Proteoglycan UDP-Galactose:β-Xylose β1,4-Galactosyltransferase I Is Essential for Viability in Drosophila melanogaster*

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Heparan and chondroitin sulfates play essential roles in growth factor signaling during development and share a common linkage tetrasaccharide structure, GlcAβ1,3Galβ1,3Galβ1,4Xylβ1-O-Ser. In the present study, we identified the Drosophila proteoglycan UDP-galactose:β-xylose β1,4-galactosyltransferase I (db4GalTTI), and determined its substrate specificity. The enzyme transferred a Gal to the β-xylene (Xyl) residue, confirming it to be the Drosophila ortholog of human proteoglycan UDP-galactose:β-xylene β1,4-galactosyltransferase I. Then we established UAS-dβGalTTI-IR fly lines containing an inverted repeat of dβGalTTI-ligated to the upstream activating sequence (UAS) promoter, a target of GAL4, and observed the F1 generation of the cross between the UAS-dβGalTTI-IR fly and the Act5C-GAL4 fly. In the F1, double-stranded RNA of dβGalTTI is expressed ubiquitously under the control of a cytoplasmic actin promoter to induce the silencing of the dβGalTTI gene. The expression of the target gene was disrupted specifically, and the degree of interference was correlated with phenotype. The lethality among the progeny proved that βGalTTI is essential for viability. This study is the first to use reverse genetics, RNA interference, to study the Drosophila glycosyltransferase systemically.

Proteoglycans are widely expressed on the cell surface and in the extracellular matrix of various tissues and play important roles in the control of growth and differentiation. Proteoglycans consist of a core protein and negatively charged glycosaminoglycans (GAGs) that interact with growth factors, the components of extracellular matrix, morphogens, and cytokines (1–3). GAGs are classified into two categories, heparin/heparan sulfate (HS) and chondroitin sulfate (CS)/dermatan sulfate (DS). The biosynthesis of GAG is initiated by the formation of the linkage tetrasaccharide structure, GlcAβ1,3Galβ1,3Galβ1,4Xylβ1-O-Ser, which is common to heparin/HS and CS/DS. In humans, all four kinds of glycosyltransferases related to the synthesis of the linkage tetrasaccharide structure have been cloned: two peptide O-xylosyltransferases (O-XylT) (4, 5), one proteoglycan β1,4-galactosyltransferase I (β4GalTTI) (6, 7), one proteoglycan β1,3-galactosyltransferase II (β3GalTII) (8), and one glucuronosyltransferase I (GlcATI) (9). In nematodes, two glycosyltransferases, β4GalTI and GlcATI, have already been cloned and characterized (10). Biochemical analysis of GAGs has demonstrated that both Caenorhabditis elegans and Drosophila melanogaster have HS and CS (11, 12). Recently, Drosophila peptidase O-XylT has been reported to transfer xylose (Xyl) to the syndecan peptide (13). But it is still unknown which Drosophila β4GalTI works as proteoglycan β4GalTI.

RNA interference (RNAi) was first recognized in C. elegans as a biological response to exogeneous double-stranded RNA (dsRNA), which induces sequence-specific gene silencing. RNAi is an evolutionarily conserved phenomenon and a multistep process involving the generation of active small interfering RNA (siRNA) in vivo through a reaction with an RNase III endonuclease, Dicer (14–16). The resulting 21–23-nucleotide siRNA mediates degradation of the complementary homologous RNA (17). RNAi has recently emerged as a powerful reverse genetics tool to study gene function in many model organisms, including plants, C. elegans and D. melanogaster in which large dsRNAs efficiently induce gene-specific silencing (18, 19). Only recently, DNA vector-based siRNA has been reported to suppress the expression of the corresponding gene in mammalian cells (20, 21).

