The EMBO Journal Vol.16 No.8 pp.1850–1857, 1997

Growth retardation and early death of β-1,4-galactosyltransferase knockout mice with augmented proliferation and abnormal differentiation of epithelial cells

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Carbohydrate chains on a glycoprotein are important not only for protein conformation, transport and stability, but also for cell–cell and cell–matrix interactions. UDP-Gal:*N*-acetylglucosamine β-1,4-galactosyltransferase (GalT) (EC 2.4.1.38) is the enzyme which transfers galactose (Gal) to the terminal *N*-acetylglucosamine (GlcNAc) of complex-type *N*-glycans in the Golgi apparatus. In addition, it has also been suggested that this enzyme is involved directly in cell–cell interactions during fertilization and early embryogenesis through a subpopulation of this enzyme distributed on the cell surface. In this study, GalT-deficient mice were produced by gene targeting in order to examine the pathological effects of the deficiency. GalT-deficient mice were born normally and were fertile, but they exhibited growth retardation and semi-lethality. Epithelial cell proliferation of the skin and small intestine was enhanced, and cell differentiation in intestinal villi was abnormal. These observations suggest that GalT plays critical roles in the regulation of proliferation and differentiation of epithelial cells after birth, although this enzyme is dispensable during embryonic development.

Keywords: epithelial cell differentiation/epithelial cell growth/galactosyltransferase/glycosyltransferase/knockout mouse

Introduction

N-linked sugar chains are associated with most proteins found on the mammalian cell surface and in secretions. Oligosaccharides on secreted glycoproteins are shown to be important for their biological activities, transport, stability and clearance from the circulation, while oligosaccharides on cell surface glycoproteins are suggested to be involved in various cellular functions including cell–cell and cell–matrix interactions during embryogenesis, immune reactions and tumor development (reviewed in Iwakura, 1989; Hakomori, 1991; Furukawa and Kobata, 1992; Varki, 1993).

Previously, we showed that tunicamycin, an inhibitor of dolichophosphate synthesis, inhibits blastocyst formation of mouse embryos, suggesting that cell surface carbohydrates play an important role in early embryogenesis (Iwakura and Nozaki, 1985). Several lines of evidence suggest that galactose (Gal)-containing complex *N*-glycans are particularly important in these processes. For example, stage-specific embryonic antigen-1 (SSEA-1) (Solter and Knowles, 1978), which contains poly-*N*-acetyllactosamine structure and is expressed specifically on pre-implantation embryos and undifferentiated embryonal carcinoma cells, has been suggested to be involved in cellular interactions during morula compaction and implantation, since oligosaccharides containing this antigenic structure can competitively inhibit these processes (Fenderson *et al.*, 1984). The observation that treatment of early embryos with endo-β-galactosidase (Rastan *et al.*, 1985) or UDP-Gal (Shur *et al.*, 1979) inhibited compaction also suggests the importance of Gal-containing molecules in these processes.

The involvement of Gal-containing carbohydrates in other processes has also been suggested. Lower galactosylation of serum IgGs is characteristic of patients with rheumatoid arthritis, suggesting involvement in the pathogenesis of this disease (Parekh *et al.*, 1985; Rademacher *et al.*, 1988). Sialyl Le^x and sialyl Le^a, which contain NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAc and NeuAcα2→3Galβ1→3[Fucα1→4]GlcNAc, respectively, are suggested to be ligands for selectins which mediate interactions between leukocytes and vascular endothelial and platelet cells (reviewed in Lasky, 1992). Recently, galectins, which particularly recognize β-galactoside sugar chains, were shown to be important for immune recognition (Perillo *et al.*, 1995; Yang *et al.*, 1996). Furthermore, some Gal-containing carbohydrates such as Le^a, Le^b, Le^x, Le^y antigens and their derivatives were shown to be associated with tumors (reviewed in Hakomori, 1989). It was shown that the expression of these antigens and tumor progression were closely correlated, suggesting that aberrant glycosylation at the cell surface perturbs normal cell–cell and cell–matrix interactions which results in uncontrolled cell growth, invagination and metastasis of tumor cells (reviewed in Hakomori, 1991). However, so far, the pathological effects of the deficiency in Gal-containing carbohydrates on an animal and its embryonic development have not been examined.

UDP-Gal:*N*-acetylglucosamine β-1,4-galactosyltransferase (GalT) is the only enzyme which is known to transfer Gal from UDP-Gal to terminal *N*-acetylglucosamine (GlcNAc) of complex *N*-glycans to form a Galβ1→4GlcNAc structure in the Golgi apparatus. In the mammary gland, GalT is known to synthesize lactose by binding to α-lactalbumin (α-lac) which modifies the acceptor specificity of the transferase from GlcNAc to glucose (reviewed in Kuhn, 1983). In addition to a

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biosynthetic role for GalT, it was suggested that a subpopulation of this enzyme is present at the cell surface and is involved directly in cellular interactions by binding to terminal GlcNAc of glycoconjugates at the opposing cell surface and matrix (reviewed in Shur, 1993). In this context, it was suggested that this enzyme is involved directly in mouse gamete interaction (Miller *et al.*, 1992), neurite extension (Begovac and Shur, 1990), mesenchymal and neural crest cell migration (Runyan *et al.*, 1986) and late morula compaction (Bayna *et al.*, 1988).

