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Expression and functional characterization of a nucleotide sugar transporter from *Drosophila melanogaster*: relevance to protein glycosylation in insect cell expression systems

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Abstract

Insect cells are used routinely to express recombinant mammalian glycoproteins. However, insect protein glycosylation pathways are not well understood and appear to differ from those of mammalian cells. One way to more clearly evaluate the protein glycosylation potential of insect cells is to use the *Drosophila melanogaster* genome to identify genes that might encode relevant functions. These genes can then be expressed and the functions of the gene products directly evaluated by biochemical assays. In this study, we used this approach to determine the function of a putative *Drosophila* nucleotide sugar transporter gene. The results showed that this gene encodes a protein that can transport UDP-galactose, but not CMP-sialic acid. Thus, *Drosophila* encodes at least some of the infrastructure needed to produce glycoproteins with complex glycans, but this particular gene product does not directly support glycoprotein sialylation. These findings are relevant to insect cell biology and to an informed consideration of insect cell expression systems as tools for recombinant glycoprotein production.

Keywords

Glycoproteins; Insect expression systems; *Drosophila*; Protein glycosylation; Nucleotide sugar transporter

The development of baculovirus-insect cell [1,2] and constitutive insect cell [3,4] expression systems has stimulated great interest in the nature of the protein glycosylation and glycan processing machinery in these cells. Currently, it seems clear that there is at least one major difference between insect and mammalian protein glycosylation pathways. Specifically, insect cells appear to be unable to produce sialylated glycoproteins (reviewed in [5–8]). The earliest indications of this difference came from a comprehensive survey, which showed that insects had no detectable sialic acids [9]. This finding was extended by biochemical studies, which showed that insect cells had no detectable sialyltransferase activity and produced no detectable sialylated *N*-glycans [10–13]. More recent studies have shown that the major *N*-glycans produced by insect cells are high mannose or paucimannose structures and their major *O*-glycans are monosaccharides or core 1 disaccharides with no terminal sialic acids (reviewed in [5–8]). In addition, recent studies have confirmed the absence of detectable sialyltransferase activity and CMP-sialic acids in insect cells [14,15]. Thus, there is substantial evidence indicating that insects cannot produce sialic acids or sialylated glycoproteins.

On the other hand, it is difficult to draw an unequivocal conclusion from these negative data and some data indicate that insects actually do have sialic acids and can produce sialylated glycoproteins (reviewed in [5]). For example, structural analysis of recombinant human plasminogen produced by baculovirus-infected lepidopteran insect cell lines indicated that this protein had acquired terminal sialic acids [16,17]. In addition, sialic acids have been detected in *Drosophila melanogaster* (Diptera) embryos [18] and in various tissues of the larval, pupal, or adult stages of lepidopteran [19,20], orthopteran [21], and homopteran [22] insects. Two transgenic lepidopteran insect cell lines engineered to express mammalian β 1,4-galactosyltransferase and α 2,6-sialyltransferase genes produced sialylated glycoproteins, indicating that they must be able to produce CMP-sialic acid and transport it into the Golgi apparatus [23,24]. Finally, three lepidopteran insect cell lines were able to produce sialylated recombinant glycoproteins when treated with a β N-acetylglucosaminidase inhibitor, indicating that they have all the machinery needed to produce complex, terminally sialylated N-glycans when the action of this putative N-glycan trimming enzyme is inhibited [25].

One way to more clearly evaluate the potential for protein glycosylation in insect cells is to use bioinformatics to search the *D. melanogaster* genome for genes that might be involved in insect glycoprotein biosynthesis. In three recent studies, this approach was used to search the *D. melanogaster* genome for genes encoding proteins involved in sialic acid biosynthesis or glycoprotein sialylation. No relevant genes were identified in the first study [26], a putative sialyltransferase gene was identified in the second [27], and putative N-acetylneuraminic acid phosphate synthase, sialyltransferase, and CMP-sialic acid/UDP-galactose transporter genes were identified in the third [28].

In March 2001, we independently searched GenBank and Flybase for *Drosophila* genes that might be involved in sialic acid biosynthesis or transport, as part of an effort to understand how our transgenic insect cells can produce sialoglycoproteins. This search revealed a putative nucleotide sugar transporter gene, which had already been identified by at least three other groups [29–31], and was identical to one reported by Kim et al. [28]. We subsequently expressed this gene and performed genetic complementation and nucleotide sugar transport assays to directly examine the function of the gene product. The results showed that this gene encodes a protein that can transport UDP-galactose, but not CMP-sialic acid. Thus, this gene product does not directly support glycoprotein sialylation and its presence in the *Drosophila* genome does not support the idea that insect cells can produce sialylated glycoconjugates.

