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Functional analysis of *Drosophila* β1,4-N-acetlygalactosaminyltransferases

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Members of the mammalian β1,4-galactosyltransferase family are among the best studied glycosyltransferases, but the requirements for all members of this family within an animal have not previously been determined. Here, we describe analysis of two *Drosophila* genes, *β4GalNAcTA* (*CG8536*) and *β4GalNAcTB* (*CG14517*), that are homologous to mammalian β1,4-galactosyltransferases. Like their mammalian homologs, these glycosyltransferases use N-acetylglucosamine as an acceptor substrate. However, they transfer N-acetylgalactosamine rather than galactose. This activity, together with amino acid sequence similarity, places them among a group of recently identified invertebrate β1,4-N-acetylgalactosaminyltransferases. To investigate the biological functions of these genes, null mutations were generated by imprecise excision of a transposable element (*β4GalNAcTA*) or by gene-targeted homologous recombination (*β4GalNAcTB*). Flies mutant for *β4GalNAcTA* are viable and fertile but display behavioral phenotypes suggestive of essential roles for GalNAc-β1,4-GlcNAc containing glycoconjugates in neuronal and/or muscular function. *β4GalNAcTB* mutants are viable and display no evident morphological or behavioral phenotypes. Flies doubly mutant for both genes display only the behavioral phenotypes associated with mutation of *β4GalNAcTA*. Thus *Drosophila* homologs of the mammalian β4GalT family are essential for neuromuscular physiology or development but are not otherwise required for viability, fertility, or external morphology.

Key words: β1,4-galactosyltransferase/behavior/development/*CG8531*/Notch

Introduction

Whole genome sequencing of *Drosophila* has revealed many predicted proteins with sequence similarity to mammalian glycosyltransferases (Adams *et al*., 2000). Recently, a number of insights into the requirements for particular glycan structures have come out of genetic studies in *Drosophila*, including identification of the roles of *O*-fucose glycans in Notch signaling (reviewed in Haines and Irvine, 2003; Haltiwanger and Stanley, 2002), appreciation of the diverse roles of heparin sulfate proteoglycans in growth factor signaling (reviewed in Nybakken and Perrimon, 2002) and identification of a role for glycolipids in Notch signaling (Schwientek *et al*., 2002, Wandall *et al*., 2003). Here we describe studies of the *Drosophila* homologs of one of the best studied families of mammalian glycosyltransferases, the β1,4-galactosyltransferases.

Seven β1,4-galactosyltransferases have been identified in mammals (Amado *et al*., 1999; Furukawa and Sato, 1999; Lo *et al*., 1998). These enzymes all share highly homologous sequence motifs within their catalytic domains and use UDP-Gal as their sugar donor. Six members of the family, β4GalT-1 to β4GalT-6 (β4GalTs), catalyze the transfer of Gal to acceptor substrates with a terminal N-acetylglucosamine (GlcNAc), to generate Galβ1,4-GlcNAc (LacNAc) (Amado *et al*., 1999; Guo *et al*., 2001). The seventh member, β4GalT-7, is the furthest diverged based on sequence similarity and has a distinct enzymatic activity, because it uses xylose rather than GlcNAc as an acceptor. β4GalT-7 participates in the synthesis of the glycosaminoglycan core linker on proteoglycans (Okajima *et al*., 1999; Quentin *et al*., 1990), and structural and functional homologs of β4GalT-7 have been identified in both *Drosophila* and *Caenorhabditis elegans* (Bulik *et al*., 2000; Herman *et al*., 1999; Nakamura *et al*., 2002; Takemae *et al*., 2003; Vadaie *et al*., 2002).

The LacNAc structure generated by β4GalTs is a common feature of mammalian glycoproteins and glycolipids (Amado *et al*., 1999; Varki *et al*., 1999). Studies in cultured mammalian cells identified a role for this linkage in the modulation of Notch signaling by the N-acetylglucosaminyltransferase Fringe (Chen *et al*., 2001). Terminal LacNAc moieties have been implicated in antibody function (Axford, 1999) and clearance of serum glycoproteins (Tozawa *et al*., 2001). LacNAc is also a major substrate for sialyltransferases (Varki *et al*., 1999), and thus is essential for the generation of a diverse array of sialylated glycans. Although these observations suggest a range of potential functions for β4GalTs, a comprehensive understanding of their requirements has remained elusive due to the potential for redundancy among the six mammalian family members. To date, only β4GalT-1 has been analyzed genetically (Asano *et al*., 1997; Lu *et al*., 1997). Gene-targeted mutations in β4GalT-1 are viable and do not exhibit the defects in Notch signaling observed in mammalian *Lunatic fringe* mutants (Evrard *et al*., 1998; Zhang and Gridley, 1998). They do exhibit some degree of neonatal lethality, have proliferation and differentiation defects in epithelial cells, and have reduced male fertility. However, the effect on male fertility is thought to reflect a role for a cell surface isoform of β4GalT-1 as a lectin-like receptor, rather its action as glycosyltransferase (Nixon *et al*., 2001; Rodeheffer and Shur, 2004).

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Two genes encoding proteins with sequence similarity to mammalian β4GalTs are encoded by the *Drosophila* and *C. elegans* genomes. The enzymatic activity of only one of these has been reported previously, the *C. elegans* enzyme encoded by Y73E7A.7 (Ce β4GalNAcT). By contrast to the β1,4GalT activity of mammalian enzymes, this *C. elegans* enzyme transfers GalNAc to GlcNAc in a β1,4 linkage, to create GalNAcβ1,4-GlcNAc (LacdiNAc) (Kawar *et al.*, 2002). A β4GalT homolog from a lepidopteran, the cabbage looper *Trichoplusia ni*, has also been characterized recently. This enzyme also efficiently transfers GalNAc to GlcNAc acceptors in a β1,4 linkage, and has only very low galactosyltransferase activity (Vadaie and Jarvis, 2004). Low levels of β4GalT activity have been reported in lepidopteran cell lines (Palomares *et al.*, 2003; Van Die *et al.*, 1996), but it is not known if the LacNAc linkage is actually present in insects. However, LacdiNAc has been identified on invertebrate glycoconjugates (Koles *et al.*, 2004; Kubelka *et al.*, 1995; Seppo *et al.*, 2000; Van Die *et al.*, 1997). These observations raise the possibility that LacdiNAc could subsume requirements for LacNAc that are conserved between invertebrates and vertebrates. Two glycosyltransferases that are predicted to act prior to the synthesis of a LacdiNAc linkage in *Drosophila* glycolipids (Seppo *et al.*, 2000) are encoded by *egghead* (*egh*) and *brainiac* (*brn*) (Schwientek *et al.*, 2002; Wandall *et al.*, 2003), mutation of which results in maternal effect neurogenic phenotypes and embryonic lethality (Goode *et al.*, 1996a,b). Mutations in *C. elegans* homologs of *egh* and *brn* (*bre-3* and *bre-5*), as well as Ce β4GalNAcT (*bre-4*), have also been identified (Griffitts *et al.*, 2001, 2003). No developmental defects have been described in these mutants, but they appear to act together to make a glycoconjugate that acts as the receptor for Crystal toxin (Griffitts *et al.*, 2003).

We report the characterization of the two *Drosophila* homologs of the mammalian β4GalTs. Like invertebrate homologs in *C. elegans* and *T. ni*, these enzymes function as N-acetylgalactosaminyltransferases. Mutation of one homolog results in behavioral phenotypes in adult flies, thus identifying an essential role for LacdiNAc-containing glycoconjugates in *Drosophila* neuronal development or physiology. However, mutation of the second *Drosophila* family member does not result in any discernible phenotypes. This lack of morphological or developmental phenotypes is not due to functional redundancy between the homologs, as doubly mutant flies exhibit only the adult behavioral phenotypes of the first mutant. The limited requirements for this gene family in *Drosophila* should facilitate future efforts to characterize the influence of LacdiNAc glycoconjugates in neuromuscular function.

Results

CG8536 and CG14517 encode Drosophila *homologs of the mammalian β4GalT family*

Sequence similarity searches identify only three predicted *Drosophila* proteins with any similarity (Blastp E values < 1) to mammalian β4GalTs. One of these is closely related to mammalian β4GalT-7 and has already been demonstrated to act as a functional homolog (Nakamura *et al.*, 2002; Takemae *et al.*, 2003; Vadaie *et al.*, 2002). The other two, encoded by *CG8536* and *CG14517*, are much more closely related to mammalian β4GalT-1 through 6 than to β4GalT-7. Amino acid sequence alignments confirm that these two proteins include all of the motifs conserved among known vertebrate and invertebrate members of this family, including pairs of conserved cysteines and the PFRXR, FNRA, DXD, FGGVSA, and (W/F)GWGGEDDD motifs (Amado *et al.*, 1999; Van Die *et al.*, 1997) (Figure 1A). Phylogenetic analysis indicates that CG8536 is most closely related to the β4GalNAcTs of *T. ni* and *C. elegans*, whereas CG14517, together with a predicted gene in the mosquito genome, appears to form a distinct branch (Figure 1B).

