# Active site mutant transgene confers tolerance to human β-glucuronidase without affecting the phenotype of MPS VII mice

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**Mucopolysaccharidosis type VII (MPS VII; Sly syndrome) is an autosomal recessive lysosomal storage disorder due to an inherited deficiency of β-glucuronidase. A naturally occurring mouse model for this disease was discovered at The Jackson Laboratory and shown to be due to homozygosity for a 1-bp deletion in exon 10 of the *gus* gene. The murine model MPS VII (*gus*$^{mps/mps}$) has been very well characterized and used extensively to evaluate experimental strategies for lysosomal storage diseases, including bone marrow transplantation, enzyme replacement therapy, and gene therapy. To enhance the value of this model for enzyme and gene therapy, we produced a transgenic mouse expressing the human β-glucuronidase cDNA with an amino acid substitution at the active site nucleophile (E540A) and bred it onto the MPS VII (*gus*$^{mps/mps}$) background. We demonstrate here that the mutant mice bearing the active site mutant human transgene retain the clinical, morphological, biochemical, and histopathological characteristics of the original MPS VII (*gus*$^{mps/mps}$) mouse. However, they are now tolerant to immune challenge with human β-glucuronidase. This ''tolerant MPS VII mouse model'' should be useful for preclinical trials evaluating the effectiveness of enzyme and/or gene therapy with the human gene products likely to be administered to human patients with MPS VII.**

Mucopolysaccharidoses (MPS) are a subgroup of lysosomal storage diseases that result from deficiencies of specific lysosomal enzymes involved in the stepwise degradation of glycosaminoglycans (GAGs) (for review, see reference 1). They are characterized by progressive intralysosomal accumulation of undegraded GAGs that eventually lead to cellular and organ dysfunction. MPS type VII (Sly syndrome) results from deficiency of β-glucuronidase activity (2). β-Glucuronidase (β-D-glucuronoside glucuronosohydrolase EC.3.2.1.31), abbreviated GUS, is a tetrameric glycoprotein acid hydrolase localized primarily in lysosomes and found in virtually all mammalian cells (for review, see refs. 3 and 4). It acts in lysosomes as an exoglycosidase to remove glucuronic acid residues from the nonreducing termini of GAGs. Many different mutations have been found in the *GUS* gene in patients with MPS VII, accounting for the considerable clinical variability among patients with MPS VII (5).

Although MPS VII was one of the last human MPS disorders identified (2), it was one of the first whose catalytic defect was ascribed to an already well characterized enzyme (3, 4), and the first for which a mouse model was identified (6–8). Murine MPS VII was shown to result from homozygosity for the autosomal recessive, naturally occurring 1-bp deletion in exon 10 of the *gus* structural gene, referred to as the *gus*$^{mps}$ mutation (9). The MPS VII (*gus*$^{mps/mps}$) mice have been studied very extensively and are found to have morphologic, genetic, and biochemical characteristics which closely mimic those of human MPS VII. Affected mice have facial dysmorphism, growth retardation, deafness, behavioral deficits, and shortened lifespan. They show widespread storage of GAGs in lysosomes of visceral organs, skeleton, and brain. The murine model of MPS VII has been widely used for evaluating the effectiveness of bone marrow transplantation (10–14), enzyme replacement (15–22), and gene therapy with retroviral (23–28), adenoviral (29–33), and adeno-associated viral vectors (34–39).

As valuable as the murine model for MPS VII has been, we felt that introducing a transgene expressing inactive human GUS might give it considerable added value if it conferred immune tolerance to human GUS on the MPS VII mouse. Immune tolerance to human GUS would allow preclinical trials in mice with the form of the human enzyme (or human gene) intended for administration to human patients. The goal would be to prevent formation of antibodies to human GUS that might be expected to alter the targeting and tissue distribution of infused enzyme, or inhibit catalytic activity of the therapeutic enzyme. Such antibodies often blunt or abrogate therapeutic responses to enzyme therapy (40) and gene therapy (41) and can force cessation of therapy by producing life-threatening reactions (18).