In the present study, we identified the Drosophila proteoglycan β4GalTTI (db4GalTTI) and performed a biochemical characterization. The protein transferred a Gal to the β-Xyl residue, confirming it to be the Drosophila ortholog of human proteoglycan β4GalTI (hβ4GalTTI). After that, we produced an inverted repeat; RpL32, ribosomal protein L32; RT, reverse transcriptase.
Table I

Gene-specific primers used for competitive RT-PCR

| Target mRNA | Sense primer | Antisense primer | Target size | Competitor size |
|-------------|--------------|-----------------|-------------|-----------------|
| d4GalTI(+1) | 5'-GGATTAGAGGACGACGTT-3' | 5'-TCAGGTTTGTACGCGGATG-3' | 348 | 401 |
| d4GalTI(+2) | 5'-GGGAAAGGGAGATCCGCTT-3' | 5'-GGGGACCACTTTGTACAAGA-3' | 207 | 240 |
| d4GalTI(1) | 5'-AAGAACTGTTATGGCACGG-3' | 5'-TCAGGTTTGTACGCGGATG-3' | 202 | 240 |
| d4GalTI(2) | 5'-ACGCCCGAGTACGAGAA-3' | 5'-TCAGGTTTGTACGCGGATG-3' | 204 | 240 |
| Rpl52     | 5'-ATGACCATCGGCGCCGACATAAG-3' | 5'-TCAGGTTTGTACGCGGATG-3' | 401 | 465 |

*Primers used for detection of d4GalTI mRNA in Act5c-GAL4UAS-d4GalTI-HR N larvae and prepupae.

**Primers used for detection of d4GalTI mRNA in Act5c-GAL4UAS-d4GalTI-HR larvae.

α-mannopyranosidoses (Man-p-Nph) were purchased from Calbiochem. Galβ1,4Xylβ1-p-methoxyphenyl (Galβ1,4Xylβ1-p-Mph) was provided by Seikagaku Corp. Uridine diphosphate-[14C]glactose (UDP-[14C]Gal) (325 mCi/mmol) was supplied by PerkinElmer Life Sciences.

Identification of the Drosophila Proteoglycan β1,4-Galactosyltransferase I—We performed a BLAST search of all Drosophila databases and identified one Drosophila proteoglycan β1,4-galactosyltransferase I gene, CG11780. The Drosophila expressed sequence tag clone CK02622 including CG11780 was obtained. The plasmid DNA was prepared from CK02622 and sequenced using an ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (ABI, Foster City, CA).

Construction and Purification of d4GalTI and hβ4GalTI Proteins Fused with FLAG Peptide—The putative catalytic domain of d4GalTI (amino acids 36-322) was expressed as a secreted protein fused with a FLAG peptide in insect cells according to the instruction manual of GATEWAY™ Cloning Technology (Invitrogen). An ~9-kb DNA fragment was amplified by two-step PCR. The first PCR used the plasmid DNA from expressed sequence tag clone CK02622 as a template, the forward primer 5'-AAAAACGAGCGTCTTCGCTCAGCGCATACGG-3' and the reverse primer 5'-AGAAAGCTGGGTTACACGGCTTTGACGC-3'. The second PCR used the first PCR product as a template, the forward primer 5'-GGGGGACCACTTTGTACAAGA-3' and the reverse primer 5'-GGGGGACCACTTTGTACAAGA-3'. The forward and reverse primers were flank with attB1 and attB2 sequences, respectively, to create the recombination sites. The amplified fragment was recombined between the attB1 and attB2 sites of the pDONR™-201 vector using the BP CLONASE Enzyme Mix (Invitrogen). Then the insert was transferred between the attR1 and attR2 sites of pvL1393-FLAG to yield pvL1393-FLAG-d4GalTI. pvL1393-FLAG is an expression vector derived from pvL1393 (Pharmlingen, San Diego, CA) and contains a fragment encoding the signal peptide of human immunoglobulin κ (MHFQVQPSFLIISAISVSRG), the FLAG peptide (DYKDDDDK), and a conversion site for the GATEWAY system. pvL1393-FLAG-hβ4GalTI was also prepared by the same procedure using the two primers, 5'-AAAAAACGAGCGTCTTCGCTCAGCGCATACGG-3' and 5'-AGAAAGCTGGGTTACACGGCTTTGACGC-3'. The forward and reverse primers were flank with attB1 and attB2 sequences, respectively, to create the recombination sites. The amplified fragment was recombined between the attB1 and attB2 sites of the pDONR™-201 vector using the BP CLONASE Enzyme Mix (Invitrogen). Then the insert was transferred between the attR1 and attR2 sites of pvL1393-FLAG to yield pvL1393-FLAG-d4GalTI.

