Expression of Three *Caenorhabditis elegans* N-Acetylglucosaminyltransferase I Genes during Development*

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UDP-N-acetylglucosamine:α-3-β-mannoside β-1,2-N-acetylglucosaminyltransferase I (GnT I) is a key enzyme in the synthesis of Asn-linked complex and hybrid glycans. Studies on mice with a null mutation in the GnT I gene have indicated that N-glycans play critical roles in mammalian morphogenesis. This paper presents studies on N-glycans during the development of the nematode *Caenorhabditis elegans*. We have cloned cDNAs for three predicted *C. elegans* genes homologous to mammalian GnT I (designated gly-12, gly-13, and gly-14). All three cDNAs encode proteins (467, 449, and 437 amino acids, respectively) with the domain structure typical of previously cloned Golgi-type glycosyltransferases. Expression in both insect cells and transgenic worms showed that gly-12 and gly-14, but not gly-13, encode active GnT I. All three genes were expressed throughout worm development (embryo, larval stages L1–L4, and adult worms). The gly-12 and gly-13 promoters were expressed from embryogenesis to adulthood in many tissues. The gly-14 promoter was expressed only in gut cells from L1 to adult developmental stages. Transgenic worms that overexpress any one of the three genes show no obvious phenotypic defects. The data indicate that *C. elegans* is a suitable model for further study of the role of complex N-glycans in development.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank*TM* EBI Data Bank with accession number(s) AF082011 (gly-12), AF082010 (gly-13) and AF082012 (gly-14). The accession numbers of the corresponding genomic DNA sequences are U28735 (gly-12), U28736 (gly-13) and Z46381 (gly-14).

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‡ The abbreviations used are: GnT I, UDP-N-acetylglucosamine:α-3-β-mannoside β-1,2-N-acetylglucosaminyltransferase I (GnT I); CDGS, carbohydrate-deficient glycoprotein syndrome; GnT II, UDP-N-acetylglucosamine:α-6-β-mannoside β-1,2-N-acetylglucosaminyltransferase II (GnT II); nt, nucleotide(s); PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; RACE, rapid amplification of cDNA ends; MES, 2-N-morpholinoethanesulfonic acid; M₆-octyl, Man₆–1Man₆–1Man₆–1Man₆–1Man₆–1Man₆–1; M₆-glycopeptide, (Man₆–16Man₆–13)Man₆–1; UTR, untranslated region; kb, kilobase pair(s).

UDN-N-acetylglucosamine:α-3-β-mannoside β-1,2-N-acetylglucosaminyltransferase I (GnT I) is a key enzyme in the synthesis of Asn-linked complex and hybrid glycans. Studies on mice with a null mutation in the GnT I gene have indicated that N-glycans play critical roles in mammalian morphogenesis. Although somatic Chinese hamster ovary cell mutants lacking the GnT I gene show essentially normal growth, mouse embryos with a null mutation in this gene do not survive beyond 10.5 days postfertilization and show severe developmental abnormalities particularly of the brain (3, 4). Mice with a homozygous null mutation in the gene encoding UDP-N-acetylglucosamine:α-6-β-mannoside β-1,2-N-acetylglucosaminyltransferase II (GnT II) survive to term but are born stunted with various congenital abnormalities and die shortly after birth (5). Carbohydrate-deficient glycoprotein syndrome (CDGS) is a group of congenital diseases in which there is a defect in protein N-glycosylation (6). Children with CDGS types 1 and 2 show severe psychomotor retardation and other multisystemic abnormalities. About 80% of CDGS type 1 children have a defect in the phosphomannomutase gene (7–9). Another variant of CDGS type 1 has been described recently with a defect in the phosphomannose isomerase gene (10). Two children with CDGS type 2 have inactivating point mutations in the GnT II gene (11–13). Several other congenital diseases are associated with defective complex N-glycan synthesis (14).

These studies indicate that although complex N-glycans are not essential for the growth of cells in tissue culture, they play critical roles in mammalian morphogenesis. Complex N-glycans are absent from bacteria (15) and yeast (16) and are present in very small amounts, if at all, in protozoa (Trypanosoma cruzi (17), Leishmania (18), and Plasmodium (19)) and Dictyostelium discoideum (20). All of the above organisms except bacteria are capable of making N-glycans of the oligomannose type. Complex N-glycans are present in most of the multicellular invertebrate and vertebrate animals that have been analyzed (nematodes (21–23), schistosomes (24–26), molluscs (27, 28), insects (29), fish (30), birds (31–34), and mammals) and in plants (35). However, a mutant Arabidopsis plant, which lacks GnT I and is unable to synthesize complex N-glycans, shows no apparent phenotype (36, 37), suggesting that complex N-glycans do not play an essential role in plant development. The data indicate that complex N-glycans appeared in evolution just prior to the appearance of the multicellular organism and that, at least in mammals, they play important roles in the interactions between a cell and its cellular and fluid environment.

Because of the complexities encountered in the study of mammalian development, we have initiated studies on the role of complex N-glycans in the development of a simpler organism, the nematode worm *Caenorhabditis elegans*. Over 80% of the *C. elegans* genome has been sequenced, and detailed information is available on the morphology, development, and physiology of this worm. The GnT I gene (MGATI) has been cloned from several mammalian and nonmammalian species (38). A

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Caenorhabditis elegans N-Acetylglucosaminyltransferase I

computer search of the C. elegans genomic DNA sequence data base for sequences similar to the rabbit GnT I protein sequence using the BLASTP algorithm (39) revealed three homologous sequences, the products of predicted genes F48E3.1, B0416.6, and M01F1.1 (40). We report in this paper the sequences of the cDNAs of these three genes which we have designated gly-12, gly-13, and gly-14, respectively; all C. elegans glycansylation-related genes are named gly (41). We present an analysis of the spatial and temporal pattern of gene expression during C. elegans development and the enzyme activities the genes encode when expressed in insect cells and in transgenic worms. Preliminary reports of this work have appeared (42–45).