Materials and methods

Cells

CHO¹-KI (ATCC CCL 61), Lec 2 (ATCC CRL-1736; [32,33]), and Lec 8 (ATCC CRL-1737; [32,34,35]) cells were obtained from the American Type Tissue Culture Collection (Manassas, VA). Each was routinely maintained as an adherent culture in Alpha Minimum Essential Medium (α -MEM) containing L-glutamine, ribonucleosides, and deoxyribonucleosides (Invitrogen Life Technologies, Carlsbad, CA). This medium was supplemented with 10% (v/v) fetal bovine serum (Hy-Clone; Logan, UT) and 0.22% (w/v) sodium bicarbonate and the cells were cultured in a humidified atmosphere containing 5% CO₂ at 37 °C. Schneider's *Drosophila* line 2 (S2; [36]) cells were also obtained from the

¹Abbreviations used: ATCC, American Type Culture Collection; CHO, Chinese hamster ovary; EST, expressed sequence tag; GS-II, *Griffonia simplicifolia* lectin; MEM, minimum essential medium; PNA, *Arachis hypogaea* lectin from peanut; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

American Type Culture Collection (ATCC CRL-1963) and maintained as suspension cultures in HyQ SFX-Insect medium (HyClone) under atmospheric conditions at 28 °C.

Construction of expression plasmids

pcDNA3.1/V5-His-TOPO is a commercial plasmid that can be used to express foreign genes in mammalian cells under the transcriptional control of a cytomegalovirus IE promoter (Invitrogen Life Technologies). A DNA fragment containing the full-length dNST-1 open reading frame was produced by PCR, gel-purified, and inserted into this plasmid. The template used for the PCR amplification was genomic DNA prepared from log-phase S2 cells with a commercial reagent according to manufacturer's instructions (Tri Reagent, Molecular Research Center, Cincinnati, OH). The sense primer was 5'-GACATGGGCGCTCCTGCCCCG-3' and the anti-sense primer was 5'-CTAGACGCGCGGCAGCAGCT-3'. PCRs were performed using a hot start, touchdown protocol, in which the annealing temperature in the first cycle was 65 °C and was reduced one degree per cycle to 55 °C, which was the annealing temperature used for the tenth through thirtieth cycles. In each cycle, template DNA was denatured at 94 °C for 50s, primers were annealed at various temperatures for 1 min, as described above, and extensions were done at 72 °C for 3 min. A mixture of Taq (Promega, Madison, WI) and Pfu Turbo (Stratagene, La Jolla, CA) polymerases was used to increase fidelity while ensuring addition of the single nucleotide overhangs needed for cloning of the PCR product by the TOPO-TA method [37]. The PCR product in multiple clones was sequenced and one clone encoding the wild type dNST-1 protein was assembled from fragments of two independent clones.

An analogous construct encoding dNST-2 was produced using a DNA fragment of an EST clone (LD37122) encoding this full-length open reading frame. The fragment was excised with *EcoRV* and *DraI*, treated with Taq polymerase, gel-purified, and subcloned into pcDNA3.1/V5-His-TOPO by the TOPO-TA method.

The genetic structures of both expression plasmids were confirmed by restriction mapping and/or sequencing. Each was extracted from large-scale bacterial cultures by the alkaline lysis method [38] and purified by equilibrium centrifugation on continuous ethidium bromide–CsCl gradients [39] for subsequent use in experiments.

Transient expression and genetic complementation assays

Freshly seeded CHO-K1, Lec 2, or Lec 8 cells were allowed to adhere to the surfaces of 75cm² culture flasks at 37 °C and then the cells were transfected with no DNA or the expression plasmids encoding dNST-1 or dNST-2 using a commercial liposome-mediated transfection kit (LipofectAMINE 2000; Invitrogen-Life Technologies) according to manufacturer's instructions. Six hours after transfection, the medium was replaced with α -MEM and the cells were incubated for another 18 h at 37 °C. Subsequently, the cells were washed with an appropriate buffer and stained with various FITC-conjugated lectins for 1 h at 4 °C. The cells were then washed with the same buffer and examined using a Zeiss Axiovert 25 microscope equipped for epifluorescence. The lectins used in this study were *Arachis hypogaea* lectin from peanut (PNA; EY Laboratories; San Mateo, CA), which is specific for β -linked galactose residues, and Griffonia simplicifolia lectin (GS-II; EY Laboratories), which is specific for terminal *N*-acetylglucosamine residues. The buffer used to wash the cells before and after PNA or GS-II staining was 10mM HEPES, pH 7.5, containing 0.15M NaCl, or 10mM sodium phosphate, pH 7.3, containing 0.15M NaCl and 0.5mM CaCl₂, respectively.