Western Blot Analysis—The enzymes purified above were subjected to 12.5% SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis. The separated proteins were transferred to a Hybond-P membrane (Amersham Biosciences). The membrane was probed with anti-FLAG M2-peroxidase conjugate (Sigma) and stained with Konica Immunostaining HRP-1000 (Konica, Tokyo, Japan). The intensity of positive bands on Western blotting was measured by densitometer to determine the amount of the purified enzyme using FLAG-BAP Control Protein (Sigma).

Assay of Galactosyltransferase Activity—To determine the galactosyltransferase activity, Xyla-p-Nph, Xyla-p-Nph, Ulex-p-Nph, GalNAc-p-Nph, GlcNAc-p-Nph, GalNAcβ-p-Nph, GlcNAcβ-p-Nph, GlcNAcβ-p-Nph, GlcNAcβ-p-Nph, Manα-p-Nph, Manβ-p-Nph,
and Gal1,4Xylβ1-pMph were utilized as acceptor substrates. With 10 nmol of each acceptor, the βGalT activity reaction was performed at both 25 and 37 °C for 2 h in 20 μl of a reaction mixture containing 14

### Table II

| Acceptor substrate | dβGalTI | hβGalTI |
|--------------------|---------|---------|
|                    | 25 °C   | 37 °C   | 25 °C   | 37 °C   |
| Xyl-βpNph          | 0.17    | 0.22    | 0.39    | 0.49    |
| XylβpNph           | 4.4     | 7.9     | 7.8     | 8.5     |
| GlcNAc-βpNph       | ND      | ND      | ND      | ND      |
| GlcNAcβ-βpNph      | ND      | ND      | ND      | ND      |
| GlcNAcβ-S-pNph     | ND      | ND      | ND      | ND      |
| Glc-pNph           | ND      | ND      | ND      | ND      |
| Gal-pNph           | ND      | ND      | ND      | ND      |
| GalβpNph           | ND      | ND      | ND      | ND      |
| GalNac-pNph        | ND      | ND      | ND      | ND      |
| Man-βpNph          | ND      | ND      | ND      | ND      |
| Gal1,4Xylβ1-pMph   | ND      | ND      | ND      | ND      |

ND, not detected.

### Table III

| Stock no. | Insertion chromosome | F1 progeny of UAS-dβ4GalTI-IR fly crossed with Act5C-GAL4 fly |
|-----------|----------------------|-------------------------------------------------------------|
| C1        | 3                    | +                                                           |
| C2        | 2                    | Lethal                                                      |
| C3        | 3                    | Lethal                                                      |
| C4        | 2                    | Lethal                                                      |
| C5        | 2                    | Lethal                                                      |
| C6        | 3                    | Lethal                                                      |
| C7        | 3                    | +                                                           |
| C8        | 3                    | Lethal                                                      |
| C9        | 3                    | +                                                           |
| C10       | 3                    | +                                                           |
| C11       | 3                    | +                                                           |
| N1        | 3                    | Lethal                                                      |
| N2        | 2                    | Lethal                                                      |
| N3        | 2                    | Semilethal                                                  |
| N4        | 3                    | Lethal                                                      |
| N5        | 2                    | Lethal                                                      |
| N6        | 3                    | Lethal                                                      |
| N7        | 2                    | Lethal                                                      |
| N8        | 3                    | Lethal                                                      |
| N9        | 3                    | Lethal                                                      |
| N10       | 2                    | Lethal                                                      |
| N11       | 2                    | Lethal                                                      |
| N12       | 3                    | Lethal                                                      |
| N13       | 2,3                  | Lethal                                                      |

