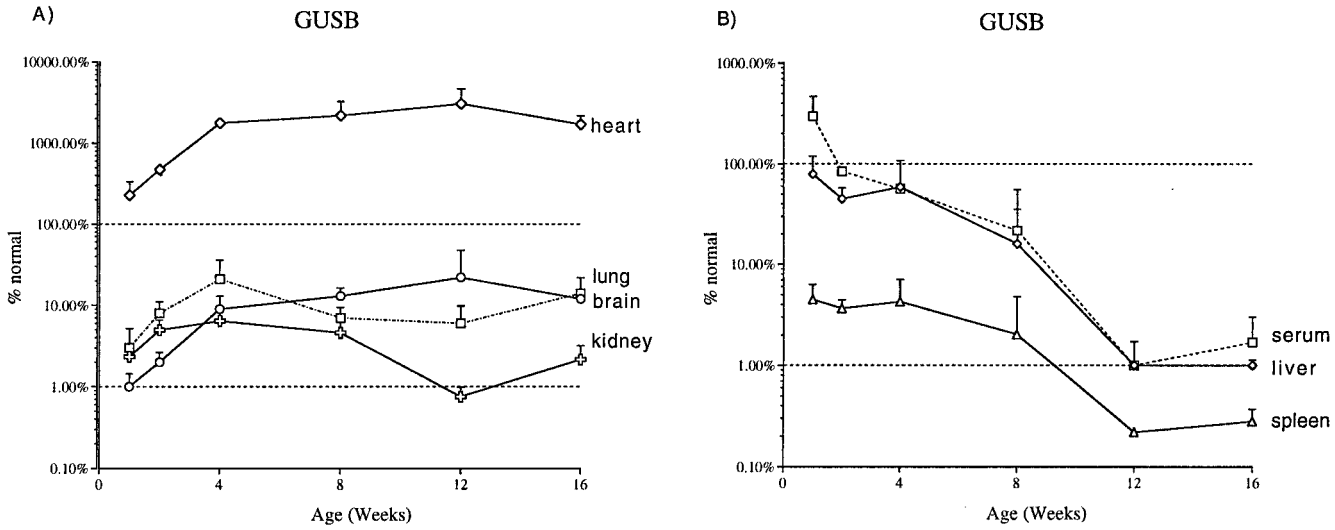


FIG. 1. Mice were killed at time points ranging from 1 to 16 weeks of age, and organs were assayed for GUSB activity with a fluorometric quantitative assay. Each point represents the average and standard deviation from three AAV-treated mice. Results are expressed as a percentage of normal GUSB activity found in the corresponding organs from three age-matched +/+ B6 mice. Dotted lines at 1% and 100% represent the "therapeutic range" of GUSB activity, because previous work with enzyme replacement therapy and bone marrow transplantation has shown that levels of approximately 1% normal are sufficient to reverse storage disease in some organs.