In prior work, we characterized the structure and active site of human GUS, and identified residue E540 as the active site nucleophile (42, 43). Expression of the cDNA containing the E540A missense mutation in COS cells or insect cells produced an inactive protein with normal turnover and stability (43). These studies suggested that a transgene expressing human GUS E540A on the MPS VII (*gus*$^{mps/mps}$) background might provide a murine MPS VII model that retains the MPS VII phenotype, but has the added desirable feature of being immune tolerant to human GUS. We reasoned that such a model would be a great asset for evaluating the benefits of enzyme therapy with human GUS or gene therapy using the human *GUS* cDNA.

## Materials and Methods

**Construction of the Transgenic Mouse.** The 2.2-kb human *GUS* E540A cDNA (43, 44) was cloned into a pBluescript KS+ vector between the 0.54-kb mouse phosphoglycerate kinase (PGK) promoter and a 3′ element including the rabbit β-globin intron 1 and the SV40 poly(A) signal. The transgene, including the PGK promoter, cDNA, and 3′ element, was removed by digestion with *Xho* and *Not*I, gel isolated, and injected into male pronuclei of C57BL/6J eggs as described (45). Of 42 pups born from injected zygotes, six contained the human transgene identified by PCR of tail DNA, in 1–4 copies as estimated by Southern blot analysis of tail DNA. Two low copy number male founders were crossed with C57BL/6 females, and progeny tested for tolerance to

Abbreviations: MPS VII, mucopolysaccharidosis type VII; GAGs, glycosaminoglycans; GUS, β-glucuronidase.

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immunizing human β-glucuronidase by ELISA as described below. One (founder 55) produced tolerant progeny, and the human *GUS* E540A transgene was placed onto the B6 MPS VII (*gus*$^{mps}$) background by additional crosses to produce a line that was C57BL/6 Tg h*GUS* E540A/Tg h*GUS* E540A, *gus*$^{mps/+}$. The colony was maintained by brother–sister matings of this genotype, and 20% of the offspring were *gus*$^{mps/mps}$ homozygotes that showed the MPS VII phenotype by 28 days of age.

All mice were genotyped by PCR of tail DNA for the *gus*$^{mps}$ allele as described (8). This method utilizes an intronic primer 5′-CCTGTGTCATTTGCATGTG-3′ and a reverse primer with a mismatched nucleotide 5′-GATAACATCCACGTACCGG-3′, which creates a restriction site in the normal allele but not in the mutant allele. Following cleavage with *Nci*I or *Scr*FI, the 77-bp restriction fragment present in the normal allele is distinguished on a 3% Nu Sieve agarose gel from the uncleaved 95-bp PCR fragment from the mutant allele. The presence of the human transgene was detected by PCR of tail DNA using forward primer 5′-GCTGGTGAATTACCAGATCTCTGT-CAA-3′ and reverse primer 5′-GGAAATAGAAAGGTTTC-CCATTGATGAGG-3′, which amplified a 312-bp fragment spanning nucleotides 750-1062 of the human cDNA (44).

**Lysosomal Enzyme Assays.** Lysosomal enzymes were assayed fluorometrically by using 4-methylumbelliferyl substrates, as described (45–47). Tissues were dissected and homogenized immediately (by Brinkmann Polytron homogenizer for 30 sec at 4°C) in 5 vol of homogenization buffer (25 mM Tris·HCl, pH 7.2, 140 mM NaCl, 1 mM PMSF). Total homogenate was diluted appropriately for assay in PBS, the final dilution in an equal volume of citrate phosphate buffer, pH 4.4, containing 0.075 M NaCl, 1.0 mg/ml human serum albumin, and 0.001% Triton X-100. Assays on dilutions of wild-type tissue extracts were for 30 min, and for MPS VII/E540A$^{Tg}$ extracts, 24 h. Units were nmol hydrolyzed per hour, and activity was expressed as u/mg protein, determined by microlowry assay (47).

**Pathology.** Multiple tissues from six MPSVII/E540A$^{Tg}$ mice from 3–9 months of age were studied morphologically as previously described (7). Tissues were evaluated for the extent of lysosomal storage and compared with those previously described in the MPS VII (*gus*$^{mps/mps}$) model. The skeletons of a transgenic and an unaffected 6-month-old mouse were radiographed as previously described (7).