The structural basis for conservation of motifs among β4GalTs is now well understood, based on crystal structures of bovine β4GalT-1 (Gastinel *et al.*, 1999; Ramakrishnan *et al.*, 2002). Notably, site-specific mutagenesis has identified a tyrosine or phenylalanine residue that lies in the donor binding pocket and is critical for recognition of the Gal moiety of UDP-Gal. Mutagenesis of this to isoleucine switches the donor substrate preference of β4GalT-1 to UDP-GalNAc, whereas mutagenesis to leucine results in an enzyme that uses UDP-Gal or UDP-GalNAc with equal efficiency (Ramakrishnan and Qasba, 2002). Interestingly, isoleucine is encoded at this position in CG8536 and in other biochemically characterized invertebrate GalNAcTs, and leucine is encoded at this position in CG14517 (Figure 1A).

Biochemical characterization of CG8536 and CG14517 proteins

To characterize the enzymatic activity of the proteins encoded by *CG8536* and *CG14517*, full-length cDNAs were cloned into vectors for expression in cultured *Drosophila* S2 cells. To facilitate visualization and purification, sequences encoding V5 epitope and hexahistidine tags were included at the 3′ end. Analysis of cells transiently transfected with a *CG8536* expression vector by western blotting revealed that a protein with a mobility of ~ 51 kDa, slightly larger than the calculated mobility of 49 kDa, was detected in the cell lysate (Figure 2A). A slightly smaller band (~ 47 kDa) was detected in the culture media, indicating that CG8536 can be cleaved and secreted from cells (Figure 2A). Hydropathy analysis of CG8536 protein (not shown) predicts the presence of an amino-terminal type II transmembrane domain or signal sequence. Although most glycosyltransferases that include amino-terminal type II transmembrane domains are resident Golgi proteins, there are also by now a number of examples in which a proteolytic cleavage can occur near the transmembrane domain, allowing a soluble, catalytically active enzyme to be secreted (Varki *et al.*, 1999). This appears to be the case for CG8536, but glycosylation is nonetheless expected to be restricted to the Golgi by the limited availability of nucleotide sugar donors.

Because CG8536 protein was found in significant quantities in the medium of transfected cells, conditioned medium was used as a source of enzyme for biochemical characterization. Because conditioned medium from S2 cells has a high endogenous galactosyltransferase activity on GlcNAc acceptors (not shown), epitope-tagged CG8536 was purified from conditioned media using anti-V5 beads (Figure 3D).

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A

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DmB4GalNAcTA  131  LLANCTDPDPRDGGPITPNTTLESLDVIEAELG--PLLRPGGAFEPENCNAQHHVAIVVPFRDRYAHLLLF
DmB4GalNAcTB  61   QE-VFECTYSEIIAENRFVYHLAHYHDAVQRGA---EIRPGGEFRPEGCQARYSTAIIVPYRQREEQLHAF
Ag317181      148  MRNGLRCDYSEVIEENLSLYRPPYTEDIINN-----NVRLGGEYYPEDCLPSFSTAIIVPYRQRERQLNQF
Ag318033      1    ----CPPIPPNLDGPITVDVAFESLSAVESRFA--DKLQPGGQYVPPDCTARNRVAIIVPYRDREKHLPIF
TnB4GalNAcT   151  VTLPLCDSMPPDLGPITLNKTELELEWVEKKFP---EVEWGGRYSPPNCTARHRVAIIVPYRDRQQHLAIF
CeB4GalNAcT   99   TLQFCNQTPPHLVGPIRVFLDEPDFKTLEKIYP---DTHAGGHGMPKDCVARHRVAIIVPYRDREAHLRIM
HsB4GalT-1    136  SLPACPEESPLLVGPMLIEFNMPVDLELVAKQN--PNVKMGGRYAPRDCVSPHKVAIIIPFRNRQEHLKYW
HsB4GalT-2    93   TLPPCPDSPPGLVGRLLIEFTSPMPLERVQREN--PGVLMGGRYTPPDCTPAQTVAVIIPFRHREHHLRYW
HsB4GalT-3    73   GLPYCPERSPLLVGPVSVSFSPVPSLAEIVERN--PRVEPGGRYRPAGCEPRSRTAIIVPHRAREHHLRLL
HsB4GalT-4    73   ELDNCPSVSPYLRGQSKLIFKPDLTLEEVQAEN--PKVSRG-RYRPQECKALQRVAILVPHRNREKHLMYL
HsB4GalT-5    110  ANHTCPERLPSMKGPIDINMSEIGMDYIHELFSKDPTIKLGGHWKPSDCMPRWKVAILIPFRNRHEHLPVL
HsB4GalT-6    104  PYLPCPEKLPYMRGFLNVNVSEVSFDEIHQLFSKDLDIEPGGHWRPKDCKPRWKVAVLIPFRNRHEHLPIF

DmB4GalNAcTA  200  LRNIHPFLMKQRIAYRIFIVEQTNGKPFNRAAMMNIGYLEALKLY--QWDCFIFHDVDLLPLDDRNLYNCP
DmB4GalNAcTB  128  LTYMHNYLPQQLIHYRIFLVEQFDHKPFNRAMLFNIGAQVAAEYG---FPCLILHDVDLLPLNSGQIYACS
Ag317181      214  LIYMHNYLRRQRIHYRIFVIEQYDPKPFNRAKLFNIGAMIAIGFD---YPCLVLHDVDLMPMNLGHLYACS
Ag318033      66   LKNIHALLMKQQLEYGIYIVEQTAGSSFNRAALMNIGFVEAMKQK--NWECMVFHDIDLLPMDDRNLYTCP
TnB4GalNAcT   219  LNHMHPFLMKQQIEYGIFIVEQEGNKDFNRAKLMNVGFVESQKLVAEGWQCFVFHDIDLLPLDTRNLYSCP
CeB4GalNAcT   167  LHNLHSLLAKQQLDYAIFIVEQVANQTFNRGKLMNVGYDVASRLYP--WQCFIFHDVDLLPEDDRNLYTCP
HsB4GalT-1    95   LYYLHPVLQRQQLDYGIYVINQAGDTIFNRAKLLNVGFQEALKD--YDYTCFVFSDVDLIPMNDHNAYRCF
HsB4GalT-2    162  LHYLHPILRRQRLRYGVYVINQHGEDTFNRAKLLNVGFLEALKED-AAYDCFIFSDVDLVPMDDRNLYRCG
HsB4GalT-3    142  LYHLHPFLQRQQLAYGIYVIHQAGNGTFNRAKLLNVGVREALRD--EEWDCLFLHDVDLLPENDHNLYVCD
HsB4GalT-4    141  LEHLHPFLQRQQLDYGIYVIHQAEGKKFNRAKLLNVGYLEALKE--ENWDCFIFHDVDLVPENDFNLYKCE
HsB4GalT-5    281  FRHLLPMLQRQRLQFAFYVVEQVGTQPFNRAMLFNVGFQEAMKD--LDWDCLIFHDVDHIPESDRNYYGCG
HsB4GalT-6    175  FLHLIPMLQKQRLEFAFYVVEQTGTQPFNRAMLFNVGFKEAMKDD-SVWDCVIFHDVDHLPENDRNYYGCG

DmB4GalNAcTA  269  RQ-PRHMSVAIDTLNFRLPYRSIFGGVSAMTREHFQAVNGFSNSFFGWGGEDDDMSNRLKHANLFISRYPV
DmB4GalNAcTB  196  ER-PRHMSSALDHWRFRLPYRGLFGGVVAINTAQYQQINGMSNLYYGWGGEDDDLYERLQALNIDICRFAM
Ag317181      282  RK-PRHMCSSLDEFRYNLPYRGLFGGAVAIESSVYLNVNGMSNMFSGWGGEDDDLYGRLQNKQIEICRFSP
Ag318033      135  DQ-PRHMSVAVDTFGFKLPYSTIFGGVSAMTEKQFRMVNGFSNAFWGWGGEDDDMSNRLKHVGFHIARYPV
TnB4GalNAcT   290  RQ-PRHMSASIDKLHFKLPYEDIFGGVSAMTLEQFTRVNGFSNKYWGWGGEDDDMSYRLKKINYHIARYKM
CeB4GalNAcT   236  IQ-PRHMSVAIDKFNYKLPYSAIFGGISALTKDHLKKINGFSNDFWGWGGEDDDLATRTSMAGLKVSRYPT
HsB4GalT-1    264  SQ-PRHISVAMDKFGFSLPYVQYFGGVSALSKQQFLTINGFPNNYWGWGGEDDDIFNRLVFRGMSISRPNA
HsB4GalT-2    132  DQ-PRHFAIAMDKFGFRLPYAGYFGGVSGLSKAQFLRINGFPNEYWGWGGEDDDIFNRISLTGMKISRPDI
HsB4GalT-3    211  PRGPRHVAVAMNKFGYSLPYPQYFGGVSALTPDQYLKMNGFPNEYWGWGGEDDDIATRVRLAGMKISRPPT
HsB4GalT-4    210  EH-PKHLVVGRNSTGYRLRYSGYFGGVTALSREQFFKVNGFSNNYWGWGGEDDDLRLRVELQRMKISRPLP
HsB4GalT-5    250  QM-PRHFATKLDKYMYLLPYTEFFGGVSGLTVEQFRKINGFPNAFWGWGGEDDDLWNRVQNAGYSVSRPEG
HsB4GalT-6    244  EH-PRHFAAKLDKYMYILPYKEFFGGVSGLTVEQFRKINGFPNAFWGWGGEDDDLWNRVHYAGYNVTRPEG