In the present study, we generated mice deficient in the GalT gene by homologous recombination in order to examine the pathological effects of the deficiency *in vivo*. We found that homozygous GalT-deficient mice can be obtained at almost the expected ratio in crosses between heterozygous mice. However, approximately half of them died before weaning, with growth retardation, indicating the importance of GalT for survival after birth.

Results

Generation of GalT-deficient (GalT$^{-/-}$) mice

The targeting vector was constructed by replacing the GalT exon 1 with a PGKneobpA cassette (Soriano *et al.*, 1991), in order to disrupt both the long and short forms of the GalT gene (Shaper *et al.*, 1988; Hollis *et al.*, 1989). In this construct, the translation initiation sites for both forms and the Golgi retention signal (Nilsson *et al.*, 1991) in exon 1 were deleted (Figure 1A). The targeting vector was electroporated into embryonic stem (ES) cells and G418-resistant colonies were picked up. Twelve positive clones were obtained out of 180 Neo^r clones by PCR, and the homologous recombination was confirmed in nine clones by Southern blot analysis using an external 3′ probe (Figure 1B) and 5′ probe (data not shown). Chimeric mice were produced using three targeted ES cell clones and the targeted allele was transmitted through a germ line in two independent ES cell clones. GalT$^{-/-}$ mice were generated by inter-crossing of GalT$^{+/-}$ mice. The phenotypes described here were identical in the two mouse lines. GalT mRNA was not detected in GalT$^{-/-}$ mice, indicating disruption of this gene (Figure 1C).

GalT activity and N-linked carbohydrate chains in GalT$^{-/-}$ mice

When GalT activity was determined using the liver homogenates as an enzyme source and asialo (As)- and agalacto (Ag)-transferrin as an acceptor, the transferase activity in GalT$^{+/-}$ mice was just half of that in GalT$^{+/+}$ mice, while that in GalT$^{-/-}$ mice was <5% of that in GalT$^{+/+}$ mice (Figure 2A). However, it should be pointed out that this level in GalT$^{-/-}$ mice was significantly higher than in the control without enzyme. A similar result was obtained using the spleen homogenates as an enzyme source (data not shown). In order to verify the galactosyltransferase activity found in this assay, the galactosylated product isolated by Bio-Gel P-6 column chromatography was digested with diplococcal β-galactosidase which cleaves the Galβ1→4GlcNAc linkage but not the Galβ1→3GlcNAc linkage (Paulson *et al.*, 1978). When the digested material was analyzed by Bio-Gel P-4 chromatography, the radioactive product was eluted at the same position as galactose, indicating that Gal was transferred at the C-4 position of the GlcNAc of AsAg-transferrin (data not shown).

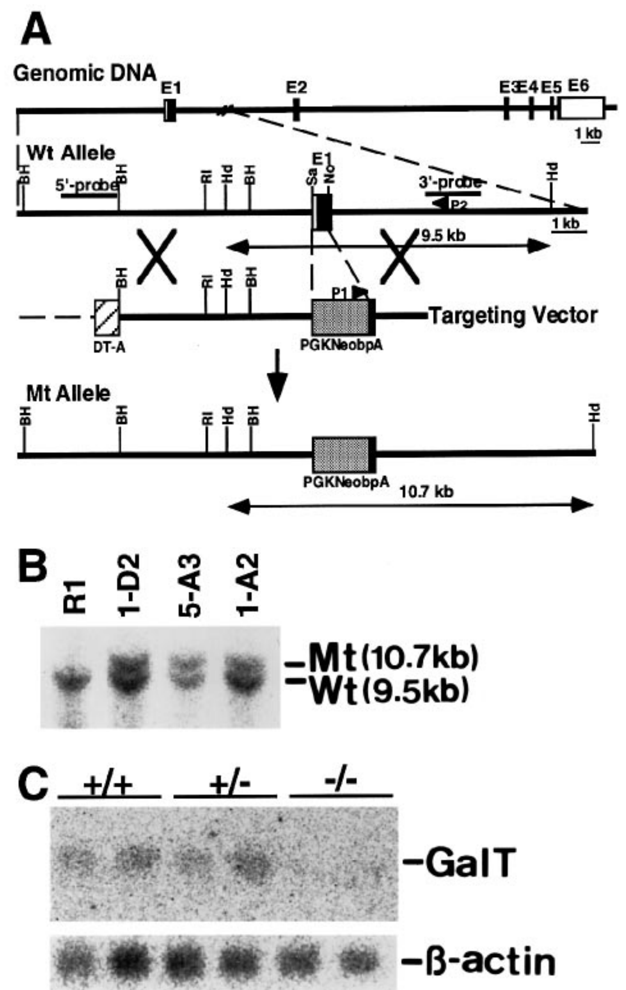

Fig. 1. Targeted disruption of the GalT gene by homologous recombination. (**A**) Targeting strategy. Coding and non-coding exons of the GalT gene are shown by closed and open boxes, and the PGKneobpA cassette and the diphtheria toxin A fragment gene cassette (DT-A) by dotted and hatched boxes, respectively. PCR primers (P1 and P2) used for screening are shown by arrowheads. 5′-Probe and 3′-probe show the positions of external probes used for Southern blot analysis and the expected *Hin*dIII fragments are indicated by arrows. BH, *Bam*HI; Hd, *Hin*dIII; RI, *Eco*RI; Sa, *Sac*II; No, *Not*I. (**B**) Southern blot analysis of ES cell clones. Genomic DNA (10 μg) from parent ES cells (R1) and targeted ES cell clones were digested with *Hin*dIII and hybridized with the 3′ probe. The expected DNA fragments for the mutant allele (Mt) and wild-type allele (Wt) are indicated. (**C**) Northern blot analysis of GalT$^{-/-}$ mice. Poly(A)$^+$ RNA (1 μg) from the liver was hybridized with exon 1 and 2 probes of the GalT gene, and rehybridized with the β-actin probe.