Transformation of CHO cell mutants and isolation of microsome fractions

Lec 2 and Lec 8 cells were transfected with no DNA or the expression plasmid encoding dNST-1, which also encodes neomycin phosphotransferase under the control of an SV40 promoter, using the same method described above. After a three-day recovery period, the growth media were replaced with fresh α -MEM containing 0.3 mg neomycin per ml α -MEM. One week later, the media were replaced with fresh α -MEM containing 1 mg neomycin per ml α -MEM. After another week in neomycin, the cells transfected with no DNA were completely dead, while the cells transfected with the expression plasmid were healthy and growing in the presence of the antibiotic. Subsequently, these polyclonal transformed cell populations were routinely maintained in α -MEM containing 0.5 mg neomycin per ml. Microsomal membrane fractions were prepared and in vitro nucleotide sugar transport assays were performed using modifications of previously described procedures [40–43]. Briefly, the cells were washed with phosphate-buffered saline (PBS; 10 mM sodium phosphate, pH 7.4, 0.14 M NaCl), Dounce homogenized in 10 mM Hepes (pH 7.2) containing 250 mM sucrose and 1 mM EDTA, and the nuclei were pelleted by low speed centrifugation. The post-nuclear supernatant was applied to step gradients consisting of 0.4 and 1.25 M sucrose prepared in 10 mM Hepes (pH 7.2) containing 1 mM EDTA. The gradients were centrifuged for 1 h at 40,000 rpm at 4 °C in an SW41 rotor in an Optima XL-100K ultracentrifuge (Beckman Coulter Instruments, Fullerton, CA). The membrane bands were harvested at the 0.4–1.25 M sucrose interphase, diluted, and pelleted in the SW41 rotor for 20 min at 26,000 rpm and 4 °C in the same centrifuge. Finally, the pellets were resuspended in 1 $\times$ transport assay buffer (STKM: 10 mM Tris-HCl, pH 7.6; 0.25 M sucrose, 150 mM KCl, and 1 mM MgCl₂) and stored at –85 °C. The bicinchoninic acid assay [44] was used to quantify total protein in aliquots of the membrane preparations treated with 1% Nonidet-P40, with bovine serum albumin as the standard. The in vitro nucleotide sugar transport assays included 10 μ g total protein from the membrane preparations in a total volume of 100 μ l STKM buffer containing 0.3 μ Ci UDP-galactose, [galactose-1-³H(N)] (Perkin-Elmer Life Sciences, Boston, MA; 9.1 Ci/mmol) or CMP-sialic acid, [sialic-9-³H] (American Radiolabeled Chemicals, St. Louis, MO; 20 Ci/mmol). These mixtures were incubated for 10 min at 30 °C and then filtered through 0.45 μ m nitrocellulose disks (Type HA; Millipore, Bedford, MA). The disks were washed with 1 $\times$ STKM buffer, dried, placed in 7 ml Ultima Gold F scintillation cocktail (Packard Instrument Company; Meriden, CT), and counted in a Model LS-6500 liquid scintillation spectrometer (Beckman Coulter).

PCR analysis of dNST-1 and dNST-2 expression

Drosophila cDNA prepared from 21 h embryos was purchased from BD Biosciences Clontech (Palo Alto, CA). This cDNA was analyzed for evidence of dNST-1 or dNST-2 expression by PCRs with sense primers that were specific for each alternatively spliced transcript and a common antisense primer. The sequences of these primers were: dNST-1-sense 5'-GACATGGCGCTCCT GCCCGC-3'; dNST-2 sense 5'-ACCATGAATAGCATACAC-3', and antisense 5'-GTCGAAGATCCTACTG CCG-3'. Hot start PCRs were performed with 1 ng cDNA and 35 cycles of (i) 30 s of denaturation at 94 °C, (ii) 30 s of annealing at 50 °C, and (iii) 2 min of extension at 72 °C. PCRs performed using genomic DNA from S2 cells served as the positive control for these experiments and PCRs performed with no template DNA, sense primer alone, or antisense primer alone served as the negative controls. Spent PCRs were analyzed by agarose gel electrophoresis with ethidium bromide staining.

