Identification of the *Drosophila* core 1 β1,3-galactosyltransferase gene that synthesizes T antigen in the embryonic central nervous system and hemocytes

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T antigen (Galβ1-3GalNAcα1-Ser/Thr), the well-known tumor-associated antigen, is a core 1 mucin-type O-glycan structure that is synthesized by core 1 β1,3-galactosyltransferase (C1β3GalT), which transfers Gal from UDP-Gal to Tn antigen (GalNAcα1-Ser/Thr). Three putative C1β3GalTs have been identified in *Drosophila*. However, although all three are expressed in embryos, their roles during embryogenesis have not yet been clarified. In this study, we used P-element inserted mutants to show that CG9520, one of the three putative C1β3GalTs, synthesizes T antigen expressed on the central nervous system (CNS) during embryogenesis. We also found that T antigen was expressed on a subset of the embryonic hemocytes. CG9520 mutant embryos showed the loss of T antigens on the CNS and on a subset of hemocytes. Then, the loss of T antigens was rescued by precise excision of the P-element inserted into the CG9520 gene. Our data demonstrate that T antigens expressed on the CNS and on a subset of hemocytes are synthesized by CG9520 in the *Drosophila* embryo. In addition, we found that the number of circulating hemocytes was reduced in third instar larvae of CG9520 mutant. We, therefore, named the CG9520 gene *Drosophila* core 1 β1,3-galactosyltransferase 1 because it is responsible for the synthesis and function of T antigen in vivo.

**Keywords:** CNS/core 1 β1, 3-galactosyltransferase/ *Drosophila*/hemocyte/T antigen

**Introduction**

Mucin-type O-glycans contain GalNAc in an α1-linkage to a serine or threonine residue. This posttranslational modification is found on many membrane-bound and -secreted proteins (Van den Steen et al. 1998). In vertebrates, eight different O-glycan core structures have been described (Brockhausen 1997). There is evidence that one of these core structures, core 1 structure (Galβ1-3GalNAcα1-Ser/Thr), called T antigen, is associated with immunosuppression, metastasis dissemination, and the proliferation of several types of cancer cells (Springer et al. 1984; Berger 1999; Brockhausen 1999).

The core 1 structure is synthesized from GalNAcα1-Ser/Thr by core 1 β1,3-galactosyltransferase (C1β3GalT). C1β3GalTs have been identified from various organisms, including rat (Ju, Cummings et al. 2002), human (Ju, Brewer et al. 2002), mouse (Xia et al. 2004), and *Caenorhabditis elegans* (Ju et al. 2006). Xia et al. recently reported that C1β3GalT (T-synthase) null mice showed embryonic lethality, defective angiogenesis, and fatal embryonic hemorrhage (Xia et al. 2004; Xia and McEver 2006). Thrombocytopenia and kidney disease have been observed in plt1 mice, which have an N-ethyl-N-nitrosourea-induced point mutation in the C1β3GalT gene and very low residual C1β3GalT activity (Alexander et al. 2006). However, most mammalian mucin-type core 1 structures are elongated or modified by sialylation or fucosylation (Brockhausen 1999). It has also been reported that T antigen is expressed in the normal placenta, seminal plasma, and the developing and adult kidney in humans (Richter et al. 2000; Toma et al. 2000; Yamaguchi et al. 2001). Recently, it was reported that three putative *Drosophila* C1β3GalTs have C1β3GalT activity in vitro (Muller et al. 2005). They showed activity on glycolipids as well as mucin proteins. However, the in vivo roles of these C1β3GalTs have not yet been clarified.

In *Drosophila*, the only mucin-type O-glycans identified to date are T antigen and Tn antigen (GalNAcα1-Ser/Thr) (Kramerov et al. 1996). Unlike vertebrates, these structures are not sialylated in *Drosophila*. It is known that the *Drosophila* α2,6-sialyltransferase cannot transfer sialic acid to T antigen (Koles et al. 2004). In addition, it has been reported that the distribution of T antigen, identified using peanut agglutinin (PNA) lectin, is regulated in tissue- and stage-specific manners during embryonic development (Fristrom DK and Fristrom JW 1982; D’Amico and Jacobs 1995). These data remind us that *Drosophila* may be a suitable model system for investigating the functions of T antigen and the C1β3GalT genes during development.