In order to determine whether or not the Galβ1→4GlcNAc structure is expressed in *N*-linked sugar chains of GalT$^{-/-}$ mouse glycoproteins, serum glycoproteins were analyzed by lectin blotting. When filters were stained with Coomassie brilliant blue (CBB), no significant difference was detected between the three genotypes (Figure 2B, CBB). When the filters were incubated with *Ricinus communis* agglutinin (RCA)-I, which binds to oligosaccharides which terminate with Galβ1→4GlcNAc/Glc groups (Baenziger and Fiete, 1979), RCA-I binding was observed for most protein bands in GalT$^{+/+}$ and GalT$^{+/-}$ mice,

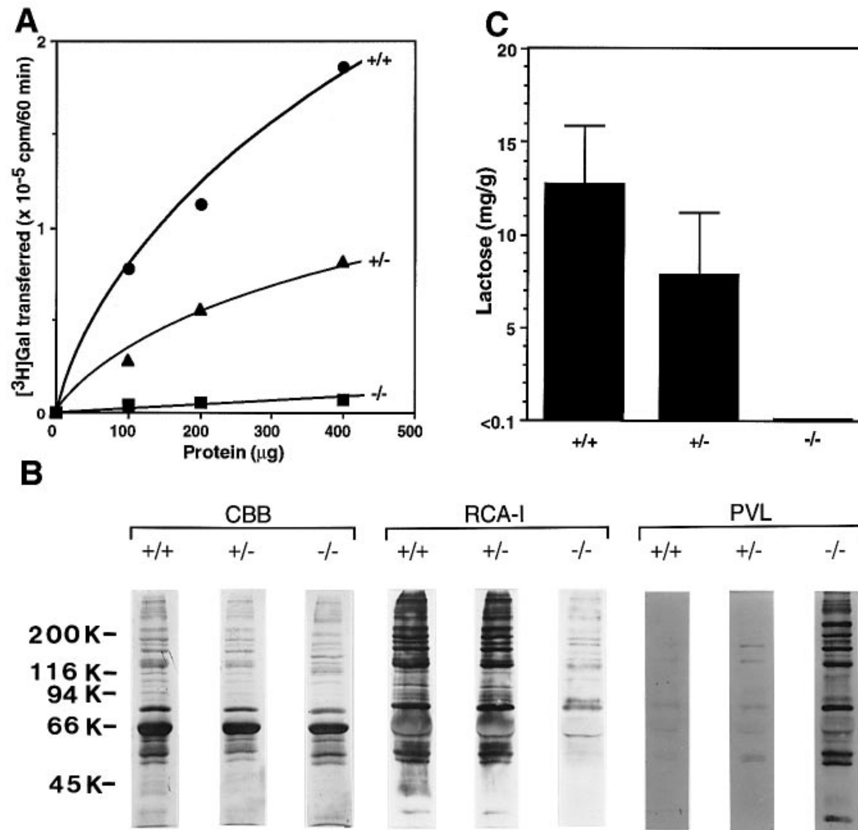

Fig. 2. (**A**) β-1,4-galactosyltransferase activity in liver homogenates from 10-week-old GalT$^{+/+}$ (●), GalT$^{+/–}$ (▲) and GalT$^{–/–}$ (■) mice. (**B**) Lectin blot analysis of serum glycoproteins from 10-week-old GalT$^{+/+}$ (+/+), GalT$^{+/–}$ (+/–) and GalT$^{–/–}$ (–/–) mice. The bars indicate the positions of molecular weight markers. CBB indicates filters stained with Coomassie brilliant blue to detect protein bands. RCA-I and PVL indicate filters incubated with RCA-I and PVL, respectively. (**C**) Lactose concentration in milk of five GalT$^{+/+}$ (+/+), five GalT$^{+/–}$ (+/–) and two GalT$^{–/–}$ (–/–) mice. The values indicate averages with standard deviations.