**MATERIALS AND METHODS**

**Molecular Biology Procedures**

Unless otherwise stated, standard molecular biology procedures were used (46, 47). Oligonucleotides were synthesized on a Pharmacia DNA synthesizer and purified by the cartridge method (Hospital for Sick Children-Amersham Pharmacia Biotech, Institute, Toronto, Canada). All cDNAs and DNA constructs were sequenced in both directions by the double strand dideoxy method (48) using the Amersham Pharmacia Biotech TT Sequencing Kit.

**Cloning of gly-13 cDNA by Phage Library Screening**

The polymerase chain reaction (PCR) was used to prepare three gly-13 gene-specific probes (based on the genomic sequence; GenBank™ accession number U28735), as follows (see Table I for PCR primer sequences): probe A (142 nt), primers CEBF1 and CEBR2; probe B (145 nt), primers CEBF3 and CEBR4; probe C (168 nt), primers CEBF5 and CEBR6. PCR products were purified by electrophoresis on a 1% agarose gel. DNA probes were labeled with [α-32P]dCTP (Amersham Pharmacia Biotech; 3000 Ci/mmol) using the Amersham Pharmacia Biotech Ready-To-Go DNA labeling kit. The labeled probes were purified using Sephadex G50 DNA grade nick columns (Amersham Pharmacia Biotech). An oligo(dT)-primed C. elegans cDNA library in λgt10 (provided by Drs. S. Kim and H. R. Horvitz, MIT) was screened with a mixture of probes A, B, and C. Positive plaques were rescreened with each probe individually. Only one plaque hybridized to all three probes, and it was purified for further analysis.

**Subcloning and Sequencing of gly-13 cDNA**

Since there is an EcoRI site in the open reading frame of gly-13 near the 3′-end, the full-length gly-13 cDNA was obtained by partial digestion of the λgt10 plaque DNA with EcoRI, subcloned into pGEM7zf+ (Promega), and sequenced. A partial cDNA was also produced by the RT-PCR/3′-RACE procedure (49) using a preparation of C. elegans total RNA as template. An oligo(dT) primer was used for reverse transcription. A gene-species forward primer (Table I) and the adaptor primer (AP2) were used for 3′-RACE (Marathon cDNA amplification kit, CLONTECH).

**Cloning of gly-14 cDNA by RT-PCR**

The gly-14 cDNA was cloned by an RT-PCR approach using the Marathon cDNA Amplification Kit (CLONTECH) as recommended by the manufacturer. Adapter-ligated double stranded cDNA was synthesized by reverse transcription of adult C. elegans total RNA followed by second strand synthesis and ligation of Marathon cDNA adaptor to both ends of the double-stranded cDNA. The Marathon cDNA adaptor has two primer binding sites: AP1 (outer) and AP2 (inner) (see Table I). PCR was then carried out three times in succession using the adaptor-ligated cDNA as template and the following primer pairs (Table I; gene-specific primers based on the genomic sequence, GenBank™ accession number Z46381): CEMR5-AP1 (once) followed by CEMF1-CEMR6 (twice). PCR was also carried out three times in succession using the following primer pairs (Table I): CEMF7-AP1 (once) followed by CEMF8-CEMR4 (twice). The PCR products could be visualized by ethidium bromide staining of agarose gels only after the third round of PCR. Fusion of the two PCR products was carried out by PCR using Vent DNA polymerase (New England Biolabs) and the primer pair CEP1/CPEM1 (Table I) to yield a cDNA fragment encoding the GLY-14 protein sequence containing the STOP codon but lacking 30 amino acids at the amino terminus, including the putative transmembrane domain. This truncated cDNA was subcloned into the pNol and pKpnI sites of the baculovirus transfer vector pV7-Bac-His (kindly donated by Dr. David Joziasse, Vrije Universiteit, Amsterdam) (38) and sequenced. Some of the missing 5′-sequence of gly-14 cDNA was obtained by PCR using the AP2-CEMR10 primer pair (Table I) and adapter-ligated cDNA as template. Attempts to determine the remainder of the 3′-end of the cDNA by 3′-RACE were not successful.

**Cloning of gly-12 cDNA by Phage Library Screening**

A 1.2-kb gly-12 hybridization probe was made by PCR using the Marathon adaptor-ligated C. elegans cDNA (described above) as template and primers CEF2-CEF3 (Table I; gene-specific primers based on the genomic sequence, GenBank™ accession number U28735). The C. elegans cDNA library was screened with this probe, as described above for gly-13. After three rounds of screening with the same probe, nine positive plaques were identified. Phage DNA from five of these plaques was prepared, and inserts were excised with EcoRI, subcloned into pGEM7zf+, and sequenced. A portion of the 3′-end of the gly-12 cDNA (88 nt) was obtained by PCR using adapter-ligated cDNA as template and three successive PCR reactions with primer pairs CEF1-AP1, CEF2-AP2, and CEF5-CEF7, respectively (Table I). Attempts to determine the remainder of the 3′-end of the cDNA by 3′-RACE were not successful.

