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Targeted Mutation in β1,4-Galactosyltransferase Leads to Pituitary Insufficiency and Neonatal Lethality

Qingxian Lu,[1] Paul Hasty,[2] and Barry D. Shur[1]

Department of Biochemistry and Molecular Biology, M. D. Anderson Cancer Center, Houston, Texas 77030

Despite much attention, the function of oligosaccharide chains on glycoproteins and glycolipids remains largely unknown. Our understanding of oligosaccharide function *in vivo* has been limited to the use of reagents and targeted mutations that eliminate entire classes of oligosaccharide chains. However, most biological functions for oligosaccharides have been attributed to specific terminal sequences on these glycoside chains; yet, there have been few studies that examine the consequences of modifying terminal oligosaccharide structures *in vivo.* To address this issue, mice were created bearing a targeted mutation in β1,4-galactosyltransferase (GalTase), an enzyme responsible for elaboration of many of the proposed biologically active carbohydrate epitopes. Most GalTase-null mice died within the first few weeks after birth and were characterized by stunted growth, thin skin, sparse hair, and dehydration. In addition, spermatogenesis was delayed, the lungs were poorly developed, and the adrenal cortices were poorly stratified. The few surviving adults had puffy skin (myxedema) and difficulty delivering pups at birth (dystocia) and failed to lactate (agalactosis). All of these defects are consistent with endocrine insufficiency, which was confirmed by markedly decreased levels of serum thyroxine. The polyglandular nature of the endocrine insufficiency is indicative of a failure of the anterior pituitary gland to stimulate the target endocrine organs. Previous *in vitro* studies have suggested that incomplete glycosylation of anterior pituitary hormones leads to the creation of hormone antagonists, which down-regulate subsequent endocrine function, producing polyglandular endocrine insufficiency. In GalTase-null mice, the anterior pituitary acquired a normal secretory phenotype during neonatal development indicative of normal glycoprotein hormone synthesis and secretion. However, as expected, the gland was devoid of GalTase activity. These results support a requirement for terminal oligosaccharide sequences for anterior pituitary hormone function. The fact that ~10% of the GalTase-null mice survive the neonatal period indicates the presence of a previously unrecognized compensatory pathway for glycoprotein hormone glycosylation and/or action.

INTRODUCTION

Of the major classes of macromolecules, we know the least about the most complex class, that of the glycoconjugates. Their potential heterogeneity is greater than that of the nucleic acids, proteins, or lipids. However, our understanding of glycoconjugate function has been limited to *in vitro* analyses, in which glycoside structure and/or expression can be manipulated using a wide range of reagents. Antibiotics, glycosidases, lectins, anti-carbohydrate antibodies, somatic cell mutants, and hapten inhibitors are just some of the many reagents that have been used to decipher oligosaccharide function. From these studies, we suspect that glycosides carry out a myriad of biological activities, impacting glycoprotein synthesis, transport, and stability; supramolecular organization; modulation of protein activity; intra- and intercellular protein trafficking; and recognition motifs for a variety of infectious agents, for cell–cell interactions and for cell–matrix interactions (Varki, 1993).

Although these *in vitro* analyses suggest that glycosides perform a multiplicity of functions, we still know very little about the actual functions of glycoside residues *in vivo.* The use of pharmaceuticals or mutations that block the synthesis of entire oligosaccharide chains have confirmed that glycosides are indeed required for normal development (Surani, 1979; Metzler *et al.*, 1994; Ioffe and Stanley, 1994). However, *in vitro* analyses suggest that the most biologi-

[1] Present address: Department of Anatomy and Cell Biology, Emory University School of Medicine, Atlanta, GA 30322.
[2] Present address: Lexicon Genetics, Inc., The Woodlands, TX 77381.

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cally relevant information is encoded in the terminal sequences of glycoside chains. Unfortunately, there have been only a few studies to date assessing the functional significance of terminal carbohydrate sequences *in vivo* (Youakim *et al.*, 1994; Maly *et al.*, 1996).

To address this issue, mice were created that are null for β1,4-galactosyltransferase (GalTase), one of the enzymes responsible for the synthesis of many of the proposed biologically significant epitopes. Surprisingly, the great majority of GalTase-null mice survived embryogenesis, but died during the neonatal period. At least one of the primary defects responsible for the neonatal lethality appears to be a failure to galactosylate the glycoprotein hormones of the anterior pituitary gland, leading to polyglandular endocrine insufficiency.

MATERIALS AND METHODS

Targeting Vectors and Homologous Recombination

Genomic DNA for mouse GalTase was isolated from a genomic DNA library (Stratagene) prepared from strain 129/Sv using a ^{32}P-labeled 531-bp cDNA fragment corresponding to exon 1 of GalTase as probe (Lopez *et al.*, 1991). An 8-kb *Eco*RI–*Sal*I fragment spanning the first exon (Hollis *et al.*, 1989) was subcloned into pKS(−) plasmid vector, from which the gene targeting vector was constructed. A PGK-neo expression cassette was inserted in reverse orientation into the *Cla*I–*Not*I sites and served as a positive selection marker as well as a mutagen. A negative selection marker, the herpes simplex virus thymidine kinase (HSV-tk) gene, was inserted into the targeting vector outside of the homologous region to allow selection against cells that underwent nonhomologous integration (Mansour *et al.*, 1988). Targeting vector was transfected into AB1 embryonic stems (ES) cells (McMahon and Bradley, 1990), and homologous recombination events were screened from the drug-resistant ES population by using a 1.1-kb *Sty*I fragment as a 5′-external probe. The targeted ES clones were microinjected into blastocysts of 3.5-day C57BL/6J embryos that further developed to chimeras (Bradley, 1987). Chimeric animals were mated to C57BL/6J to create heterozygous animals that were intercrossed to generate homozygous animals.

Analysis of Viability

Heterozygous females were housed with one heterozygous male. The plug day was considered to be E0.5. Fetal viability was determined by embryonic movement in response to stimulus or by the presence of a beating heart. Embryos (E18.5), newborn pups, and neonatal pups were genotyped by Southern analysis (Sambrook *et al.*, 1989) using the 1.1-kb *Sty*I fragment as probe and by PCR analysis using primers that distinguished the wild-type (301-bp PCR product) and mutant (243-bp PCR product) alleles [wild-type allele: 5′-primer: 5′-ACACTCCCGCCCCCCCAGGTT-3′, 3′-primer: 5′-GCTCACGAAACCTAAGCTTCCCGCC-3, corresponding to the −286th and 15th nucleotides, respectively, relative to the first GalTase ATG codon; mutant allele: 5′-primer: 5′-GGTGGATGTGGA-ATGTGTGCGAGG-3′, corresponding to the −401st nucleotide in the *pgk*-1 promoter of the *neo* cassette (Ada *et al.*, 1987), 3′-primer: 5′-AGAAGCTGCACCAGGGCTGGA-3′, corresponding to the 318th nucleotide, relative to the first GalTase ATG codon]. Pups were weighed once a week beginning on the day of birth. Eye color and sex or pen labeling were used to distinguish pups until they were toe-cut and genotyped.

