Transporters of nucleotide sugars regulate the availability of these substrates required for glycosylation reactions in the lumen of the Golgi apparatus and play an important role in the development of multicellular organisms. Caenorhabditis elegans has seven different sugars in its glycoconjugates, although 18 putative nucleotide sugar transporters are encoded in the genome. Among these, SQV-7, SRF-3, and CO3H5.2 exhibit partially overlapping substrate specificity and expression patterns. We now report evidence of functional redundancy between transporters CO3H5.2 and SRF-3. Reducing the activity of the CO3H5.2 gene product by RNA interference (RNAi) in SRF-3 mutants results in oocyte accumulation and abnormal gonad morphology, whereas comparable RNAi treatment of wild type or RNAi hypersensitive C. elegans strains does not cause detectable defects. We hypothesize this genetic enhancement to be a mechanism to ensure adequate glycoconjugate biosynthesis required for normal tissue development in multicellular organisms. Furthermore, we show that transporters SRF-3 and CO3H5.2, which are closely related in the phylogenetic tree, share a simultaneous and independent substrate transport mechanism that is different from the competitive one previously demonstrated for transporter SQV-7, which shares a lower amino acid sequence identity with CO3H5.2 and SRF-3. Therefore, different mechanisms for transporting multiple nucleotide sugars may have evolved parallel to transporter amino acid divergence.

Approximately 50% of eukaryotic proteomes are comprised of glycoproteins and proteoglycans (1), which together with glycolipids play many essential cellular functions. These include molecular recognition events that are critical to cell growth and differentiation, inflammation, immune defense, fertilization, and parasitic infection. Assembly of the glycan moieties takes place in the lumen of the endoplasmic reticulum and Golgi apparatus. In this latter organelle, different glycosyltransferases transfer sugars, from nucleotide sugars, to the appropriate acceptors. Nucleotide sugars are mostly synthesized in the cytosol and therefore need to be translocated into the Golgi apparatus before becoming substrates for the corresponding transferases. Studies on these transporter mutants in yeast, mammals, and invertebrates have shown that transport of nucleotide sugars regulates their availability in the Golgi apparatus lumen and thereby the quantity and quality of their reaction products (2–4).

Nucleotide sugar transporters from every organism analyzed to date share common features such as being very hydrophobic proteins with 6–10 transmembrane domains that appear to be homodimers in the membrane (5–7). Their substrate specificity is difficult to predict based on their primary sequence. For example, the highly substrate-specific UDP-GlcNAc transporters from Madin-Darby canine kidney cells and the yeast Kluyveromyces lactis are only 22% identical (8), whereas the substrate-specific mammalian transporters of CMP-sialic acid, UDP-Gal, and UDP-GlcNAc are 40–50% identical (4). Although initial studies suggested that these transporters were specific for only one substrate, more recent studies have shown that several substrates can be transported by a given transporter (4).

The Caenorhabditis elegans genome encodes 18 putative nucleotide sugar transporters based on amino acid sequence homology with the corresponding transporters from other species (Fig. 1). However, analyses of sugars occurring in this nematode suggest that only seven nucleotide sugars should be required for their glycoconjugate biosynthesis. A similar situation occurs in humans (9). This observation raises the question of whether, in multicellular organisms, different proteins transport the same nucleotide sugars in different tissues, whether the transporters have redundant activities in the same tissues, or whether different nucleotide sugar transporters may act in distinct compartments of the secretory pathway.

To date, in-depth biochemical and biological studies have been done with only three C. elegans nucleotide sugar transporters. We showed that SQV-7 transports UDP-Gal, UDP-glucuronic acid (UDP-GlcA), and UDP-GalNac in a competitive manner (10). Recently we characterized the transporter encoded by the CO3H5.2 gene and found that it translocates...
UDP-GlcNAc and UDP-GalNAc in a novel manner that is non-competitive, simultaneous, and independent (11). Previously we had shown that SRF-3 transports UDP-Gal and UDP-GlcNAc (12). In this instance and in virtually all other cases where multisubstrate transporters of nucleotide sugars were described (13–18), a competitive transport mechanism was assumed but not demonstrated.

The partial overlap in substrate specificity among the above three C. elegans transporters, namely UDP-Gal for SQV-7 and SRF-3, UDP-GalNAc for SQV-7 and C03H5.2, and UDP-GlcNAc for SRF-3 and C03H5.2, suggests the possibility that this redundancy may have significant biological implications, particularly for multicellular organisms. We now show that reducing the activity of the C03H5.2 gene product by RNAi in SRF-3 transporter mutants results in oocyte accumulation and abnormal gonad morphology, whereas RNAi in wild type or RNAi hypersensitive C. elegans strains does not cause a detectable phenotype. We further show that transport of substrates via transporter SRF-3 occurs in a non-competitive, independent, and simultaneous manner, similarly to that of C03H5.2 but differently from the competitive transport of SQV-7. Based on the phylogenetic tree of C. elegans nucleotide sugar transporters, we hypothesize that different transport mechanisms may have evolved parallel to amino acid sequence divergence.