**Determination of 5′-Ends of gly-12, gly-13, and gly-14 cDNAs**

Total cDNA was prepared by RT-PCR using as substrate total RNA prepared from the L2 larval stage. The following PCR reactions were carried out using this cDNA as template and the SL1 primer (Table I) as the forward primer and reverse primers as shown in Table I. gly-12—PCR was carried out using CEF15 as the reverse primer. This primer was synthesized as the nested primer CEF4R. A PCR product of the expected size was observed and further amplified with reverse primer CEF10. gly-13—PCR was carried out using CEF4R as the reverse primer. A PCR product of the expected size was seen and reamplified with the nested primer CEF8R. gly-14—A procedure similar to that used for gly-12 was carried out using CEMR6, CEMR10, and CEMR2, respectively, as reverse primers. The three final PCR products were sequenced and all three messages showed trans-splicing to SL1.

**Expression of GnT I in the Baculovirus/Sf9 Insect Cell System**

C. elegans GnT I was expressed in the baculovirus/Sf9 insect cell system as described previously (38, 50, 51). DNA fragments encoding truncated GLY-12, GLY-13, and GLY-14 GnT I proteins lacking the amino-terminal cytoplasmic and transmembrane domains and parts of the stem region were synthesized by PCR amplification using Vent DNA polymerase and GnT I cDNAs as templates (43, 39, and 31 amino acids were removed from the amino-terminal end, respectively). The primer pairs used for gly-13 and gly-14 are shown in Table I. The PCR products were subcloned into the baculovirus transfer vector pV7-Bac-His (39) downstream from and in frame with the ATG start site of the plasmid using restriction enzyme sites introduced by the primers (Table I). This vector encodes a cleavable signal sequence for secretion from the Sf9 cells. Full-length gly-12 cDNA was excised from plasmid p78F-Myc (see below) and subcloned into pBlueBacHis C (Invitrogen) downstream from and in frame with the ATG start site of the plasmid to create a recombinant transfer vector encoding full-length GLY-12 (51). Recombinant plasmids were sequenced and co-transfected with BacuGold linearized baculovirus DNA (PharMingen) to produce recombinant baculovirus by homologous recombination in Sf9 cells (50). Sf9 cells were infected with baculovirus, and after 5–6 days, cells were harvested, and lysed in 0.5 M of 25 mM MES, pH 6.5, 0.1% Triton X-100, 0.02% sodium azide.

**GnT I Enzyme Assays**

Sf9 cell lysates and culture medium and worm lysates were assayed for GnT I activity using as acceptor substrate 0.5 mM Man[α-1–6]Man–l–3)Man–O–octyl (M–octyl, kindly provided by Dr. Hans Paulsen, University of Hamburg) (50, 52) and as donor substrate 1 mM UDP-[3H]GlcNac (NEN Life Science Products) diluted with nonradioactive UDP-GlcNac (Sigma) to a specific activity of 2500 dpm/nmol for recombinant protein assays, 15 000–140 000 dpm/nmol for kinetic studies. The assay mixture also contained 2.5 mM AMP, 50 mM GlcNac, 20 mM MnCl2, 1 mg ml−1 bovine serum albumin, 0.1% Triton X-100, 0.1 M MES buffer, pH 6.1, and 0.010 ml of enzyme in a 0.050-ml total volume (50). Time of incubation was 0.5–1 h at 37 °C for all baculovirus/Sf9-expressed enzymes and at 28 °C for enzymes expressed in C. elegans. Kinetic parameters were determined
on the recombinant rabbit (50) and C. elegans enzymes by a series of reciprocal velocity-substrate plots at four concentrations of both substrates (0.5, 1.0, 1.5, and 2.0 mM M3-octyl and 0.02, 0.04, 0.06 and 0.08 mM UDP-GlcNAc) (50, 53). Kinetic analyses were also carried out using (Manα1-6Manα1-3Manα1-6Manα1-3Manα1-4GlcNAc2→6GlcNAc2→3GalNAc2→ [Nae-Aae (N-acetylglucosamidase) 54] as acceptor substrate (0.22, 0.44, 0.66, and 0.88 mM). Control Gnt I assays were carried out with uninfected S9 cells and cells infected with wild type baculovirus. Metal requirements (20 mM divalent cation) and pH and temperature optima were determined. Protein content was measured by the BCA assay (Pierce).

Northern Analysis of C. elegans mRNA

Total (20 μg) and poly(A)+ RNA (2.5 μg) from a mixed stage population of N2 hermaphrodites and total RNA from staged synchronized populations was subjected to Northern analysis by electrophoresis in a denaturing 1.0% agarose gel (10% formaldehyde) (55). DNA size markers were prepared by digestion of λ phage DNA (5 μg) with EcoRI and HindIII; DNA fragments were end-labeled with [α-32P]dATP (3000 Ci/mmol, NEN Life Science Products) and Klenow DNA polymerase (New England Biolabs). Probes for gly-12 (126–1504 nt relative to the ATG start codon at +1) and gly-13 (114–1350 nt relative to the ATG start codon at +1) were made by excision of the truncated gly-12 and gly-13 inserts from the respective recombinant baculovirus transfer vectors (see above). Probes were randomly labeled with [α-32P]dATP (3000 Ci/mmol, NEN Life Science Products). After hybridization of Northern blots with these probes (1 and 1), the blots were stripped with 0.1% SDS at 100 °C and reprobed with a [32P]-labeled probe for the fen-1 gene (1–2× 106 cpm/ml) as a sample loading control (56).