**Immunization Method and Analysis of Sera from Immunized Mice by ELISA.** Four MPS VII (*gus*$^{mps/mps}$) and four MPS VII/E540A$^{Tg}$ mice were immunized with purified human GUS beginning at 2 months of age. Each mouse received 50 μg human GUS in 0.2 ml complete Freund's adjuvant intraperitoneally as an initial challenge, and two subsequent boosts with 50 μg human GUS in 0.2 ml of incomplete Freund's adjuvant intraperitoneally (the first boost at 28 days and the other at 42 days after the initial challenge). Blood was collected by eye bleed to measure antibodies to human GUS by ELISA 12 days after each boost.

Analysis of sera from immunized mice was done by ELISA assay on microtiter aliquots. The wells of 96-well microtiter plates were coated overnight at 4°C with 10 μg/ml purified recombinant human β-glucuronidase in 15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, 0.02% $NaN_3$, pH 9.6. The wells were washed three times with TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20), then blocked for 1 h at room temperature with 3% casein in PBS (pH 7.2). After washing three times with TBST, 100 μl of serial 10-fold dilutions of mouse plasma ($10^{-2}$–$10^{-8}$) in TBST were added to the wells and incubated at 37°C for 2.5 h. The wells were washed four times with TBST, then 100 μl of TBST containing a 1:500 dilution of peroxidase conjugated goat anti-mouse IgG was added to the wells and incubated at room temperature for 1 h. The wells were washed three times with TBST and two times with TBS (10 mM Tris, pH 7.5, 150 mM NaCl). Peroxidase substrate (ABTS solution, Roche Molecular Biochemicals) was added (100 μl per well) and plates were incubated at room temperature for 10 min. The reaction was stopped with the addition of 2.5 μl of 20% SDS and the plates read at OD 400 nm on an automatic ELISA plate reader.

## Results

We previously reported (48) that the normal human *GUS* cDNA transgene fully corrected the mutant phenotype when bred onto the MPS VII (*gus*$^{mps/mps}$) background. These transgenic mice produce several times normal levels of human β-glucuronidase, and were naturally tolerant to immune challenge with the human enzyme. To determine whether the inactive human *GUS* E540A cDNA transgene could confer tolerance, the hGUS/E540A transgene was first introduced into C57BL/6 mice as described in *Materials and Methods*, and offspring of founders were tested for tolerance to immunization with purified human GUS.

A low copy number transgenic line was identified that was tolerant to immunization with human GUS, and the h*GUS*/E540A transgene in this line was crossed onto the B6 *gus*$^{mps/+}$ stock to derive a line homozygous for the chromosome carrying the transgene, and heterozygous for the *gus*$^{mps}$ allele. The colony of C57BL/6$^{TghGUS/E540A\ mps/+}$ mice was maintained by brother–sister matings and genotyped by enzymatic analysis of extracts of tail samples for GUS activity and by PCR analysis of genomic DNA for the wild-type and mutant mouse *gus*$^{mps}$ alleles and for the h*GUS*/E540A$^{Tg}$ allele. MPS VII/E540A$^{Tg}$ offspring from this colony were analyzed for morphologic, biochemical, and histopathologic phenotypes and tested for tolerance to immune challenge with human GUS.

**Mutant Phenotype of the h*GUS*/E540A Transgenic MPS VII Mice.** Homozygote MPS VII (*gus*$^{mps/mps}$) mice carrying the h*GUS*/E540A transgene, herein referred to as MPS VII/E540A$^{Tg}$ mice, were not distinguishable from *gus*$^{mps/+}$ and +/+ littermates at birth without genotyping, but could easily be identified visually by the time of weaning from their shortened face and slightly smaller size. As they aged, their growth retardation, shortened extremities, and facial dysmorphism became more prominent. Figure 1*A* shows the difference in phenotype of wild-type and mutant mice at age 6 months. By this age, radiographic analysis of the axial and appendicular skeleton of MPS VII/E540A$^{Tg}$ mice demonstrated marked dysplasia with shortened, broad, sclerotic long bones, a narrow thorax, and sclerosis of the calvarium (Fig. 1*B*). Other aspects of the MPS VII mutant phenotype (which include deafness, failure to reproduce, and shortened survival) were also retained. The MPS VII/E540A$^{Tg}$ mice had a mean survival of 200 days ($n$ = 27; SD ± 61 days). The longest survivor lived 301 days. The cause of death was unclear. However, typically, the mutant mice became progressively less active, stopped eating, and underwent a sharp drop in body weight in the few days before death. Collectively, these findings indicate that the MPS VII/E540A$^{Tg}$ mice retained the complete mutant clinical phenotype described for the original MPS VII (*gus*$^{mps/mps}$) mice, which do not carry the transgene (6, 7).