**Functional Redundancy of Nucleotide Sugar Transporters**

**Strain Maintenance and Genetics**—Saccharomyces cerevisiae strain PRY225 (ura3-52, lys2-801am, ade2-1020c, his3, leu2, trplΔ1) was grown at 30 °C in liquid yeast extract/peptone/dextrose or on solid yeast extract/peptone/dextrose media containing 2% Bacto-agar. Strains derived from PRY225 transformed with URA plasmids were grown at 30 °C in synthetic complete medium lacking uracil (SC-URA) prepared using SCM-URA (Sigma). All C. elegans strains were grown as described previously. The following strains were used: Bristol N2; srf-3 (yj10); ref-3 (pk1426) (provided by CGC); srf-3 (e2689), srf-3 (e2797) (provided by J. Hodgkin), and BC14237 (C03H5.2 promoter-GFP fusion construct) (provided by C. elegans bioinformatics via the C. elegans expression patterns site). Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the National Center for Research Resources (NCRR) at the National Institutes of Health.

**Radioactive Substrates**—The following radioactive substrates were used in all purchased from American Radiolabeled Chemicals (St. Louis, MO): UDP-[14C]GlA (196 mCi/mmol), UDP-[14C]Gal (258 mCi/mmol), UDP-[1H]GlcNAc (60 Ci/mmol), UDP-[3H]GalNAc (60 Ci/mmol).

**Nucleotide Sugar Transport Assay**—The theoretical basis for the translocation assay of nucleotide sugars into Golgi apparatus-derived vesicles has been described previously (19). Transport assays of C. elegans nucleotide sugar transporter expressed in S. cerevisiae and analyses of the samples were carried out as described previously. Radioactivity was detected using a liquid scintillation spectrometer.

**RNAi Experiments**—RNAi was performed by feeding as described (20). All the RNAi experiments were done using clones from Open Biosystems. These clones contain the entire open reading frame for the corresponding gene, from start to stop, cloned into an RNAi feeding vector (pLa4400-dest-RNAi) and transformed into the compatible host (HT115 (DE3)). Briefly, bacteria transformed with empty plasmid or the corresponding constructs were grown in LB media supplemented with 50 µg/µl ampicillin and 12.5 µg/µl tetracycline for 16 h. Cultures were diluted and induced for 4 h with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside when they reached an A600nm = 0.5. Induced bacteria, resuspended in 50% fresh media, were used to seed nematode growth medium plates containing 1 mM isopropyl-1-thio-β-D-galactopyranoside, 50 µg/µl carbenicillin.

**EXPERIMENTAL PROCEDURES**

**Subcellular Fractionation**—S. cerevisiae transformed with pG426, pG426-SQV-7, or pG426-SRF-3 were grown in SCM-URA (Sigma). All complete medium lacking uracil (SC-URA) prepared using SCM-URA. Strains derived from PRY225 transformed with URA plasmids were grown at 30 °C. Strain PRY225 (ura3-52, lys2-801am, ade2-1020c, his3, leu2, trplΔ1) was grown at 30 °C in liquid yeast extract/peptone/dextrose or on solid yeast extract/peptone/dextrose media containing 2% Bacto-agar. Strains derived from PRY225 transformed with URA plasmids were grown at 30 °C in synthetic complete medium lacking uracil (SC-URA) prepared using SCM-URA (Sigma). All C. elegans strains were grown as described previously. The following strains were used: Bristol N2; srf-3 (yj10); ref-3 (pk1426) (provided by CGC); srf-3 (e2689), srf-3 (e2797) (provided by J. Hodgkin), and BC14237 (C03H5.2 promoter-GFP fusion construct) (provided by C. elegans bioinformatics via the C. elegans expression patterns site). Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the National Center for Research Resources (NCRR) at the National Institutes of Health.