Quantitation of gly-12 and gly-14 Messages by Competitive RT-PCR

We were able to detect gly-14 mRNA only by RT-PCR, not by Northern blot analysis, suggesting that gly-14 is expressed at lower levels than gly-12 and gly-13. We used competitive quantitative RT-PCR to estimate the relative abundance of both gly-12 and gly-14 mRNA during development. Total cDNA was obtained by oligo(dT)-primed reverse transcription of total RNA from all six developmental stages in the presence of 1 μCi of [α-32P]dCTP (800 Ci/mmol, Amersham Pharmacia Biotech) using the Advantage RT-for-PCR kit (CLONTECH). Competitor cDNA was made by PCR with primer pairs (Table I) CEF/DEL/CEF3 (gly-12, 660 nt product, internal deletion of 233 nt) and CEMF7/CEMR-DEL (gly-14, 726 nt product, internal deletion of 213 nt) using the respective gly-12 and gly-14 cDNAs as templates. PCR was then carried out with gene-specific primer pairs (Table I) CEF/5/CEF3 (gly-12) and PRBR4027/PRBM5 (gly-14) on IVS of plasmid pPD49.57. PCR products were then used in a competitive PCR reaction using as template a mixture of [α-32P]dCTP-labeled total cDNA (at a constant concentration) and purified competitor cDNA (at variable concentrations). The PCR products were resolved in a 1.5% agarose gel stained with ethidium bromide. Since the amount of added competitor cDNA is known, and on the assumption that the molar ratio of wild type cDNA to competitor cDNA remains approximately constant throughout the amplification, estimates can be made of the amount of gly-12 and gly-14 cDNA present at the start of the PCR reaction by scanning of the agarose gels. The expression level of message at each worm developmental stage was normalized with the amount of [α-32P]dCTP-labeled total cDNA added to each PCR reaction.

Preparation of DNA Constructs for Promoter Analysis

We used transcriptional fusion of Gnt I genomic DNA to the lacZ reporter gene to examine the spatial pattern of GnT I expression during C. elegans development. Plasmids encoding lacZ were provided by Dr. A. Fire, Carnegie Institute of Washington (Baltimore, MD). 2 gly-13—Cosmid B0416 (40) was grown overnight in 500 ml of LB medium containing 50 μg/ml of ampicillin. DNA was prepared by using the Qiagen Maxi-prep kit, and two gene fragments were cut out using SalI and PstI, respectively. A DNA fragment (1008 nt) containing the putative promoter region immediately upstream of the first exon, the complete 3′-UTR, and the first 8 nucleotides of the open reading frame (the initiation codon ATG was mutated to TTG) was obtained by PCR using the SalI fragment from cosmid B0416 as a template and primers PRBF618 and PRBR4027 (Table I). The PCR product was subcloned into the SpeI and ApII sites of plasmid p11B/prom, downstream of the lacZ gene, creating plasmid p11B/prom-ORF.

gly-14—Cosmid M01F1 (40) DNA was prepared as above. A DNA fragment (3.4 kb) containing the complete open reading frame except for the first three nucleotides and the 3′-UTR was amplified by PCR from cosmid M01F1 using primers PRMF1158 and PRMR4530 (Table I) and subcloned into the SpeI and ApII sites of plasmid pD95.11 downstream of the lacZ gene to create plasmid p11M/ORF. A DNA fragment (2.6 kb) containing the putative promoter region immediately upstream of the first exon, the complete 5′-UTR, and the first nine nucleotides of the open reading frame (the initiation codon ATG was mutated to AGC) was amplified by PCR from cosmid M01F1 DNA with primers PRMF33 and PRMR2664 (Table I) and subcloned into the SalI and BamHI sites of plasmid p11M/ORF upstream of the lacZ gene to create plasmid p11M/prom-ORF. Plasmid p57M/prom-2.6, containing the same 2.6-kb putative promoter region as p11M/prom-ORF but in which the region downstream of the lacZ gene was replaced with the 3′-UTR of the unc-54 gene, was constructed by inserting the 2.6-kb PCR product from the promoter region (see above) into the SalI and BamHI sites of pD95.57. Deletion of −1.3- and −2.0-kb fragments from the 5′-end of p57M/prom-2.6 with EcoRI and NheI resulted in plasmids p57M/prom1.3 and p57M/prom0.6, respectively.

gly-12—Cosmid F48E3 (40) DNA was prepared as above. A DNA fragment (1740 nt) containing the putative promoter region immediately upstream of the first exon, the complete 5′-UTR, and four nucleotides from the open reading frame (the initiation codon ATG was mutated to AGG) was amplified by PCR from the cosmid DNA with NheI and PrB2 (Table I) and subcloned into the SalI and BamHI sites of pD95.57 upstream of the lacZ gene to create plasmid p57P/prom.

DNA Constructs for Heat-Shock-induced Expression of GnT I by Transgenic Worms

gly-13 Expression Construct—A Not I DNA fragment was excised from the gly-13 baculovirus transfer plasmid (see above) encoding the open reading frame downstream of the transmembrane domain (nucleotides 114–1350 relative to the initiation ATG codon at +1), blunt-ended, and digested with ApaLI. A DNA fragment covering the first exon, first intron, and second exon was amplified by PCR from cosmid B0416 using primers BEXF1 and BEXR2 (Table I) and digested with Nhel and ApaLI. These two fragments were then subcloned into the NotI site of plasmid pPD49.57.73 downstream of the heat shock promoter (57), producing plasmids p78B and p83B, respectively. Myc spacer was amplified from plasmid AS13097 using primers SC001 and SC0002 (Table I). This fragment contains sequences encoding a Myc epitope tag (underlined) and 18 amino acids from the PEM-1 amino terminus (italics) (MAAEQKLISEEDLGR...)