**Biochemical Phenotype of the MPS VII/E540A$^{Tg}$ Mice.** Table 1 summarizes data comparing the tissue levels of β-glucuronidase and α-galactosidase in MPS VII/E540A$^{Tg}$ mice and C57BL/6 control mice. The MPS VII/E540A$^{Tg}$ mice showed the profound deficiency of β-glucuronidase characteristic of MPS VII (*gus*$^{mps/mps}$) mice (6). They also showed the secondary elevations in tissue levels of α-galactosidase (10, 48). This secondary elevation has been shown to be a convenient measure of lysosomal storage secondary to β-glucuronidase deficiency

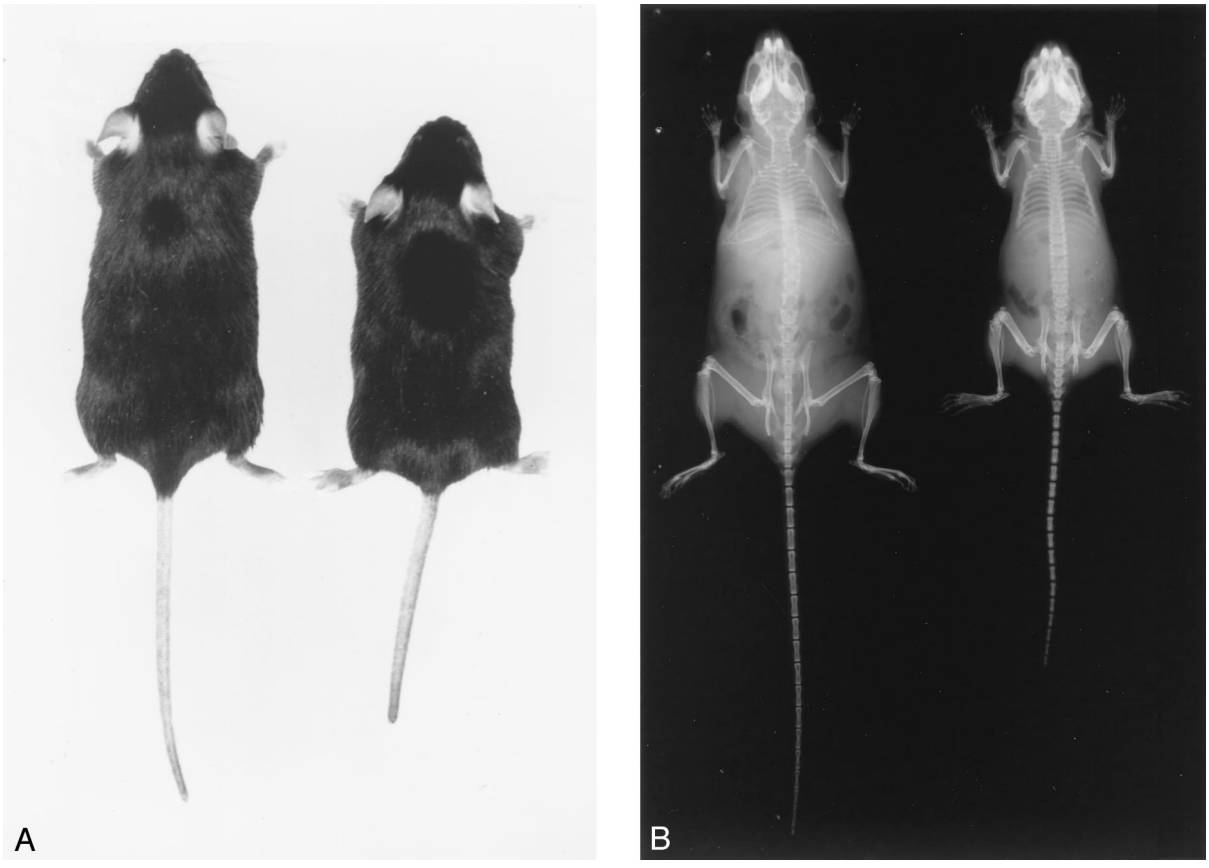


**Fig. 1.** Morphological and radiographic phenotype of the MPS VII/E540$^{Tg}$ mouse. (*A*) A 6-month-old female MPS VII/E540A$^{Tg}$ mouse (*Right*) is smaller and has dysmorphic features, compared with a normal female mouse (*Left*) of the same age. The affected mouse has a small head with blunted nose, short limbs, and a hobbled gait. (*B*) Radiograph of the axial skeleton of the MPS VII/E540A$^{Tg}$ mouse. The skeleton of a 6-month-old female MPS VII/E540A$^{Tg}$ mouse (*Right*) has generalized abnormalities including narrow rib cage, broad ribs, flared metaphyses, shortened, broad sclerotic long bones, and sclerosis of the calvarium. The skeleton of a normal female mouse of the same age (*Left*) is shown for comparison.

and provided a means to follow the biochemical response to therapy. Reductions in the levels of secondary elevations of $\alpha$-galactosidase were shown to be associated with correction of lysosomal storage by bone marrow replacement (10), enzyme therapy (16), and gene therapy (23).

