N-Glycans of Caenorhabditis elegans Are Specific to Developmental Stages*

We have examined the N-glycans present during the developmental stages of Caenorhabditis elegans using two approaches, 1) a combination of permethylation followed by MALDI-TOF mass spectrometry (MS) and 2) derivatization with 2-aminobenzamide followed by separation by high-performance liquid chromatography and analyses by MALDI-TOF MS, post source decay (PSD) MS, and MALDI-QqTOF MS/MS. The N-glycan profile of each developmental stage (Larva 1, Larva 2, Larva 3, Larva 4, and Dauer and adult) appears to be unique. The pattern of complex N-glycans was stage-specific with the general trend of number and abundance of glycans being Dauer > L1 > adult > L4 > L3 = L2. Dauer larvae contained complex N-glycans with higher molecular masses than those seen in other stages. MALDI-QqTOF MS/MS of Hex₃HexNAc₂ showed an N-acetyllactosamine substitution not previously observed in C. elegans. Phosphorylcholine (Pc)-substituted glycans were also found to be stage-specific. Higher molecular weight Pc-containing glycans, including fucose-containing ones such as difucosyl Pc-glycan (Pc₂dHex₂Hex₂HexNAc₂) seen in Dauer larvae, have not been observed in any organism. Pc₂Hex₂HexNAc₂, from Dauer larvae, when subjected to PSD MS analyses, showed Pc may substitute both core and terminally linked GlcNAc; no such structure has previously been reported in any organism. C. elegans-specific fucosyl and native methylated glycans were found in all developmental stages. Taken together, the above results demonstrate that in-depth investigation of the role of the above N-glycans during C. elegans development should lead to a better understanding of their significance and the ways that they may govern interactions, both within the organism during development and between the mobile nematode and its pathogens.

Carbohydrate-mediated interactions between cells and their environment are important in differentiation, embryonic development (1), inflammation and immunity (2), and cancer metastasis (3). Errors of glycosylation manifest themselves throughout cell lineages, resultant tissue formation, and cell-interactive processes such as those enumerated above. In humans and mice, mutations that prevent proper formation of N-glycans result in multisystem defects that can be traced to developmental processes and, thus, demonstrate that appropriate glycosylation is essential for development.

Caenorhabditis elegans is a genetically and developmentally well-characterized multicellular eukaryote with a short life cycle, invariant cell lineage, and distinct stages of development in which growth, reorganization, and switching between vegetative and developmentally arrested states occur. Knowledge of its sequenced genome and mapped cell fates, as well as accessibility of expression data bases (4) and gene ablation consortia, make this organism attractive for study of the roles of N-glycosylation in development and nematode-pathogen interactions.

To date N-glycans only from mixed developmental stages have been examined (5–12). N-Glycans released from glycoproteins from mixed stages will naturally be enriched in glycans from the largest and most represented developmental stages, and will, thus, likely contain little glycan originating from stages that are less represented. The most abundant oligosaccharides observed are high mannose type (Man₃–9GlcNAc₂) with minor amounts of C. elegans fucosyl-type (Me₀–3Fuc₁–4Hex₀–₂Man₃–6GlcNAc₂, where Hex = Gal or Man), mammalian-type hybrid and complex (Fuc₁Man₃–5GlcNAc₂–₈₉) and phosphorylcholine-substituted glycans (PC₁–₅Man₃GlcNAc₃–₇). The higher order Pc and complex-type oligosaccharides have very low abundance and have only been observed by two groups (6, 9, 13). Genetic and biochemical evidence exists to suggest that complex oligosaccharides other than those detected thus far exist in this metazoan. For example, in vitro characterization of C. elegans glycosyltransferases CeβGalNAcT and Ce3FucT suggests that LacdiNAc (14), fucosylated LacNAc, and possibly Lewis X-containing oligosaccharides may exist in this organism (15).