gly-14 Expression Construct—A KpnI DNA fragment was excised from the gly-14 baculovirus transfer plasmid (see above) encoding the open reading frame downstream of the transmembrane domain (nucleotides 90–1314 relative to the initiation ATG codon at +1), blunt-ended and digested with HindIII. A DNA fragment covering the first three exons and, introns 4, and exon 5 of intron 4 was amplified by PCR from cosmid M01F1 using primers MEXF1 and MEXR2 (Table I) followed by digestion with Nhel and HindIII. These two fragments were then subcloned into the NotI and EcoRV sites of plasmids pPD49.78 and pPD49.83 downstream of the heat shock promoter, producing plasmids p78M and p83M, respectively. Myc spacer was subcloned into these two plasmids, as described above, immediately upstream of the gly-14 sequence, producing plasmids p78B-Myc and p83B-Myc, respectively.

gly-12 Expression Construct—The amino terminus of the gly-12 open reading frame was amplified by PCR using as template one of the gly-12 cDNA clones isolated from the cDNA library (see above) and primers FEXF1 (Table I) and CEFR4 (Table I). An NsiI site was introduced near the ATG start codon. The carboxyl terminus of gly-12 was similarly amplified by PCR using primers CEFF5 (Table I) and FEXR2 (Table I). The middle of the gly-12 cDNA was obtained directly from the cDNA

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2 A. Fire, S. Xu, J. Ahn, and G. Seydoux, personal communication.

3 J. Gaudet and A. Spence, unpublished results.
clone. These three fragments and Myc spacer were subcloned into the Nhel and SacI sites of pPD49.78 and pPD49.83, producing plasmids p78F-Myc and p83F-Myc, respectively.

Preparation of Transgenic Worms

Promotor Analysis—DNA injection into the C. elegans germ line was carried out as described by Mello et al. (57, 58). Transgenic lines were established from F2 descendants of animals injected with 10 or 50 ng/µl of GnT I::lac Z constructs and 50 ng/µl of plasmid pRF4, which carries the dominant rol-6 allele rol-6 (sa1006) that serves as a transformation marker. The total DNA concentration of the injection mixture was adjusted to 100 ng/µl by the addition of pBlueScript SK− (Stratagene). In some experiments, the F1 progeny of injected animals were analyzed directly for reporter expression; in these experiments, the concentration of GnT I::lac Z constructs was 100 ng/µl. LacZ expression was examined in a smg-1 (e1228) background. The smg-1 mutation stabilizes aberrant transcripts with long 3′-UTRAs (59). Transgenic lines or F1 progeny were cultured at 25°C. For F1 lac Z assays, 15–20 adult hermaphrodites were injected on each of three consecutive days. β-galactosidase staining of late larvae and adults was carried out as described by Xie et al. (60). Staining of embryos and young larvae was carried out by the method of Fire (61). All animals were co-stained with 1 µg/ml of 4,6-diamidino-2-phenylindole to visualize the cell nuclei. In the figures, anterior is to the left and dorsal is up.

Heat Shock-induced Overexpression—Heat shock plasmids (see above) were injected in the following mixtures: (i) p78B/p83B mixture (p78B (10 ng/µl), p83B (10 ng/µl), pRF4 (50 ng/µl), pBlueScript II− (30 ng/µl)); (ii) p78B-Myc/p83B-Myc mixture (p78B-Myc (25 ng/µl), p83B-Myc (25 ng/µl), pRF4 (50 ng/µl), pBlueScript II− (30 ng/µl)); (iii) p78M/p83M mixture (p78M (10 ng/µl), p83M (10 ng/µl), pRF4 (50 ng/µl), pBlueScript II− (30 ng/µl)); (iv) p78M-Myc/p83M-Myc mixture (p78M-Myc (10 ng/µl), p83M-Myc (10 ng/µl), pRF4 (50 ng/µl), pBlueScript II− (30 ng/µl)); (v) p78F-Myc/p83F-Myc mixture (p78F-Myc (10 ng/µl), p83F-Myc (10 ng/µl), pRF4 (50 ng/µl), pBlueScript II− (30 ng/µl)). Transgenic lines carrying the injected DNA on extrachromosomal arrays were established from F2 Rol progeny of injected N2 animals.

C. elegans Culture, Heat Shock, and Worm Lysis

The standard laboratory wild type strain N2 or a smg-1 (e1228) mutant derived from N2 nematodes was grown on MYOB (62) agar plates seeded with Escherichia coli strain OP50 (a leaky uracil-requiring strain). To examine the consequences of overexpressing GnT I, gravid adults were allowed to lay eggs for 2–3 h at 20°C. Eggs were incubated for a further 6 h at 20°C and subjected to heat shock treatment at 33°C for 1 h at 12-h intervals until the animals reached adulthood.

To measure the activity of overexpressed enzyme, heat shock was carried out at 33°C for 2 h followed by recovery at 20°C for a further 2 h. Worms from 10–15 agar plates were harvested by washing off the plates with M9 buffer, pelleted by centrifugation at 700 rpm for 2 min, washed with 10 ml of M9 buffer and suspended in 5 ml of ice-cold M9 buffer. An equal volume of ice-cold 60% (w/w) sucrose was added, and the suspension was mixed by inversion and centrifuged at 700 rpm for 5 min to remove bacteria. Worms were collected; washed twice with 10 ml of M9 buffer; resuspended in 1 ml of buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM sucrose, and protease inhibitor mixture (Boehringer); and stored at −70°C. Worms were lysed by sonication, five times with 5-s pulses at 30-s intervals. The sonicate was centrifuged at 3500 rpm in a Beckman JA17 rotor for 10 min, and the supernatant was centrifuged at 55,000 rpm for 1 h (Beckman 100.3 rotor). The microsomal pellet was resuspended in lysis buffer (25 mM MES, pH 6.1, 1% Triton X-100 and protease inhibitor mixture).