while no binding was observed in a GalT$^{–/–}$ mouse, with the exception of a few weak bands (Figure 2B, RCA-I). RCA-I binding was completely lost upon digestion of glycoprotein samples with diplococcal β-galactosidase or *N*-glycanase prior to the analysis (data not shown), showing that these bands actually represented *N*-glycans containing β-linked Gal. In contrast, when filters were incubated with *Psathyrella velutina* lectin (PVL), which binds to oligosaccharides which terminate with β-linked GlcNAc (Endo *et al*., 1992), PVL did not bind to most protein bands in GalT$^{+/+}$ and GalT$^{+/–}$ mice, but did so in a GalT$^{–/–}$ mouse (Figure 2B, PVL). No PVL binding was observed upon digestion of glycoprotein samples with jack bean β-*N*-acetylhexosaminidase or *N*-glycanase prior to the analysis (data not shown), showing that these bands actually represented *N*-glycans containing β-linked GlcNAc. These results indicate that *N*-linked sugar chains of most serum glycoproteins from GalT$^{–/–}$ mice are not galactosylated and terminated with β-GlcNAc, although a few protein bands were RCA-I positive and contained β-1,4-linked Gal in their *N*-linked sugar chains. In order to rule out the possibility that the RCA-I-positive glycoproteins were produced by bacteria in the gut, we analyzed proteins from intestinal bacteria in GalT$^{–/–}$ mice and found that these bacteria have no RCA-I-reactive glycoproteins upon lectin binding (data not shown). In addition, no change has been detected in the RCA-I-reactive glycoprotein pattern on lectin blotting after treatment of these mice with antibiotics (data not shown).

No lactose was detected in GalT$^{–/–}$ mouse milk, while its concentration in the milk of GalT$^{+/–}$ mice was about half as much as that of wild-type mice (Figure 2C). These results confirm that GalT is a component of lactose synthetase (Kuhn, 1983). As a result, these GalT$^{–/–}$ mothers could not rear their offspring (data not shown).

Semi-lethality of GalT$^{–/–}$ mice

GalT$^{–/–}$ mice were born healthy at almost the expected ratio of 20% in heterozygous mating, suggesting normal embryonic development, although GalT$^{–/–}$ mice were slightly smaller than GalT$^{+/–}$ and GalT$^{+/+}$ mice at birth (body weight of GalT$^{–/–}$ mice was 80–90% of that of control littermates at birth). Most of them, however, developed severe skin lesions within a few days after birth (Figure 3A). Horny cells continued to come off from the flaky skin for ~2 weeks, and the skin lesions eventually healed. GalT$^{–/–}$ mice also showed growth retardation and nearly half of them died before 4 weeks of age (Figure 3B). The body weight of GalT$^{–/–}$ survivors was only 60–70% of that of GalT$^{+/+}$ mice at 4 weeks old, while GalT$^{+/–}$ mice were normal. After weaning, GalT$^{–/–}$ mice gained weight normally, but they still continued to die, and only 20% of them survived beyond 16 weeks (Figure 3B). Both mature female and male GalT$^{–/–}$ mice were fertile (Table I). Sperm from GalT$^{–/–}$ males was able to fertilize. Although GalT$^{–/–}$ females appeared to be able to ovulate, and maintain pregnancy to term, the litter size

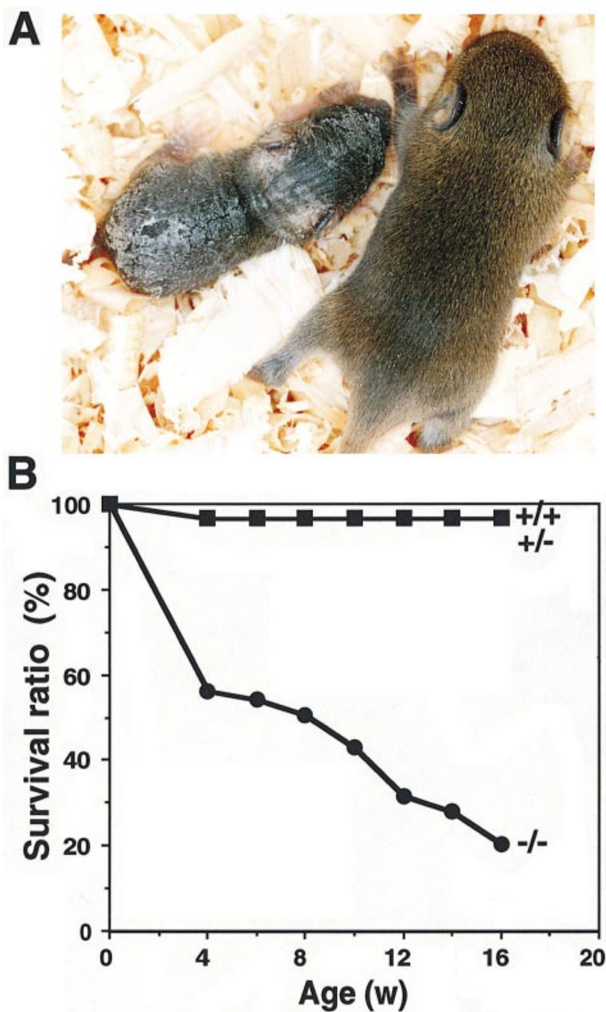

Fig. 3. Semi-lethality in GalT$^{-/-}$ mice. (**A**) A 10-day-old GalT$^{-/-}$ pup (left) with a control littermate (right). Note that the GalT$^{-/-}$ pup is smaller than the normal littermate and the hair is lost from the back with the presence of flaky skin. (**B**) Survival ratio of GalT$^{-/-}$ mice after birth. The genotype ratios at birth in heterozygous mating were 20% (–/–), 49% (+/–) and 31% (+/+) from 59 pups analyzed. The number of mice at birth is taken as 100% and the ratios of survivors in 30 mice are shown.