In this study, we demonstrate that the CG9520 gene, one of the *Drosophila* orthologs of human C1β3GalT, is involved in synthesis of T antigen in the central nervous system (CNS) and a subset of hemocytes during the developmental process of *Drosophila*.
Results

Candidate for synthesizing T antigen in Drosophila embryos

Recently, the Drosophila genome was reported to have at least three $\beta$3GalT genes, CG9520, CG8708, and CG13904 (Muller et al. 2005). To estimate which C1$\beta$3GalTs synthesize T antigen in vivo, we built a phylogenetic tree from the sequences of the three Drosophila C1$\beta$3GalTs, human C1$\beta$3GalT (Ju, Brewer, et al. 2002), mouse T-synthase (Xia et al. 2004), C. elegans T-synthase (Ju et al. 2006), rat C1$\beta$3GalT (Ju, Cummings, et al. 2002), and putative chicken, Xenopus and zebrafish C1$\beta$3GalTs (Figure 1A). The phylogenetic tree indicated that CG9520 and CG8708 are the orthologs of mammalian C1$\beta$3GalTs responsible for the synthesis of T antigen. Then, we selected the two as the first candidates for synthesizing T antigen in vivo. To distinguish which of the two is responsible for the synthesis of T antigen, we investigated the expression patterns of the CG9520 and CG8708 genes during embryonic development and also in several tissues and cultured cell lines by quantitative real-time PCR (Figure 1B). In embryos, the highest level of CG9520 transcript was found at 2 h and was maternally derived. On the other hand, CG8708 transcripts were strongly expressed in the larval salivary glands and testis of adult male flies. Recently, Muller et al. used an in situ hybridization analysis to show that the CG9520 transcript is maternally expressed and also expressed in the amnioserosa of late-stage embryos. In addition, they found that the CG8708 transcript is only expressed in the salivary glands of the embryos. They also showed that CG9520 exhibits more than 100 times higher C1$\beta$3GalT activity than CG8708 (Muller et al. 2005). In embryos, the levels of CG8708 transcripts were less than 10 times of those of CG9520 transcripts. Therefore, CG9520 is a good candidate for synthesizing T antigen in embryos.

T antigen is present on the embryonic CNS of Drosophila

To investigate the expression patterns of T antigen during development, we used an anti-T antigen monoclonal antibody to immunostain Drosophila embryos. T antigen was first clearly detected at the head and tail region of stage 11 embryos (Figure 2A). By stage 16, T antigen expressing cells were evenly dispersed throughout the embryos (Figure 2D and G). Interestingly, T antigen was strongly expressed on the CNS during late embryogenesis (Figure 2G). To confirm that T antigen is expressed on the CNS, we coimmunostained embryos with an anti-T antigen antibody and mAb BP102, a CNS marker (Figure 2B, E and H). We did not detect colocalization of T antigen and BP102 on the CNS at stage 11 (Figure 2C), but did find colocalization after stage 13 (Figure 2F and I). These observations show that T antigen is expressed in the developing CNS during embryogenesis.