Immunolocalization of Overexpressed C. elegans GnT I in Heat-shocked Transgenic Worms

Heat-shocked transgenic worms were prepared as described above. Worms were fixed and stained as described by Finney and Ruvkun (63). Mouse monoclonal anti-Myc antibody 9E10 (64) was used at 1.5 dilution, followed by biotinylated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) was used at 1:50 dilution. The worms were also co-stained with 4,6-diamidino-2-phenylindole.

Western Blot Analysis

Worm lysates were subjected to SDS-polyacrylamide gel electrophoresis (10%) (65) followed by Western blot analysis with anti-Myc antibody 9E10 (1:50 dilution). Horseradish peroxidase-labeled second-
GLY-14 sequences, respectively. The three *C. elegans* GnT I protein sequences show no similarity to the mammalian GnT I proteins in the cytoplasmic, transmembrane, and stem regions (Fig. 1). The *gly-12* and *gly-14* genes share 9 of 11 intron positions, whereas four introns occur at the same positions in all three genes (Fig. 1). Genes *gly-12* and *gly-13* are on *C. elegans* chromosome X, and *gly-14* is on chromosome III. The data indicate that the mammalian GnT I genes and three *C. elegans* GnT I genes are derived from a common ancestor.

**Expression of *C. elegans* GnT I cDNAs in the Baculovirus/Sf9 System**—Sf9 cell lysates have been reported to contain GnT I activity (68), but endogenous GnT I activity was low in both cell lysates and supernatants under our assay conditions (0.03–3.4 nmol/10³ cells/h at 48–120 h after infection). The culture medium of Sf9 cells infected with recombinant baculovirus encoding truncated GLY-14 contained levels of enzyme activity equivalent to those previously obtained with mammalian GnT I expression (data not shown). Expression of GLY-12 either as a truncated or full-length protein yielded enzyme activities above background levels, but this activity was appreciably less than the intracellular activity of truncated GLY-14 (data not shown). Western blot analysis using mouse monoclonal antibody raised against the enterokinase cleavage site (Anti-Xpress antibody kit, Invitrogen) (38) showed the dominant protein bands at the expected molecular weights for GLY-12 (weak) and GLY-14 (strong). Attempts to express recombinant baculovirus encoding truncated GLY-13 were not successful; we could not detect a protein band by Western analysis, nor could we detect any enzyme activity either in cell lysates or supernatants (data not shown). Kinetic analysis of GLY-14 in cell
supernatant and GLY-12 in Sf9 cell extracts gave linear $1/v$ versus $1/S$ plots (where $v$ is the initial velocity and $S$ is the substrate concentration) consistent with an ordered sequential Bi Bi mechanism if one assumes steady state rather than rapid equilibrium kinetics (53). Kinetic constants (data not shown) indicate no major differences between C. elegans and previously published data on rabbit GnT I (54); GLY-12 was assayed only with M3-octyl, whereas GLY-14 and the rabbit enzyme were assayed with both M3-octyl and M5-glycopeptide. Rabbit GnT I had a higher temperature optimum (37 °C) than GLY-12 and GLY-14 (20–30 °C, data not shown), and the rabbit enzyme remained active at pH 5.0–5.5, whereas GLY-14 did not (data not shown). The rabbit and GLY-14 enzymes showed very similar metal requirements (data not shown); there was an absolute requirement for Mn$^{2+}$ and little (20% of maximum activity) or no activity with Mg$^{2+}$, Ni$^{2+}$, Ba$^{2+}$, Ca$^{2+}$, Cd$^{2+}$, Fe$^{2+}$, or Cu$^{2+}$.

The products of GLY-12 and GLY-14 with M3-octyl were analyzed by thin layer chromatography and shown to comigrate with standard Man$\alpha_1$–6(GlcNAc$\beta_1$–2Man$\alpha_1$–3)Man$\beta$-O-octyl (69) in the following solvent systems: (i) acetonitrile/water (5:1) and (ii) dichloromethane/methanol/water (55:35:6) (data not shown).

Expression of GnT I mRNA at Various Stages in C. elegans Development—As a first step toward understanding the role of N-glycans in development, we studied the expression of GnT I mRNA in six developmental stages of C. elegans, an embryo fraction containing a mixture of embryo stages, the four larval stages L1–L4, and adult worms (70). Northern analysis detected messages for both gly-12 (a major band at 2.1 kb, Fig. 2A) and gly-13 (a major band at 1.9 kb, Fig. 2B) in all six developmental stages. Assuming that the 3′-untranslated regions of gly-12 and gly-14 are not excessively long, the mRNA sizes are consistent with the cDNA lengths determined by sequencing (1982, 1719, and 1322 nt for gly-12, gly-13, and gly-14, respectively). We used quantitative RT-PCR (Figs. 3, A–C) to establish the relative abundance of gly-12 and gly-14 mRNA. There were no major variations between different stages of development except for a higher level of gly-12 message at the embryo stage (Fig. 3, A and C). The gly-12 mRNA levels are 6–38 times higher than the gly-14 mRNA (Fig. 3C).