DmB4GalNAcTA  338  NIARYKMLKHQKEKANPK-----RYENLQNGMSKIEQDGINSIKYSIYSIKQFPTFTWYLAELKNSERKS
DmB4GalNAcTB  266  EFSKYTMLKHKQEQPNAN-----RVALLRSATLRQHSDGLNSLVYTEMERRMHSLFTHILVDT-------
Ag317181      352  TYSQYSMLKHRKETPNKD-----RVAFLRNGKQRYHTDGLNSLVYKQVGFKLHNLFTHVLVET-------
Ag318033      205  NIARYTMLSHKKEKANPK-----RYEKLVNGAKRFDSDGLNSLHYQLVNLIRKPLYTWIHADISPD----
TnB4GalNAcT   360  SIARYAMLDHKKSTPNPK-----RYQLLSQTSKTFQKDGLSTLEYELVQVVQYHLYTHILVNIDERS---
CeB4GalNAcT   306  QIARYKMIKHSTEATNPVN--KCRYKIMGQTKRRWTRDGLSNLKYKLVNLELKPLYTRAVVDLLEKDCRR
HsB4GalT-1    334  VVGRCRMIRHSRDKKNEPN--PQRFDRIAHTKETMLSDGLNSLTYQVLDVQRYPLYTQITVDIGTPS---
HsB4GalT-2    302  RIGRYRMIKHDRDKHNEPN--PQRFTKIQNTKLTMKRDGIGSVRYQVLEVSRQPLFTNITVDIGRPPSWP
HsB4GalT-3    282  SVGHYKMVKHRGDKGNEEN--PHRFDLLVRTQNSWTQDGMNSLTYQLLARELGPLYTNITADIGTDPRGP
HsB4GalT-4    280  EVGKYTMVFHTRDKGNEVN--AERMKLLHQVSRVWRTDGLSSCSYKLVSVEHNPLYINITVDFWFGA---
HsB4GalT-5    320  DTGKYKSIPHHHRGEVQFLG---RYALLRKSKERQGLDGLNNLNYFAN-ITYDALYKNITVNLTPELAQV
HsB4GalT-6    314  DLGKYKSIPHHHRGEVQFLG---RYKLLRYSKERQYIDGLNNLIYRPK-ILVDRLYTNISVNLMPELAPI
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B

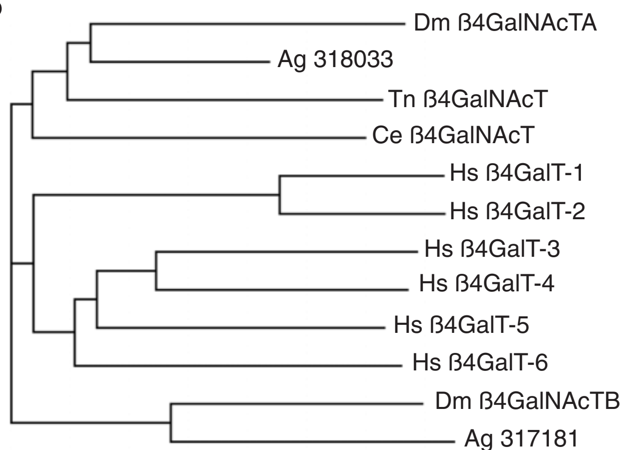

Fig. 1. Similarity of invertebrate β4GalNAcTs to mammalian β4GalTs. (**A**) The predicted amino acid sequences of β4GalNAcTs and β4GalTs from *Drosophila melanogaster* (Dm), *Anopheles gambiae* (Ag), *Trichoplusia ni* (Tn), *Caenorhabditis elegans* (Ce), and *Homo sapiens* (Hs) aligned by ClustalW. The two uncharacterized predicted proteins from *A. gambiae* are referred to by their GenBank reference numbers (Ag318033 = XP_318033, Ag317181 = XP_317181). Black boxes identify amino acids identical among at least six of the proteins, gray boxes identify similar amino acids among at least six of the proteins. Lines above demarcate the conserved sequence motifs identified in the text, the vertical arrow identifies an amino acid in the donor binding pocket that can influence recognition of Gal versus GalNAc, the bent arrow identifies the predicted first potential start codon in β*4GalNAcTA*$^{4.1}$ and *b4GalNAcTA*$^{7.1}$ mutations, and the asterisk marks the last amino acid in β*4GalNAcTB* mutations. (**B**) A phylogenetic tree, based on the full-length protein coding sequences of the proteins listed.

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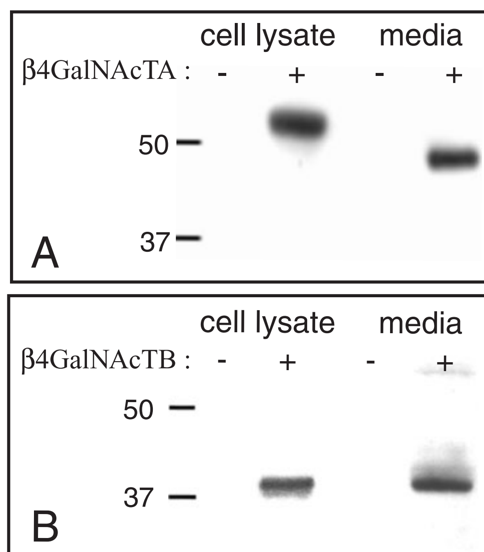

Fig. 2. Expression of β4GalNAcTA and β4GalNAcTB in *Drosophila* S2 cells. Western blots of cell lysate and conditioned medium from S2 cells transfected with (**A**) pMTWB- *β4GalNAcTA*:V5:His (+) or control (vector alone, –) or (**B**) pMTWB-*β4GalNAcTB*:V5:His (+), or control (–) expression plasmids, probed with anti-V5 antibody. Because *β4GalNAcTB* is not secreted as well, the conditioned medium in (**B**) was concentrated ~ 10-fold for this experiment.

CG8536 protein-coated beads were then used as an enzyme source in assays with a simple β-linked GlcNAc acceptor substrate, pNP-β-D-GlcNAc, which is an effective substrate for previously characterized mammalian β4GalTs and invertebrate β4GalNAcTs. Robust glycosyltransferase activity was detected using UDP-GalNAc as a donor (Figure 3B). A low signal was detected using UDP-Gal as a donor, however, it was not significantly different from that detected in control experiments using beads loaded with green fluorescent protein (GFP), or without a pNP-β-D-GlcNAc acceptor, indicating that CG8536 has little or no Gal transferase activity (Figure 3A). No activity was detected with a UDP-GlcNAc donor (Figure 3C). Thus, like the recently described *T. ni* β4GalNAcT (Vadaie and Jarvis, 2004), to which CG8536 is closely related, CG8536 protein acts as a GalNAc transferase. Based on this activity and the sequence similarity, we will henceforth refer to CG8536 protein as β4GalNAcTA.

Analysis of cells transiently transfected with a *CG14517* expression vector by western blotting revealed that a protein of the expected mobility, ~ 38 kDa, was detected in the cell lysate (Figure 2B). A band of similar mobility was detected in conditioned medium, but it appears to be secreted less efficiently than β4GalNAcTA (not shown). Using the same assays as for β4GalNAcTA, a low level of GalNAc transferase activity could be detected using anti-V5 beads loaded with CG14517 protein from conditioned medium (Figure 3F). Attempts to detect more substantial activity of CG14517 using protein isolated from cell lysates, protein efficiently secreted into the medium with a BiP signal peptide, or protein without a C-terminal V5 tag all proved negative, and no activity could be detected in assays using UDP-Gal or UDP-GlcNAc donors, nor with pNP-xylose, pNP-galactose, or pNP-glucose acceptors (Figure 3E, G, and data not shown). Our inability to detect more substantial levels of glycosyltransferase activity associated with CG14517 likely results from a requirement for an additional factor (Hans Bakker personal communication), which might be limiting in S2 cells. This GalNAcT activity, together with the sequence similarity to members of the β4GalT/β4GalNAcT family, suggest that CG14517 protein is a β4GalNAcT, and we henceforth refer to it as β4GalNAcTB.