RT-PCR

Total tissue RNA was isolated with RNeasy reagent (Qiagen) and quantified by UV absorbance at 260 nm. One microgram of each RNA sample was random primed and reverse transcribed with Superscript II reverse transcriptase (BRL) at 42°C for 50 min in the presence of 1× first strand buffer (BRL), 10 m*M* DTT, 0.5 m*M* each dNTP, and 5 U RNase inhibitor (Ambion). One-twentieth of synthesized cDNA from each sample was subjected to 100 μl of PCR amplification. The PCR reaction mixture was denatured at 94°C for 5 min and amplified for 35 cycles of 94°C, 30 sec; 58°C, 30 sec; 72°C, 1 min. PCR products were fractionated on a 2% agarose gel. Sense primer (5′-GGCGTCACCCTCGTCTATTA-3′) and antisense primer (5′-ACGGAATGGGATGATGATGG-3′) correspond to the 104th and 559th nucleotides, respectively, of the cDNA, relative to the first ATG. In some instances, primers were used to assess the presence of sequences encoding the COOH-terminal catalytic domain (sense: 5′-AAGGCATGTCTATAT-CACGT-3′; antisense: 5′-TGTCCCGATGTCCACTGTGA-3′) corresponding to the 974th and 1191st nucleotides, respectively, of the cDNA, relative to the first ATG. β-actin primers (sense: 5′-TGTGATGGTGGGAATGGGTCAG-3′; antisense: 5′-TTTGAT-GTCACGCACGATTTCC-3′) were included in all reactions, producing a 514-bp fragment as an internal control.

Analysis of GalTase Activity and Reaction Product

Tissues were isolated from wild-type and *gt* −/− mice at 4 weeks of age, homogenized on ice in 0.5 ml of medium B, and assayed for GalTase activity toward endogenous and exogenous (i.e., *N*-acetylglucosamine) substrates as described (Shur, 1982).

To determine the glycosidic linkage of the ^{3}H-galactosylated reaction product in testis or brain homogenates, equal cpm of product were treated with *Streptococcus pneumoniae* β1,4-galactosidase (Oxford GlycoSystems) in a volume of 50 μl reaction containing the indicated amount of enzyme. The reaction was incubated at 37°C for 2 hr. Remaining ^{3}H products were determined by high-voltage borate electrophoresis as described (Shur, 1982).

The presence of terminal β1,4-galactosyl residues in tissue lysates was determined by either lectin blotting or reactivity toward α2,6-sialyltransferase. For lectin blotting, 6 μg of each sample were heat-inactivated at 85°C for 10 min and divided into three groups. One group was incubated without enzyme (control). The other two groups were incubated with 20 mU of *Clostridium perfringens* neuraminidase (Sigma) at 37°C for 6 hr, after which the neuraminidase was heat-inactivated and cooled. One of these groups was further incubated at 37°C overnight with 4 mU of β1,4-galactosidase. Two micrograms of each sample was loaded on each slot of Hybond-N^{+} membrane (Amersham). The blots were labeled with RCA-I lectin specific for terminal β1,4-galactosyl residues (Nicolson and Blaustein, 1972) following the manufacture's instructions (Vector).

Alternatively, tissue homogenates were treated with either neuraminidase or β1,4-galactosidase as above prior to incubation with α2,6-sialyltransferase (Sigma). Treated samples were incubated at 37°C for the indicated time in the presence of 0.5 mU sialyltransferase and 0.2 μCi of CMP-^{3}H-NeuAc (Dupont-NEN) for each time

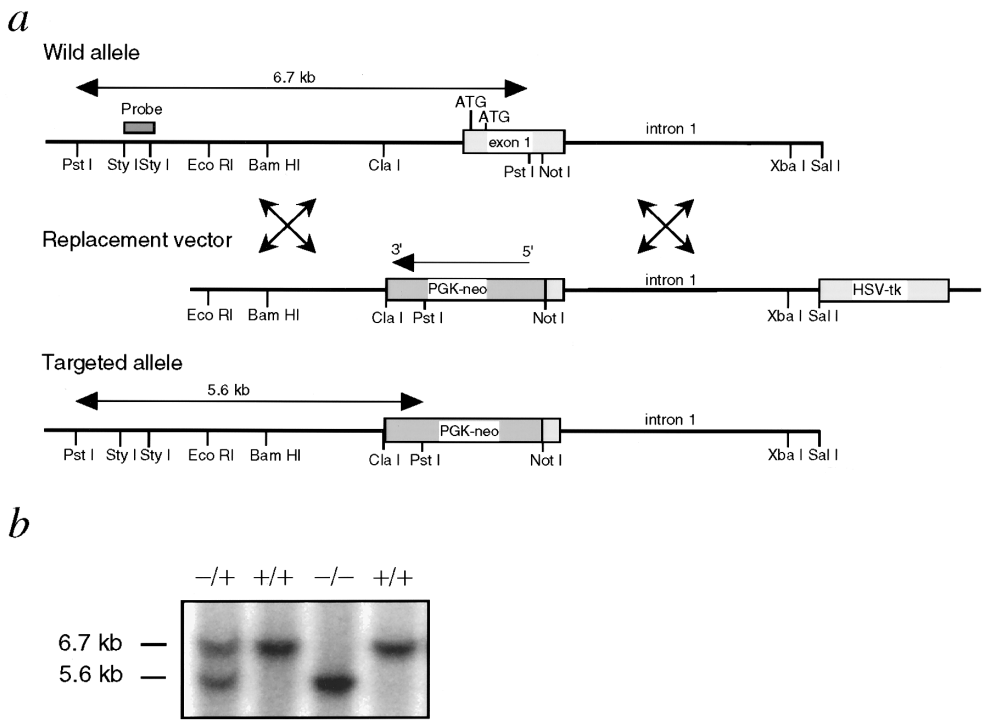

FIG. 1. Generation of GalTase-deficient mice. (a) Targeted disruption of the mouse GalTase gene by homologous recombination. Schematic diagrams show the wild-type GalTase allele (top), the replacement vector (middle), and the predicted targeted mutated allele (bottom). Exon 1 includes the two ATG codons for translation of the two GalTase isoforms. The targeting construct was prepared from an 8.0-kb fragment of 129/Sv genomic DNA. After recombination (crossed arrows), the PGK-neo expression cassette replaces a 1.9-kb fragment containing the coding region for the entire cytoplasmic and transmembrane domains and a portion of the catalytic domain. (b) Tail DNA from neonatal pups was digested with *Pst*I, fractionated on 0.8% agarose, transferred to Hybond-N^+ membrane, and probed with a ^{32}P-labeled *Sty*I fragment diagramed in (a). *Pst*I fragments reflecting the wild-type (6.7 kb) and mutant (5.6 kb) alleles are shown.

point. The labeled products were analyzed by high-voltage borate electrophoresis as described (Shur, 1982).