Table I. Litter sizes of –/–×+/– matings

Female×male	Litter size (No. of litters examined)
–/–×+/–	5.1 ± 2.1[a] (24)
+/–×–/–	6.2 ± 2.3[b] (51)
+/+×+/+	7.2 ± 2.6 (53)

[a]$P<0.001$.
[b]$P<0.05$.

was slightly smaller compared with that of the wild-type control (Table I).

Enhanced proliferation of epithelial cells in the skin and small intestine

Histological examination revealed that the epidermis was thickened in the skin lesions of GalT$^{-/-}$ mice (Figure 4A and B). At higher magnification, hyperplasia of the prickle cell layer and granular layer was observed, and the horny layer was also thickened in GalT$^{-/-}$ mice (Figure 4C and D). Many keratohyalin granules were deposited not only in the granular layer but also in the prickle cell layer in GalT$^{-/-}$ mice (Figure 4C and D). These histological observations indicate that acanthosis and hyperkeratosis occurred in the skin of GalT$^{-/-}$ mice. This result suggests that proliferation of epithelial stem cells located in the basal layer of the epidermis is augmented in GalT$^{-/-}$ mice.

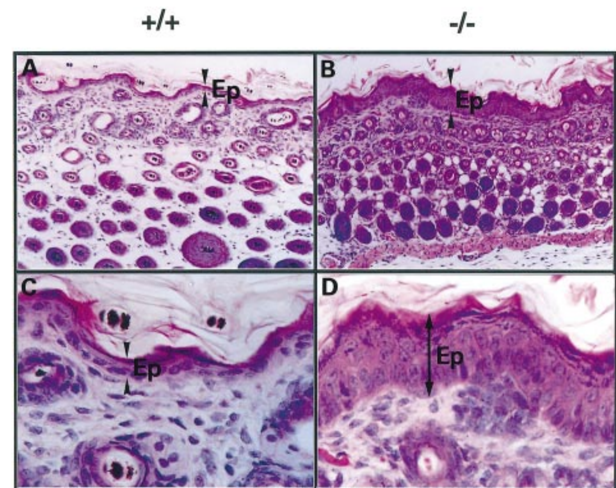

Fig. 4. Histological analysis (HE staining) of the skin of 10-day-old pups. (**A** and **C**) A GalT$^{+/+}$ mouse; (**B** and **D**) a GalT$^{-/-}$ mouse. Note that the prickle cell layer and granular layer are much thicker in the GalT$^{-/-}$ mouse than in the normal mouse. Ep: epidermis. Original magnification: ×100 (A and B), ×400 (C and D).

In the small intestine, the crypts of GalT$^{-/-}$ mice were found to be enlarged 3–4 times compared with those of wild-type (Figure 5A and B), indicating that proliferation of epithelial stem cells located in the crypt is also enhanced in GalT$^{-/-}$ mice. RCA-I staining showed that Galβ1→4GlcNAc-containing sugar chains were not expressed in the crypts and villi of GalT$^{-/-}$ mice, while the surface of crypt and villus cells was stained strongly with RCA-I in GalT$^{+/-}$ mice (Figure 5C and D). The number of metaphase cells was counted after vincristine injection to confirm the augmented proliferation of epithelial cells. The numbers of metaphase cells per crypt per 90 min were 21.7 ± 5.6 (–/–) versus 5.5 ± 1.4 (+/–) in the duodenum and 6.9 ± 1.8 (–/–) versus 2.0 ± 1.1 (+/–) in the ileum, indicating that the mitotic indices of the small intestine crypt cells were increased ~4 times in GalT$^{-/-}$ mice as compared with those of GalT$^{+/-}$ mice. These results suggest that the Galβ1→4GlcNAc structure may be involved in growth control of epithelial cells in the small intestine and skin. However, these abnormalities were less prominent in adult GalT$^{-/-}$ mice.

Abnormal differentiation of small intestinal villus cells

Interestingly, differentiation of small intestine villus cells was also found to be abnormal in GalT$^{-/-}$ mice using disaccharidases as markers. Although lactase was expressed strongly over the entire surface of villus epithelial cells of 7-day-old GalT$^{+/-}$ mice (Figure 6A), lactase localization was restricted to the top of the villi in GalT$^{-/-}$

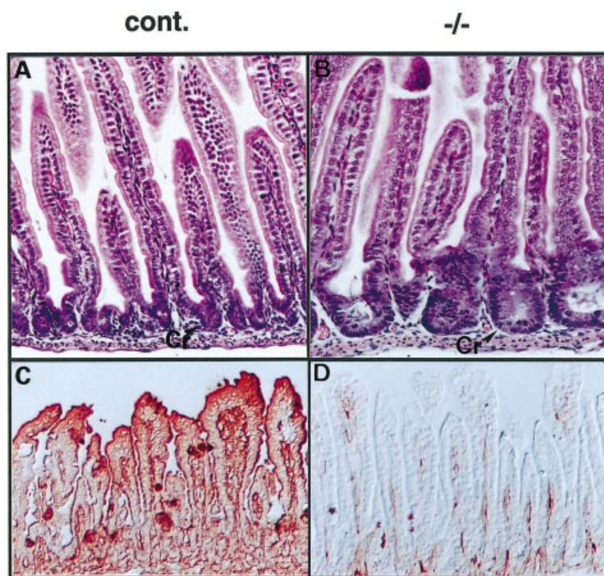

Fig. 5. Histological analysis of the small intestine of pups. (A and B) HE staining; (C and D) RCA-I staining. (**A**) A 10-day-old $GalT^{+/+}$ mouse; (**B**) a 10-day-old $GalT^{-/-}$ mouse; (**C**) a 7-day-old $GalT^{+/-}$ mouse; (**D**) a 7-day-old $GalT^{-/-}$ mouse. Note that the crypt size is much larger in the $GalT^{-/-}$ mouse than in the control mouse and RCA-I-reactive sugar chains are not expressed in crypt and villus cells of the $GalT^{-/-}$ mouse. Cr: crypt. Original magnification: ×400 (A and B), ×200 (C and D).