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Functional Redundancy of Nucleotide Sugar Transporters

RESULTS

Tissue Expression and Localization of C. elegans Nucleotide Sugar Transporters CO3H5.2 and SRF-3—In previous studies, the SQV-7 transporter was localized to the vulva, seam cells, distal tip cells, and oocytes, whereas the SRF-3 transporter was detected in seam cells, pharyngeal gland cells, and the spermatheca (12, 21). We used a C. elegans strain BC 14237, which carries an extrachromosomal array with a promoter CO3H5.2-GFP construct, to identify the tissue expression pattern of this nucleotide sugar transporter gene. As shown in Fig. 2, left and right panels, B, D, and F, the GFP fluorescence, driven by the above promoter, suggests that the CO3H5.2 gene has a broad expression pattern, consistent with that reported in WormBase. This includes expression in the pharynx and pharyngeal gland cells, seam cells, spermatheca, stomatointestinal muscle, vulva, and body wall muscle. Therefore, the C. elegans CO3H5.2 and SRF-3 transporters appear to have a partial overlap in tissue expression (pharynx and pharyngeal gland cells, seam cells, and spermatheca), as well as nucleotide sugar transport specificity, that of UDP-GlcNAc.

Functional Redundancy between C. elegans Nucleotide Sugar Transporters CO3H5.2 and SRF-3—We previously showed that mutants lacking SRF-3 activity exhibit only a mild locomotion phenotype and are virtually indistinguishable from wild type animals when grown on E. coli (12). These characteristics, as well as the above partial overlap in tissue and substrate specificity with transporter CO3H5.2, stimulated our interest in determining whether or not functional redundancy occurred between these transporters. We therefore determined the effects of CO3H5.2 RNAi inactivation in wild type, RNAi hypersensitive strain rrf-3, and srf-3 animals when grown on E. coli. In all cases, we used a culture of the same strain fed with bacteria transformed with an empty plasmid as a control.

CO3H5.2 RNAi inactivation in wild type or the RNAi hypersensitive strains resulted in neither morphological defects (Fig. 3, left panel) nor a surface phenotype such as that of srf animals (data not shown) and is consistent with a previous report (22). In contrast, CO3H5.2 inactivation in srf-3 mutants resulted in striking morphological phenotypes. These consisted of an increased accumulation of oocytes in proximal gonad arms, when compared with wild type animals (Fig. 3, right panel, D and F when compared with negative controls A and B). No evidence of sterility or differences in brood size was detected, and each oocyte appeared to have normal morphology with only one nucleus with an intact membrane per cell. We also observed abnormal gonad arms, such as twists and overturns resembling morphological gonad distortion caused by defects during gonad migration (Fig. 3, right panel, F when compared with negative control C). Importantly, no abnormal phenotypes were observed when N2 or srf-3 animals were subject to RNAi against the closely related (43% identical) nucleotide sugar transporter encoded by the ZK896.9 gene (Fig. 1; data not shown). This last experiment rules out that a closely related nucleotide sugar transporter is a secondary target of CO3H5.2 RNAi.

It was also important to rule out the possibility that the above observed defects in srf-3 mutants were the result of unknown mutations in the particular srf-3 strain used. Therefore, the same RNAi experiments were also performed with two independently isolated srf-3 alleles. Fig. 4A shows scores of abnormal morphology of the gonad arms and of oocyte accumulation for each of the three srf-3 alleles. Twenty-three percent of the yj10 animals (n = 65), 25% of the e22689 animals (n = 52), and 19.6% of the e2797 (n = 56) animals displayed misshapen gonad arms (extra turns of distal gonad arms or intersecting gonad arms), whereas less than 4% of control srf-3 animals (empty plasmid RNAi) showed this phenotype. Eleven percent of the yj10 animals, 17.3% of the e2689 animals, and 3.6% of the e2797 animals showed accumulation of oocytes (Fig. 4B), whereas neither CO3H5.2 RNAi in wild type animals nor empty plasmid RNAi in srf-3 animals showed this defect (Fig. 4B). The above experiments therefore strongly suggest that simultaneous inactivation of CO3H5.2 and SRF-3 causes the observed abnormal phenotypes and are consistent with functional redundancy between these nucleotide sugar transporters. The low percentage of e2797 animals showing accumulation of oocytes after...
SRF-3 transport activity is not completely abolished. Animals are barely resistant to this infection, suggesting that the transporter SRF-3, which is closely related to transporter CO3H5.2 in the phylogenetic tree, is also closely related in its function. SQV-7 also shares substrate specificity with SRF-3 (UDP-Gal) and CO3H5.2 (UDP-GalNAc) (10). Furthermore, SQV-7, SRF-3, and CO3H5.2 were shown to be expressed in seam cells (12, 21), whereas both SQV-7 and CO3H5.2 are also expressed in the vulva (21). Nevertheless, the low fertility of sqv-7 mutants does not allow us to use the RNAi approach with these animals to test whether there is also functional redundancy among these transporters.