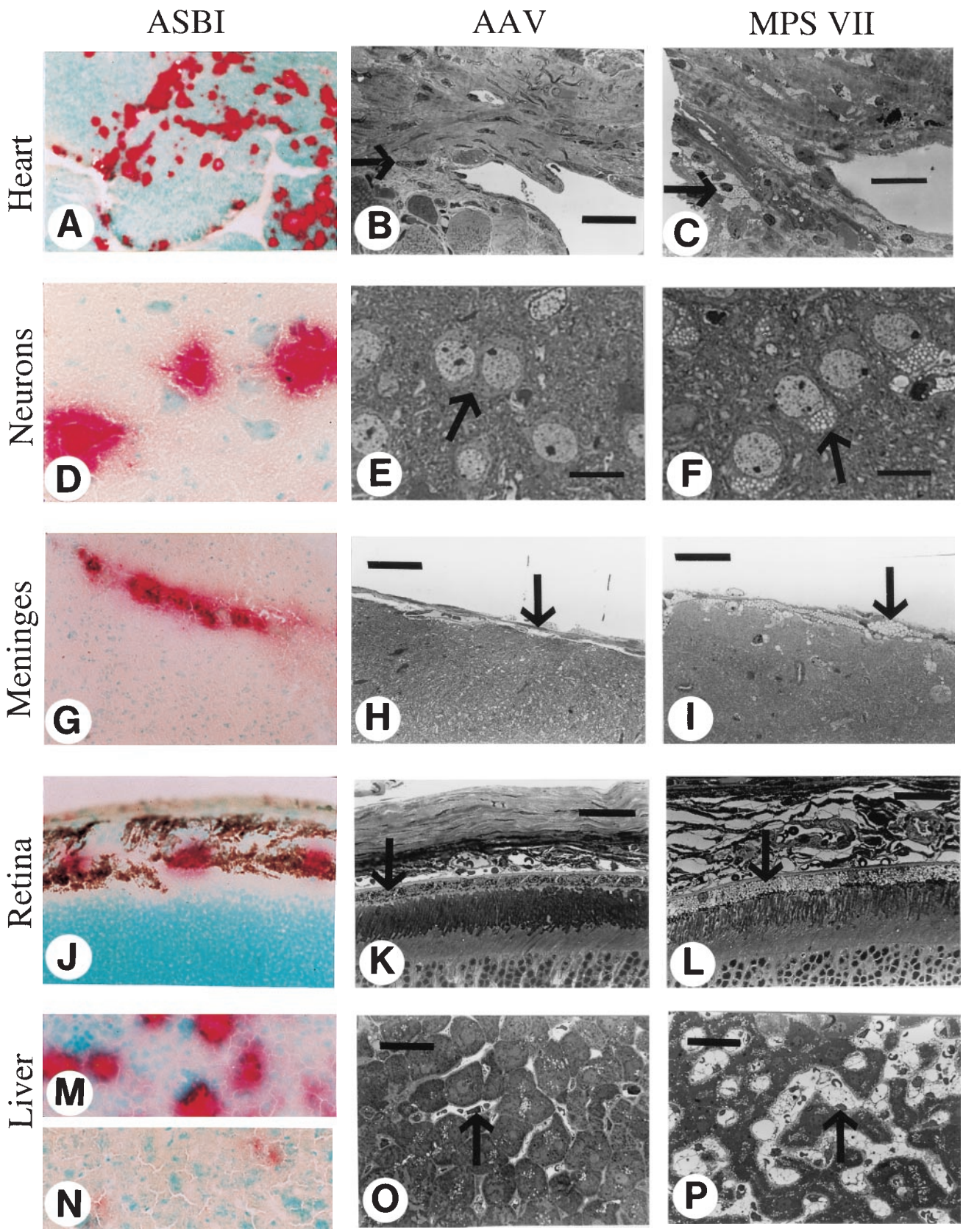


FIG. 2. Tissue sections were examined for GUSB activity and evidence of therapeutic response to AAV treatment. (*Left*) The column labeled ASBI (naphthol-AS-BI β-D-glucuronide, see *Methods*) shows histochemical staining for GUSB activity. Cells that stain red contain GUSB. (*Center* and *Right*) The other columns show histopathological analysis of 16-week-old treated (AAV) and untreated (MPS VII) mutant mice. Widespread GUSB activity is present in cardiac muscle at week 16 (*A*), and a corresponding reduction of disease in cardiac-valve stromal fibroblasts is seen (*B*, arrow) when compared with untreated mutants (*C*, arrow). GUSB activity is also present in sections of polygonal neurons of the basis pons (*D*), meninges (*G*), and retinal pigmented epithelium (*J*) from 16-week-old AAV-treated mice, leading to the nearly complete elimination of lysosomal storage in these organs when compared with the corresponding untreated controls (*Center and Right*). In the liver,

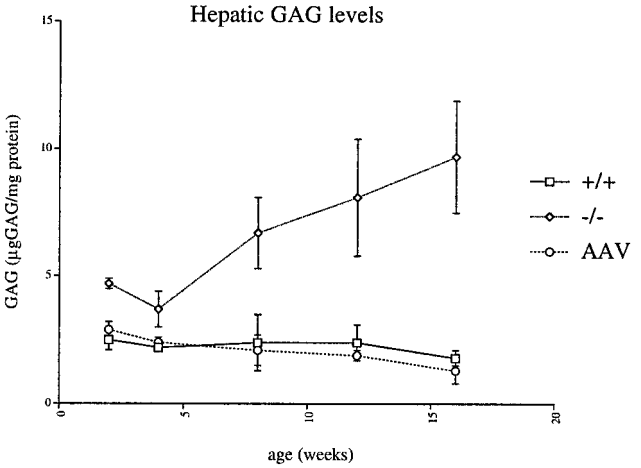


FIG. 3. The total glycosaminoglycan content of hepatic tissue from the normal, mutant, and AAV-treated mice shown in Fig. 2 *M–P* was compared. Each point represents the average GAG content from three mice. Vertical error bars represent the standard deviation.