Results

Computer-assisted analysis of a putative *Drosophila* nucleotide sugar transporter gene

Our initial searches of Flybase and GenBank identified a cosmid clone containing a fragment of the *D. melanogaster* X chromosome that encodes eight putative genes [29]. Among these, one (CG2675) had been identified in both databases as a putative nucleotide sugar transporter gene. The splicing pattern used to produce the open reading frame had been determined in silico using Gene Finder. We independently obtained the same result with Genscan [45] and named this open reading frame dNST-1 (Fig. 1A). However, a Flybase error report indicated that this gene might have three exons, instead of two [46], and we named the open reading frame resulting from this latter splicing pattern dNST-2 (Fig. 1A). The database correction was apparently based on the fact that the 5' end of the dNST-1 open reading frame did not match that of an expressed sequence tagged (EST) clone (LD37122) from the Berkeley *Drosophila* Genome Project [47]. Thus, dNST-1 and dNST-2 encode slightly different proteins, which begin with either 17 or 6 unique amino acids, respectively, followed by an identical sequence of 351 amino acids (Fig. 1B). In December 2001, there were at least 10 different EST clones in GenBank representing dNST-2 and none representing dNST-1 (data not shown). Thus, dNST-2 appears to be the major spliced transcript derived from the putative nucleotide sugar transporter gene, but the possibility that dNST-1 is also produced by differential splicing has not been eliminated.

The results of a BLAST-P [48] analysis confirmed that the theoretical proteins encoded by dNST-1 and dNST-2 are most closely related to UDP-galactose, UDP-*N*-acetylglucosamine, and CMP-sialic acid transporters. A CLUSTAL W [49] alignment of the theoretical *Drosophila* proteins with these human nucleotide sugar transporters is shown in Fig. 2. Generally, all known nucleotide sugar transporters belong to a family of structurally related type III transmembrane proteins [50]. The highly conserved regions found in these proteins are also found in both of the putative *Drosophila* proteins, as well. However, none of these conserved amino acids are found at the extreme N-termini of the putative *Drosophila* proteins, suggesting that the differences in their amino acid sequences would not directly influence their putative transport functions. One would expect the putative dNST-1 and dNST-2 proteins to transport the same nucleotide sugar because the substrate specificities of the human UDP-galactose and CMP-sialic acid transporters are known to be determined by internal transmembrane helices, not their terminal regions [51]. Statistical analyses of the overall amino acid sequence comparisons predicted that the putative fly proteins would most likely transport UDP-galactose (Table 1). The phylogenetic tree shown in Fig. 3 supports this conclusion. However, it is important to note that amino acid sequence conservation does not necessarily reveal the substrate specificities of nucleotide sugar transporters, so these must be determined experimentally [50].

Genetic complementation analysis of putative *Drosophila* nucleotide sugar transporter genes

The functions of the putative *Drosophila* gene products were determined by assessing their ability to rescue the deficiencies in certain well-characterized Chinese hamster ovary (CHO) mutant cell lines [52]. The specific mutant cell lines used in this study were Lec 2, which has no functional CMP-sialic acid transporter [32,33], and Lec 8, which has no functional UDP-galactose transporter [32,34,53]. CHO, Lec 8, and Lec 2 cells were transfected with no DNA or with expression plasmids encoding dNST-1 or dNST-2 and then the cells were stained with various FITC-conjugated lectins and examined under a fluorescence microscope, as described in Materials and methods. The results of these assays demonstrated that dNST-2 rescued the Lec 8, but not the Lec 2 phenotype. That is, Lec 8 cells were intensely stained with GS-II, which is specific for the terminal *N*-acetylglucosamine

residues found on the surface glycoproteins of these cells, but staining was greatly reduced when these cells were transfected with dNST-1 or dNST-2 (Fig. 4A). This result indicated that expression of either dNST-1 or dNST-2 restored the ability of Lec 8 cells to transport UDP-galactose, which allowed these cells to produce cell surface glycoproteins with galactose-capped glycans, which, in turn, reduced GS-II staining. Similarly, Lec 2 cells were intensely stained with PNA, a lectin specific for the terminal galactose residues on the surface glycoproteins of these cells, but, in this case, the staining pattern was unaltered when the cells were transfected with dNST-1 or dNST-2 (Fig. 4B). This indicated that neither dNST-1 nor dNST-2 could restore the ability of Lec 2 cells to transport CMP-sialic acid, which would have restored their ability to produce cell surface glycoproteins with sialic acid-capped glycans and eliminated PNA staining, as was observed with the wild type CHO cell control. Together, the results of the genetic complementation assays suggested that the putative *Drosophila* nucleotide sugar transporter gene encodes a UDP-galactose, not a CMP-sialic acid transporter, and either the dNST-1 or the dNST-2 protein can provide this function.