Histology

Tissues were isolated from 3-week-old mice, fixed, embedded in paraffin, and sectioned using routine histological procedures. Five micrometer sections were stained with hematoxylin and eosin or with Heidenhain's aniline blue stain (Thompson, 1966).

Thyroxine RIA

Mice were sacrificed by carbon dioxide asphyxiation and blood was withdrawn by cardiac puncture. Serum was collected, frozen, and shipped to Ani Lytics (Gaithersburg, MD) for T4 and T3 assays.

RESULTS

Creation of GalTase-null Mice

GalTase was inactivated in AB1 embryonic stem (ES) cells by introduction of the *neo* selectable marker into the first exon of GalTase (Fig. 1a). The *neo* cassette replaced 1.9 kb of genomic GalTase DNA encoding the complete cytoplasmic and transmembrane domains of GalTase and a portion of the extracellular stem region (Russo *et al.*, 1990). The *neo* insertion also disrupted the reading frame of the remaining GalTase sequences.

Five independent ES clones (1.6%) were identified that underwent homologous recombination as determined by Southern analysis. Two independent ES clones bearing the targeted GalTase allele were transmitted through the germ line after embryo injection. There were no obvious defects in heterozygous mutant mice (*gt* +/−), which were crossed to produce homozygous mutant animals (*gt* −/−). Litter size averaged 6.9 pups, and the genotypes of all animals were determined by Southern (Fig. 1b) or PCR analysis.

Most GalTase-null Mice Are Neonatal Lethals

The majority of homozygous mutant mice progressed through embryonic development, since 22.7% of 18.5-day-old embryos were *gt* −/−. However, homozygotes died soon after birth and during the subsequent 2–3 weeks (Fig. 2a). Only 10% of *gt* −/− mice (2.6% of total mice) survived this period of neonatal lethality. All *gt* −/− mice showed a simi-

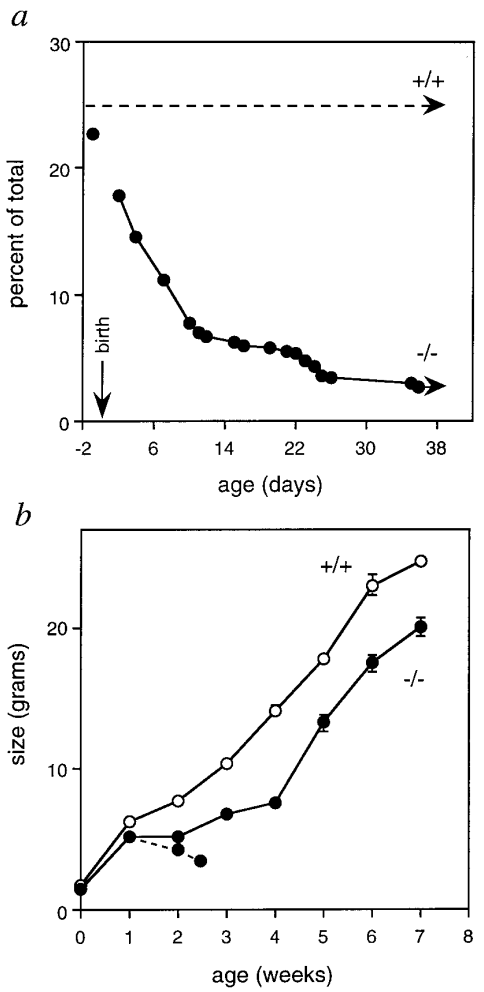

FIG. 2. Viability of GalTase homozygous null mice. (a) The majority of *gt* −/− embryos develop to term as judged by their presence at 18.5 days of gestation [22.7% observed (22/97) vs 25% expected]. Some newborn pups were lost during or soon after delivery as indicated by the open line between Day −1 (E18.5) and Postnatal Day 2, however, most *gt* −/− pups died during neonatal development. The theoretical viability of wild-type pups is indicated by the dashed line. (b) Only 10% of *gt* −/− mice (2.6% of total) survive past the neonatal period, after which they grow at normal rates. Values represent the mean weight ±SE of four wild-type (○) and three *gt* −/− (●) males. A male pup who died at 17 days of age is also shown (dashed line).

lar phenotype as described below, including stunted growth and defective skin and hair. Surprisingly, those that survived the neonatal period recovered a normal growth rate and overall appearance between 3 and 4 weeks of age (Fig. 2b).

Analysis of GalTase mRNA, Activity, and Reaction Product

The expression of both GalTase mRNA and enzyme activity toward *N*-acetylglucosamine (GlcNAc) substrates was examined in multiple tissues from *gt* −/− animals. Tissues assayed included salivary gland, stomach, adrenals, small intestine, pancreas, testis, spleen, lung, thymus, liver, kidney, heart, brain, and skeletal muscle. RT-PCR of sequences within exon 1 demonstrated the loss of the GalTase mRNA in all tissues (Fig. 3a). The integrity of all RNA samples was confirmed through the use of PCR primers for β-actin.

GalTase enzyme activity was undetectable in all *gt* −/− tissues except testis and brain, which exhibited 4 and 30%, respectively, of wild-type enzyme activities (Fig. 3b). Although the residual activity in *gt* −/− testis and brain reflects different percentages of wild-type activity in these organs, their specific activities were very low and similar to one another (<100 cpm ^{3}H-Galβ1,4GlcNAc product/25 μg prt/hr). The galactosylated product in both testis and brain was partially sensitive to digestion with β1,4-specific galactosidase from *S. pneumoniae*, suggesting the presence of authentic GalTase-like activity in these two tissues. The residual β1,4-galactosidase-resistant material in brain, but not testis, was sensitive to digestion by bovine testis galactosidase, which hydrolyzes β1,4-, β1,3-, and β1,6-galactosyl residues (Sheares *et al.*, 1982). This indicates the presence of other non-β1,4-galactosyltransferase activities toward GlcNAc (e.g., Galβ1,3GlcNAc) in brain lysates (data not shown). Tissues from *gt* +/− heterozygotes showed intermediate levels of enzyme activity (data not shown).