β4GalNAcTA and β4GalNAcTB are broadly expressed

To assess the potential for β4GalNAcTA and β4GalNAcTB to contribute to the development of different tissues, their expression was analyzed by *in situ* hybridization to mRNA. Both genes are expressed ubiquitously, although at relatively low levels, throughout embryonic and larval development, as well as in adult tissues (Figure 4). Their expression is first detected in very early embryos, prior to the onset of zygotic transcription, implying that maternally expressed transcripts are deposited into the egg (Figure 4A, I). Consistent with this, expression is detected in the nurse cells during oogenesis (Figure 4M, S). In addition to low-level ubiquitous expression, *β4GalNAcTA* is strongly expressed in the garland cells during embryonic stages, beginning around stage 12 (Figures 4B–D). The garland cells are thought to function as nephrocytes, taking up waste materials from the hemolymph by endocytosis, and are known for their high levels of exocytosis and endocytosis (Kosaka and Ikeda, 1983; Wigglesworth, 1972).

Generation of null mutations in β4GalNAcTA

To identify biological functions for *Drosophila* β4GalNAcTs, genetic studies were initiated. A P transposable element insertion in the 5′ untranslated region of *β4GalNAcTA*, *EP2551*, was identified by the Berkeley *Drosophila* Genome Project (Rorth *et al.*, 1998; BDGP unpublished data). Thus we took advantage of the fact that mobilization of P elements often results in deletion of flanking DNA (imprecise excisions). Subsequent to introduction of transposase, flies were first screened for excision of the P insertion by loss of the *white*$^{+}$ marker gene, and then screened for imprecise excisions by Southern blotting using a *β4GalNAcTA* cDNA as a probe. Five candidate mutants were identified and then further analyzed by polymerase chain reaction (PCR) and DNA sequencing. Two lines, *β4GalNAcTA*$^{4.1}$ and *β4GalNAcTA*$^{7.1}$ have deletions that lie exclusively within the *β4GalNAcTA* transcription unit (Figure 5). The deletions in *β4GalNAcTA*$^{4.1}$ and *β4GalNAcTA*$^{7.1}$ are 610 and 568 bases, respectively (see supplementary material), and are predicted to delete the first 143 and 129 amino acids, respectively, of *β4GalNAcTA*. However, the first in-frame ATG that could then serve as a translation start site in either deletion mutant encodes amino acid 208 (Figure 1A). Such a truncated protein would lack the conserved PFRXR motif (Figure 1A), which makes critical contacts with substrates (Gastinel *et al.*, 1999), and hence would be expected to behave as a null allele. Another line, *Df(2R)β4GalNAcTA[20.1]* is a 4.4-kb deletion that removes the entire *β4GalNAcTA* transcription unit as well as the neighboring gene, *CG8531*, and hence

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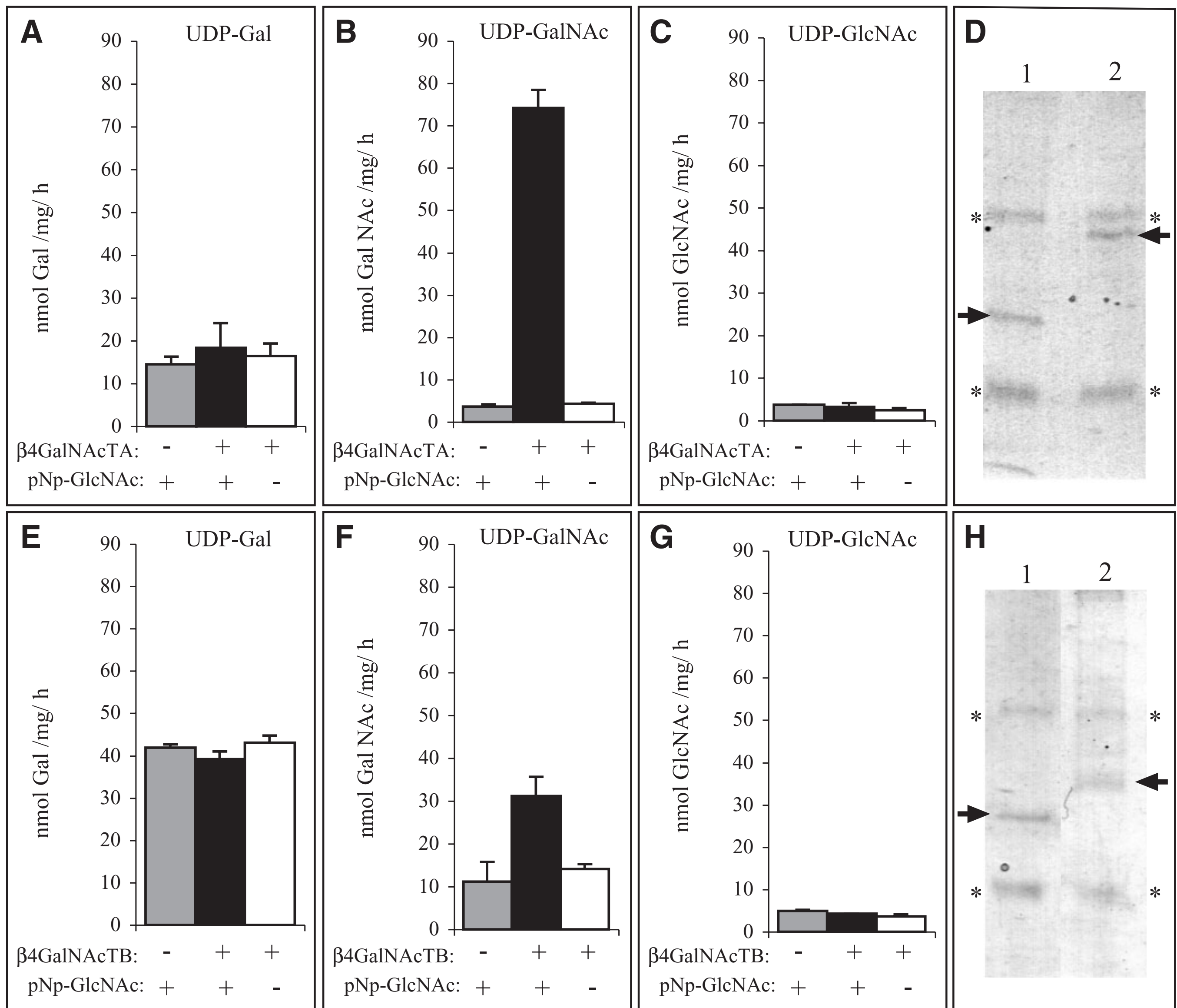

Fig. 3. Glycoslytransferase activity of β4GalNAcTA and β4GalNAcTB. V5-tagged β4GalNAcTA (**A**–**C**), β4GalNAcTB (**E**–**G**), or GFP (–controls) were isolated from conditioned media on anti-V5 agarose beads and assayed for glycosyltransferase activity using a pNP-GlcNAc acceptor and (**A**, **E**) UDP-Gal donor (**B**, **F**) UDP-GalNAc donor or (**C**, **G**) UDP-GlcNAc donor. The higher background in **E**–**G** than in **A**–**C** reflects the fact that less protein was on the beads, and the results are presented normalized to the amount of protein; that is, the background is largely proportional to the amount of beads. (**D**, **H**) Show total protein (Coomassie blue) stains of beads loaded with purified proteins that were used in the assays. Arrows point to bands of the expected mobility, asterisks mark bands corresponding to anti-V5 IgG heavy and light chains. Lane 1 in each case shows a GFP:V5 control, lane 2 shows β4GalNAcTA (**D**) or β4GalNAcTB (**H**).

constitutes a true null allele for both genes (Figure 5, S1). The two remaining imprecise excision lines were determined to be larger deletions that removed additional genes on either side of *β4GalNAcTA* and were not analyzed further.

β4GalNAcTA mutants display adult behavioral phenotypes

Animals homozygous for each of the three deletion alleles of *β4GalNAcTA*, as well as transheterozygous combinations between alleles, were examined for mutant phenotypes. In all cases adult flies with normal external morphology could be recovered. Most allelic combinations are also both male and female fertile. *Df(2R)β4GalNAcTA[20.1]* homozygous males are sterile. However, because this sterility is not observed in homozygous or transheterozygous combinations of other alleles, it most likely results from the deletion of the adjacent gene, *CG8531* (Figure 5A). This gene is expressed in testis (Andrews *et al.*, 2000), and encodes a protein with a DNAJ domain, suggesting that it might function as a chaperone (Walsh *et al.*, 2004).