In genetic terms, C. elegans has retained the biosynthetic components required for the formation of high mannose and the abbreviated mammalian-type complex glycans. Three N-acetylglucosaminyltransferase I (16–18) and an N-acetylglucosaminyltransferase V (19) homologues have been expressed and characterized. A homologue of N-acetylglucosaminyltransferase II also appears to exist (17). However, no strong homologies to N-acetylglucosaminyltransferases III, IV, or VI have been identified, although some structural studies suggest that GlcNAc is present in linkages identical to those catalyzed by these enzymes (6, 7, 9). C. elegans also possesses insect-like pathways to process glycans (20). Insect cell lines have a Golgi N-acetylglucosaminidase, which has been shown to remove N-acetylglucosaminyltransferase I-added GlcNAc, thus preventing formation of complex glycan, and this activity appears to be developmentally related (21). The presence of insect-like pathways suggests that the low abundance of complex glycans seen in C. elegans N-glycans may be related to the activity of this enzyme. Therefore, it is possible that, during some stages of glycoconjugate biosynthesis, enzyme balance is shifted to
favor the formation of more complex glycans in some tissues. Here, we present the N-glycan structures correlated to the developmental stages of *C. elegans*. These findings may provide important clues derived from the glycosylation patterns of these developmentally distinct stages, which should be relevant to differentiation, embryonic development, inflammation, and immunity in this nematode.

**MATERIALS AND METHODS**

*Isolation of Developmental Stages of C. elegans*—The temporal progression of life stages was approximated using synchronized cultures by the observation of the cessation of pharyngeal pumping, which precedes each developmental stage. Nematodes were grown in liquid culture and harvested when 90–95% of individuals were of the desired developmental stage, as observed by light microscopy.

*Isolation of N-Glycans*—The glycoprotein-rich fraction was isolated from 2-g batches of *C. elegans*, as previously described (6). Briefly, following treatment of extracted proteins with 1-1-rosamidino-2-phenyl-ethyl chloromethyl ketone trypsin, N-glycans were released from 0.5-ml samples with 8000 units/ml of PNGase F (New England Biolabs) overnight at pH 8.5 and 37 °C. Free glycans were separated from the tryptic peptides by precipitation of the peptides (23) with 50% methanol at pH 5.5 followed by centrifugation at 3500 × g. The solutions, containing free glycans and some peptides, were subjected to rotary evaporation, and the resulting precipitate was suspended in distilled water and applied to the MALDI target with an equal volume of 2,5-dihydroxybenzoic acid (20 mg/ml) in 20% acetonitrile. Oligosaccharides were applied to chromatography paper (Whatman), dried, and separated from reaction products by ascent in a chromatography tank containing 100 ml of acetonitrile. The aminated products were visualized using the phenol sulfuric assay for neutral hexose standardized with mannose (24).

**Permethylation of Oligosaccharides**—Permethylation was carried out using a slight modification of the method of Ciucanu (25) as previously described (6).

**Reductive Amination and Chromatographic Separation**—Oligosaccharides were dried in a Savant speed evacuation device and reconstituted in 15 μl of MeSO. To the reconstituted samples were added 100 μl of 2 μM cyanoborohydride and 35 μl of 0.5 M 2-amino benzamide, both of which were solubilized in MeSO. Glacial acetic acid (50 μl) was added. The reaction was performed at 65 °C for 2 h. The aminated glycans were applied to chromatography paper (Whatman), dried, and separated from reaction products by ascent in a chromatography tank containing 100 ml of acetonitrile. The aminated products were visualized using a BLAK-RAY 366 nm UV lamp and eluted into a 4% butanol solution. The glycan products were filtered by centrifugation, dried by speed evacuation, reconstituted in 4% butanol, and chromatographed on a Waters high-performance liquid chromatography system equipped with a Waters 2475 fluorescence detector and Breeze™ software. The aminated glycans were separated on a Varian C18 column using a gradient of 10 m ammonium acetate 0.1 to 1% butanol applied over 72 min.