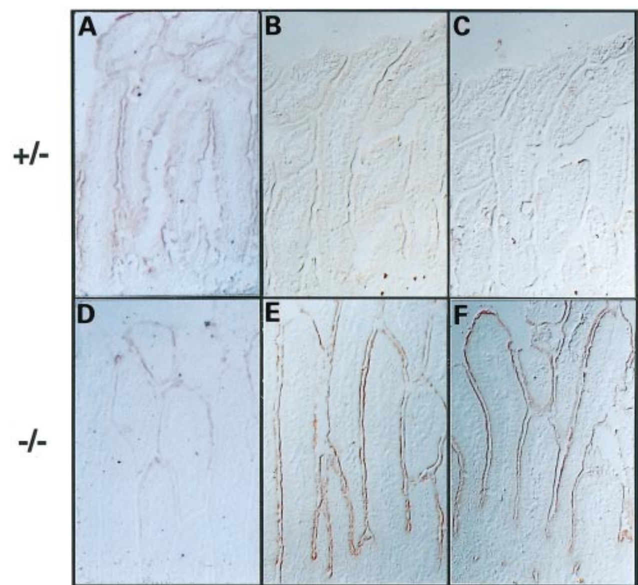

Fig. 6. Analysis of small intestine disaccharidases. Immunohistochemical localization of lactase (**A** and **D**), maltase (**B** and **E**) and the sucrase–isomaltase complex (**C** and **F**) in the duodenum of $GalT^{+/-}$ (A, B and C) and $GalT^{-/-}$ (D, E and F) mice. Note that the expression of lactase is much weaker in the $GalT^{-/-}$ mouse compared with the normal mouse, whereas maltase and sucrase expression is stronger in the $GalT^{-/-}$ mouse. Original magnification: ×100.

mice (Figure 6D). Additionally, maltase and the sucrase–isomaltase complex appeared precociously in $GalT^{-/-}$ mice (Figure 6E and F). These enzymes had not yet been expressed on day 7 in $GalT^{+/-}$ mice (Figure 6B and C). Since no RCA-I binding was detected in villus cells of $GalT^{-/-}$ mice (Figure 5D), it is suggested that the lack of Galβ1→4GlcNAc structure is responsible for the abnormal differentiation of villus cells. Consistent with the immunohistochemical findings, lactase activity in $GalT^{-/-}$ small intestine homogenates was markedly low, while maltase and isomaltase activities were higher in $GalT^{-/-}$ mice compared with $GalT^{+/-}$ mice (Table II). Sucrase activity was barely detectable in both types of mice at day 7, and was still not detected in $GalT^{-/-}$ mice by day 16, in contrast to $GalT^{+/-}$ mice in which the activity was detected at day 16 (data not shown).

Table II. Disaccharidase activities (nmol/mg protein/min) in 7-day-old mice

	+/–	–/–
Lactase	2.80, 2.27	0.86, 0.78
Maltase	1.89, 1.17	9.62, 5.56
Isomaltase	0.00, 0.00	0.35, 0.76

Discussion

In this study, we have generated GalT-deficient mice and shown that >95% of the galactosyltransferase activity was inactivated and that most Galβ1→4GlcNAc residues on glycoprotein carbohydrate chains were absent in these mice. The origin of the low residual galactosyltransferase activity is not clear. Since the translation initiation sites and Golgi retention signal were deleted in the targeting construct, the galactosyltransferase activity of the GalT gene is considered to be lost. It is unlikely that GalT activity and these Gal-containing glycoproteins are derived from colostrum because they were detected in $GalT^{-/-}$ mice even at 10 weeks of age. One possibility is that this residual activity could derive from previously unknown galactosyltransferases encoded by genes distinct from GalT. In this context, the presence of another β-1,4-galactosyltransferase in porcine trachea was suggested previously (Sheares and Carlson, 1984). However, we cannot rule out the possibilities that galactosyltransferases, which are involved in glycolipid or glycosaminoglycan synthesis, or other linkage specificity, might be responsible for this activity. We are now trying to discriminate between these possibilities.