Although mutant flies are viable and appear morphologically normal, they are sluggish and uncoordinated and exhibit occasional tremors. The adult flies are also short-lived, but this might result simply from their tendency to

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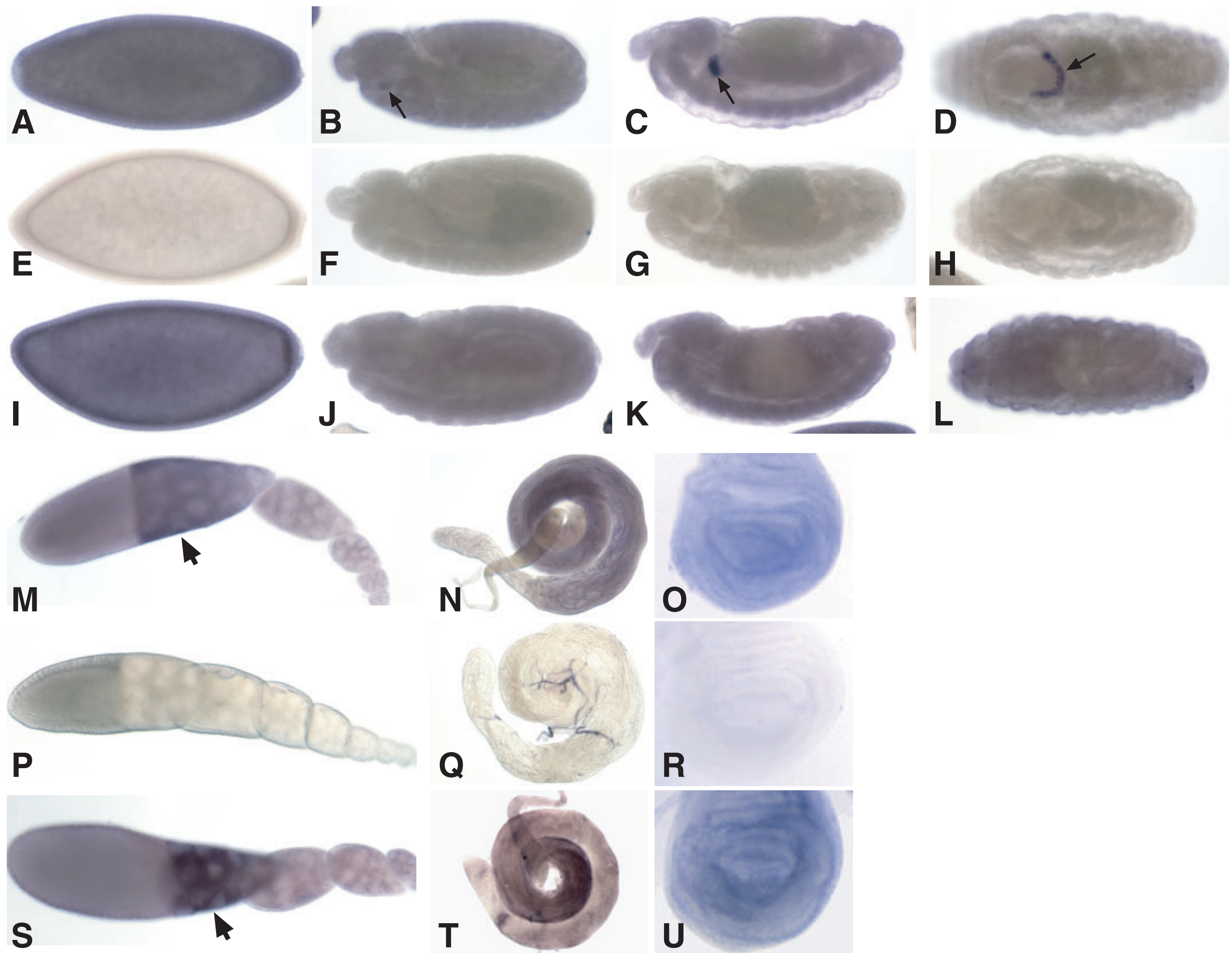

Fig. 4. Expression of β*4GalNAcTA* and β*4GalNAcTB*. *In situ* hybridization to *Drosophila* tissues using RNA probes. (**A**–**D**, **M**–**O**) Tissues stained for expression of β*4GalNAcTA*; **E**–**H**, **P**–**R**) tissues stained with sense strand control probes; and (I-L, S-U) show embryos stained for expression of β*4GalNAcTB.* Embryos are shown at stage 5 (**A**, **E**, **I**), stage 12 (**B**, **F**, **J**), stage 13–14 (**C**, **G**, **K**), stage 15–16 (**D**, **H**, **L**). **D**, **H**, and **L** are horizontal views, the remaining are embryos are sagital views. **M**, **P**, **S** are ovarioles, **N**, **Q**, **T** are testes, and **O**, **R**, **U** are wing imaginal discs. In general expression is ubiquitous, but arrows in **B**–**D** point to elevated expression in Garland cells, and arrows in **M** and **S** point to nurse cells within the most mature follicle.

become immobilized in the fly media. To further characterize this adult phenotype, β*4GalNAcTA* mutants were examined in simple behavioral assays (Figure 6) (Richards *et al.*,1996). A test of locomotion examines the ability of flies to climb up the sides of a vial. *Drosophila* are negatively geotropic, and if tapped to the bottom of an empty vial, wild-type flies will rapidly climb up to the top. However, mutant flies are significantly more sluggish ($p < 0.05$ using Student unpaired *t*-test) and often fail to climb at all (Figure 6A). In a test of coordination, a mechanical shock was administered to a fly in an empty vial, which sometimes results in the fly being knocked onto its back. The number of falls and the time taken for a fly to right itself were analyzed. Mutant flies are knocked on their backs as often as wild type (Figure 6B) but take significantly longer to right themselves after a fall ($p < 0.05$ using Student unpaired *t*-test) (Figure 6C).

To confirm that these behavioral phenotypes are due specifically to mutation of β*4GalNAcTA*, a construct that includes the wild-type β*4GalNAcTA* genomic region was cloned into a P element vector and transformed into *Drosophila* (Figure 5). This genomic construct rescues the behavioral phenotypes of β*4GalNAcTA* mutants, as assayed both by visual inspection and by the locomotion and coordination assays (Figure 6). Thus the behavioral deficits can be ascribed specifically to mutation of β*4GalNAcTA*, and by inference, to the absence of one or more glycoconjugates that it synthesizes.

Generation of null mutations in β4GalNAcTB

Because no P element insertions near β*4GalNAcTB* have been isolated, we instead employed gene-targeted homologous recombination to create a mutation in this gene (Gong

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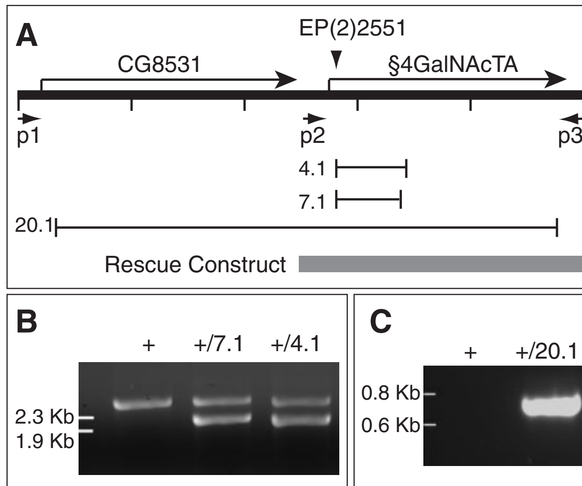

Fig. 5. β*4GalNAcTA* genomic region. (**A**) The *CG8531* and β*4GalNAcTA* transcription units are indicated by horizontal arrows, vertical slashes mark 1-kb intervals, the *EP*(*2*)*2551* insertion by an arrowhead, the extent of deletions in β*4GalNAcTA* mutations by bars, and the genomic rescue construct by the gray line. PCR primers P1-P3 are indicated by small arrows. (**B**) Agarose gels showing bands amplified by PCR from wild-type (+), β$4GalNAcTA^{4.1}$/wild-type heterozygote, and β$4GalNAcTA^{7.1}$/wild-type heterozygote genomic DNA using primers P2 and P3. The smaller bands from the 4.1 and 7.1 deletion alleles are visible. (**C**) Bands amplified using primers P1 and P3 from wild-type and β$4GalNAcTA^{20.1}$/wild-type heterozygote DNA. No bands are visible from the wild-type chromosome, because the region is too large (5.5 kb) to be amplified under these conditions, but a 700-bp band specific to this deletion allele is visible. Mutant bands were isolated and sequenced to identify the endpoints of the deletions (see supplementary material).

and Golic, 2003). An ends-out targeting construct was designed that would create a null allele by deleting 200 bp in the middle of the transcription unit, removing the FGGVSA and (W/F)GWGGEDDD motifs, and inserting a copy of the *white*$^+$ gene in their place (Figures 1, 7). These motifs play essential roles in interactions with substrates and catalysis (Gastinel *et al.*, 1999; Ramakrishnan *et al.*, 2002), and hence the resulting mutations could not encode functional enzymes. Nine independent insertions of the targeting donor construct to the third chromosome were recovered. Eight of these were identified as correctly targeted events to β*4GalNAcTB* by PCR and Southern blot analysis (Figure 7 and data not shown).