**Mass Spectrometric Analysis**—MALDI-TOF MS analysis was performed on a Bruker Reflex IV MS mass spectrometer in positive reflectron mode. Between 20 and 50 pmol of sample dissolved in 20% acetonitrile was applied to the MALDI target with an equal volume of 2,5-dihydroxybenzoic acid, and typically the signal from 50–200 laser shots was summed for each spectrum. The laser power used was 30–33 μJ. Nitrogen (3 psi) was used as the collision gas for MS/MS experiments. The range of operator-controlled collision voltages was 35–90 V. Nomenclature is that of Domon and Costello unless otherwise indicated.

**RESULTS**

*General Workup Strategy*—Profiling of permethylated oligosaccharides was initially undertaken to compare amounts of high mannose-, complex-, and *C. elegans*-specific fucoyl N-glycans of different developmental stages of *C. elegans*. This was achieved with MALDI-TOF MS analysis. Although this procedure does not distinguish among isomers, it allows relative quantitation of individual isoforms based on their spectral intensities. Standard errors of the mean of individual isoform peaks were typically less than 5% of peak intensity.

More detailed analyses of N-glycans, from different developmental stages, were carried out following derivatization of the glycans with 2-aminobenzamide (2AB). This allowed detection of glycans after their separation by C18 high-performance liquid chromatography. Thereafter, samples were pooled and analyzed by MALDI-TOF MS, PSD MS, and MALDI-QcTOF MS/MS. The combination of these analytical techniques has proven useful for glycan identification (26–28). This approach led to the detection of a broader range of *C. elegans* oligosaccharides than had been previously described. In the next section we first present an overview of the N-glycan patterns of the different *C. elegans* developmental stages and follow this with in-depth analyses of the different glycan species.

**Overview of N-Glycan Patterns in C. elegans Developmental Stages**—The general patterns of 2-aminobenzamide-labeled N-glycans of different developmental stages can be seen in Fig. 1. These results led to two general conclusions: 1) the N-glycan profile of each developmental stage is unique and 2) the profile from mixed worms resembles that of adults, a not-too-surprising result, because the mixed animal population is enriched in adults, on the basis of mass. In all stages abundant ions correspond to Man$_n$GlcNAc$_2$-2AB, [M + Na$^+$] $m/z$ 1053.5 (50–52 min), Man$_n$GlcNAc$_2$-2AB, [M + Na$^+$] $m/z$ 1377.6 (46–48 min), Man$_n$GlcNAc$_2$-2AB, [M + Na$^+$] $m/z$ 1701.7, 1863.7, and 2025.8 (38–40 min),Hex$_3$GlcNAc$_2$-2AB, [M + Na$^+$] $m/z$ 1539.6 (42–44 min), and Fuc$_1$Man$_n$GlcNAc$_2$-2AB, [M + Na$^+$] $m/z$ 1199.5 (66–68 min). All of the above glycans are consistent with those seen by the permethylation analyses described below.

The pattern of complex N-glycans was stage-specific, as shown in Fig. 2. The general trend of the number and abundance of complex N-glycan ions was Dauer $\sim$ L1 $>$ adult $\sim$ L4 $>$ L3 $\sim$ L2. Detailed listings of all detected glycans are shown in Tables I–IV, whereas important glycan molecular ions of each developmental stage are discussed under “Results” (see “Detailed Analyses of Complex Oligosaccharides,” “Detailed Analyses of Phosphorylcholine Oligosaccharides,” and “Detailed Analyses of C. elegans-specific Fucoyl and Methylated Oligosaccharides” below).