T antigen is also expressed on a subset of embryonic hemocytes

The expression pattern of T antigen during embryogenesis was similar to the distribution of Drosophila embryonic hemocytes (Tepass et al. 1994; Cho et al. 2002), except for the CNS. Drosophila embryonic hemocytes derive exclusively from the mesoderm of the head region of stage 8 embryos and disperse along several invariant migratory paths throughout the embryo during late embryogenesis (Cho et al. 2002). To investigate whether T antigen is expressed in embryonic hemocytes, we carried out coimmunostaining of embryos carrying Cg25C-lacZ with an anti-T antigen antibody and an anti-β-galactosidase antibody. This fly line has the enhancer trap insertion, A109.1F2, inserted into the promoter region of the collagen IV gene, located cytologically at 25C (Cg25C), and expresses β-galactosidase in embryonic hemocytes (Bellen et al. 1989; Wilson et al. 1989). A subset of hemocytes expressing Cg25C-lacZ coexpressed T antigen in their cytoplasm; there were also indications that the antigen might also be expressed on hemocyte cell surfaces (Figure 3A–F). In Figure 3A–F, T antigen is simultaneously expressed on the CNS but, because of the narrow focal plane of confocal microscopy, it is out of focus.

The S2 cell line was derived from a primary culture of late-stage Drosophila embryos (Schneider 1972). It has macrophage-like phagocytic properties and is believed to be derived from embryonic hemocytes (Rame et al. 2001). Interestingly, our real-time PCR data showed that the CG9520 transcript is highly expressed in the S2 cells. To investigate whether T antigen was expressed in the S2 cells, we stained them using an anti-T antigen antibody. T antigen expression was detected on the S2 cells; it is also worth noting that a large focus of T antigen was often observed at the contact point between two S2 cells (Figure 3G).

CG9520 synthesizes T antigen on the CNS during embryonic development

As described above, CG9520 and CG8708 were the candidates for synthesizing T antigen in vivo. Two mutant lines, CG9520$^{EY13370}$ and CG9520$^{KG02976}$, have a P-element insertion in the CG9520 gene region. Although the P-elements are located in the first intron of the CG9520 gene in both lines (Figure 4A), CG9520$^{KG02976}$ homozygotes develop normally whereas some CG9520$^{EY13370}$ homozygotes die during development and escape homozygous CG9520$^{EY13370}$ adult flies have abnormal legs (data not shown). One CG8708 mutant line, CG8708$^{KG05736}$, contains a P-element inserted into the second exon of the CG8708 gene (Figure 5A). The levels of the CG9520 and CG8708 transcripts in mutant third instar larvae were measured by quantitative real-time PCR. In third instar larvae homozygous for CG9520$^{KG02976}$ or CG9520$^{EY13370}$, the level of the CG9520 transcript was, respectively, reduced to 60% and 15% of that of wild-type third instar larvae (Figure 4B). In addition to the decrease in the CG9520 transcript, the extracts of CG9520$^{EY13370}$ third instar larvae had a galactosyltransferase activity of 2.8 pmol/h mg protein (44% of that of wild-type) using GalNAc–pNph as the acceptor (Figure 4C). Third instar larvae homozygous for CG8708$^{KG05736}$ had less than 28% of the CG8708 transcript present in wild type (Figure 5B).

To determine whether CG9520 or CG8708 synthesizes T antigen during embryogenesis, we carried out immunostaining of CG9520 (Figure 4D–F) and CG8708 (Figure 5C and D) mutant embryos with an anti-T antigen monoclonal antibody. Although the expression pattern of T antigen on hemocytes was similar in CG9520$^{KG02976}$ embryos to that of wild-type embryos, the expression level of T antigen on the CNS was slightly decreased (Figure 4E). In CG9520$^{EY13370}$ embryos, we could only detect low levels of the expression of T antigen on hemocytes and did not detect any expression on the CNS (Figure 4F). Furthermore, the expression pattern of T antigen was normal in CG9520$^{KG02976}$ embryos following precise excision of the insertion into the promoter region of the collagen IV gene, located cytologically at 25C (Cg25C), and expresses β-galactosidase in embryonic hemocytes (Bellen et al. 1989; Wilson et al. 1989).
Fig. 1. Phylogenetic tree of *Drosophila*, *C. elegans*, and vertebrate C1β3GalTs and quantitative analyses of the CG9520 and CG8708 transcripts. (A) Dendrograms showing the relationship of human, mouse, rat, *C. elegans* and *Drosophila* C1β3GalTs, and putative chicken, Xenopus and zebrafish C1β3GalTs. The dendrograms were constructed using the amino acid sequences with the Clustal X program. The branch length indicates the evolutionary distance between each member. The scale at the bottom represents evolutionary distance. (B) mRNAs were prepared from embryos and larvae at several developmental stages, from pupae and adult flies (top and bottom left panels) and from tissues and cultured cells (top and bottom right panels). The expression levels of the CG9520 and CG8708 transcripts were normalized to that of the RpL32 transcript, which was measured in the same cDNAs. Experiments were repeated three times.