We examined the possibility that the low levels of residual GalTase-like activity in brain and testis resulted from cryptic promoter activity in the inverted *neo* cassette. Even in the absence of the cytoplasmic and signal sequence/transmembrane domains, it is conceivable that such transcripts could permit translation from downstream AUGs leading to low levels of a soluble, catalytic domain. PCR analysis of 3′-COOH-terminal sequences encoding the catalytic domain showed low and variable levels of these sequences in testis (data not shown). However, it is not known if these sequences are, in fact, translated, which would account for the low level of enzyme activity. Furthermore, we can not eliminate the possibility of a novel β1,4-galactosyltransferase gene product responsible for this low level of activity.

The presence or absence of GalTase activity in *gt* −/− mice was further examined by analyzing tissue homogenates for the presence of the GalTase product, i.e., β1,4-galactosyl residues. Reactivity toward RCA-I lectin and α2,6-sialyltransferase was assayed, since both preferentially recognize terminal β1,4-galactosyl groups (Nicolson and Blaustein, 1972; Paulson *et al.*, 1977). RCA-I-reactive glycoconjugates were readily detectable in tissues from wild-type animals; representative results are shown for intestine, heart, testis, and lung (Fig. 4a). RCA-I reactivity was increased following neuraminidase treatment (N) to expose additional β1,4-galactosyl groups and was eliminated by treatment with β1,4-specific galactosidase (G), as expected for authentic RCA-I reactivity. Similarly, wild-type tissues had substrate activity (i.e., terminal β1,4-galactosyl residues) toward α2,6-sialyltransferase, which was eliminated

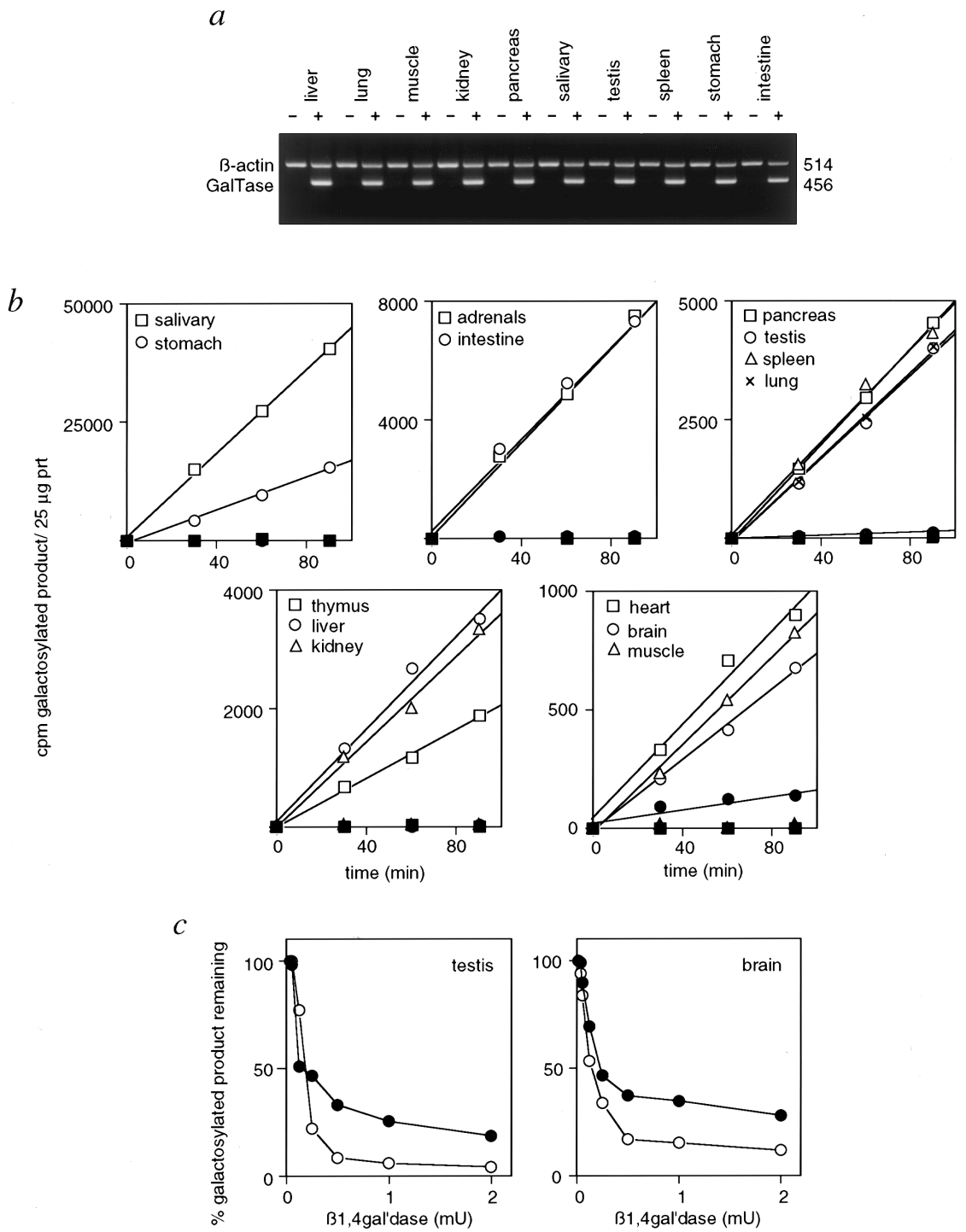

FIG. 3. Characterization of GalTase expression. (a) RT-PCR analysis of GalTase expression in various tissues. GalTase-specific amplification of a 456-bp fragment was detected in all wild-type tissues (+), but not in *gt* −/− tissues (−). Primers for β-actin were used to assess the integrity of the RNA samples. (b) GalTase activity in wild-type (open symbols) and *gt* −/− (filled symbols) tissues. Enzyme activity was undetectable in all *gt* −/− tissues, except testis and brain, which exhibited 4 and 30%, respectively, of wild-type enzyme levels. Tissues with similar specific activities are graphed together. (c) Approximately 70% of the ^{3}H-galactosylated product in *gt* −/− testis and brain was sensitive to β1,4-galactosidase digestion, indicating the presence authentic GalTase activity in these tissues, although of very low specific activity.