Expression of GnT I Promoters at Various Stages in C. elegans Development—Expression of the β-galactosidase reporter gene in the F1 progeny of worms injected with the gly-12 promoter construct p57F/prom was observed throughout all developmental stages and in many tissues (intestine, muscle, hypodermis, and other epithelial cells and in ganglia in the head and tail region) (Fig. 4, A–C, and data not shown). From L2 to adulthood, β-galactosidase was expressed in many different tissues, including gut, muscle, hypodermis, and other epithelial cells and the nervous system (ganglia in the head and tail region and the ventral nerve cord) (Fig. 4, E and F, and data not shown).

Transgenic animals carrying the gly-14 promoter construct

![Fig. 1. Alignment of GnT I amino acid sequences from mammals (mouse, rat, Chinese hamster, rabbit, and human) and C. elegans (gly-12, gly-13, and gly-14) using the GCG PileUp program (78) (Genetics Computer Group Inc., Madison, WI). Dashed line, same as consensus sequence; dotted line, gap, vertical bar, exon-exon boundaries; asterisk, STOP codon.](image-url)
We did not detect reporter expression in embryos, despite finding that embryos contain approximately the same amount of mRNA as other stages (Fig. 3, panels A–C). Expression of gly-14 mRNA was maternally contributed to the embryo. Injection of p11M/prom-ORF (lacking the 3'-UTR of the unc-54 gene) and the truncated constructs p57M/prom1.3 and p57M/prom0.6 did not result in reporter gene expression.

Heat Shock-induced Overexpression of GnT I—Transgenic worms that overexpress gly-12, gly-13, or gly-14 under the control of the hsp-16 heat shock promoters show no obvious phenotypic defects. To test whether functional GnT I was produced in these transgenic worms, microsomal fractions were prepared to determine enzyme activity. Heat shock induction of gly-13 resulted in little or no increase in GnT I activity compared with wild type N2 worms (wild type GnT I activity = 0.1 nmol/h/mg) (Table II). Dramatic increases in enzyme activity were observed on heat shock induction of both gly-12 (27–157-fold) and gly-14 (39–182-fold) (Table II). Lysates of all three transgenic lines overexpressing GnT I showed protein bands of the expected size by Western blotting (Fig. 5). This finding suggests that the low GLY-13 activity is not due to poor expression but rather to a low specific activity, at least with the acceptor substrate used for assay; GLY-13 may have a high activity with an as yet unknown physiological acceptor. Immunolocalization experiments showed that all three GnT I gene products stained as focal areas in the perinuclear region of the cytoplasm, suggesting a Golgi complex location (Fig. 6 and data not shown).
Caenorhabditis elegans N-Acetylglucosaminyltransferase I

TABLE II

| Heat shock constructs | Transgenic lines | GnT I activitya | Increaseb |
|----------------------|-----------------|-----------------|------------|
| None                 |                 | 0.065 ± 0.033   | 1.0        |
| (n = 5)              |                 | (n = 5)         | (n = 4)    |
| Myc-GLY-12           | 2b              | 2.6             | 27         |
| GLY-13               | 10              | 15.1            | 157        |
| GLY-13               | 15              | 0.1             | 1          |
| Myc-GLY-13           | 16              | 0.2             | 2          |
| GLY-14               | 1b              | 0.07            | 0.7        |
| Myc-GLY-14           | 2b              | 0.09            | 0.9        |
| GLY-14               | 8               | 10.2            | 106        |
| GLY-14               | 12              | 17.5            | 182        |
| Myc-GLY-14           | 2c              | 4.3             | 45         |
|                    | 4c              | 3.7             | 39         |

a All enzyme assays were done in duplicate.
b Relative to heat shock-treated N2 animals.

No heat shock treatment. All other values were heat shock-induced GnT I activities. The n value refers to the number of separate worm extracts.

FIG. 5. Western blot analysis of Myc-tagged C. elegans GnT I overexpressed in transgenic worms under the control of heat shock promoter. Left, gly-14 (Ex[HS-GLY-14]) and gly-13 (Ex[HS-GLY-13]) transgenic worms and wild-type worms (N2) were analyzed before (-) and after (+) heat shock induction. About 280 µg of total protein was loaded in each lane. This corresponds to 0.96 nmol/h of GLY-14 activity after heat shock. The GLY-13 protein does not show GnT I activity (Table II). Before (1) and after (+) heat shock induction. Sample loading was as follows: ~420 µg of uninduced GLY-13 lysate, ~280 µg of heat shock-induced GLY-12 lysate (worm line 2b) corresponding to 0.73 nmol/h GnT I activity, and ~140 µg of heat shock-induced GLY-12 lysate (worm line 2c) corresponding to 2.2 nmol/h enzyme activity. The calculated molecular weights of Myc-tagged GLY-12, GLY-13, and GLY-14 are 58.0, 55.7, and 54.9 kDa, respectively. Protein bands of approximately the expected sizes are seen for all three genes after heat shock induction but not in wild type or uninduced transgenic worms.