Ability of putative *Drosophila* nucleotide sugar transporter genes to drive nucleotide sugar transport in vitro

The function of the putative *Drosophila* nucleotide sugar transporter gene was examined further using biochemical assays that directly measure the transport of UDP-galactose or CMP-sialic acid into microsomal vesicles in vitro [42]. Lec 8 and Lec 2 cells were transfected with the expression plasmid encoding dNST-1, which also encodes a selectable marker, and neomycin-resistant transformants were selected, as described in Materials and methods. Microsomal membrane fractions were then prepared from the parental, mutant, and transformed cells and each was used for in vitro nucleotide sugar translocation assays, as described in Materials and methods. The results of these assays showed that membranes from Lec 8 cells transformed with dNST-1 could transport UDP-galactose about as well as the parental CHO cells (Fig. 5A). In contrast, membranes from Lec 2 cells transformed with the same gene failed to transport CMP-sialic acid any better than membranes from the original mutant cell line (Fig. 5B). The transformed cells had the same lectin staining patterns as the transiently transfected cells. That is, transformation of the Lec 8 cells eliminated GS-II staining, while transformation of the Lec 2 cells did not change the PNA staining pattern (data not shown). These data showed directly that the putative *Drosophila* nucleotide sugar transporter gene encodes a UDP-galactose, not a CMP-sialic acid transporter.

Expression of the *Drosophila* UDP-galactose transporter gene

Our next goal was to confirm that the *Drosophila* UDP-galactose transporter gene is expressed, as indicated by the presence of EST hits in the databases. Polymerase chain reactions (PCRs) were performed using a commercial *Drosophila* cDNA preparation and primers were designed to specifically amplify the two alternatively spliced gene products, as described in Materials and methods. The results provided no evidence of dNST-1 (Fig. 6A), but clear evidence of dNST-2 expression (Fig. 6B). These results showed that this *Drosophila* nucleotide transporter gene is expressed and supported the conclusion that dNST-2 is the major spliced gene product. Conversely, dNST-1 appears to be an artifact of the splicing algorithms used to analyze this gene, or is expressed at extremely low levels, or is expressed in a developmental stage or tissue that was not examined in this experiment.

Discussion

While there is significant interest in using insect cells to produce recombinant mammalian glycoproteins, this approach is potentially limited by the inability of insect cells to produce

structurally authentic carbohydrate side chains. This controversial issue focuses on the ability of insect cells to produce sialic acids and terminally sialylated glycoproteins. Unfortunately, our current knowledge of the glycan processing machinery in insect cells precludes a clear analysis. One way to gain additional knowledge is to use bioinformatics to analyze the *Drosophila* genome for genes involved in glycoprotein biosynthesis. Recently, this approach provided some interesting results that are sure to stimulate further discussion on this topic [26–28].

Our study began with an examination of the *Drosophila* genome for evidence of genes involved in sialic acid biosynthesis and transport. This search was motivated by our previous finding that transgenic lepidopteran insect cells engineered to express mammalian glycosyltransferases were able to produce sialylated *N*-glycoproteins [23,24]. This result indicated that these cells must have some endogenous infrastructure for glycoprotein sialylation, which would allow them to acquire or produce CMP-sialic acid and transport it into the Golgi apparatus. We reasoned that the identification of fly genes encoding this machinery, which might be accomplished relatively easily, would strengthen the idea that lepidopteran insect cells might have this machinery, as well.

Our search revealed that *D. melanogaster* does encode a putative nucleotide sugar transporter with amino acid similarity to known CMP-sialic acid transporters. The gene encoding this protein had been identified previously as CG2675 in GenBank, Flybase, and in the literature [28–31]. In fact, a report on this gene product appeared in the literature while we were preparing this paper, as will be discussed further below [54]. Two alternatively spliced transcripts were predicted by computer analysis. The one we designated dNST-2 is represented by multiple EST clones in the database [47] and analysis of a *Drosophila* cDNA preparation supported the idea that it is the major spliced transcript derived from CG2675. While we did not detect the alternatively spliced transcript (dNST-1), this negative result does not eliminate the possibility that it, too, might be produced. The results of functional analyses indicated that the slightly different proteins encoded by the two alternatively spliced transcripts would have the same biochemical activity, if produced.