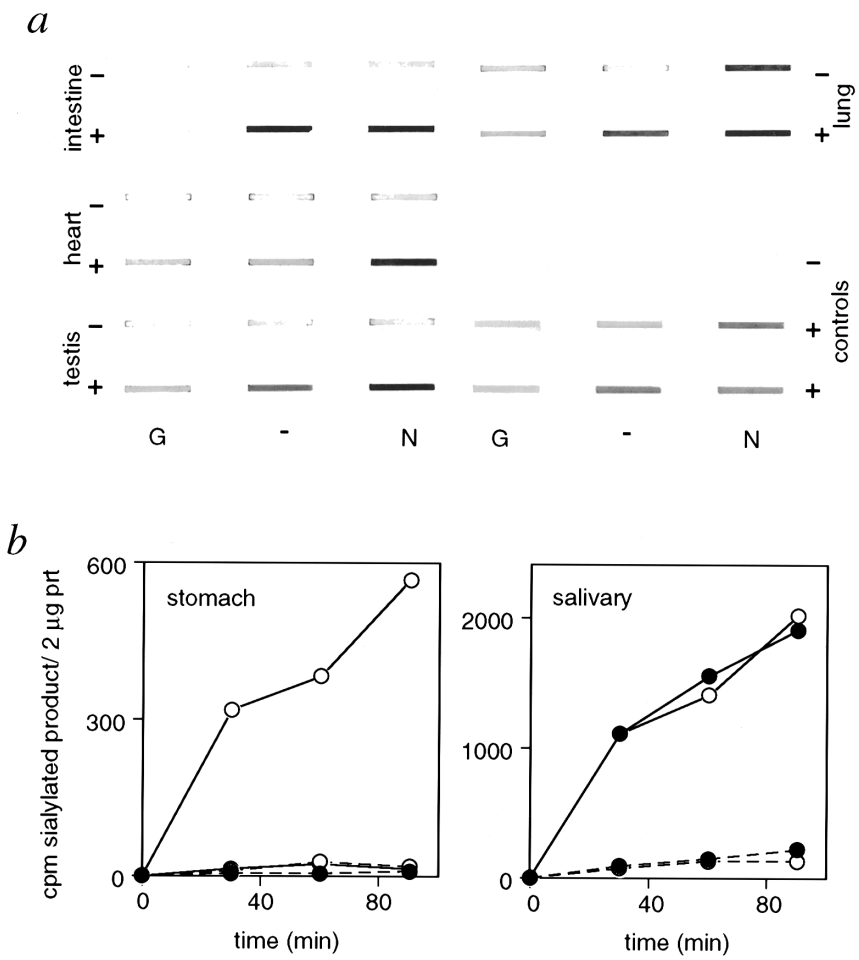

FIG. 4. Analysis of GalTase reaction product. (a) RCA-I reactivity of tissue homogenates. RCA-I specifically recognizes Galβ1,4GlcNAc linkages and binds to glycoconjugates in tissue homogenates from wild-type (+) mice. Tissue homogenates were reacted with RCA-I under one of three conditions: either without prior treatment (middle), or following neuraminidase treatment (N) to expose β1,4-galactosyl groups, or following β1,4-galactosidase (G) digestion to remove β1,4-galactosyl groups. Most tissues from *gt* −/− (−) animals failed to react with RCA-I even after neuraminidase treatment to expose potential β1,4-galactosyl groups. However, neuraminidase treatment exposed reactivity in lung. Negative (−, carboxypeptidase Y) and positive (top +, fetuin; bottom +, asialo-fetuin) control proteins are included. (b) Detection of terminal β1,4-galactosyl groups by reactivity to α2,6-sialyltransferase. Homogenates of wild-type (○) stomach are shown to illustrate reactivity to sialyltransferase, whereas *gt* −/− (●) stomach had no reactivity. Wild-type reactivity was sensitive to pretreatment with β1,4-galactosidase (dashed lines), indicative of authentic β1,4-galactosyl groups. In contrast, salivary gland homogenates from both wild-type (○) and *gt* −/− (●) mice had similar reactivity to sialyltransferase, all of which were eliminated by pretreatment with β1,4-galactosidase (dashed lines), thus suggesting an alternative pathway for β1,4-galactosylation in salivary gland.

FIG. 5. Phenotypic analysis of GalTase-deficient mice. (a, b) Views of 3-week-old wild-type and *gt* −/− C57BL/6J black and albino mice. The *gt* −/− mice (right of each pair) are dwarf with sparse hair and thin skin. Note the intestinal contents visible through the albino skin as is the scalp; the hair is missing on the dorsal neck of the black *gt* −/− animal. (c, d) Sections through age-matched dorsal, thoracic–lumbar skin from wild-type (c) and *gt* −/− (d) mice. Note the reduced development of hair follicles and subdermal adipose tissue. (e, f) Sections of testis from wild-type (e) and *gt* −/− (f) males. Spermatogenesis is delayed in the mutant as evidenced by the smaller seminiferous tubules and by the predominance of pachytene spermatocytes in *gt* −/− mice (arrowhead) relative to the predominance of round and late spermatids in wild-type (arrowhead). (g, h) Sections through lungs of newborn wild-type (g) and *gt* −/− (h) mice. Note the thick trabeculae in *gt* −/− and the smaller alveoli as compared to wild-type. (i) Views of dissected whole adrenal glands from wild-type (left) and *gt* −/− (right) mice. (j, k) Sections of adrenal glands through their widest diameter (equator) illustrating the reduced size and stratification of the adrenal cortex. Most conspicuous is the dramatically reduced differentiation of the inner zona reticularis and intermediate zona fasciculata.