* In F1, dsRNA of dβ4GalTI is expressed ubiquitously under the control of the cytoplasmic actin promoter.  
  + , viable and morphologically normal.  
  a The N13 line, which has two copies of the IR, was made by crossing N2 stock with N4 stock.
FIG. 4. Quantitative analysis of dβ4GalTI mRNA in each inducible dβ4GalTI RNAi fly by competitive RT-PCR. The mRNA levels of all Drosophila β4GalTIs (dβ4GalTI, dβ4GalTA, and dβ4GalTB) in each dβ4GalTI RNAi fly were determined by competitive RT-PCR. The actual amount of each β4GalT mRNA was divided by that of RpL32 mRNA for normalization. The relative amount of each β4GalT mRNA to RpL32...
mRNA in F1 progeny of the \( \omega^{118} \) crossed with Act5C-GAL4 fly. Act5C-GAL4+/C, which corresponds to the wild type, was presented as 1. Amounts of 1 and 0.5 \( \mu l \) of synthesized cDNA were used for the quantitation of \( b4GalTI \) and \( Rpl32 \), respectively. The CDSs for each \( b4GalTI \) mRNA and \( Rpl32 \) mRNA were amplified together with 0.5–1000 \( \times \) copies and 2.5–10 \( \times \) copies, respectively, of the corresponding competitor DNAs. A, the mRNA levels of the three kinds of \( b4GalT \) in the third instar larvae of the \( F_1 \) progeny of each \( N \) line of \( UAS-d4GalTI-IR \) fly crossed with Act5C-GAL4, designated as Act5C-GAL4/N. Each \( N \) line has the IR of the sequence encoding the N-terminal region of \( d4GalTI \). Act5C-GAL4/N12, Act5C-GAL4/N4, and Act5C-GAL4/N13 showed lethality at the pupal stages, whereas Act5C-GAL4/N6 was viable and morphologically normal. The \( N \) lines of the IR on chromosomes 2 and 3 B, the mRNA levels of the three kinds of \( b4GalT \) in the third instar larvae of the \( F_1 \) progeny of each \( C \) line of \( UAS-d4GalTI-IR \) fly crossed with Act5C-GAL4, designated as Act5C-GAL4/C. Each \( C \) line has the IR of the sequence that encodes the C-terminal region of \( d4GalTI \). Act5C-GAL4/C5 and Act5C-GAL4/C6 were lethal. C, the mRNA level of \( d4GalTI \) in the prepuce of Act5C-GAL4/N4 and Act5C-GAL4/N13.

RESULTS

Identification of the Drosophila Proteoglycan \( b1,4\)-Galactosyltransferase I—When human \( b4GalTI \) was used as the query sequence for a TBLASTN search of the Berkeley Drosophila Genome Project, one highly homologous gene, \( CG11780 \), was obtained as \( d4GalTI \). The complete CDS of the \( d4GalTI \) gene and the predicted amino acid sequence are shown in Fig. 1A. The putative protein, consisting of 323 amino acids, was a type II transmembrane protein with a hydrophobic domain in the N-terminal region (Fig. 1B).

When the other members of the human \( b1,4\)-galactosyltransferase family, \( h4GalTI \) to -6, were used as query sequences for the TBLASTN search, two highly homologous genes, \( CG8536 \) and \( CG14517 \), were also obtained as members of the Drosophila \( b1,4\)-galactosyltransferase family, \( d4GalTI A \) and \( d4GalTA \). The \( d4GalTA \) and \( d4GalTB \) showed much lower homology of amino acids to \( h4GalTI \) (33 and 29%, respectively) than \( d4GalTI \) to \( h4GalTI \) (48%).

The ClustalW alignment of the human and Drosophila \( b4GalTI \) families showed that the three \( b4GalT \) motifs found in the human family, including the DXD motif, a metal binding site, were also conserved in the Drosophila \( b4GalTI \) family (Fig. 2A) (24, 25). A phylogenetic tree of the three Drosophila \( d4GalTI \), \( d4GalTA \), and \( d4GalTB \) and seven human \( b4GalT \)s (\( h4GalTI \) to -6 and \( h4GalTI \)) was generated based on the amino acid sequences (Fig. 2B). The \( d4GalTI \) was confirmed to be the Drosophila ortholog of \( h4GalTI \). Both \( d4GalTA \) and \( d4GalTB \) showed higher homology to \( h4GalTI \) to -6 than \( h4GalTI \).

Characterization of the Galactosyltransferase Activity of \( d4GalTI \)—The FLAG-tagged recombinant \( d4GalTI \) was expressed in insect cells to determine whether or not \( d4GalTI \) has galactosyltransferase activity. The soluble form was prepared by replacing the N-terminal region including the cytoplasmic and transmembrane domains, amino acids 1–35, with an IgG signal sequence and FLAG peptide sequence. The secrete enzyme was purified with anti-FLAG M1 gel and quantitated by Western blotting analysis using Anti-FLAG antibody. FLAG-tagged recombinant \( h4GalTI \) was also prepared by the same procedure.