Phosphorylcholine oligosaccharides were found in all developmental stages, and selected spectra containing some of these glycans are shown below in Fig. 4. The general trend of the number and abundance of the glycan species eluting between 52 and 54 min of the chromatograms shown in Fig. 1 was Dauer $\sim$ L1 $>$ adult $\sim$ L4 $>$ L3 $\sim$ L2. These glycans were observed as both the [M + H$^+$] and [M + Na$^+$] forms despite doping of the sample matrix with sodium acetate. This is most likely the result of the zwitterionic nature of phosphorylcholine. Detailed descriptions of the different structures, including novel high molecular mass species and some glycoforms not previously described in any organism, are presented under “Detailed Analyses of Complex Oligosaccharides.”
All traces are kept at constant scale. Approximately 15 μg of glycans from mixed and each individual developmental stage was subjected to C-18 chromatographic separation. The source of each pool is indicated above each trace.

Detailed Analyses of Complex Oligosaccharides—The pattern of complex glycans was stage-specific (Fig. 1), the general trend of the number and abundance being Dauer > L1 > adult > L4 > L3 > L2. All stages contained a Hex$_2$HexNAc$_5$-2AB, [M+Na]$^+$ $m/z$ 1256.5, in the 48–50 min fraction except for L2 were it was found in an adjacent pool (not shown). A dHex$_2$Hex$_2$HexNAc$_5$-2AB, [M+Na]$^+$ $m/z$ 1767.7, component was seen in the spectra of L1, L4, adult, and Dauer larvae, whereas L4 and Dauer larvae spectra also contained ions for dHex$_2$Hex$_2$HexNAc$_5$-2AB, [M+Na]$^+$ $m/z$ 1970.8, as shown in Fig. 2. Hex$_3$HexNAc$_5$-2AB[M+Na]$^+$ $m/z$ 1663.2, was only observed in L4 larvae.

We detected higher molecular weight complex glycans in Dauer larvae than in other stages. Fig. 5B shows the MALDI-QTOF MS spectrum of fractions collected at 58–60 min, which included ions consistent with dHex$_2$Hex$_2$HexNAc$_5$-2AB, [M+Na]$^+$ $m/z$ 2116.8, dHex$_2$Hex$_2$HexNAc$_5$-2AB, [M+Na]$^+$ $m/z$ 2279.1, dHex$_2$Hex$_2$HexNAc$_5$-2AB, [M+Na]$^+$ $m/z$ 2319.9, and dHex$_2$Hex$_2$HexNAc$_5$-2AB, [M+Na]$^+$ $m/z$ 2522.9.

Hex$_3$HexNAc$_5$-2AB, [M+Na]$^+$ $m/z$ 1621.7, observed in Fig. 5D, was subjected to MALDI-QTOF MS/MS analysis, and the structure deduced for this glycan will be shown to be a structure not previously observed in C. elegans (Fig. 3). Evidence for two structures was obtained: one contained a lacNAc substitution, whereas the other had a core bisecting GlcNAc substitution. Evidence for the former structure is supported by the following fragments: 1) B$_{3nm}$, C$_{5nm}$, and B$_{2}$Y$_{3nm}$ ions at $m/z$ 550.20. These ions cannot originate from Structure II, whose presence is supported by ion B$_{2}$, at $m/z$ 347.11. Further support for the presence of Structure II comes from the observation that a biantennary Gal$_1$Man$_3$GlcNAc$_3$ standard, prepared from bovine IgG, did not give rise to ions of $m/z$ 347.11. Other possible isomers that by composition must contain a chito-, LacdiNAc, or HexNAc-Hex-HexNAc-Hex moiety would produce ions of $m/z$ 429.16 and 592.21 for the first two and 591.21 and 753.26 for the latter two. None of these ions was detected. It is possible that the higher mass Pc oligosaccharides found within the sample fraction (Pc$_1$deoxyHexHexHexNAc$_5$ [M+H]$^+$ $m/z$ 2095.0 and Pc$_1$deoxyHexHexHexNAc$_5$ [M+H]$^+$ $m/z$ 2280.9) may have contributed to some ions isolated at $m/z$ 1621.7 through prompt decay. However, because the Gal$_1$Man$_3$GlcNAc$_3$ standard did not show evidence of prompt decay under the experimental conditions such fragmentation seems unlikely.
As mentioned above, phosphorylcholine oligosaccharides were observed in all developmental stages with a generalized composition of $\text{Pc1–2Fuc0–2Hex2–5HexNAc2–8}$ (Table I). While all stages contained $\text{Pc1Hex3NAc3-2AB}$, $[\text{M}/\text{H}]^{+}$ $m/z$ 1399.5, L1, L4, adult, and Dauer larvae spectra showed ions consistent with $\text{Pc1Hex3HexNAc4-2AB}$, $[\text{M}/\text{H}]^{+}$ $m/z$ 1602.6, and $\text{Pc1Hex3HexNAc3-2AB}$, $[\text{M}/\text{H}]^{+}$ $m/z$ 1421.5.