P-element (Figure 4G). In contrast, T antigen was not decreased in *CG8708KG05736* embryos (Figure 5D). These results clearly demonstrated that CG9520 synthesizes T antigen on the CNS in *Drosophila* embryos, including on hemocytes.

We performed PNA-lectin blot analyses of wild-type and *CG9520EY13370* embryo extracts at several developmental stages (Figure 4H–J). The expression level and pattern of T antigen changed during embryogenesis. In particular, the zygotic expression of T antigen was strong at late embryogenesis between 13 and 19 h (stages 16 and 17) (Figure 4H–J, lane 3). Moreover, the expression of T antigen was drastically reduced in *CG9520EY13370* embryos (Figure 4H–J, lane 4). These results
are consistent with the expression pattern of T antigen obtained from the immunohistochemical analyses (Figures 2 and 4D–G) and also strongly support our conclusion that CG9520 synthesizes T antigen on the CNS. T antigen was strongly expressed on the CNS in late embryos at stage 16 (Figure 2G and I). We detected six bands carrying T antigen (i–vi) at stages 16 and 17 (Figure 4H–J, lane 3). Consequently, the new bands detected in late embryos (i–vi) at 173, 151, 119, 113, 59.0, and 56.6 kD were good candidates for core-proteins carrying T antigen expressed on the CNS.

The above results showed that CG9520 synthesizes T antigen expressed in Drosophila embryos. Therefore, we have named the CG9520 gene as Drosophila core 1 β1,3-galactosyltransferase 1 (dC1β3GalT1).

The number of circulating hemocytes is reduced in third instar larvae of dC1β3GalT1 mutant

Although T antigen is normally expressed on the Cg25c-positive embryonic hemocytes (Figure 3A–F), T antigen on hemocytes was partially reduced in CG9520EY13370 mutant embryo (Figure 4F). To investigate whether T antigen functions in hemocyte development, we carried out immunostaining of embryos with 5H7 monoclonal antibody, which recognizes MDP-1/Papilin, one of the components of basement membrane deposited by hemocytes (Hortsch et al. 1998). In CG9520EY13370 mutant embryo, there were no apparent defects on the staining pattern of hemocytes at stage 11 (Figure 6A and B). It is reported that embryonic hemocytes persist through metamorphosis and that larval hemocytes are released by larval lymph glands just at the onset of pupation (Holz et al. 2003). To confirm whether the lack of T antigen affects embryonic hemocyte development by the late third instar larval stage, we counted the number of plasmatocytes in CG9520EY13370 mutant larvae. In embryo, hemocytes are composed of approximately 700 plasmatocytes, which migrate through the embryo, and about 30 crystal cells that localized around the proventriculus (Tepass et al. 1994; Lebestky, Chang, et al. 2000). Therefore, most of T antigen-positive hemocytes are considered as plasmatocytes.
In this study, we have demonstrated that T antigen is normally expressed on the embryonic CNS and that dC1β3GalT1 synthesizes the antigen in Drosophila melanogaster. In the dC1β3GalT1 mutant, CG9520EY13370, we could not detect T antigen on the CNS (Figure 4F). This defect was rescued by precise excision of the P-element inserted into the CG9520 gene (Figure 4G). Moreover, CG9520EY13370 embryos that survived later in development showed a dramatic reduction of T antigen on their core proteins (Figure 4H–J, lane 4). In addition, the galactosyltransferase activity of CG9520EY13370 third instar larvae was 44% of that of wild type (Figure 4C). The Berkeley Drosophila Genome Project gene expression database indicates that the CG9520 transcripts are expressed in the CNS (http://www.flyexexpress.net/), while Muller et al. (2005) did not detect its transcript in the CNS by in situ hybridization. Due to the weak mRNA expression on CNS, this might have been overlooked. A small amount of mRNA might be enough to synthesize T antigens on mucin proteins and glycolipids because of high enzymatic activity of dC1β3GalT1 (Muller et al. 2005). Taken together, these data demonstrate that CG9520 synthesizes T antigen during development.