The purified enzymes were used for a galactosyltransferase assay with various acceptor substrates (Table I). The determined amounts of \( d4GalTI \) and \( h4GalTI \) and the same amount of each substrate were used for the enzyme reactions, so we could determine relative activities that were comparable. \( h4GalTI \) had strong activity toward the \(-\beta-Xyl \) residue, whereas it had only slight activity toward \(-\alpha-Xyl \) and NAc activity toward \(-\beta-GlcNAc \), \(-\beta-Glc \), \(-\beta-Gal \), and \(-\beta-GalNAc \) as reported previously (6, 7). \( d4GalTI \) also showed strong activity toward the \(-\beta-Xyl \) residue; only slight activity toward \(-\alpha-Xyl \); and no activity toward \(-\beta-GlcNAc \), \(-\beta-Glc \), \(-\beta-Gal \), and \(-\beta-GalNAc \). These results demonstrated that \( d4GalTI \) was the Drosophila Proteoglycan \( b1,4\)-Galactosyltransferase I.
sophila ortholog of hβ4GalTI in view of their activities. But the β4GalTI activity of dβ4GalTI toward the β-Xyl residue was almost half that of hβ4GalTI at both 25 and 37 °C.

Viability of Inducible dβ4GalTI RNAi Flies—Proteoglycan β4GalTI contributes to the synthesis of the common carbohydrate-protein linkage structure, Glcβ1,3Galβ1,3Galβ1,4Xyl β1-O-Ser, of proteoglycan including heparin/HS and CS/DS. If proteoglycan β4GalTI is inactivated, every proteoglycan lacks GAG, and a severe biological phenotype is expected. To test this hypothesis, we tried to make inducible dβ4GalTI RNAi flies according to the method described under “Experimental Procedures.”

A scheme of the heritable and inducible RNAi system is shown in Fig. 3. In this report, we used Act5C-GALA as a GALA driver to induce dβ4GalTI gene silencing in all cells of the fly. The Act5C-GALA fly has a transgene containing yeast transcriptional factor GALA, the expression of which is under the control of the cytoplasmic actin promoter. 24 UAS-dβ4GalTI-IR fly stocks having a transgene containing two types of the IR of dβ4GalTI ligated to the UAS promoter, a target of GALA, were established (Table III). The IR of dβ4GalTI was separated by an unrelated DNA sequence that acts as a spacer to give a hairpin loop-shaped RNA. C-1 to C-11 have a transgene containing the IR of the sequence encoding the C-terminal region of the catalytic domain (amino acids 209–322). N-1 to N-13 have a transgene containing the IR of the sequence encoding the N-terminal region (amino acids 1–167). In the F1 generations of the Act5C-GALA fly and the UAS-dβ4GalTI-IR fly, dsRNA of dβ4GalTI is expressed ubiquitously under the control of the cytoplasmic actin promoter to induce dβ4GalTI gene silencing.

The phenotypes of the F1 of each UAS-dβ4GalTI-IR fly crossed with the Act5C-GALA fly are shown in Table III; 65% (15 of 23) of these crosses caused lethality in the progeny (i.e. the flies could not develop into adults). The expression of dβ4GalTI dsRNA by crossing the N13 line carrying two copies of UAS-dβ4GalTI-IR to the Act5C-GALA fly also led to lethality at the pupal stages. These results clearly demonstrated that proteoglycan β4GalTI is essential for the viability of flies.

Quantitative Analysis of dβ4GalTI mRNA in Each Inducible dβ4GalTI RNAi Fly by Competitive RT-PCR—To test the efficiency and specificity of RNAi in this system, the mRNA levels of all Drosophila β4GalTs (dβ4GalTI, dβ4GalTA, and dβ4GalTB) were determined in each dβ4GalTI RNAi fly by competitive RT-PCR (Fig. 4). The relative amount of each β4GalT mRNA to RpL32 mRNA in F1 progeny of w1118 crossed with the Act5C-GALA fly, Actin5C-GAL4AI+, which corresponds to the wild type, was presented as 1. The F1, progeny of each N or C line of the UAS-dβ4GalTI-IR fly crossed with Act5C-GALA fly was designated as Act5C-GALA/N or Act5C-GALA/C, respectively.