Each of the eight gene-targeted alleles of β*4GalNAcTB* was tested for mutant phenotypes. In all cases, homozygous mutant adult flies could be recovered that are fertile, appear morphologically normal, and do not display evident behavioral phenotypes.

Drosophila *homologs of mammalian β4GalTs are dispensable for development*

Because β*4GalNAcTA* and β*4GalNAcTB* encode structurally related proteins that can both catalyze the formation of a GalNAc-GlcNAc linkage and are expressed in largely overlapping patterns, we considered the possibility that they might be functionally redundant. Flies stocks mutant for both genes were generated by crossing two different β*GalNacTA* alleles, β$GalNacTA^{4.1}$ and β$GalNacTA^{20.1}$ to two independent isolates of flies with gene-targeted mutations

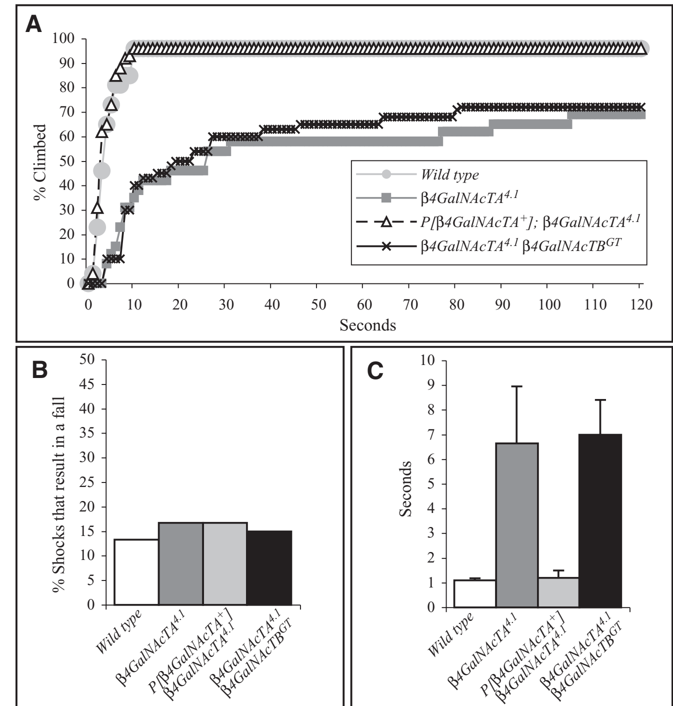

Fig. 6. Behavioral phenotypes of β*4GalNAcT* mutants. (**A**) Climbing assay. Flies of the indicated genotypes were scored for their ability to climb 5 cm up a vial. Chart shows percentage of flies reaching 5 cm at each second of the assay. (**B**) Mechanical agitation assay one. Flies of each genotype were scored for their tendency to fall over in response to mechanical shock of the vial. (**C**) Mechanical agitation assay two. Flies of each genotype were scored for their ability to right themselves after falling on their back in response to mechanical agitation. Error bars are SEM.

in β*4GalNAcTB* (β$4GalNAcTB^{GT}$). Animals homozygous for mutations in both genes could be readily identified using standard genetic markers (see *Materials and methods*), and in addition their identity was confirmed by PCR analysis (data not shown). Except for the male sterile phenotype associated with *Df*(*2R*)β*4GalNAcTA*[*20.1*] males, all allelic combinations were viable, fertile, and morphologically normal. The double-mutant flies do display the behavioral phenotypes associated with β*4GalNAcTA* mutants, but the severity of the phenotypes is similar to that of β*4GalNAcTA* single mutants (Figure 6). These observations suggest that β*4GalNAcTA* andβ*4GalNAcTB* are not functionally redundant and indicate that this gene family is not required for the development or fertility of *Drosophila*.

Discussion

β4GalNAcTA and β4GalNAcTB are members of the invertebrate β4GalNAcT family

β4GalNAcTA and β4GalNAcTB proteins show significant similarity to enzymes of the mammalian β4GalT family, and include all of the motifs common to β4GalT family members (Figure 1A). However, like two other recently described invertebrate members of this family (Kawar *et al.*, 2002; Vadaie and Jarvis, 2004), they preferentially transfer GalNAc rather than Gal to GlcNAc. This change in donor

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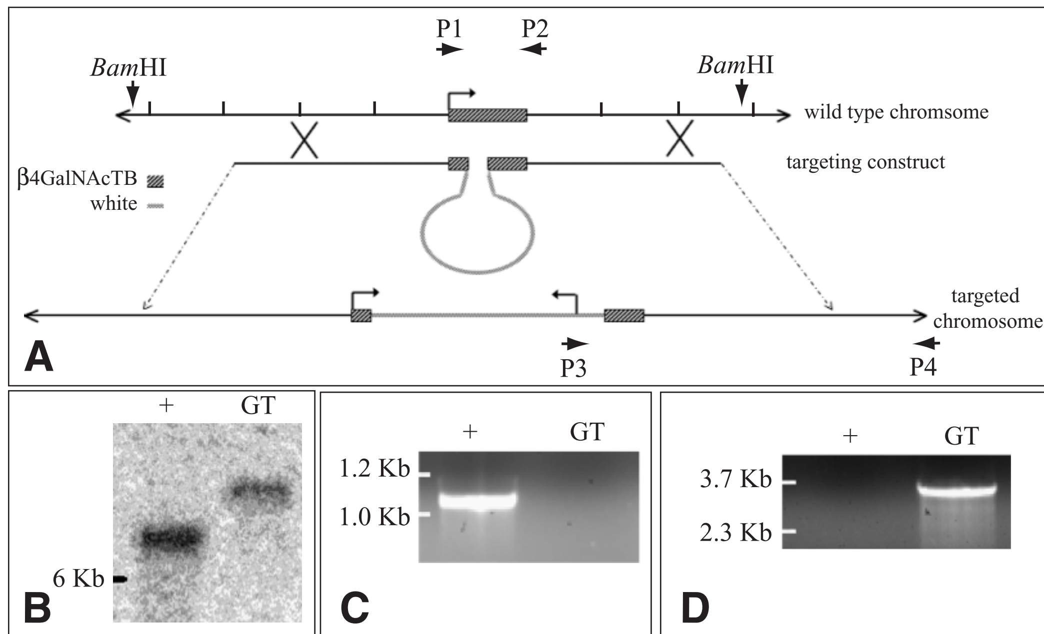

Fig. 7. Construction of a gene-targeted allele of β*4GalNAcTB*. (**A**) Diagrammatic representation of the genomic region, gene-targeting construct, and the gene-targeted mutation obtained. Vertical slashes mark 1-kb intervals, hatched box represents the β*4GalNAcTB* gene, bent arrows indicate transcription start sites, and gray line represents the *white* gene. Note that *white* is inserted in opposite orientation to β*4GalNAcTB*. (**B**) Southern blot on genomic DNA digested with *Bam*HI, using a probe generated from the P1–P2 PCR fragment. In wild type (+) this band is 8 kb; in the gene target allele (GT) it is shifted to 11 kb. (**C**) Primers P1 and P2, which flank the β*4GalNAcTB* transcription unit, amplify a 1.1-kb band from wild-type genomic DNA, but in gene-targeted mutants this band is absent. (**C**) Primers P3, within the *w*+ transcription unit, and P4, in the genomic region adjacent to β*4GalNAcTB*, amplify a 3.3-kb fragment from gene-targeted alleles but not from wild type.

specificity is consistent with the substitution of a tyrosine or phenylalanine in the donor binding pocket for isoleucine or leucine, as site-specific mutagenesis of bovine β4GalT-1 has demonstrated that single amino acid substitutions at this site can switch the substrate specificity from Gal to GalNAc (Ramakrishnan and Qasba, 2002). Although we have not conducted extensive characterization of the activity of these glycosyltransferases on different substrates, or of the products formed, based on the amino acid sequence and GalNAcT activity it can be anticipated that the activity profile of β4GalNAcTA will closely resemble that of the recently characterized *T. ni* β4GalNAcT (Vadaie and Jarvis, 2004). In the case of β4GalNAcTB, detailed analysis will require characterization of an additional factor required for normal activity (H. Bakker personal communication).