**Histopathology of the MPS VII/E540A$^{Tg}$ Mouse.** Multiple tissues from six transgenic mice from 3–9 months of age were studied morphologically as described (7). Tissues were evaluated for the extent of lysosomal storage and alterations were compared with those described in the MPS VII (*gus*$^{mps/mps}$) mouse model (6, 7). Widespread lysosomal storage was seen throughout the fixed tissue macrophage system. The liver (Fig. 2*A*) and spleen (not shown) had marked lysosomal storage in sinus-lining cells. Renal tubular cells and glomerular visceral epithelial cells were also altered with abundant lysosomal storage (Fig. 2*B*). The brain had lysosomal storage in cells of the leptomeninges, neurons, and glial cells (data not shown). The eye showed corneal fibrocytes and endothelial cells distended with lysosomal storage (Fig. 2*C*) and enlarged lysosomes in the retinal pigment epithelial cells (Fig. 2*D*). Osteoblasts were distended with cytoplasmic vacuoles as were bone marrow sinusoidal cells (Fig. 2*E*). The epiphyseal plates of limb bones were hypercellular and irregular (Fig. 2*F*). The joints showed synovial proliferation, articular-synovial synechiae, and vacuolated synovial cells. Similar bone alterations affected the middle ear (not shown). The cardiac valves and endocardial fibroblasts also showed storage. In summary, all tissues examined showed the histopathology characteristic of the MPS VII (*gus*$^{mps/mps}$) mouse. In fact, in no tissue from the MPS VII/E540A$^{Tg}$ mouse could changes be distinguished from those in the MPS VII (*gus*$^{mps/mps}$) mouse.

**Table 1. Tissue levels of $\beta$-glucuronidase and $\alpha$-galactosidase activity in MPS VII/E540A$^{Tg}$ and B6 control mice**

| Tissue | Animal | $\beta$-glucuronidase units/mg | $\alpha$-galactosidase units/mg |
|---|---|---|---|
| Liver | E540A$^{Tg}$ | 0.053 ± 0.004 | 44.5 ± 2.2 |
| | B6 | 148 ± 10.7 | 18.1 ± 0.9 |
| Kidney | E540A$^{Tg}$ | 0.25 ± 0.005 | 79.8 ± 9.3 |
| | B6 | 81 ± 6.9 | 23.2 ± 1.3 |
| Brain | E540A$^{Tg}$ | 0.073 ± 0.013 | 31.3 ± 4.7 |
| | B6 | 20 ± 1.5 | 17.8 ± 1.2 |