Although T antigen disappeared from the CG9520EY13370 CNS (Figure 4F), there was no significant structural defect, even in heterozygous combination with CG9520 deficient chromosome Df(2L)Exel7040. The ratios of defective embryos in the formation of CNS were as follows: 1.4% of Oregon R (n = 648), 3.9% of Canton S (n = 257), 2.2% of CG9520EY13370/CG9520EY13370 (n = 503), and 5.3% of CG9520EY13370/Df(2L)Exel7040 (n = 376). These data suggest that dC1β3GalT1 may have redundant functions or only partly participate in the embryonic CNS development. As proteins or lipids modified by T antigen localize on the surfaces of cells, T antigen may regulate the binding affinity of adhesion molecules. It is also generally assumed that many null mutants having a defect in a cell adhesion protein do not show obvious phenotypes during embryonic CNS development. Mutations in the armadillo, Fasciclin 2, Fasciclin 3, Neuroractin, Neurexin IV, or Neuroglian genes (Patel et al. 1987; Peifer and Wieschaus 1990; Baumgartner et al. 1996; Schuster et al. 1996; Speicher et al. 1998) have phenotypes restricted to defasciculation of

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**Discussion**

In this study, we have demonstrated that T antigen is normally expressed on the embryonic CNS and that dC1β3GalT1 synthesizes the antigen in Drosophila melanogaster. In the dC1β3GalT1 mutant, CG9520EY13370, we could not detect T antigen on the CNS (Figure 4F). This defect was rescued by precise excision of the P-element inserted into the CG9520 gene (Figure 4G). Moreover, CG9520EY13370 embryos that survived later in development showed a dramatic reduction of T antigen on their core proteins (Figure 4H–J, lane 4). In addition, the galactosyltransferase activity of CG9520EY13370 third instar larvae was 44% of that of wild type (Figure 4C). The Berkeley Drosophila Genome Project gene expression database indicates that the CG9520 transcripts are expressed in the CNS (http://www.flyexpress.net/), while Muller et al. (2005) did not detect its transcript in the CNS by in situ hybridization. Due to the weak mRNA expression on CNS, this might have been overlooked. A small amount of mRNA might be enough to synthesize T antigens on mucin proteins and glycolipids because of high enzymatic activity of dC1β3GalT1 (Muller et al. 2005). Taken together, these data demonstrate that CG9520 synthesizes T antigen during development.

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particular axons, partial disorganization of major tracts and nerve roots, or other localized adhesion defects. Such localized effects might explain why $CG9520^{EY13370}$ embryos showed no obvious defects in the CNS.

In addition to the embryonic CNS, we found that T antigen is expressed on embryonic hemocytes. While it was hard to detect T antigen on the CNS of $CG9520^{EY13370}$ mutant embryos, we were still able to detect T antigen on hemocytes.
The \textit{CG9520}\textsuperscript{EY13370} allele resulted from insertion of a P-element into the first intron of the \textit{dCl\beta3GalT1} gene (Figure 4A). \textit{CG9520}\textsuperscript{EY13370} is not a null allele (Figure 4B) and the low level of the \textit{dCl\beta3GalT1} transcript that it produces may be sufficient for synthesis of T antigen on hemocytes, but not on the embryonic CNS. The activity of the \textit{dCl\beta3GalT1} might be different between acceptor substrates on hemocytes and on the embryonic CNS. Alternatively, it is possible that another \textit{Cl\beta3GalT} may be involved in synthesis of T antigen on hemocytes. One of putative \textit{Cl\beta3GalTs}, \textit{CG8708}, could be a candidate for such synthesis as the \textit{CG8708} gene is expressed during embryogenesis (Figure 1B). In order to elucidate the reason why T antigen is expressed on hemocytes of \textit{CG9520}\textsuperscript{EY13370} mutant embryos, we are currently attempting to isolate a \textit{dCl\beta3GalT1} null mutant by imprecise excision of the P-element from the \textit{CG9520}\textsuperscript{EY13370} allele.