The N lines have a transgene including the IR of the sequence encoding the N-terminal region of β4GalTI. N13 having two copies of the IR on chromosomes 2 and 3 was made from the N2 and N4 lines. The degree of expression of the transgene is known to depend on its sites of insertion on the chromosome. Act5C-GALA/N2, Act5C-GALA/N4, and Act5C-GALA/N13 were pupal lethal, whereas Act5C-GALA/N6 was viable and morphologically normal (Table III). First, we determined the amounts of three kinds of dβ4GalTs mRNA in the third instar larvae of these four RNAi flies and the wild-type fly, Act5C-GALA/. The ratios of reduction in dβ4GalTI mRNA of Act5C-GALA/N13, Act5C-GALA/N4, Act5C-GALA/N2, and Act5C-GALA/N6 were 0.26, 0.32, 0.36, and 0.76, respectively, demonstrating a correlation with the severity of the phenotype (Fig. 4A). F1 progeny of the N13 line having two copies of the IR had less dβ4GalTI mRNA than those of the N2 line and N4 line, which were crossed to make the N13 line. Reductions in dβ4GalTA mRNA and dβ4GalTB mRNA were not observed in all RNAi flies. It was clearly demonstrated that the dβ4GalTI mRNA was disrupted specifically, and the ratio of degraded dβ4GalTI mRNA was well correlated with the severity of the phenotype.

Similar analyses were performed for the F1 progeny of C lines crossed with Act5C-GALA fly, Act5C-GALA/C5, and Act5C-GALA/C6. Both RNAi flies showed lethality at the pupal stages (Table III). The dβ4GalTI mRNAs in the third instar larvae were also interfaced with specifically, and the ratios of degraded dβ4GalTI mRNA of Act5C-GALA/C5 and Act5C-GALA/C6 (0.35 and 0.45, respectively) were almost the same as those of Act5C-GALA/N2 and Act5C-GALA/N4 (Fig. 4B). The efficiency of RNAi did not depend greatly on the target sequences using the constructions of IR.

We also determined the amount of dβ4GalTI mRNAs in prepupa of Act5C-GALA/N4 and Act5C-GALA/N13 (Fig. 4C). The target efficiency in the prepupa was almost the same as that in the third instar larvae.

The above results clearly demonstrated that the expression of the target gene was specifically reduced by RNAi in this Drosophila RNAi system to induce the phenotype.

DISCUSSION

We identified the Drosophila proteoglycan β4GalTI by molecular and biochemical analyses and then made the RNAi fly to investigate dβ4GalTI function in vivo. The expression of the target gene was disrupted specifically in the RNAi fly and the degree of interference was correlated to phenotype. The reduction of dβ4GalTI mRNA caused lethality, indicating an essential function of dβ4GalTI for viability. This is the first example of a reverse genetics approach to the systematic study of Drosophila glycosyltransferase.

Drosophila has three members of the β4GalT family, dβ4GalTI, dβ4GalTA, and dβ4GalTB (Fig. 2A). The phylogenetic tree (Fig. 2B) and acceptor substrate specificities (Table II) of the β4GalT family clearly demonstrated that dβ4GalTI is the Drosophila ortholog of hβ4GalTI, dβ4GalTA, and dβ4GalTB showed higher homology to hβ4GalTI to -6 than to hβ4GalTI, but the Drosophila ortholog of the six hβ4GalTs could not be identified. So there is a possibility that dβ4GalTA and dβ4GalTB share acceptor substrates, to which hβ4GalT1 to -6 can transfer Gal. Recently, hβ4GalT1 has been reported to transfer Gal to fringe-modified O-fucose glycans on the Notch protein and the elongation of glycans was necessary to modulate Notch signaling. A novel Drosophila galectin has also been isolated (26). It is still unknown which of the two dβ4GalTs works to elongate O-fucose glycans on Notch or synthesize the ligands of Drosophila galectin. We are now attempting to determine the substrate specificity of the two dβ4GalTs.

The three members of the dβ4GalT family also conserved the three β4GalTI motifs found in the hβ4GalTI family (Fig. 2A)(25). The crystal structure of the bovine β4GalTI has already been reported (24, 27, 28). The DXD motif is a Mn2+-binding site, and the other two motifs expose the surface of the catalytic pocket. The FNRA motif is involved in UDP-Gal binding and the negatively charged residues of the GWGXXED/(D/E) motif contribute to UDP-Gal and UDP-glucose binding. The three motifs conserved between human and Drosophila had amino acid sequences that related to the binding of metal or donor substrates.

dβ4GalTI showed roughly half the activity of hβ4GalTI toward each substrate at both 25 and 37 °C, although the sub-
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strate specificities of the two were similar (Table II). The breeding temperature of flies and the body temperature of humans are 25 and 37 °C, respectively. Under physiological conditions, hβ4GalTTI performed a more efficient reaction than dβ4GalTTI. Very recently, two papers about dβ4GalTTI have been published (29, 30) reporting similar enzymatic activity to ours.