While computer-assisted analyses of the putative nucleotide sugar transporter gene product indicated that it was related to known CMP-sialic acid transporters, they also indicated that it was more closely related to known UDP-galactose transporters. In addition, it is clear from the literature that the specificities of nucleotide sugar transporters cannot necessarily be determined in silico [50]. Therefore, we performed genetic complementation [52] and in vitro nucleotide sugar transport [42] assays to determine the actual function of the CG2675 gene product. These assays were done in conjunction with two well-characterized mutant CHO cell lines, lacking functional UDP-galactose or CMP-sialic acid transporters. The results demonstrated that the *Drosophila* CG2675 gene encodes a UDP-galactose transporter, not a CMP-sialic acid transporter. Transfection of Lec 2 cells with this gene had no influence on their PNA staining pattern, while transfection of Lec 8 cells clearly altered their GS-II staining pattern. Furthermore, transformation of Lec 2 cells with this gene did not restore the ability of their microsomes to transport CMP-sialic acid, whereas transformation of Lec 8 cells did restore the ability of their microsomes to transport UDP-galactose in vitro. Finally, it must be noted that the results obtained in this study are perfectly consistent with those of a similar functional analysis of the CG2675 gene product by another group, which were published while we were finishing this paper [54].

The experimental demonstration that CG2675 encodes a UDP-galactose transporter certainly does not preclude the possibility that *D. melanogaster* encodes a CMP-sialic acid transporter. However, our search failed to identify any other gene with this putative function. One might speculate that CG2675 encodes a promiscuous transporter that can

recognize both UDP-galactose and CMP-sialic acid as substrates. However, the results of our functional assays showed that this is not true. Furthermore, this speculation would be inconsistent with our current understanding of nucleotide sugar transporters, which is that each is specific for an individual nucleotide sugar or can transport substrates with different sugar, but not different nucleotide, moieties [50].

In the final analysis, the results of this study neither provided support for the notion that insects can produce or transport CMP-sialic acid nor did they help us explain how our transgenic lepidopteran insect cells can produce sialylated glycoproteins. However, these results did confirm that *Drosophila* encodes a functional UDP-galactose transporter, which is consistent with the ability of this organism to produce galactosylated glycoconjugates including *O*-glycoproteins [55], glycosaminoglycans [56], and glycolipids [57]. Thus, this study contributed to our growing knowledge of the machinery available for glycoconjugate processing in insect cell systems, which is needed for informed consideration of these systems as tools for recombinant glycoprotein production. In this regard, it is noteworthy that the *N*-glycoproteins produced by *Drosophila* contain no galactose [58,59]. This finding suggests that this insect lacks some other function, independent of the UDP-galactose transporter that is needed to process this subclass of glycoproteins. A prime candidate for the missing function is β 4-galactosyltransferase I. Bioinformatics analyses have identified three putative β 4-galactosyltransferase genes in the *Drosophila* genome [27] and it will be interesting to see if any of these genes encodes an enzyme that functions in *N*-glycoprotein biosynthesis.