**Evolutionary Relationship among C. elegans Nucleotide Sugar Transporter Mechanisms**—We have recently demonstrated that transporter CO3H5.2 translocates UDP-GlcNAc and UDP-GalNAc in an independent and simultaneous manner (11). This novel translocation mechanism is different from the competitive and non-cooperative transport of multiple substrates by SQV-7 (10) and prompted us to examine the transport mechanism of the multisubstrate transporter SRF-3 (12). SRF-3 was identified as a C. elegans nucleotide sugar transporter with specificity for UDP-Gal and UDP-GlcNAc (12). It was initially assumed, without detailed studies, that these substrates compete for transport as reported previously for SQV-7 (10).

Examination of the C. elegans nucleotide sugar transporter phylogenetic tree (Fig. 1) indicates that SRF-3 has a high amino acid sequence identity with CO3H5.2 (37.8%) and shares lower identity with SQV-7 (13%). This observation led us to hypothesize that mechanisms of transport of substrates by different nucleotide sugar transporters may have evolved parallel to the divergence among the transporter proteins as shown in Fig. 1. Thus, we postulated that transport of nucleotide sugars via SRF-3 might be similar to that of transporter CO3H5.2 and different from that reported for SQV-7.

To test whether UDP-GlcNAc and UDP-Gal can be transported via SRF-3 in an independent and simultaneous manner as CO3H5.2, we incubated Golgi apparatus-enriched vesicles from S. cerevisiae, which expressed SRF-3, with 2 μM UDP-[14C]Gal alone or with the mixture of 2 μM UDP-[14C]Gal and a 10-fold excess (20 μM) UDP-[3H]GlcNAc. Following the incubation, we measured the radioactivity corresponding to each radionuclide within the vesicles. As can be seen in Fig. 5A, the amount of UDP-[14C]Gal entering the vesicles is not significantly affected by the presence of a 10-fold excess of UDP-[3H]GlcNAc. In addition, this latter substrate is entering the vesicles at the same rate as if it were the only substrate in the reaction mixture (Fig. 5A). This result resembles the one previously described for transporter CO3H5.2 and suggests that transporter SRF-3, which is closely related to transporter CO3H5.2 in the phylogenetic tree, is also closely related in the

C03H5.2 RNAi inactivation is consistent with e2797 mutants displaying a temperature-sensitive Bus phenotype (bacterial unswollen) when grown on *Microbacterium nematophilum* (12). At 16 °C, the temperature used in our experiments, e2797 animals are barely resistant to this infection, suggesting that the SRF-3 transport activity is not completely abolished.
manner by which it transports substrates, namely a non-competitive, independent, and simultaneous manner (11).

It was important to demonstrate that the experimental design shown above could indicate a competitive transport mechanism, had it occurred. Accordingly, we next incubated *S. cerevisiae* Golgi apparatus-enriched vesicles containing the SQV-7 transporter (previously shown via a different approach to have a competitive transport mechanism) with 1 mM UDP-[3H]GalNAc alone or with the mixture of 1 mM UDP-[3H]GalNAc and 10 mM UDP-[14C]GlcA. Following incubations for 4 min, the radioactivity of each radionuclide was measured within vesicles. These are two of the substrates previously shown to be translocated by SQV-7 in a competitive manner (10). Following incubations for 4 min, the radioactivity of each radionuclide was measured within vesicles. Fig. 5B shows that the addition of a 10-fold excess of UDP-GlcA when compared with UDP-GalNAc to the reaction mixture decreased by almost 90% the amount of UDP-GalNAc being transported. This experiment corroborates the competitive transport mechanism described previously for SQV-7 using an entirely different approach (10). The results further demonstrate that the experimental approach used to study the transport mechanism of CO3H5.2 (11) and SRF-3 (this study) would detect a competitive transport mechanism should it occur. Together, our collective studies suggest the possibility that the competitive transport mechanism exhibited by SQV-7 reflects an evolutionary divergence from that of nucleotide sugar transporters CO3H5.2 and SRF-3.