**Tolerance of the MPS VII/E540A$^{Tg}$ Mice to Immune Challenge with Human $\beta$-Glucuronidase.** Having established that the transgene expressing the cDNA for hGUS/E540A did not alter the phenotype of the MPS VII mouse, we next tested the hypothesis that the transgene would confer tolerance to human $\beta$-glucuronidase on the MPS VII mouse, which has no endogenous murine GUS. To provide a maximum immunogenic challenge, we used i.p. injection of human GUS in complete Freund's adjuvant as the initial challenge, followed by two boosts with human GUS in incomplete Freund's adjuvant at 28 and 42 days. As a control for the effect of the transgene, we used homozygous B6 MPS VII (*gus*$^{mps/mps}$) mice, which do not carry the transgene that received

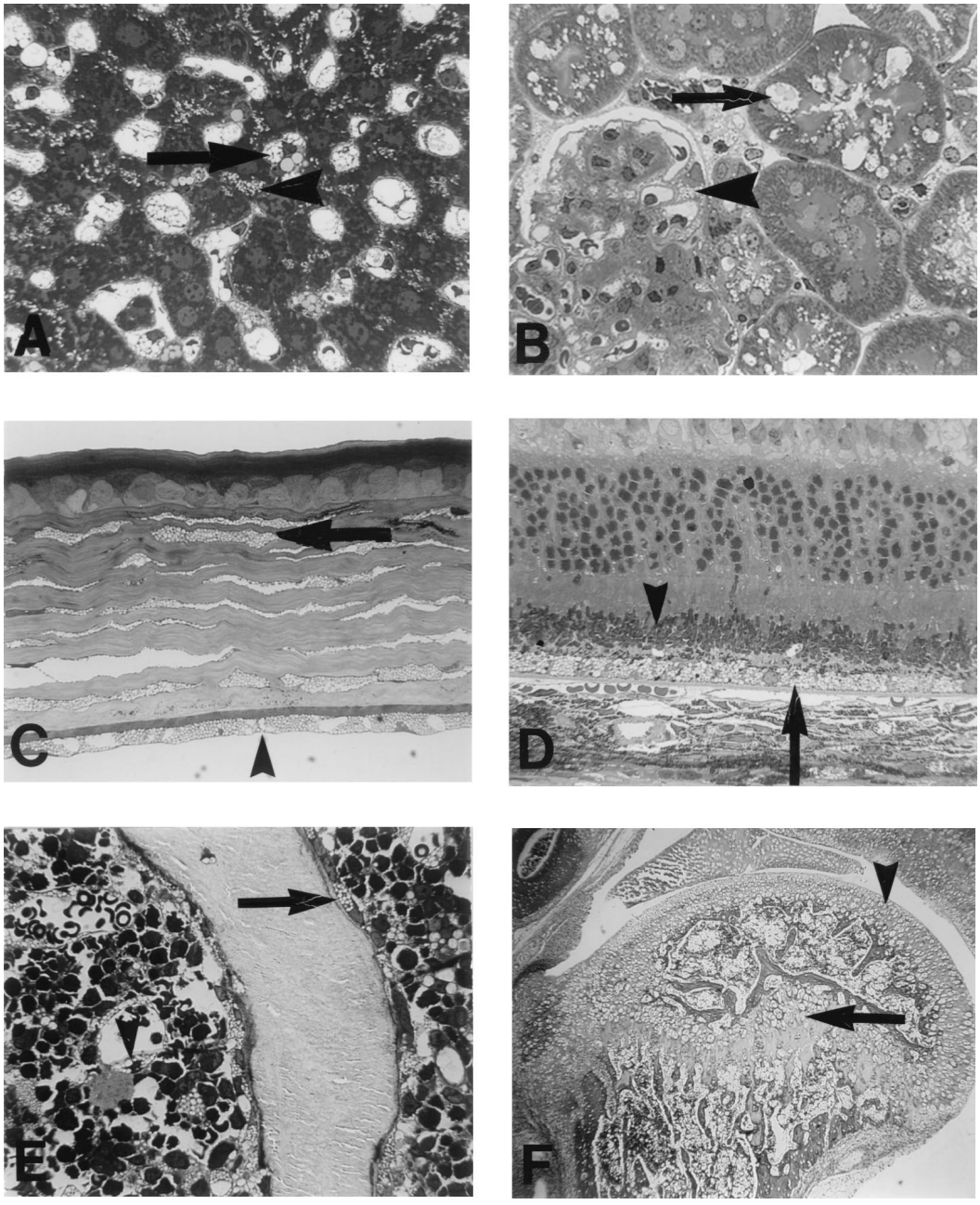


**Fig. 2.** Histopathology of the MPS VII/E540A$^{Tg}$ mouse. (*A*) The liver from a 9-month-old MPS VII/E540A$^{Tg}$ mouse has sinus-lining cells (arrow) distended by lysosomal storage. The hepatocytes also have a small amount of cytoplasmic vacuolization in a pericanalicular distribution (arrowhead) (Toluidine blue, 1 cm = 23.8 microns). (*B*) Renal tubular epithelial cells (arrow) in the kidney of a 9-month-old MPS VII/E540A$^{Tg}$ mouse contained very large cytoplasmic vacuoles representing lysosomal storage. Glomerular visceral epithelial cells (arrowhead) and interstitial cells also have storage, although their cytoplasmic vacuoles are smaller than those seen in the tubular epithelial cells (Toluidine blue, 1 cm = 23.8 microns). (*C*) The cornea of a 3-month-old MPS VII/E540A$^{Tg}$ mouse is altered with fibrocytes (arrow) and endothelial cells (arrowhead) distended with cytoplasmic vacuolization representing lysosomal storage (Toluidine blue, 1 cm = 23.8 microns). (*D*) Retinal pigment epithelial cells (arrow) from the eye of a 9-month-old MPS VII/E540A$^{Tg}$ mouse have extensive storage and the outer segments of the photoreceptors (arrowhead) are disorganized (Toluidine blue, 1 cm = 23.8 microns). (*E*) The