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Fig. 1. Analysis of a putative nucleotide sugar transporter gene from *D. melanogaster*. (A) shows two putative splicing patterns proposed to yield the open reading frames designated dNST-1 and dNST-2. The open boxes show the relative positions of the exons encoding the putative nucleotide sugar transporter, with the starting and ending nucleotide sequences shown above. (B) shows an amino acid alignment of the putative nucleotide sugar transporters encoded by dNST-1 and dNST-2. The uppercase white letters in black boxes indicate amino acid identities and the lowercase black letters with no boxes indicate amino acid differences.

```

dNST-1      -----mallpapvty---syshrtvntantLKYISLltLtLQnAilGLsmRYaRTr
dNST-2      -----mnsihmnanntLKYISLltLtLQnAilGLsmRYaRTr
Hu UDP-GlcNAc-T -----mfan-LKYVSLgiLvfQttsLvLtmRYsRTl
Hu UDP-Gal-T maavgaggstaapggpavagalepgtasaahrrLKYISLlavLvQnAsLiLSiRYaRTl
Hu CMP-SA-T -----maaprdnvtllfKlYcLlavmtlmaAvyttialRYtRTs
consensus   -----lKyisL--l-lq-a-l-lsmRY-RT-

dNST-1      --pGdiflSsTAVlmaEfaKlitClflVfneegkdaqkfvrsLHkttiianPmdTLKvcVP
dNST-2      --pGdiflSsTAVlmaEfaKlitClflVfneegkdaqkfvrsLHkttiianPmdTLKvcVP
Hu UDP-GlcNAc-T keeGprylSsTAVvvAEllKimaCillVvykdkcsrlalnrvLHdeilnkPmdTLKlaip
Hu UDP-Gal-T    --pGdrffatTAVvmaEvlKglTClilLlfaqkrgnvkhlvlfLHeavlvqyvdtTLKlaVP
Hu CMP-SA-T     --dkelyfStTAVcitEviKlllsvglaketg-slgrfkasLrenvlgspkelLKlsVP
consensus     ---gd-f-sstAVlmaE--Kli-cl-lvf-e-----Lh--ii--pmdtLKv-vP

dNST-1      SLvYivQNNLLYVsaShLDAATYQVTYQLKILTTAmFavviLrRkLlntQWgaLllLvmG
dNST-2      SLvYivQNNLLYVsaShLDAATYQVTYQLKILTTAmFavviLrRkLlntQWgaLllLvmG
Hu UDP-GlcNAc-T SgiYtLQNNLLYValSnLDAATYQVTYQLKILTTAlFsvsmLskkLgvyQWlsLviLmtG
Hu UDP-Gal-T    SLiYtLQNNLLqYVaiSnLPAATfQVTYQLKILTTAlFsvlmlnRslsrlQWasl11LftG
Hu CMP-SA-T     SLvYavQNNmaflalSnLDAAvYQVTYQLKILpCTAlctVlmlnRtLsklQWsvvmfLcaG
consensus     SlvY-vQNNl-yv--S-LdAATYQVTYQLKILtTAmf-VviL-r-L---QW--l11L--G

dNST-1      ivlVQlaqtgptsgsaggaaaaataassggapeqNrmlGLwAalgACflSGFAGiYFEK
dNST-2      ivlVQlaqtgptsgsaggaaaaataassggapeqNrmlGLwAalgACflSGFAGiYFEK
Hu UDP-GlcNAc-T vafVQwpsdsqlds-----kelsag-----sqfvGLmAvltACfsSGFAGvYFEK
Hu UDP-Gal-T    vaiVQaqqaggggpp-----rpldq-----NpgaGLaAvvasClsSGFAGvYFEK
Hu CMP-SA-T     vtlVQwkpaqatk-----vvveq-----NpllGfgAiaiaVlcSGFAGvYFEK
consensus     i-lVQ-----g-----n--lG1-A-l-ac--SGFAGiYFEK

dNST-1      ILKgaeiSVWmRNvQLsLlsipfGLltcfvndGsrifdqGFFKGYdlfVWylVlLQAgGG
dNST-2      ILKgaeiSVWmRNvQLsLlsipfGLltcfvndGsrifdqGFFKGYdlfVWylVlLQAgGG
Hu UDP-GlcNAc-T ILKketkqSVWiRNiQLgffgsifGLmgvviyDGelvsknGFFqGYnrlrtWivVvLQALGG
Hu UDP-Gal-T    ILKgsagSVWlRNiQLgLfgtalGLvglwaeGtavatrGFFfGYtpaVWgvVlnQAfGG
Hu CMP-SA-T     vLKssdtSLWvRNiQmyLsgiivtLagvylsDGaeikeKGFFYGYtyyVWfvifLasvGG
consensus     iLK----SvWmRNvQ1-l-----g1l--fv-dG--i---GFF-GY---vW-lv11qa-GG

dNST-1      LivAVVVKYADNiLKGFATSLaIiisCvAS-iyiFdFnltlqFsfGAGLViLasifLYGyd
dNST-2      LivAVVVKYADNiLKGFATSLaIiisCvAS-iyiFdFnltlqFsfGAGLViLasifLYGyd
Hu UDP-GlcNAc-T LviAAviKYADNiLKGFATSLsIilStliSyfwlqdfvptsvfflGAILViltatfLYGyd
Hu UDP-Gal-T    LlvAVVVKYADNiLKGFATSLsIvlStvAS-irlFgFhvdplFalGAGLVigavyLYslp
Hu CMP-SA-T     LytsVVVKYtDNimKGFsaaalIvlStiAS-vmlFglqiTltFalGtlLVicvsyLYGlP
consensus     LivavVvKYADNiLKGFatsl-Iiis-vas-i-if-f-lt--F--Lvi--ifLYg--

dNST-1      p-----arsapkptmhgPgGdee-----Kllprv---
dNST-2      p-----arsapkptmhgPgGdee-----Kllprv---
Hu UDP-GlcNAc-T p-----k-----PaGnpt-----Ka-----
Hu UDP-Gal-T    rgaakaiasasasagpcvhqqPpGqppppqlsshrgdlitepflpKlltkvkgs
Hu CMP-SA-T     r-----qdttsiqqgetaske-----rvigv-----
consensus     -----p-g-----kll-----