We made 24 UAS-dβ4GalTTI-IR fly lines and observed the F₁ generation of each UAS-dβ4GalTTI-IR fly crossed with the Act5C-GAL4 fly. The severity of the phenotype differed between the stocks. Approximately 65% of the flies died at the pupal stages (Table III), but some lived to become adults, similar in morphology to the wild type. Because the degree of expression of the transgene is known to depend on its sites of insertion on the chromosome, it is reasonable that the phenotypes differed. The reduction in dβ4GalTTI mRNA was correlated with the severity of the phenotype (Fig. 4A). The severest phenotype should be considered to represent the real phenotype of the mutant. Although we have no data indicating that cell, tissue, or organ abnormalities caused the death of individual flies, finer analyses of the phenotype should reveal the in vivo function of dβ4GalTTI.

We analyzed the amounts of three dβ4GalTTI mRNAs (dβ4GalTTA, dβ4GalTA, and dβ4GalTB) to estimate the specificity and efficiency of RNAi in our Drosophila-inducible RNAi system. The RNAi occurred only on dβ4GalTTI and had no effect on the other members of the dβ4GalTT family, dβ4GalTTA and dβ4GalTB (Fig. 4, A and B). During the process of RNAi, 21–23-nucleotide siRNA mediates the degeneration of the complementary homologous RNA (17). If even one nucleotide differs between the siRNA and target mRNA, siRNA cannot mediate the degeneration of target RNA. Comparing the DNA sequence of dβ4GalTTI with the sequences of dβ4GalTTA and dβ4GalTB, we could not find any identical regions longer than 21 nucleotides. This must be the reason why the RNAi of dβ4GalTTI was specific with no cross-effect for dβ4GalTTA and dβ4GalTB.

The efficiency of RNAi largely did not depend on the target sequences using the constructions of IR (Fig. 4, A and B), and the ratio of degraded dβ4GalTTI mRNA was well correlated with the severity of the phenotypes. These findings demonstrate that our Drosophila-inducible RNAi system has the potential to be a powerful tool for analyses of the biological roles of glycans. As mentioned above, D. melanogaster will become a powerful tool for analysis of the biological roles of glycans.

In this report, we demonstrated the first systematic reverse genetics approach using RNAi of Drosophila glycosyltransferase and showed that RNAi worked well in the case of a glycosyltransferase. We found almost 70 Drosophila glycosyltransferases by performing a TBLASTN search of the Drosophila data bases using mammalian glycosyltransferases as the query sequence. It is possible to make a RNAi fly for each of the 70 glycosyltransferases, whereas knock-out mice cannot be made. The inducible glycosyltransferase RNAi fly obtained using the GALA-UAS system will open the way for the analysis of the biological role of glycans.

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REFERENCES

1. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Linecum, J., and Zako, M. (1999) Annu. Rev. Biochem. 68, 729–777

2. Selleck, S. R. (2000) Trends Genet. 16, 206–212

3. Lin, X., and Perrimon, N. (2000) Matrix Biol. 19, 303–307

4. Gotting, C., Kuhn, J., Zahn, R., Brinkmann, T., and Klesseik, K. (2000) J. Mol. Biol. 304, 517–528

5. Kuhn, J., Gotting, C., Schnolzer, M., Kempf, T., Brinkmann, T., and Klesseik, K. (2001) J. Biol. Chem. 276, 4940–4947

6. Okajima, T., Yoshida, K., Kondo, T., and Furukawa, K. (1999) J. Biol. Chem. 274, 22915–22918

7. Almeida, R., Rever, S. B., Mandel, U., Kresse, H., Schwientek, T., Bennett, E. P., and Clausen, H. (1999) J. Biol. Chem. 274, 26165–26171