bone spicules from the rib of a 9-month-old MPS VII/E540A$^{Tg}$ mouse are lined by osteoblasts (arrow) with cytoplasmic vacuolization. Similar lysosomal storage affects the bone marrow sinus-lining cells (arrowhead) (Toluidine blue, 1 cm = 23.8 microns). (*F*) A stifle joint from a 6-month-old MPS VII/E540A$^{Tg}$ mouse has marked lysosomal storage in chondrocytes with distortion of the epiphyseal plate (arrow), articular cartilage (arrowhead), and periarticular articular connective tissue. The bone marrow also contains vacuolated cells (hematoxylin and eosin, 1 cm = 384 microns).

the same immunogen on the same schedule. At the first bleed (12 days after the first boost), all four of the MPS VII (*gus*$^{mps/mps}$) controls, but none of the MPS VII/E540A$^{Tg}$ mice, showed anti-human GUS antibodies by ELISA (data not shown). Fig. 3 shows the ELISA plate assay on blood taken 12 days following the second boost (i.e., 54 days after the initial challenge). All four MPS VII control mice had titers of $10^5$ or greater. By contrast, none of the MPS VII/E540A$^{Tg}$ mice showed any response. These data demonstrated two important points: (*i*) The MPS VII (*gus*$^{mps/mps}$) mice that do not carry the transgene are capable of mounting a strong antibody response to human GUS when challenged in this manner. (*ii*) The h*GUS*/E540A transgene

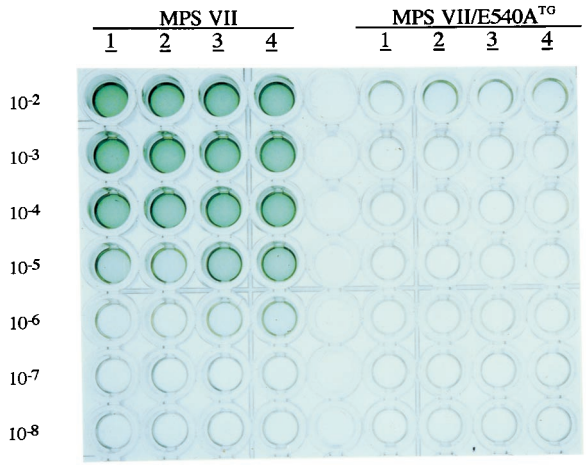


**Fig. 3.** Immune tolerance of MPS VII/E540A$^{Tg}$ mice to human GUS. ELISA plate assay of antibodies in serum of control MPS VII (*gus*$^{mps/mps}$) mice (*Left*) and MPS VII/E540A$^{Tg}$ mice (*Right*) following primary immunization with human GUS in complete Freund's adjuvant and two boosts with human GUS and incomplete Freund's adjuvant as described in *Materials and Methods*. Control mice have antibodies detectable at $10^5$ dilutions or greater, whereas MPS VII/E540A$^{Tg}$ mice show no antibody response.

clearly conferred tolerance to human GUS, even when provided with this extreme immunogenic challenge.