*Behavioral phenotypes of β*4GalNAcTA *mutants*

The behavioral phenotypes observed in β*4GalNAcTA* mutants imply that one or more LacdiNAc-containing glycoconjugates synthesized by β4GalNAcTA has an important role in *Drosophila*. The phenotypes suggest some deficit in neuronal or muscular physiology, although it is also possible that they result from an earlier defect during nervous system development. Clues as to the potential nature of the requirement for β*4GalNAcTA* can come from comparison to other mutants that share related phenotypes. One such mutant is *uncoordinated*, which lacks transduction in mechanosensory neurons because of defective ciliogenesis (Baker *et al*., 2004; Eberl *et al*., 2000; Kernan *et al*., 1994). Another mutant with similar behavioral phenotypes, including lack of motility in the climbing assay and lack of coordination in the fall assay, is *purity of essence* (*poe*), also known as *pushover* (Richards *et al*., 1996). *poe* encodes a protein that binds calmodulin and contains an N-recognin type Zn-finger, which is suggestive of a role in ubiquitination. Mutations in *poe* cause spontaneous fusion of synaptic vesicles and increased neurotransmitter release, potentially accounting for the behavioral phenotypes (Richards *et al*., 1996).

Although a role for β*4GalNAcTA* in membrane vesicle dynamics remains speculative, it is worth noting that β*4GalNAcTA* is highly expressed in the Garland cells, which are known for their high levels of endo- and exocytosis (Kosaka and Ikeda, 1983; Wigglesworth, 1972). Indeed, mutants that affect neurotransmitter release or synaptic function often have behavioral phenotypes, but in most cases behavioral phenotypes are observed in hypomorphic alleles, because null alleles are lethal (Babcock *et al*., 2004; Eberl *et al*., 2000; Kernan *et al*., 1994; Wang *et al*., 2004). Indeed, it is not clear that any of the extent *poe* alleles are null. By contrast, because only behavioral phenotypes are observed in β*4GalNAcTA* null alleles, the contribution of its products to such processes would have to be more restricted.

Although β*4GalNAcTA* transfers GalNAc rather than Gal, it is conceivable that it could share biological functions with members of the mammalian β4GalT family, if for example, a *Drosophila* LacdiNAc-containing glycoconjugate has a role equivalent to a mammalian LacNAc-containing glycoconjugate. β4GalT-1 mutant mice develop almost

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normally but exhibit neonatal lethality (Asano *et al*., 1997; Lu *et al*., 1997). Among other deficits, they appear to have impaired endocrine function (Lu *et al*., 1997). A human congenital disorder of glycosylation has been identified in an individual with a mutation of β4GalT-1 (Hansske *et al*., 2002). This individual was diagnosed with mental retardation, hydrocephalus, and myopathy. The molecular basis for these mammalian phenotypes remains as unknown, and the degree of functional redundancy among members of the mammalian β4GalT family remains uncertain. Thus once the molecular basis for the behavioral phenotypes of *β4GalNAcTA* mutants is understood, it will be important to determine whether it is reflective of a conserved biological function.

Drosophila *homologs of β4GalTs are not essential for development*

In mammals, members of the β4GalT family modify a diverse array of glycans, including *N*-linked, *O*-linked, and glycolipids. However, the large number of potentially redundant family members complicates efforts to catalog the biological functions of these glycosyltransferases. Although there are significant differences between mammalian and insect glycans, there are an increasing number of examples of conserved functions, such as the roles of proteoglycans in growth factor signaling (Nybakken and Perrimon, 2002) and the roles of *O*-fucose glycans in Notch signaling (Haines and Irvine, 2003). Thus one premise of this study was that analysis of β4GalT homologs in *Drosophila* might uncover essential conserved functions of this gene family. In particular, we note that studies in cultured mammalian cells identified a requirement for a β4GalT in modulation of Notch signaling by Fringes (Chen *et al*., 2001).

Sequence analysis of the mutations created in *β4GalNAcTA* and *β4GalNAcTB* indicates that they are nonfunctional alleles. Despite this, mutant flies are viable and morphologically normal. Because null mutations in *fringe* are lethal in *Drosophila*, and even weak alleles have obvious morphological phenotypes in adult flies (Correia *et al*., 2003; Irvine and Wieschaus, 1994), it is clear that *Drosophila* homologs of β4GalTs are not required for Notch signaling, nor for other essential developmental process. Thus the functions of this gene family in *Drosophila* appear to be largely physiological rather than developmental. Similarly, mutations in the closest *C. elegans* homolog of *Drosophila* *β4GalNAcTA* have recently been isolated as Bt toxin resistant mutants (*bre-4*) (Griffitts *et al*., 2003); these mutants do not display obvious developmental phenotypes.

The absence of visible phenotypes is unexpected not only in terms of the previously proposed function for this gene family in Notch signaling but also in terms of requirements for *Drosophila* glycolipids. Synthesis of invertebrate arthroseries glycolipids requires a β1,4GalNAc transferase (Seppo *et al*., 2000), and the two enzymes that act just prior to the addition of GalNAc in glycolipid synthesis are encoded by *egh* and *brn* (Schwientek *et al*., 2002; Wandall *et al*., 2003). Both of these are essential genes, mutation of which results in lethality and impairment of Notch signaling during oogenesis and embryonic development(Goode *et al*., 1996a,b). *β4GalNAcTA* or *β4GalNAcTB* single or double mutants do not share the developmental phenotypes of *egh* and *brn*. The only other predicted *Drosophila* genes with any similarity to known mammalian *β4GalNAcT*s are *CG12913* and *CG9220*, both of which, based on sequence comparisons, are predicted to function specifically in chondroitin synthesis. Thus the present data suggest that either the essential functions of *brn* and *egh* do not require the further elongation of glycolipids, or that *Drosophila* encodes an additional *β4GalNAcT* that lacks significant sequence similarity to previously characterized glycosyltransferases. Future studies of glycan structures in mutant flies should help resolve this question.

Materials and methods

Sequence analysis

Full-length protein sequences were aligned using ClustalW, and the phylogenetic tree was generated using GeneBee (www.genebee.msu.su/clustal). Peptide cleavage and transmembrane prediction carried out online at www.cbs.dtu.dk/services/SignalP and www.sbc.su.se/~miklos/DAS/tmdas.cgi.

DNA constructs

DNA encoding full-length *β4GalNAcTA* was amplified by PCR from cDNA clone SD05469 (Rubin *et al*., 2000) using primers forward 5′ATGGTACCCTGGACCATGTACCTC and reverse 5′CGTCTAGAGATTTGCGCTCGGAGTTC. Restriction enzyme sites *Kpn*I and *Xba*I (underscored) were included in the PCR primers to facilitate cloning of the fragment downstream of the metallothionein promoter in pMTWB (A. Xu). In the resulting construct, pMTWB-*β4GalNAcTA:V5:His*, *β4GalNAcTA* is in frame with downstream hexahistidine and V5 tags present in the pMTWB vector. pMT/Bip-GFP-V5-6xHis (pMT-GFP) (Invitrogen, Carlsbad, CA), in which a V5 and hexahistidine-tagged GFP is under control of the metallothionein promoter and expressed as a secreted protein via a Bip signal sequence was used as a control. For genomic rescue, a 2.4-kb fragment containing the entire *β4GalNAcTA* region, but none of the neighboring transcription units, was amplified from *white*⁻ genomic DNA by PCR using primers forward 5′CTGAATTCACTTCGGACTTTAGAATA and reverse 5′ATTCTAGAGCGATGAACTGTTTGAAT, and cloned into pCasper4 using *Eco*RI and *Xba*I restriction sites included on the primers, to generate pCasper4-*β4GalNAcTA*. This construct was transformed into *Drosophila* using standard techniques, and insertions on the first and second chromosomes were obtained.

DNA encoding full-length *β4GalNAcTB* was amplified by PCR from wild type (Oregon-R) genomic DNA (this gene lacks introns) using primers forward 5′ATGGTACCCTGCCAACAATGTTCG and reverse 5′GCTCTAGATCGGTACAACCAGGATG. Restriction enzyme sites were included in the PCR primers to facilitate cloning into pMTWB, generating pMTWB-*β4GalNAcTB*:V5:His. In an alternative construct, a BIP signal sequence was fused to amino acid 39 of *β4GalNAcTB* to improve its secretion by using an alternative forward primer, ATGGTACCTGACTACATCGAGGAATC,

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to generate pMTIB-Tmβ*4GalNAcTB:*V5:His. Untagged versions of *β4GalNAcTB* constructs were made by using a reverse primer including the stop codon (ATCTCGAGCGAGATACCGACTACG), to generating pMTWB-β*GalNAcTB* and pMTIB-Tmβ*4GalNAcT*. All constructs were confirmed by DNA sequencing.