**TABLE I**

| Assignment                  | Observed $m/z$ | Calculated $m/z$ |
|-----------------------------|----------------|------------------|
| $\text{Pc1dHex2Hex2HexNAc2}$ | 1348.7         | 1348.5           |
| $\text{Pc1dHex1Hex3HexNAc2}$ | 1364.7         | 1364.5           |
| $\text{Pc1Hex3HexNAc3}$     | 1399.5 $^{a,b}$ | 1399.5           |
| $\text{Pc1Hex3HexNAc3}$     | 1421.5 $^{a}$   | 1421.5           |
| $\text{Pc1Hex4HexNAc3}$     | 1583.8          | 1583.6           |
| $\text{Pc2Hex3HexNAc4}$     | 1602.6 $^{b}$   | 1602.6           |
| $\text{Pc1Hex3HexNAc4}$     | 1624.7 $^{b}$   | 1624.6           |
| $\text{Pc2dHex1Hex4HexNAc2}$ | 1691.7          | 1691.6           |
| $\text{Pc1Hex3HexNAc5}$     | 1742.8          | 1742.6           |
| $\text{Pc2Hex4HexNAc3}$     | 1748.5          | 1748.6           |
| $\text{Pc2Hex4HexNAc3}$     | 1754.4 $^{b}$   | 1754.4           |
| $\text{Pc2Hex3HexNAc4}$     | 1790.0          | 1790.7           |
| $\text{Pc2Hex3HexNAc5}$     | 1827.9          | 1827.7           |
| $\text{Pc2Hex3HexNAc5}$     | 1832.6          | 1832.5           |
| $\text{Me2dHex1Hex2HexNAc5}$ | 1874.7          | 1874.6           |
| $\text{Pc1dHex1Hex5HexNAc4}$ | 1973.6          | 1973.7           |
| $\text{Pc1dHex2Hex4HexNAc5}$ | 1974.7          | 1974.6           |
| $\text{Pc1dHex1Hex4HexNAc6}$ | 2095.0          | 2094.8           |
| $\text{Pc1dHex2Hex5HexNAc5}$ | 2119.7          | 2119.8           |
| $\text{Pc1dHex2Hex4HexNAc6}$ | 2136.0 $^{a}$   | 2135.8           |
| $\text{Pc1dHex2Hex4HexNAc7}$ | 2173.7          | 2173.8           |
| $\text{Pc1dHex2Hex5HexNAc5}$ | 2280.9          | 2280.8           |
| $\text{Pc1dHex2Hex5HexNAc6}$ | 2338.9          | 2338.9           |
| $\text{Pc1dHex2Hex6HexNAc6}$ | 2484.9          | 2484.9           |
| $\text{Pc1dHex2Hex6HexNAc6}$ | 2504.1          | 2503.9           |
| $\text{Pc1dHex3HexNAc5}$     | 2598.6          | 2598.0           |
| $\text{Pc1dHex3HexNAc6}$     | 2646.8          | 2646.0           |
| $\text{Pc1dHex3HexNAc6}$     | 2650.0          | 2650.0           |

$a$ Indicates the presence of a glycan of the same mass in an adjacent pool.