```

Fig. 2.

Comparison of putative and known nucleotide sugar transporters. This figure shows an alignment of the putative *Drosophila* proteins encoded by dNST-1 and dNST-2 with various human nucleotide sugar transporters. The alignment was created using CLUSTAL-W [49]. The uppercase white letters in black boxes indicate amino acid identities among all the proteins, the uppercase black letters in gray boxes indicate amino acid identities among some of the proteins, and the lowercase black letters with no boxes indicate amino acid differences. The GenBank Accession Nos. are AB021981 for human UDP-*N*-acetylglucosamine transporter (Hu_UDP-GlcNAc-T), D88146 for human UDP-galactose transporter (Hu_UDP-Gal-T), and D87969 for human CMP-sialic acid transporter (Hu_CMP-SA-T).

Fig. 3. Relationships among putative and known nucleotide sugar transporters. This figure shows a phylogenetic analysis of the putative *Drosophila* proteins encoded by dNST-1 and dNST-2 and various human nucleotide sugar transporters, which was created using PHYLIPS Drawtree [60].

Fig. 4. Genetic complementation assays. CHO, Lec 8 (A) or Lec 2 (B) cells were transfected with no DNA or with expression plasmids encoding dNST-1 or dNST-2, as described in Materials and methods. At 48 h after transfection, the cells were washed, treated with FITC-conjugated GS-II (A) or PNA (B), and examined by fluorescence microscopy.

Fig. 5.

In vitro nucleotide sugar transport assays. Lec 8 and Lec 2 revertants were selected by neomycin treatment after transfection of each cell line with an expression plasmid encoding dNST-1 and a neomycin phosphotransferase, as described in Materials and methods. Microsomal membrane fractions were then isolated from CHO, Lec 8, Lec 2, or the revertants (Lec 8 +dNST-1 or Lec 2 +dNST-1) and used for in vitro nucleotide sugar transport assays with [^{3} H]UDP-galactose (A) or [^{3} H]CMP-sialic acid (B), as described in Materials and methods. The results represent the average specific activities measured in triplicate transport assays with standard deviations indicated by the error bars.

Fig. 6.

Expression of dNST-1 and dNST-2. Expression of the *Drosophila* UDP-galactose transporter gene was examined by PCR analysis. The sense primer used for the reactions shown in (A) was specific for dNST-1, whereas that used for the reactions shown in (B) was specific for dNST-2. The reactions shown in lane 1 contained no template, those in lanes 2–4 contained *Drosophila* cDNA, and those shown in lane 5 were positive controls containing *Drosophila* genomic DNA. The reactions shown in lanes 1, 4, and 5 contained both sense and antisense primers, while those shown in lanes 2–3 contained sense or antisense primers alone, respectively. The expected sizes of the products amplified from genomic DNA were 1305 and 1369 bp for dNST-1 and dNST-2, respectively. The expected sizes of the products amplified from cDNA were 791 and 755 bp for dNST-1 and dNST-2, respectively.

Table 1
Comparison of putative *Drosophila* gene products to known human nucleotide sugar transporters

	dNST-1			dNST-2		
	<i>E</i> ^a	<i>B</i> ^b	% ^c	<i>E</i>	<i>B</i>	%
Hu UDP-Gal-T ^d	3e-71	269	50	5e-71	268	51
Hu UDP-GlcNAc-T	2e-68	259	47	2e-68	260	47
Hu CMP-SA-T	3e-56	219	40	2e-56	220	40

^a *E*-value.
^b Bit score.
^c Percentage amino acid identity.
^d Accession numbers for known nucleotide sugar transporters are given in the legend to Fig. 2.