Homozygous GalT-deficient mice were obtained at almost the expected ratio from crosses between heterozygous mice, suggesting that the development of $GalT^{-/-}$ embryos is normal. This is in clear contrast to *N*-acetylglucosaminyltransferase (GlcNAcT)-I knockout mice which die on day 10.5 of embryonic development due to defects in neural tube formation and vascularization (Ioffe and Stanley, 1994; Metzler *et al.*, 1994). Furthermore, we found that both male and female $GalT^{-/-}$ mice were fertile. The litter size from homozygous mothers, however, was slightly smaller compared with normal mice, suggesting a possible deficiency in uterus function. These observations suggest that neither the carbohydrate structures containing Gal nor GalT itself are absolutely necessary for cell–cell interactions during fertilization and embryonic development, although previous studies suggested the involvement of Gal-containing oligosaccharides and/or GalT in these processes (Eggens *et al.*, 1989; Shur, 1993). However,

besides GalT, two other proteins, sp56 (Cheng *et al.*, 1994) and a 95 kDa peptide (Leyton *et al.*, 1992), are known as zona-binding proteins during fertilization, and E-cadhelin/uvomorulin is also suggested to play an important role in morula compaction (Vestweber and Kemler, 1985). Thus, it is possible that these molecules might have substituted for the function of GalT in these processes. It is also possible that embryonic Gal-containing carbohydrates such as SSEA-1 are synthesized by enzymes other than GalT or that enzymes which are usually not involved in the synthesis of this structure substitute for GalT and allow development of sperm and embryos. We are now examining these possibilities.

GalT-deficient mice showed semi-lethality after birth, and almost 80% of them died before 4 months of age. Thus, it was shown that GalT plays essential roles in the growth and survival of young mice.

Interestingly, epithelial cells of the skin and small intestine of GalT-deficient mice showed marked enhancement of proliferation. Moreover, the age dependency of the disaccharidase expression pattern in intestinal epithelial cells was abnormal, suggesting abnormal differentiation. Thus, it was suggested that the Galβ1→4GlcNAc structure is important for the control of growth and differentiation of epithelial cells. In agreement with this notion, Metzler *et al.* (1994) reported that proliferation of neural epithelial cells was enhanced in GlcNAcT-I-deficient mice in which the expression of Gal on *N*-linked sugar chains is perturbed due to the lack of an acceptor GlcNAc for GalT.

The abnormalities of the intestine, especially the lack of lactase in suckling mice, may cause malnutrition of infant mice and result in their growth retardation and early death, although these mice did not show obvious diarrhea which might be expected in lactase deficiency. Analysis of nutritional absorption efficiency as well as feeding of these mice with nutrients other than lactose will clarify this point. However, the cause of semi-lethality of adult $GalT^{-/-}$ mice is not clear because these abnormalities were not prominent in the adult animals.

The mechanism for the growth augmentation of epithelial cells is not known at present, although several possibilities are conceivable. One possibility is that the deficiency in *N*-linked sugar chains might cause activation of epithelial cell growth factors or their receptors due to conformational changes. It is also possible that the Galβ1→4GlcNAc group in *N*-linked sugar chains of a cell surface has suppressive effects on growth and differentiation of opposing cells through receptors that recognize terminal Gal residues. In this context, β-linked terminal Gal in *N*-linked sugar chains of a cell surface glycoprotein, contactinhibin, is suggested to be involved in the contact-dependent inhibition of human lung fibroblast cell growth (Wieser *et al.*, 1991).

Another possible explanation is that GalT by itself participates in the regulation of cell growth. The cell surface GalT has been shown recently to deliver a growth inhibitory signal using cell lines which overexpress either the intact or truncated GalT gene (Hilton *et al.*, 1995). In addition, involvement of this enzyme in cell growth signal transduction was suggested by its direct association with the epidermal growth factor receptor (Hilton *et al.*, 1995) and CDC2-related kinase (Bunnell *et al.*, 1990). In any case, our results indicate the importance of GalT in the regulation of cellular growth and differentiation.

Lactose was not detected in the milk of $GalT^{-/-}$ mothers as was also the case in the milk of α-lac$^{-/-}$ mothers (Stinnakre *et al.*, 1994; Stacey *et al.*, 1995). This is because GalT is an essential component of lactose synthetase together with α-lactalbumin (Kuhn, 1983). The milk of α-lac$^{-/-}$ mothers has been reported to be too viscous to be sucked by pups because lactose regulates the osmotic pressure and volume of the milk (Stinnakre *et al.*, 1994; Stacey *et al.*, 1995). This is probably the reason why $GalT^{-/-}$ mothers could not rear their offspring.

No apparent arthritis developed in $GalT^{-/-}$ mice, suggesting that galactose deficiency *per se* is not arthritogenic. However, it is possible that the life span of the mutant mice is too short to develop arthritis. Detailed analyses of the carbohydrate chains of IgG, joint pathology and autoantibody production are necessary to evaluate this issue fully.

Materials and methods

Targeting vector construction

The GalT gene isolated from a 129/SvJ genomic library (Stratagene) was used to construct the targeting vector. The PGKneobpA cassette (Soriano *et al.*, 1991), in which the neomycin resistance gene was ligated under the phosphoglycerate kinase I promoter and the polyadenylation site from bovine growth hormone was ligated downstream of the *neo* gene, was inserted between the *Sac*II and *Not*I sites in GalT exon 1 for positive selection. The DT-A cassette (Yagi *et al.*, 1993), in which the diphtheria toxin A fragment gene was ligated under the MC1 promoter, was ligated at the 5′ end of the targeting vector for negative selection. Most of exon 1 (420 bp from *Sac*II to *Not*I) was deleted, and homologous regions at the 5′ and 3′ ends were of 5.7 and 1.7 kb, respectively.