We found that T antigen is expressed on S2 cells, which are derived from late-stage embryos and have hemocyte cell-like properties. Previously, it was reported that mucin-D carrying mucin-type O-glycan is expressed in cell lines including the S2 cell line, embryo, imaginal disc, testis, fat body, and larval brain and is localized at cytoplasmic bridges in various germline and somatic tissues (Kramerov et al. 1996, 1997; Kramerova and Kramerov 1999; Theopold et al. 2001). Here, we showed the expression of T antigen in the focal contacts between S2 cells (Figure 3G), although we did not confirm whether the cells in Figure 3G were dividing. Mucin-D localized at a contractile ring might carry T antigen.

Previous studies showed that embryonic hemocytes have a role during CNS condensation in the embryo (Olofsson and Page 2005). We investigated whether T antigen plays a role in embryonic CNS condensation by examining \textit{CG9520}\textsuperscript{EY13370} embryos. In wild-type embryos, condensation results in shortening of the VNC from approximately 80% of embryo length at stage 15 to around 60% at stage 17 (Olofsson and Page 2005). Although some \textit{CG9520}\textsuperscript{EY13370} embryos died during embryogenesis, escaper \textit{CG9520}\textsuperscript{EY13370} embryos showed no observable difference in VNC condensation compared to wild-type embryos (data not shown).
**Drosophila C1β3GalT synthesizes T antigen on the CNS**

**Fig. 5.** *CG8708* locus and mutant analyses. (A) The exon–intron structure of the *CG8708* gene. Open boxes indicate noncoding regions. The scale bar is 200 bp. In *CG8708KG05736*, the P-element (indicated by a triangle) is inserted into the second exon of the *CG8708* gene. The arrow indicates the transcriptional direction of gene. (B) Relative amount of the *CG8708* transcript in *CG8708KG05736* third instar larvae. The expression level of the *CG8708* transcript in wild type is shown as 100%. Experiments were repeated three times. (C and D) T antigen was stained with PNA lectin. Stage 16 embryos: wild type (C), *CG8708KG05736* (D). Dorsal is up and anterior is left. Scale bar: 100 µm.

**Fig. 6.** Number of hemocytes in wild type and *CG9520EY13370*. (A, B) SH7 stained stage 11 embryos. (A) Wild-type embryo and (B) *CG9520EY13370* embryo. (C) Number of circulating plasmatocytes per third instar larva (*n* = 20). The means of wild-type and *CG9520EY13370* were 1975 (SD 1858) and 620 (SD 610), respectively. There is a significant difference between each genotype (*t*-test, *P* = 0.005).

In *CG9520EY13370* mutant, we could not detect obvious difference in the hemocyte development at stage 11 (Figure 6A and B). In contrast, the number of hemocytes was 620, which is small compared to that of wild type, at the third instar larvae stage (Figure 6C). It is believed that the most of embryonic hemocytes derive exclusively from procephalic and gnathal mesoderm, which proliferate up to about 700 cells by stage 11 and that this number remains constant throughout embryogenesis (Tepass et al. 1994). In addition, it is revealed that the number of hemocytes is progressively increased during the larval stage by postembryonic proliferation of embryonic hemocytes (Lanot et al. 2001; Holz et al. 2003). Taken together, these data suggested that the lack of T antigen on hemocytes leads to impede the proliferation or to induce the apoptosis of hemocytes. These facts remind us that both of the thrombocytopenia in C1galt1 mice (Alexander et al. 2006) and Tn-syndrome in human (Berger 1999) exhibit the reduction of platelets. On the other hand, *Drosophila* hemocytes are the macrophage-like cells responsible for the disposal of apoptotic cells and invading microorganisms (Franc et al. 1996, 1999). Our findings indicate that dC1β3GalT1 mutant may have some defects in immune response.