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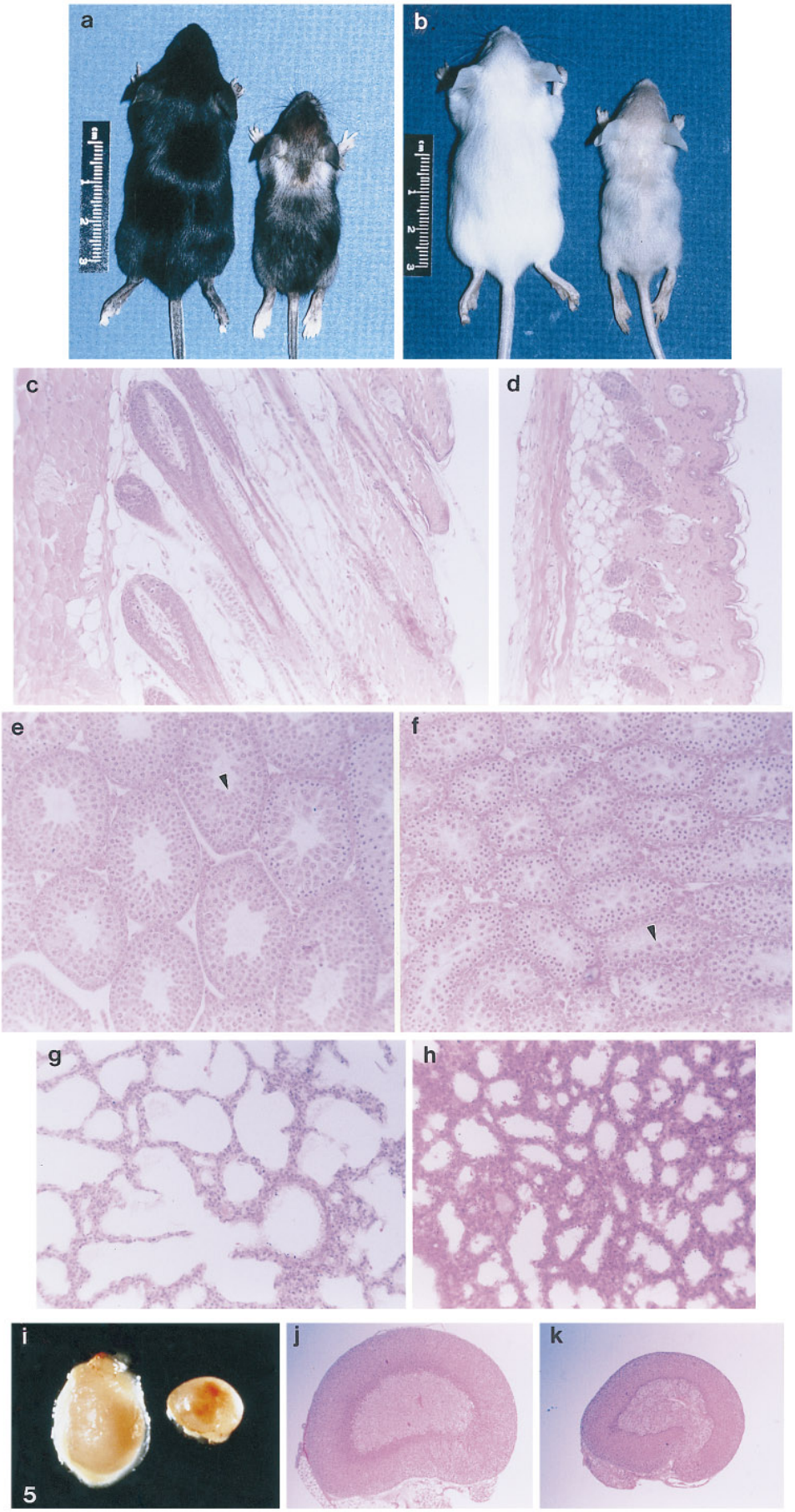

by pretreatment with β1,4-specific galactosidase; results for stomach and salivary gland are shown (Fig. 4b).

Some tissue homogenates from *gt* −/− mice retained variable amounts of reactivity toward RCA-I and/or α2,6-sialyltransferase, indicative of either residual GalTase activity or alternative pathways for β1,4-galactosylation. For example, neuraminidase treatment of *gt* −/− lung lysates exposed cryptic RCA-I reactivity (Fig. 4a). *gt* −/− salivary glands had normal levels of reactivity toward α2,6-sialyltransferase, the product of which was sensitive to β1,4-specific galactosidase digestion (Fig. 4b). Intestinal lysates also showed very low levels of residual β1,4-galactosyl groups by both RCA-I and α2,6-sialyltransferase reactivity (data not shown).

Endocrine Insufficiency in GalTase-null Mice

The *gt* −/− animals that survived the first week grew much slower than wild-type or heterozygous littermates. Their hair was sparse (Figs. 5a and 5b) and the skin was often thin enough to allow visualization of the intestinal contents (Fig. 5b). The reduced size and defective skin and hair are indicative of endocrine insufficiency, particularly hypothyroidism (Wilson *et al.*, 1991). Histological examination revealed marked reduction in the density of hair follicles and subdermal adipose tissue (Figs. 5c and 5d). Further evidence for endocrine insufficiency is the delayed spermatogenesis in the testis (Figs. 5e and 5f), the poorly differentiated lung parenchyma containing thick septa and small alveoli (Figs. 5g and 5h), and the reduced size (Fig. 5i) and stratification (Figs. 5j and 5k) of the adrenal cortex. All other tissues examined histologically appeared normal, including salivary gland, stomach, intestine, pancreas, spleen, thymus, liver, kidney, heart, and skeletal muscle, although they all were reduced in overall size. The brain appeared grossly normal in size.

In addition to the physical and histological phenotype seen in the neonates, the few surviving adults demonstrated puffy faces (hypothyroid myxedema), an inability to deliver pups (dystocia), and an inability to lactate (agalactosis). Collectively, this spectrum of neonatal and adult phenotypes is indicative of the syndrome of polyglandular endocrine insufficiency (Gill, 1991). As a marker of endocrine insufficiency, serum levels of thyroxine (T4) were assayed; levels were dramatically reduced in all *gt* −/− mice that were examined, compared to control littermates (Fig. 6). Consistent with this contributing to the neonatal *gt* −/− phenotype, thyroxine levels returned to normal in those animals that survived the neonatal period (Fig. 6). Serum levels of T3 showed a similar trend (data not shown). Liver enzymes were elevated approximately twofold, consistent with endocrine insufficiency, as was the marked dehydration of the homozygous null mice prior to death.