Cell culture

S2 cells adapted to *Drosophila* serum-free media (Invitrogen) were transiently transfected using Cellfectin (Invitrogen), according to the manufacturer's instructions. Cells were cultured to a density of ~ 5 × 10^6/ml in 12 ml serum-free *Drosophila* media, and then induced with 0.7 mM $CuSO_4$. After 96 h, cells or media were collected for analysis.

Enzyme assays

Twelve milliliters conditioned media from cells transfected with pMTWB-β*4GalNAcTA:V5:His*, pMTWB-β*4GalNAcTB:V5:His*, or pMT-GFP (V5-tagged) was incubated with 50 μl anti-V5 agarose bead slurry (Abcam, Cambridge, UK) for 1 h at room temperature. Beads were pelletted by centrifugation at 1000 rpm for 2 min, washed 5 times over 1 h in phosphate buffered saline 0.1% Triton-X100, washed a final time in assay buffer (25 mM HEPES, pH 7.2, 10 mM $MnCl_2$, and 0.1% Triton-X100), and then resuspended in 70 μl assay buffer. Five microliters of bead slurry (~ 10–30 ng protein) was assayed with 4 mM p-nitrophenly N-acetyl-B-D-glucosaminide (pNP-GlcNAc) (Sigma, St. Louis, MO) as the acceptor, and 0.1 μCi of UDP[^{14}C]-Gal (278 mCi/mMol, NEN Life Sciences Products, Boston, MA), UDP[^{3}H]-GalNAc (20 Ci/mMol, American Radiolabeled Chemicals Inc.), or UDP[^{14}C]-GlcNAc (266 mCi/mmol, Amersham Biosciences, Piscataway, NJ) as the donor, supplemented with unlabeled nucleotide sugars where needed to bring the donor concentration to 7.5 μM, in a total reaction volume of 50 μl. The reactions were incubated for 1 h at 28°C with rotary agitation, then terminated by addition of 500 μl 0.5M ethylenediamine tetra-acetic acid. Reactions were then applied to Superclean LC-18 (sigma) solid phase extraction tubes that had been equilibrated with 1 ml methanol, and washed twice with 1 ml water. Extraction tubes were washed twice with 1 ml water, and products were eluted with 1 ml 80% methanol. The elutants were added to 5 ml scintillation cocktail (Scinti Safe Econo 1, Fisher, Silver Spring, MD), and incorporated radioactivity was measured in a scintillation spectrometer. Assays were carried out in triplicate.

In situ *hybridization and tissue staining*

In situ hybridization to mRNA was carried out as described previously (Irvine and Wieschaus, 1994). The template for β*4GalNAcTA* was cDNA SD05469 (Rubin *et al*., 2000). The template for β*4GalNAcTB* was a genomic clone, pBS-CG14517 generated by PCR using 5′ATGGATCCGCGAAGATTGCGTCCG and 5′ATCTCGAGCGAGATACCGACTACG primers.

*Creation of mutations in β*4GalNAcTA

Excisions of *EP2551*{w+} were generated by crossing to a transposase-expressing line (*Sp*/*CyO; ry Sb* Δ*2–3*/*TM6B, Ubx*). One hundred and seven male progeny from this cross, of the genotype *w*; *EP2551*{*w*+}/*CyO ; +*/*ry Sb* Δ*2–3*, were then crossed to *S⁴ Bl*/*CyO* females, and 38 potential excision events were identified by the loss of the *w*⁺ marker. Of these, 33 were identified by Southern blotting with β*4GalNAcTA* SD005405 cDNA as a probe as precise excision events, whereas, as described in *Results*, 5 deleted all or part of β*4GalNAcTA*. Breakpoints for the *β4GalNAcTA*$^{4.1}$ and β*4GalNAcTA*$^{7.1}$ alleles were obtained by PCR from homozygous flies using primers P2 (Figure 5) 5′GACAACCGCTGTCAGGAT and P3 5′ATTCTAGAGCGATGAACTGTTTGAAT and for *Df*(*2R*)β*4GalNAcTA*[*20.1*], primers P1 5′GCGTGCCAGAGTTGTCAA and P3. PCR fragments were TA cloned into pGEMeasy (Promega, Madison, WI) and then sequenced.

The original *EP2551* chromosome and the excision lines initially generated were viable but both male and female sterile. This phenotype could have been due to a mutation on the chromosome unrelated to the P element, or the sterility could been due to a mutation of β*4GalNAcTA* (i.e., if *EP2551* were a mutant allele). Two independent approaches established that the sterility was unrelated to β*4GalNAcTA*. First, the P element in line *EP2551* is inserted in the 5′ untranslated region. Thus, if this line were mutant for β*4GalNAcTA*, it would be expected to influence transcription, rather than the protein product. However, β*4GalNAcTA* expression is normal in *EP2551* homozygotes. Second, the *EP2551* line was recombined with a viable and fertile *white*⁻ chromosome. Using the *w*⁺ marker on the *EP* element to follow the recombinants carrying *EP25515*, male and female fertile flies were obtained, demonstrating that the sterility was unlinked to the insertion site. Following these discoveries, the β*4GalNAcTA*$^{4.1}$ and *Df*(*2R*)β*4GalNAcTA*[*20.1*] imprecise excisions were recombined over a fully fertile *EP2551* chromosome to remove the unlinked sterile mutation(s) on the second chromosome.

*Gene targeting of β*4GalNAcTB

Two 3-kb fragments, one including the first 186 codons and extending upstream, the other including codon 255 and extending downstream, were isolated from *w*^{1118} genomic DNA by PCR, using as primers 5′AACTGCAGACAGTCTGAACAGCATCC, 5′AAGCATGCGCCCTGAATATCGACATT and 5′ATACGCGTAATTCAGTGGCAGGAGGT, 5′AAGGTACCGACCTTCGAAATCGATCC. The fragments were cloned into pPEndsOut (Sekelsky unpublished) using *Pst*I, *Sph*I and *Mul*I, *Kpn*I restriction sites included on the primers. A *white*+ gene was cloned from pHS70w (Sekelsky unpublished) and inserted in opposite orientation between the two genomic fragments to generate pEndsOut-β*4GalNAcTB*. Each insertion line was crossed to a source of FLPase, and the ability of the construct to excise was assayed by scoring the number of progeny with *w*– eyes or *w*+/*w*– mosaic eyes. Five of the lines yielded greater than 95% *w*– progeny, and three of these, one on the second chromosome and two on the first were then used as donors in gene targeting crosses as described by Rong and Golic (2001). Flies carrying targeted events were recovered at ~ 1/5000 (donor 1), 1/20,000 (donor 2), and 0/30,000 (donor 3) gametes. Further analysis was conducted by PCR using

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primers (see figure) P1 5′TTATTATTCGCCAACCGGCC, P2 5′AACACTTCGAGATACCGACT, P3 5′CTCGCATATCTGGCTCTAAGAC, P4 5′AGTGCCGTGTATGACGATAACC, and by Southern blotting using β*4GalNAcTB*. In total nine targeting events were isolated, eight of which were confirmed to be correctly targeted by PCR and Southern analysis.

Creation of double-mutant stocks

Double mutants were generated using *GalNAcTA* alleles β*4GalNAcTA*$^{4.1}$ or *Df*(*2R*)β*4GalNAcTA*[*20.1*], in combination with two of the independently isolated gene-targeted alleles of β*4GalNAcTB*. In each case, *w*; β*4GalNAcTA* ; +/TM6B females were crossed to +/CyO; β*4GalNAcTB*^{GT} males. Stocks were then established by crossing together *w*; β*4GalNAcTA*/CyO; β*4GalNAcTB*^{GT}/TM6B males and females. Double-mutant flies were identified by absence of the CyO and TM6B balancers. The presence of both mutations was further confirmed by PCR analysis of each allele as described.

Behavior assays

Behavior assays were based on those described by Richards *et al.* (1996). For motility, a single male fly 4 days posteclosion was placed in an empty vial that was marked with a line 5 cm from the bottom and allowed to rest for 15 minutes before been tapped gently to the bottom of the vial. The number of seconds taken to cross the 5 cm line was recorded with a cutoff of 2 min. Twenty-six flies of each genotype were scored. For the mechanical agitation assay, a vial containing a single fly was banged onto a rubber mat on a table five times. The number of falls onto the back and time taken for a fly to right itself after a fall was recorded. Twenty-four flies of each genotype were assayed (120 rounds of agitation in total).

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Abbreviations

GFP, green fluorescent protein; PCR, polymerase chain reaction.

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