Recently, Xia et al. (2004) suggested that capillary fragility in T-synthase knockout mouse embryos is a consequence of the separation of endothelial cells from supporting pericytes and extracellular matrix. It is also reported that *Drosophila*...
hemocytes secrete ECM (Fessler et al. 1994; Yasothornsrikul et al. 1997; Evans et al. 2003; Wood and Jacinto 2007) that binds to epithelial cells (Kiger et al. 2001). Laminin in hemocyte-like S2 cells has been shown to carry T antigen (Schwientek et al. 2007). These facts suggested that Drosophila hemocytes would be a helpful model system to understand the roles of T antigen in ECM. To fully elucidate the function of T antigen during development, it will be important to identify the core protein and the binding molecules of T antigen. Currently, we are undertaking an investigation to identify these molecules.

Material and methods

Fly stocks
Canton S was used as the Drosophila melanogaster wild type. All fly stocks were raised at 25°C and embryos were collected at the same temperature. We used the following mutant alleles: CG9520KGO2976 (Bloomington), CG9520ETY13370 (Exelixis), CG8708KG05736 (Bloomington), Cg25CA109.1F2 (Kyoto), and Df(2L)Exel7040 (Exelixis). Mutant homozygote were isolated using CyO, wg-lacZ or CyO, Act-GFP balancer chromosome.

Cell culture
Drosophila cell line S2 was cultured in Schneider’s Drosophila medium (Invitrogen), supplemented with 10% FBS at 24°C. The cell line ML-DmBG3A-C2 (BG3A-C2) (gifted from K. Ui-Tei) from Drosophila larval central nervous system was cultured in a M3 (BF) medium with 10% FBS and with 10 mg/mL insulin at 24°C.

Histocytochemistry
For immunohistochemical staining, embryos were dechorionated and fixed in 4% paraformaldehyde with heptane for 20 min. Embryos were then devitellinized in heptane/methanol, transferred into methanol for more than 1 h at −20°C, permeabilized in 0.3% Triton X-100 in PBS (PBST), and blocked in 10% normal goat serum for 15 min. We used following antibodies as a primary antibody: an anti-T antigen (mouse, 1:20, DAKO), an anti-Elav (Rat, 1:10, Developmental Studies Hybridoma Bank; DSHB, University of Iowa, IA), BP102 mAb (mouse, 1:100, DSHB), HPA-Rhodamine (1:300, EY Laboratories, San Mateo, CA), an anti-β-galactosidase (rabbit, 1:1500, Cappel), and 5H7 (mouse, 1:400, Hortsch). Secondary antibodies (1:500) were used

![Diagram](image.png)
obtained from Molecular Probes. FITC-PNA (1:300, Seikagaku, Tokyo, Japan) was also used for the staining of T antigen.

S2 cells were subcultured at a density of 1.0 \times 10^5 cells/mL onto a chamber slide (Nunc) in Schneider’s Drosophila medium containing 10% FBS. After 48 h, the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. The cells were then washed and permeabilized with PBST, blocked in 10% normal goat serum in PBST for 20 min, and incubated with primary antibody for 16 h at 4°C. After being washed, the cells were incubated with secondary antibody for 1 h at room temperature. Confocal images were taken on a LSM 5 Pascal (Carl Zeiss).