**DISCUSSION**

We have obtained evidence for functional redundancy between nucleotide sugar transporters in a multicellular organism and hypothesize this to be a mechanism required to ensure adequate glycoconjugate formation, which in turn may be necessary for proper tissue development. This requirement might reflect complete redundancy. Both transporters perform exactly the same function, e.g. they translocate the same nucleotide sugar into the same compartment, or alternatively, the transporters may sequentially regulate glycosylation of the same target(s) by translocating different nucleotide sugars or the same nucleotide sugar into different compartments. So far, corresponding changes in glycoconjugates were detected in every instance where mutants defective in nucleotide sugar transport were studied. Unfortunately, such analyses are not feasible in the current study because of the incomplete penetrance of phenotypic defects by RNAi and the lack of a CO3H5.2 mutant. Interestingly, MIG-23, a luminal nucleoside diphosphate phosphatase implicated in the nucleotide sugar transport/antiport cycle (23), is required for proper gonad development due to its role in the glycosylation of MIG-17 (24). The fact that mutations that reduce MIG-23 activity and MIG-17 glycosylation (24) result in gonad abnormalities, similar to CO3H5.2 inactivation of *srf-3* mutants, supports our hypothesis that the combined requirement of CO3H5.2 and SRF-3 for proper gonad morphology may be related to their role in regulating glycosylation. Nevertheless, at this time, we cannot completely rule out that a function of these transporters, unrelated to nucleotide sugar transport, may be the immediate cause for the observed phenotype.

Although genetic enhancement and functional redundancy have been described for many *C. elegans* genes (25, 26), they have not, to our knowledge, been shown for genes encoding proteins of glycosylation pathways in multicellular organisms. In the present instance, nucleotide sugar transporters CO3H5.2 and SRF-3 share partial substrate and tissue specificity (11, 12).
Although srf-3 mutants have no morphological phenotype when grown on E. coli (12) and CO3H5.2 RNAi inactivation does not cause visual morphological defects in wild type animals, inactivation of CO3H5.2 in srf-3 animals leads to abnormal gonad morphology and oocyte accumulation in the proximal gonad arms, strongly suggesting genetic enhancement between these transporters. In C. elegans hermaphrodites, each gonad arm adopts a U-shape, resulting from the migration path of the distal tip cells, a process in which, as mentioned above, the glycoprotein MIG-17 is known to play a role (24). Ovulation requires the passage of oocytes through the spermatheca, a process that requires integrin INA-1, also a glycoprotein, suggesting an additional possible mechanism for the phenotype observed upon inactivation of CO3H5.2 in srf-3 mutants (27). However, it is possible that several target sites occur downstream of the nucleotide sugar transport, resulting in the described phenotype. CO3H5.2 is expressed in the spermatheca, gonad distal tip cell, and body wall muscle (Fig. 2). The phenotypes observed when this transporter is targeted by RNAi might reflect lower transport activity in one or more of these tissues by specifically affecting glycosylation of extracellular or secreted signaling ligand(s) or membrane bound receptor(s). Thus, further studies will be required to determine the specific tissues where functional redundancy occurs and which particular glycoconjugates are subject to this regulation.

Inactivation or mutations in only 20–35% of C. elegans genes result in observable phenotypes (28). This observation implies that many genes encode proteins with functions that may be fully or partially substituted by the activity of other gene products. Thomas (28) suggests four different possible reasons for evolution to have maintained genetic redundancy: (a) to increase the quantity of a particular gene product, (b) to ensure high fidelity for some particular process, (c) to preserve non-overlapping functions of the corresponding gene products, or (d) an emergent property that depends on genetic redundant genes. Thus, genetic redundancy between transporters CO3H5.2 and SRF-3 may be understood in terms of any, several, or all of the above selection mechanisms.

We had previously characterized SQV-7 as a multsubstrate nucleotide sugar transporter in which substrate translocation occurred via a competitive, non-cooperative mechanism (10). Since then, multsubstrate transporters of nucleotide sugars have been described in several organisms, including Leishmania donovani (29), Drosophila melanogaster (16), humans (14), and Entamoeba histolytica (30). In all these instances, a competitive translocation mechanism of substrates has been assumed but not demonstrated.

Very recently, a new nucleotide sugar multisubstrate transporter from C. elegans was characterized, CO3H5.2, with a novel, simultaneous, and non-competitive transport mechanism (11). We have now demonstrated that SRF-3, another C. elegans multisubstrate transporter, has a translocation mechanism that is similar to the one described for CO3H5.2 and different from the one previously shown for SQV-7 (10).

Our collective results regarding different transport mechanisms of the nucleotide sugar transporters of C. elegans raise the intriguing possibility that different nucleotide sugar transport mechanisms may have evolved parallel to the divergent evolution of the transporter proteins themselves in terms of their amino acid sequence. Consistent with this hypothesis is the fact that the phylogenetic tree of the putative nucleotide sugar transporters from the C. elegans genome shows that SRF-3 and CO3H5.2 are more closely related from an evolutionary point of view than SQV-7 with the former two. Further support, or lack thereof, for this hypothesis will be obtained as substrate specificities and mechanisms of other C. elegans nucleotide sugar transporters are elucidated.

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