FIG. 6. Immunolocalization of C. elegans heat shock-induced Myc-GLY-12. The transgenic worm was stained with antibody 9E10, which recognizes the Myc epitope tag (right panel) and with 4,6-diamidino-2-phenylindole to visualize nuclei (left panel). A portion of the intestine has extruded, thereby permitting a clear view of three gut cells. It is seen that the Myc epitope is localized to punctate perinuclear areas, suggestive of localization in the Golgi complex.

detected by Western analysis when we attempted to express gly-13 in Sf9 insect cells. Expression of gly-13 in transgenic worms yielded a protein of the expected size on Western blots, but this protein showed no GnT I enzyme activity. The data suggest that gly-13 may encode a glycosyltransferase with a specificity different from GnT I. Similarly, only 5 of the 11 C. elegans UDP-GalNAc:polypeptide N-acetylglactosaminyltransferase cDNA homologues cloned by Hagen and Nehrke (41) were shown to possess enzyme activity. Bakker et al. (72) attempted to clone a snail UDP-GalNAc:GlcNAcβ-R β4-GalNAc-transferase by screening a snail cDNA library with a UDP-Gal-GlcNAcβ-R β4-GalNAc-transferase probe but instead cloned a novel UDP-GlcNAc:GlcNAcβ-R β4-GlcNAc-transferase. Studies are under way on the large scale expression of gly-13 so that a search can be made for other enzyme activities.

In contrast to the mammalian GnT I genes in which the entire open reading frame is on a single exon, the gly-12, gly-13, and gly-14 genes have multiple exons (14, 12, and 12, respectively). However, although the identity between the C. elegans and mammalian GnT I amino acid sequences is less than 50%, the GLY-12 and GLY-14 enzymes show kinetic parameters very similar to the rabbit enzyme. The major differences detected were the pH profiles (rabbit GnT I maintains its activity to a significantly lower pH than GLY-14) and the lower temperature optimum for GLY-12 and GLY-14 relative to rabbit GnT I. C. elegans GalNAc-transferase also has a lower temperature optimum than the mammalian enzyme (41).

We have previously shown that removal of 106 amino acids from the N terminus of rabbit GnT I does not inactivate the enzyme (38). This region contains the cytoplasmic, transmembrane, and stem domains and shows marked differences in amino acid sequence between the mammalian enzymes and each of the three C. elegans enzymes (Fig. 1). The catalytic domain of GnT I contains 341 amino acids for the mammalian enzymes and 350–394 amino acids for the C. elegans enzymes. Comparison of mammalian and C. elegans sequences indicates five highly conserved regions that are probably essential for catalytic activity (118–159, 200–211, 221–265, 277–330, and 431–461 in Fig. 1). Of the five cysteine residues in the consensus sequence (128, 137, 159, 256, and 322 in Fig. 1), only two are conserved for all mammalian and C. elegans sequences (256 and 322). Site-directed mutagenesis of invariant amino acids is being carried out to determine whether these residues are indeed essential for enzyme activity.

All three gene messages (gly-12, gly-13, and gly-14) are ex-
pressed in all six stages of worm development. Except for a relative increase of gly-12 expression in the embryo, there is no significant difference in expression between the various developmental stages. Analysis of reporter gene expression in transgenic animals confirms the expression of gly-12 and gly-13 at all stages of development and shows that the gly-12 and gly-13 promoters are expressed in most cell types. Expression of the gly-14 promoter was detected only postembryonically and only in gut cells, suggesting a tissue-specific expression of enzyme activity. Confirmation of its gut-specific expression will require detection of endogenous gly-14 gene products.

Overexpression of the three C. elegans GntI genes in transgenic worms under the control of the worm heat shock promoter caused no obvious phenotypic changes despite marked increases in the enzyme activities of GLY-12 and GLY-14 in worm lysates. This is perhaps not surprising, since mammalian GntI is probably a housekeeping gene (55) and is expressed in non-rate-limiting amounts. However, mice with a null mutation in the GntI gene do not survive beyond 10 days of embryonic life, and it is therefore of great interest to study the effects of null mutations in the three C. elegans genes. Attempts to create mutant worms lacking GntI by injection of single-stranded and double-stranded RNA have to date been unsuccessful in demonstrating any obvious phenotypes. Attempts to create mutant worms by other methods are under way.

The C. elegans genomic DNA and expressed sequence tag data bases contain sequences that show significant homologies to at least 17 enzymes involved in the synthesis of N- and O-glycans, glycosyl-phosphatidylinositol anchors, and proteoglycans, e.g. UDP-GalNAc-polypeptide N-acetylglucosaminyltransferase (41), UDP-GlcNAc-polypeptide N-acetylglucosaminyltransferase (73), GntT (this study), UDP-GalGlcNAc-R β1,4-galactosyltransferase, β1,6-N-acetylglucosaminyltransferase V, and α1,3-fucosyltransferase. The only enzymatically active C. elegans glycosyltransferases published to date are the UDP-GalNAc-polypeptide N-acetylglucosaminyltransferases (41) and GntT (this study). Lectins such as wheat germ agglutinin (74, 75) and concanavalin A (76) have been shown to bind to C. elegans tissues, suggesting the presence of glycoproteins in these organisms. Although no detailed glycan structures have as yet been reported for C. elegans, both N- (21–23) and O-glycan (77) fine structures have been determined for several parasitic nematodes using mass spectrometric analysis. Some of these nematode N-glycan structures contain the GlcNAcβ1,2-Manα1,3-Manβ-R moiety indicative of a functional GntI enzyme. The data available to date therefore show that C. elegans is highly active in the synthesis of glycoproteins and is an excellent organism in which to study the role of protein glycosylation in the development of a multicellular organism.

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Note Added in Proof—Catalytic activity has been reported on expression of C. elegans genes encoding an α1,3-fucosyltransferase (De Bose-Boyce, R. A., Kwame Nayame, A., and Cummings, R. D., 1998 Glycobiology 8, 905–917) and p1,6-N-acetylglucosaminyltransferase V (Warren, C. E., Roy, P. J., Kriuse, A., Cullott, J., Joulli, J., Genn, J., De这样做，您就能够理解该文档的主要内容。