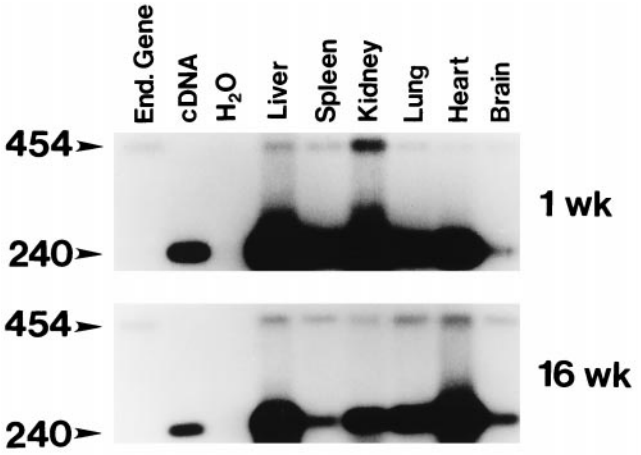


FIG. 4. Tissues were analyzed for the presence of viral cDNA with primers specific for exons 6 and 7 of human GUSB. These primers amplify a 240-bp band from the viral cDNA and a 454-bp band from the endogenous murine GUSB gene. Mismatches between the murine sequence and the human PCR primers, the interruption of the murine coding sequences by an intron, and the use of a human-specific probe cause the human cDNA to be detected more efficiently than the endogenous murine band. At 1 week of age, relatively high levels of viral cDNA are present in all tissues when normalized to the endogenous murine band. At 16 weeks of age, the amount of viral cDNA varies more widely between tissues.

meninges, cortex, and vessels of AAV-treated mice. A more detailed survey at week 16 showed continued expression in central gray matter, meninges (Fig. 2*G*), cortex, and vessels. In addition, GUSB-positive areas were observed in the choroid plexus, pyramidal cell layer of the hippocampus, and molecular layer of the cerebellum. Although specific cell types could not always be determined, in areas such as the polygonal neurons of the basis pons, clear GUSB staining of morphologically distinct neuronal cell bodies was evident (Fig. 2*D*). This observation is in agreement with a recent study that showed preferential infection of neurons in the CNS of rodents by AAV vectors (21). Histopathological analysis showed widespread reduction of lysosomal storage in parenchymal neurons of the cortex (Fig. 2 *E* and *F*) as well as in meninges (Fig. 2 *H* and *I*). Histochemical analysis also showed persistent GUSB activity in the retina, with elimination of the characteristic lysosomal storage in retinal pigment epithelial cells by 16 weeks of age (Fig. 2 *J–L*). Lysosomal storage in the cornea of treated MPS VII mice, however, was reduced only slightly (data not shown).

In the liver, the decline in GUSB activity from 1 to 16 weeks of age seems to be caused by a sharp decrease in the percentage of GUSB-expressing cells (Fig. 2 *M* and *N*). However, the remaining activity at week 16 was still sufficient to reduce lysosomal distention in Kupffer cells of AAV-treated mice when compared with untreated controls (Fig. 2 *O* and *P*). Quantitation of total hepatic GAG content confirmed that the livers of AAV-treated mice had no meaningful accumulation of GAGs over the course of the study, unlike untreated MPS VII mice (Fig. 3). Although the level of GUSB activity in the spleen was less than 1% of normal levels at week 16, a significant reduction of lysosomal storage in the sinus-lining cells was observed (data not shown). There was minimal to no effect on disease in the skeletal system, as evidenced by the development of the characteristic MPS VII phenotype (shortened limbs and facial dysmorphism) in the AAV-treated mice (data not shown).

**Presence of Viral cDNA in AAV-Treated Mice.** PCR analysis of treated mice showed the presence of viral cDNA in most tissues at 1 week of age (Fig. 4). At week 16, strong viral cDNA signals persisted in heart, lung, brain, kidney, and liver. Levels of viral cDNA in the spleen seemed to decrease somewhat by week 16 of age when normalized to the internal control of the endogenous murine GUSB gene.