## Discussion

The original MPS VII (*gus*$^{mps/mps}$) mouse has been widely used as a model for testing experimental therapies for lysosomal storage disorders (10–39), and the value of the model is amply demonstrated by these studies. Syngeneic bone marrow transplantation in the MPS VII (*gus*$^{mps/mps}$) mice, if performed in the newborn period before the clinical manifestations became pronounced, prolonged life, improved hearing and growth, and corrected lysosomal storage in many organs, although it did not correct the lysosomal storage in brain (11). Enzyme replacement therapy with purified murine GUS, if begun in the first few days of life, produced impressive reductions in visceral lysosomal storage, normalized the phenotype, and lengthened the lifespan (16–18). It also improved storage in brain if begun before 14 days of age (20). Several approaches to gene therapy for MPS disorders have been studied in this model. Retroviral (23–28), adenoviral (29–33), and adeno-associated virus vectors (34–39) have all been used. Most gene therapy trials with this model have used the human cDNA to express human GUS in the MPS VII mouse, but none have evaluated the effects of antibodies to human GUS on the duration of expression or the magnitude of response.

The MPS VII (*gus*$^{mps/mps}$) mouse was recently reported to have an impaired immune response to foreign proteins (22). Given this observation, one might question whether it would be naturally tolerant to human GUS. Results presented here show this is clearly not the case: the MPS VII (*gus*$^{mps/mps}$) mouse developed antibodies to human GUS. However, this paradox can be explained. The immune defect in the MPS VII mouse was attributed to inhibition by the accumulated GAGs of proteases required for antigen processing. Activity of these proteases on foreign proteins is required to provide peptides for antigen presentation. However, the same report (22) showed that providing purified murine GUS *in vitro* corrected this defect. One would expect, then, that the large dose of human GUS delivered as the antigenic challenge would also correct the immune defect *in vivo*. This, in turn, would enable the MPS VII (*gus*$^{mps/mps}$) control mice to develop an immune response to the corrective human GUS, which would be recognized as foreign. The data presented here argue that this is the case. The MPS VII (*gus*$^{mps/mps}$) control mice developed a strong antibody response to human GUS.

On the other hand, the MPS VII mouse carrying a transgene expressing the E540A mutant form of human GUS did not develop antibody. In fact, it was tolerant to an extraordinary challenge with human GUS. From these results, we conclude that the MPS VII/E540A$^{Tg}$ mouse should provide a valuable model for preclinical studies of enzyme therapy with purified human GUS and of gene therapy with vectors expressing human GUS, because antibodies to the corrective protein will not complicate the interpretation of the results or abrogate the therapeutic responses to the corrective enzyme.

The approach used here to produce an improved murine model of MPS VII should be generalizable to other enzyme deficiency disease models. The first step involves determination of one or more catalytically essential residues of the human enzyme in question. Next, one determines which essential residue can be replaced by an inactivating mutation, yet still allow expression of a stable, inactive enzyme. The next step involves creating a transgenic mouse expressing the inactive human gene product. Once it has been established that one of the transgenic founders expresses enough inactive human enzyme to confer tolerance on the wild-type mouse background, the tolerance-conferring transgene can be crossed onto the strain carrying the mouse null mutant. Finally, the tolerance of the homozygous null strain carrying the transgene must be confirmed by repeating the immune challenge, as done here with human GUS.

Once established, the tolerant mouse model of the disease of interest can be propagated by conventional means. Given the rapidly growing list of knockout mouse models of human diseases, and the interest in using these models in preclinical trials to evaluate the safety and effectiveness of gene products to evaluate experimental therapies using products that might be administered to humans, there should be many opportunities to use "tolerant mouse models."


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