Generation of $GalT^{-/-}$ mice

The linearized targeting vector (20 μg) was electroporated (250 V, 500 μF) into 10^7 R1 ES cells (Nagy *et al.*, 1993) and selected with 180 μg (active form)/ml G418 (Gibco/BRL) for 7–10 days. Homologous recombinants were screened by PCR and confirmed by Southern blot hybridization. The forward primer (P1) in the PGKneobpA cassette was CTCTATGGCTTCTGAGGCGGAAAG and the reverse primer (P2) outside the targeting vector was CACAGTCCCTCATATTTCAGCAGG. PCR was carried out for 40 cycles at 94°C for 1 min, 60°C for 2 min and 72°C for 3 min in a volume of 50 μl containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM P1 and P2 primers and 2.5 U of *Taq* DNA polymerase. Chimeric mice were generated by the aggregation method (Nagy *et al.*, 1993) with some modifications. Chimeras were mated with C57BL/6J females, and homozygous mutant mice were generated by inter-crossing of heterozygotes. Genotypes were determined by tail DNA dot-blot hybridization using the GalT gene exon 1 as a wild-type allele-specific probe and the *neo* gene as a mutant allele-specific probe. Mice were kept under specific pathogen-free conditions in an environmentally controlled clean room at the Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo. The experiments were conducted according to institutional ethical guidelines for animal experiments and safety guidelines for gene manipulation experiments.

Southern and Northern blot analysis

Genomic DNA prepared from ES cells was digested with restriction enzymes, electrophoresed through a 0.7% agarose gel and transferred to nylon membranes (Gene Screen Plus, NEN). Total RNA was prepared from the liver by the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987) and poly$(A)^+$ RNA was purified using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech). Poly$(A)^+$ RNA was electrophoresed through a 0.8% denatured agarose gel and transferred to nylon membranes. Hybridization was carried out according to standard methods (Sambrook *et al.*, 1989) using ^{32}P-labeled DNA probes made by Multiprimed DNA labeling system (Amersham).

β-1,4-galactosyltransferase assay

The transferase assay was conducted in a final volume of 50 μl containing 20 mM MES buffer (pH 6.5), 100 μM UDP-[^{3}H]Gal (323 μCi/mmol), 0.05% NP-40, 3 mM 5′-AMP, 5 mM 2,3-dimercapto-1-propanol, 10 mM $MnCl_2$ with 100–400 μg of liver homogenate proteins as an enzyme source and 250 μg of human AsAg-transferrin as an acceptor which was described previously (Furukawa *et al.*, 1990). After the mixtures were incubated at 37°C for 60 min, the galactosylated product was separated by paper electrophoresis in the presence of borate, and radioactivity was determined using a liquid scintillation counter.

Measurement of lactose concentration in milk

Milk samples were collected from oxytocin-injected (0.2 U/kg weight) mothers between days 3 and 6 of lactation. The lactose concentration was determined using a lactose/D-galactose analysis kit (Boehringer Mannheim) according to the manufacturer's instructions.

Lectin blot analysis

Mouse serum and homogenized tissues were defatted with acetone and then with chloroform and methanol mixtures (2:1 and 1:2, v/v) twice for each. The defatted protein samples were lyophilized, subjected to SDS–polyacrylamide gel (7.5%) electrophoresis and transferred to nitrocellulose filters. Each lane contained ~10 μg of protein. Since most Gal residues of serum glycoproteins are sialylated, the filters initially were treated with *A.ureafaciens* sialidase prior to the analysis. Lectin blot analysis using RCA-I or PVL was performed as described previously (Sato *et al.*, 1993).

Histological analysis

Tissues were fixed in 4% formaldehyde, dehydrated and embedded in paraffin according to the standard procedure. Sections of 6 μm were made and stained with hematoxylin–eosin (HE).

Measurement of mitotic indices

Ten-day-old pups were injected with vincristine (1 mg/kg weight) intraperitoneally and tissues were collected after 90 min. Samples were fixed with Carnoy's solution and stained with Shiff's solution (Okada *et al.*, 1994). The number of cells in metaphase was counted in 20 crypts per sample.

Immunohistochemistry and lectin staining

Immunohistochemistry was performed as described previously (Matsumoto *et al.*, 1992). In brief, cryo-sections were fixed with acetone and then incubated with a rabbit polyclonal antibody against lactase (Goda *et al.*, 1984), sucrase–isomaltase complex or maltase (Umesaki *et al.*, 1982) for 90 min at room temperature. Finally, sections were visualized with 3-amino-9-ethylcarbazole. For RCA-I staining, fixed cryo-sections were reacted with biotinated RCA-I and visualized with streptavidin–peroxidase.

Assay of disaccharidase activities

The entire small intestine of 7-day-old mice was homogenized in ice-cold phosphate-buffered saline by using a Potter–Elvejhem homogenizer and the disaccharidase activities were measured as described previously (Dahlqvist, 1964). The enzyme activities of each of two mice are shown.

Acknowledgements

The authors thank Drs S.Mori and T.Kuroki for their valuable comments, and S.Kakuta and N.Uetani for their help in the experiments. We also thank Drs A.Nagy, R.Nagy and W.Abramow-Newerly for R1 cells, Dr T.Yagi for the DT-A cassette, Dr T.Goda for anti-lactase antibody, and all the members of Laboratory Animal Research Center for excellent animal care. This work was supported by a grant from the Ministry of Education, Science, Sports and Culture of Japan.

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Received on October 29, 1996; revised on December 31, 1996