Polyglandular endocrine insufficiency is most often associated with dysfunction of the anterior pituitary, the gland responsible for the synthesis of the hormones that stimulate the target endocrine glands (Gill, 1991). In *gt* −/− mice, the anterior pituitary gland was reduced in size, as were most of the other organs described above (Fig. 7a). The posterior

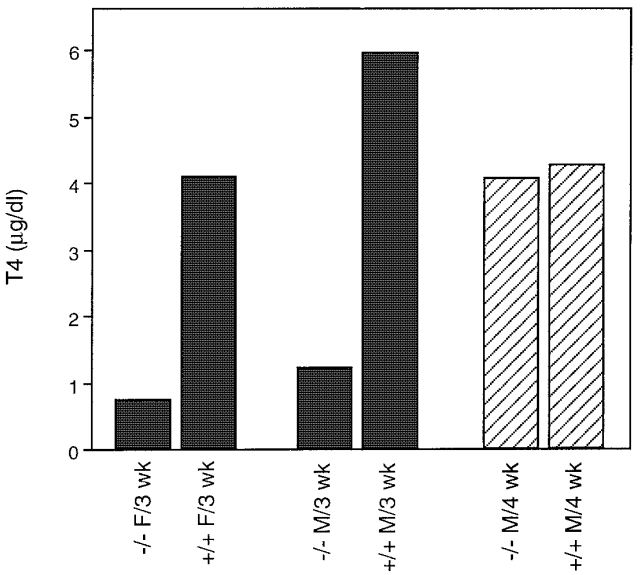

FIG. 6. GalTase-deficient mice have reduced serum levels of thyroxine. T4 levels are greatly reduced in 3-week-old *gt* −/− (−/−) mice relative to wild-type (+/+) littermates (shaded bars). A pair of females and a pair of males are shown out of a total of five pairs assayed; all produced similar results. Serum levels of T3 were also reduced in *gt* −/− mice (data not shown). T4 levels return to control values (hatched bars) in males that survive the neonatal period (4 weeks of age).

pituitary appeared normal in size (Fig. 7a). The protein mass of isolated, intact pituitaries (i.e., anterior, intermediate, and posterior lobes) from *gt* −/− mice was only 32% that of wild-type littermates (11.4 μg protein/15-day pituitary vs 35.8 μg protein). However, despite its reduced size in neonates, the constituent cells of the anterior pituitary had a characteristic chromophilic phenotype indicative of active hormone secretion (as in Fig. 7b). This was confirmed by detecting TSH and GH in serum of 14- to 21-day-old *gt* −/− mice (unpublished observations).

In newborn mice, the anterior pituitary appeared developmentally delayed, since the cells in *gt* −/− mice were chromophobic with clumped nuclei and little cytoplasm, indicative of a quiescent, poorly secretory phenotype (Snell, 1984) (Fig. 7c). Whether the developmental delay of the anterior pituitary is a primary effect of the GalTase deficiency, or is a secondary effect of dwarfism, is unknown. Nevertheless, the cells of the anterior pituitary acquired a normal secretory phenotype during neonatal development, at which time the mice showed characteristic polyglandular endocrine insufficiency.

It was of interest, therefore, to determine whether the glycoprotein hormones secreted by the anterior pituitary in *gt* −/− mice lacked the characteristic terminal disaccharide, sialic acid–galactose. The limited volume of serum obtained from 14-day-old pups precluded this analysis. As an alternative, GalTase activity was assayed directly in pituitaries isolated from *gt* −/− and control littermates, espe-

cially in light of the low levels of residual GalTase-like activity in total brain homogenates (Fig. 3b). As expected, pituitaries isolated form *gt* −/− animals were totally devoid of GalTase activity (+/+: 2294 cpm ^{3}H-Galβ1,4GlcNAc product/25 μg prt/hr vs −/−: background cpm). Thus, the hormones themselves appeared to be made normally, at least in proportion to the size of the pituitary, as evidenced by their presence in serum and by the chromophilic, secretory phenotype of the anterior pituitary cells. However, the complete lack of GalTase activity would result in hormones with incompletely glycosylated side chains.

DISCUSSION

Results presented here show that the elimination of GalTase activity from mice leads to polyglandular endocrine insufficiency. There was no evidence of any other histological abnormality that could account for the neonatal lethality. In this regard, we failed to detect any evidence of intestinal epithelial pathology in *gt* −/− mice, as suggested by others in preliminary reports (Asano *et al.*, 1996).

The polyglandular endocrine insufficiency is most readily explained by the incomplete glycosylation of the anterior pituitary hormones (Baenziger and Green, 1988; Thotakura and Blithe, 1995). The pituitary hormones TSH, FSH, and LH, as well as chorionic gonadotropin, are heterodimers composed of a common α subunit and a hormone-specific β subunit. Both subunits have N-linked oligosaccharide chains; some are of the complex-type bearing terminal sialic acid–galactose–GlcNAc trisaccharides, whereas others have an unusual sulfated *N*-acetylgalactosaminyl (GalNAc) residue (Baenziger and Green, 1988; Thotakura and Blithe, 1995). The sulfated GalNAc moiety has been shown to be critical for clearance of the circulating hormone by hepatic receptors that recognize this sulfated epitope (Fiete *et al.*, 1991). In contrast, the sialylated oligosaccharides on the α subunit, particularly those linked to Asn-52, are thought to be important for activating adenylate cyclase in the target cell (Sairam and Bhargavi, 1985; Flack *et al.*, 1994). *In vitro* studies have shown that incomplete glycosylation of the pituitary hormones results in the creation of hormone antagonists that can bind to their respective receptors, but are unable to activate adenylate cyclase. In this regard, sequentially removing terminal monosaccharide residues *in vitro* has shown that β1,4-galactosyl moieties are required for adenylate cyclase activation (Moyle *et al.*, 1975).

These results suggest that the anterior pituitary hormones in *gt* −/− animals most likely behave as biologically inactive receptor antagonists leading to hypogonadal and hypothyroid function. Interestingly, other pituitary hormones, e.g., ACTH and prolactin, are also glycosylated and recent evidence suggests that their degree of glycosylation impacts their biological activity (Skelton *et al.*, 1992; Hoffman *et al.*, 1993). It is of interest that the phenotype of the GalTase-null mouse is quite similar to that produced by a targeted mutation in the α subunit of the pituitary hormones (Kendall *et al.*, 1995). Both null mutations produce dwarf animals with hypothyroid and hypogonadal function; however, the GalTase-null mouse has reduced anterior pituitary size and is neonatal lethal.

Although the elimination of GalTase activity in the anterior pituitary can readily account for the polyglandular endocrine insufficiency, it is surprising that the anterior pituitary is reduced in size in the *gt* −/− neonate. In fact, one would expect that the lack of circulating thyroid and gonadal hormones would result in hyperplasia of the anterior pituitary as is seen in the α-subunit null mouse (Kendall *et al.*, 1995). Whether this results from delayed hypothalamic stimulation of the anterior pituitary or due to a previously undefined autocrine feedback loop awaits further investigation.