**Quantitative analysis of the CG9520 and CG8708 transcripts by real-time PCR**

Total RNA was extracted from CG9520<sup>EY13370</sup>, CG9520<sup>KG02976</sup>, and CG8708<sup>*KG05736</sup> third instar larvae and from wild-type tissues throughout development. Total RNA was also prepared from S2 and BG3-A2 cells. First-strand cDNA was synthesized using RevaTra Dash (TOYOBO, Osaka, Japan). The following gene-specific primers were used: CG9520 forward, 5′-GCCTGGCGAGCTTGTTCC-3′; reverse, 5′-GGCCATCGTATGGCATGAA-3′; probe, 5′-CTATCCACCGGAGCGGAATGA-3′ and CG8708 forward, 5′-GCACTGATACGAGGATCTATACGA-3′; reverse, 5′-GGAGTCCATTTTGCCAGCATG-3′; probe, 5′-ACGACCGAAAAGCCAAAGGCCC-3′. The amount of Ribosomal protein L32 (RpL32) mRNA in each cDNA sample was used to normalize the efficiency of cDNA preparation. The following primers were used: RpL32 forward, 5′-GCAAGCCCAAGGTATCGA-3′; reverse, 5′-CGATGTGGGCGTACGATCTG-3′; probe, 5′-AACAGAGTGCCGTCGCCGCTTCA-3′. The probes were labeled at the 5′-end with the reporter dye, 3FAM, and at the 3′-end with the quencher dye TAMRA (Nippon EGT, Toyama, Japan). Amplifications were performed using 40 cycles of 94°C for 30 s and 60°C for 4 min, with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

**Lectin blot analysis**

Wild-type and CG9520<sup>EY13370</sup> embryos were collected, dechorionated, and then homogenized in 50 mM Tris–HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.1% sodium deoxycholate, 5 mM 2-mercaptoethanol with the protease inhibitors (1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL pepstatin A, 1 μg/mL leupeptin, 2 mM benzamidine) (100 μL for every 10 larvae). The supernatant was obtained by centrifugation at 10,000 × g for 10 min and used as the larval extracts. Uridine diphosphate-[<sup>3</sup>H]galactose (UDP-[<sup>3</sup>H]Gal) (20 Ci/mmol) was supplied by American Radiolabeled Chemicals Inc. p-Nitrophenyl-N-acetyl-α-galactosaminide (GalNAc α-pNph) was purchased from Calbiochem. An assay of galactosyltransferase activity was performed referring to Cummings’ paper (Ju, Cummings et al. 2002). The reaction mixture contained 15 μg of larvae extracts, 100 mM MES (pH 6.8), 2 mM ATP, 20 mM MnCl<sub>2</sub>, 0.2% Triton X-100, 1 mM GalNAc α-pNph, 0.4 mM UDP-Gal (including 1.25 μM UDP-[<sup>3</sup>H]Gal) in volume of 20 μL. After incubation at 25°C for 2.5 h, the reaction was terminated with the addition of 500 μL of water. The reaction mixture was applied to a Sep-Pak C18 cartridges and unreacted UDP-[<sup>3</sup>H]Gal was washed out with water. The products on the column were eluted with methanol and measured the radioactivity using a liquid scintillation counter.

**Collection and counting of circulating hemocytes**

We drew upon the experimental methodology of Zettervall et al. (2004). Wandering late third instar larvae were washed in PBS, and then bled with fine scissors in 20 μL of PBS for 30 s on a siliconized glass plate. Hemocytes-containing PBS was then loaded onto an improved Neubauer hemocytometer (Digital Bio, Seoul, Korea) by a siliconized tip. Twenty larvae of each genotype were counted. Student’s t-test was performed with the statistical package R (http://www.r-project.org/).

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**Conflict of interest statement**

None declared.

**Abbreviations**

C1β3GalT1, core 1 β1,3-galactosyltransferase; CNS, central nervous system; ECM, extracellular matrix; GalNAcα-pNph, p-nitrophenyl-N-acetyl-α-galactosaminide; HPA, helix pomatia; PNA, peanut agglutinin; RpL32, Ribosomal protein L32; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VNC, ventral nerve cord.
References