## DISCUSSION

Our study shows that neonatal animals are suitable targets for intravenous AAV-mediated gene transfer and that the rapid growth and differentiation of tissues after birth does not limit expression from these vectors in the first 16 weeks of life. Several advantages of neonatal treatment are shown in this study. First, therapeutically relevant levels of enzyme can be achieved early in life, which may prevent the development of disease as opposed to reversing established disease. Second, neonatal administration of AAV provides access to the CNS by an intravenous route. This access is especially noteworthy, as it has been difficult to achieve persistent therapeutic levels of GUSB in the CNS by noninvasive means. Although AAV has been shown to infect neurons and retinal cells efficiently after localized injection, widespread infection of these organs was not seen after intravenous injections in adult animals. One possible cause for this improved transduction of the CNS in neonatal mice after intravenous injection could be because of the incompletely formed blood–brain barrier, which is not fully intact until 10–14 days of life in rodents (22). Alternatively, the relatively small size of neonatal mice results in a larger dose of virus per kilogram than can be achieved easily in adult animals. This virus load may reach a threshold for CNS infection that was not achieved in previous studies that used adult mice.

For heart, lung, kidney, and brain, persistent viral cDNA PCR signals correlated with sustained expression of GUSB in these tissues. In contrast, although viral cDNA also persisted in the liver from week 1 to week 16, hepatic GUSB activity sharply decreased during this time. This result suggests that the decline in GUSB expression in the liver occurs at a transcriptional or translational level. This decline may be caused by transcriptional silencing of the cytomegalovirus enhancer el-

10–20% of hepatocytes showed evidence of GUSB activity at week 1 (*M*). Although a much smaller percentage of cells were positive at week 16 (*N*), the residual activity is sufficient to reduce lysosomal distention greatly in treated animals at 16 weeks of age when compared with age-matched untreated controls (*O* and *P*, arrows). (For *A–C* and *G–P*, bars = 34 μm; for *D–F*, bars = 14.5 μm.)

ement (23). In the spleen, however, both viral cDNA and GUSB expression have decreased by week 16, suggesting that this organ is not stably transduced. This distribution of viral cDNA after neonatal injection suggests that the tropism of AAV in neonatal mice is similar to that described in adult mice (24, 25). In addition, the persistence of viral cDNA in multiple organs provides evidence that the primary source of GUSB activity in heart, lung, brain, and kidney is direct infection by AAV and not cross-correction by secreted enzyme. This hypothesis is supported further by the fact that GUSB activity in these organs remains relatively stable, whereas circulating GUSB levels decrease dramatically from 1 to 16 weeks of age.

Although direct infection of tissues by AAV seems to be the primary source of GUSB in most organs studied, some element of cross-correction by circulating enzyme undoubtedly plays a role, especially at early time points when high serum levels are present. Evidence for cross-correction also can be seen in the neurons of the brain. Although only a small percentage of parenchymal cells stained positive for GUSB, lysosomal distention was reduced throughout the cortex. It is unclear whether the corrective enzyme is being supplied from meningeal secretion into the cerebrospinal fluid, axonal transport from neighboring transduced cells, or GUSB circulating early in life before closure of the blood–brain barrier. However, the combination of high circulating levels of GUSB early in life and lower persistent levels that are maintained as the animals age results in the reduction of lysosomal storage in numerous tissues. It is interesting to note that the GUSB activity in the serum and spleen parallels the activity in the liver, suggesting that the liver is the primary source of secreted GUSB in AAV-treated animals. This result has important therapeutic implications. Unlike the muscle, which does not secrete large amounts of GUSB (15), the liver apparently has the capacity to secrete lysosomal enzymes more efficiently. The development of AAV vectors that constitutively express at high levels in the liver may result in more complete correction of disease in tissues that responded only partially in this study, such as the cornea, spleen, and skeletal system. It also will be crucial to determine the short-term and long-term effects of AAV-mediated gene transfer on mental, auditory, and visual functions, as well as on longevity. These kinds of neonatal therapies will be important for the treatment of many childhood genetic diseases, where early treatment is required to prevent long-term developmental damage.

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