As expected, most tissues were devoid of β1,4-galactosyl residues. Some tissues (lung, intestine, and salivary gland), however, had residual β1,4-galactosyl groups as judged by RCA-I and α2,6-sialyltransferase reactivities. Similarly, residual β1,4-galactosyltransferase-like activity toward free GlcNAc substrates was detectable in brain and testis. RT-PCR analysis suggested that these low specific activities may have resulted from inappropriate transcription from the *neo* cassette, allowing translation from downstream AUG codons. We can not eliminate the possibility, however, that these low activities reflect a unique polypeptide or that an alternative tissue-specific pathway for β1,4-galactosylation may exist that has not been previously recognized. A unique glycolipid galactosyltransferase has been identified and cloned, but this enzyme catalyzes the transfer of galactose from UDPGal to ceramide, rather than to GlcNAc (Schulte and Stoffel, 1993). To date, the only mammalian gene product known to synthesize the Galβ1,4GlcNAc linkage is the GalTase gene inactivated in this study. Interestingly, preliminary reports suggest that the chicken genome contains two distinct functional GalTase genes (Shaper *et al.*, 1995).

The availability of GalTase-deficient mice now allows the utilization of these animals and derivative cell lines to explore previously unapproachable problems in glycobiology in which β1,4-galactosyl residues are thought to be critical. Among these, fibroblasts from galactosemic patients and immunoglobulin chains from rheumatoid arthritic patients are characterized by incomplete β1,4-galactosylation (Ornstein *et al.*, 1992; Tsuchiya *et al.*, 1994). Fibroblasts and blood cells from GalTase-deficient mice can determine whether these syndromes are associated with unique GalTase-like activities or they may serve as useful animal models for these diseases. Deficient glycosylation of the selectin ligands (i.e., sialyl Lewis X epitopes) on endothelial and blood cells in GalTase-deficient mice should result in phenocopies of LAD 2 (leukocyte adhesion deficiency type 2) syndrome, analogous to that produced by targeted mutations in the selectins themselves (Frenette *et al.*, 1996) or in the α1,3-fucosyltransferase responsible for synthesizing the selectin ligand (Maly *et al.*, 1996). Furthermore, these animals can also be used to isolate GalTase-deficient cell lines that thus far have been refractory to lectin-based selection procedures.

It is noteworthy that GalTase-deficient animals progress through embryogenesis apparently normally, although we

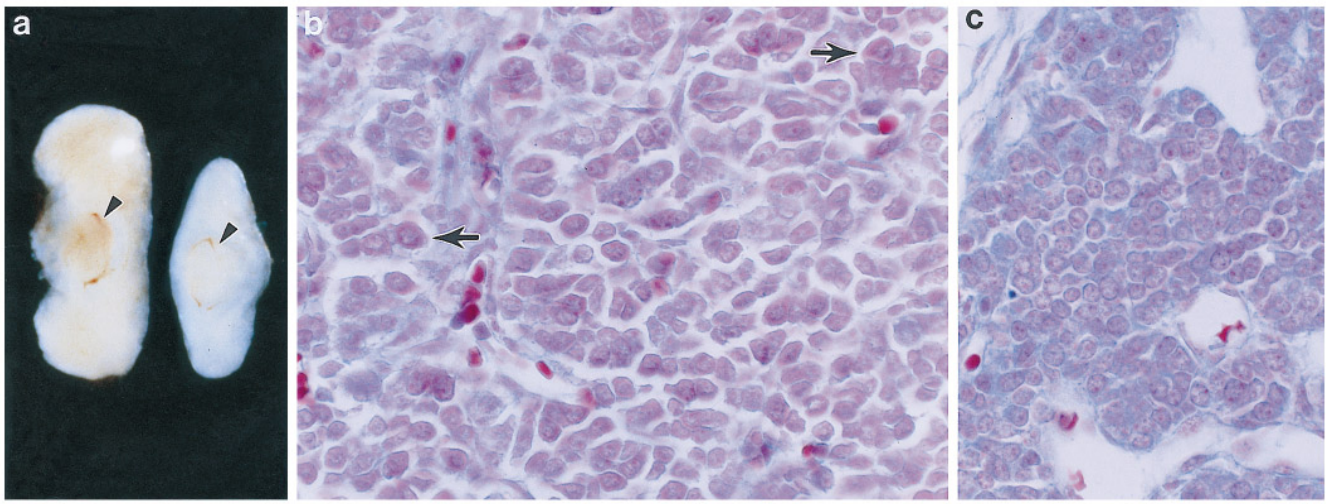

FIG. 7. (a) Views of isolated pituitary glands from wild-type (left) and *gt* −/− (right) mice. The *gt* −/− anterior pituitary is greatly reduced in size and is much thinner as evident by the transillumination through the tissue. In contrast, wild-type tissue is larger and thicker, with a fleshy appearance. The posterior lobes (arrowheads) are similar in size in both animals. (b, c) In newborn pups, the cells of the anterior pituitary appear richly acidophilic (arrows) in wild-type (b), whereas *gt* −/− cells are clumped together and nonsecretory (c). However, *gt* −/− glands acquire a normal secretory phenotype similar to that seen in (b) during neonatal development.

cannot account for all *gt* −/− animals at birth. Previous *in vitro* studies have suggested that GalTase is required for secondary aspects of morula compaction preceding implantation (Bayna *et al.*, 1988) as well as for morphogenesis of some tissue types (Hathaway and Shur, 1992). It is not necessarily surprising that *gt* −/− embryos progress normally through these events, since embryos develop normally for the first 10 days of gestation even in the complete absence of N-linked oligosaccharide chains (Metzler *et al.*, 1994; Ioffe and Stanley, 1994). Whether the absence of GalTase in *gt* −/− embryos leads to subtle morphogenetic defects awaits more direct analysis. In any event, 90% of the *gt* −/− mice die during the neonatal period and demonstrate a relatively specific defect in pituitary function, thus representing a new model for pituitary insufficiency and polyglandular endocrine disease. Perhaps more surprising, is that 10% of the *gt* −/− animals survive the neonatal period, suggesting a previously unrecognized compensatory mechanism may exist for pituitary hormone glycosylation and/or action. Whether this survival of *gt* −/− mice reflects the action of a second modifier gene is unknown; attempts to introduce the *gt* −/− mutation onto an isogenic inbreed background is in progress.

Finally, GalTase has been suggested to function as a sperm surface receptor for oligosaccharide ligands in the egg coat, thus facilitating sperm–egg binding (Miller and Shur, 1994). Some of the few *gt* −/− males that survive to young adulthood are fertile in that they impregnate females; however, *in vitro* assays show that sperm from *gt* −/− mice fail to bind their egg coat ligand or undergo an acrosome reaction consistent with GalTase functioning as a gamete receptor (Lu and Shur, in preparation).

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