8. Bai, X., Zohu, D., Brown, J. R., Crawford, B. E., Hennet, T., and Esco, J. D. (2001) J. Biol. Chem. 276, 48189–48195

9. Kitagawa, M., Tone, Y., Tamura, J., Neumann, K. W., Ogawa, O., Oka, S., Kawasaki, T., and Sugahara, K. (1998) J. Biol. Chem. 273, 6615–6618

10. Bulk, D. A., Wei, G., Toyoda, H., Kinoshita-Toyoda, A., Waldrip, W. R., Esco, J. D., Robbins, P. W., and Selleck, S. B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10838–10843

11. Toyoda, H., Kinoshita-Toyoda, A., and Selleck, S. B. (2000) J. Biol. Chem. 275, 2269–2275

12. Yamada, S., Okada, Y., Ueno, M., Iwata, S., Deepa, S. S., Nishimura, S., Fujita, M., Van Die, I., Hirabayashi, Y., and Sugahara, K. (2002) J. Biol. Chem. 277, 42488–42495

13. Wilson, I. B. (2002) J. Biol. Chem. 277, 21207–21212

14. Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., Bailie, D. L., Fire, A., Borvuk, G., and Mello, C. C. (2001) Cell 106, 23–34

15. Huttunen, G., McLaughlan, J., Pasquinelli, A. E., Balint, E., Tuschi, T., and Zamore, P. D. (2001) Science 293, 834–838

16. Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001) Nature 409, 361–368

17. Huttunen, G., and Zamore, P. D. (2002) Curr. Opin. Genet. Dev. 12, 225–232

18. Giordano, E., Rendina, R., Peluso, I., and Furia, M. (2002) Genetics 156, 159–168

19. Giordano, E., Rendina, R., Peluso, I., and Furia, M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 1443–1448

20. Giordano, E., Rendina, R., Peluso, I., and Furia, M. (2002) Genetics 156, 159–168

21. Sui, G., Soohoo, C., Affar, E. B., Gay, F., Shi, Y., and Forrester, W. C. (2002) J. Biol. Chem. 277, 10838–10843

22. Sui, G., Soohoo, C., Affar, E. B., Gay, F., Shi, Y., and Forrester, W. C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5515–5520

23. Spradling, A. C. (1986) in Drosophila: A Practical Approach (Roberts, D. B., ed) pp. 175–197, IRL Press, Oxford

24. Comenyski, W., and Saechi, N. (1987) Anal. Biochem. 162, 156–159

25. Gastineau, N., Cambillau, C., and Bourne, Y. (1999) EMBO J. 18, 3548–3557

26. Amado, M., Almeida, R., Schwientek, T., and Clausen, H. (1999) Biochim. Biophys. Acta 1473, 35–53

27. Pace, K. E., Lebestky, T., Hummel, T., Archz, P., Kwan, K., and Baum, L. G. (2002) J. Biol. Chem. 277, 13091–13098

28. Ramakrishnan, B., and Qasba, P. K. (2001) J. Biol. Chem. 310, 205–218

29. Ramakrishnan, B., Shah, P. S., and Qasba, P. K. (2001) J. Biol. Chem. 276, 37665–37671

30. Nakamura, Y., Haines, N., Chen, J., Okajima, T., Furukawa, K., Urano, T., Stanley, P., and Irvine, K. D. (2002) J. Biol. Chem. 277, 46280–46288

31. Vadaie, N., Fujita, M., Van Die, I., Hirabayashi, Y., and Sugahara, K. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 834–838

32. Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R.,
33. Bruckner, K., Perez, L., Clausen, H., and Cohen, S. (2000) *Nature* 406, 411–415
34. Tsuda, M., Kamimura, K., Nakato, H., Archer, M., Staatz, W., Fox, B., Humphrey, M., Olson, S., Futch, T., Kaluza, V., Siegfried, E., Stam, L., and Selleck, S. B. (1999) *Nature* 400, 276–280
35. Ten Hagen, K. G., and Tran, D. T. (2002) *J. Biol. Chem.* 277, 22616–22622
36. Schwientek, T. J., Bennett, E. P., Flores, C., Thacker, J., Hollman, M., Reis, C. A., Behrens, J., Mandel, U., Keck, B., Schafer, M. A., Hazelmann, K., Zubarev, R., Roepstorff, P., Hollingsworth, M. A., and Clausen, H. (2002) *J. Biol. Chem.* 277, 22623–22638