**TABLE II**

| Assignment                  | Observed $m/z$ | Calculated $m/z$ |
|-----------------------------|----------------|------------------|
| $\text{Hex3HexNAc4}$       | 1256.6 $^{b}$  | 1256.4           |
| $\text{Hex4HexNAc3}$       | 1399.5          | 1399.5           |
| $\text{Me2dHex1Hex2HexNAc5}$ | 1402.1          | 1402.5           |
| $\text{Hex5HexNAc3}$       | 1459.5          | 1459.5           |
| $\text{Hex6HexNAc3}$       | 1580.6          | 1580.6           |
| $\text{Hex7HexNAc3}$       | 1621.6          | 1621.6           |
| $\text{Hex8HexNAc3}$       | 1682.6          | 1682.6           |
| $\text{Me2dHex1Hex2HexNAc5}$ | 1742.8          | 1742.6           |
| $\text{Hex9HexNAc3}$       | 1742.6          | 1742.6           |
| $\text{dHex1Hex4HexNAc5}$  | 1970.7          | 1970.7           |
| $\text{dHex1Hex4HexNAc5}$  | 1970.8          | 1970.7           |
| $\text{dHex1Hex4HexNAc5}$  | 2116.8 $^{b}$   | 2116.8           |
| $\text{dHex1Hex4HexNAc5}$  | 1985.8          | 1985.8           |
| $\text{dHex1Hex4HexNAc5}$  | 2043.8          | 2043.8           |
| $\text{dHex1Hex4HexNAc5}$  | 2279.1          | 2279.1           |
| $\text{dHex1Hex4HexNAc5}$  | 2319.9          | 2319.9           |
| $\text{dHex1Hex4HexNAc5}$  | 2466.0          | 2466.9           |
| $\text{dHex1Hex4HexNAc5}$  | 2522.9          | 2522.9           |

All ions correspond to $[\text{M}/\text{H}]^{+}$. $^{a}$ Indicates the presence of a glycan of the same mass in a non-adjacent pool.

Detailed Analyses of Phosphorylcholine Oligosaccharides—
As mentioned above, phosphorylcholine oligosaccharides were observed in all developmental stages with a generalized composition of $\text{Pc1–2Fuc0–2Hex2–5HexNAc2–8}$ (Table I). While all stages contained $\text{Pc1Hex3NAc3-2AB}$, $[\text{M}/\text{H}]^{+}$ $m/z$ 1399.5, L1, L4, adult, and Dauer larvae spectra showed ions consistent with $\text{Pc1Hex3HexNAc4-2AB}$, $[\text{M}/\text{H}]^{+}$ $m/z$ 1602.6, and $\text{Pc1Hex3HexNAc3-2AB}$, $[\text{M}/\text{H}]^{+}$ $m/z$ 1421.5.
were most abundant in L1 and Dauer larvae. Difucosyl Pc glycans weight Pc glycans (Fig. 5, B) relativelly high molecular weight compounds observed in Dauer these have not been previously observed in any organism. The Pc1dHex1Hex3HexNAc2-2AB, [M+Na]+ lacks Golgi added GlcNAc were also observed such as chito-, and/or GlcNAc trisubstituted Man may be present containing more Pc than Golgi GlcNAc substitutions, and thereby suggests that Pc may substitute residues other than antennary GlcNAc.

Fragmentation studies were performed on those Pc glycans whose quantities were sufficient for structural studies, including determination of the sites of the Pc substitutions. Thus, PcHex,HexNAc2-2AB, [M+H]+ m/z 1748.5, from Dauer larvae was analyzed by PSD as shown in Fig. 6. This glycan was shown to have a structure not previously reported in any organism. The parent ion produced a PSD profile that can be consistent with three isomers where Pc substitutes one core and one antennary GlcNAc or both core GlcNAc residues. The three possible structures are shown in Fig. 6. The assignments of the fragment ions are shown above each ion peak in the spectrum. Regions within the glycans where fragmentation is observed in the spectrum are indicated. Some key fragment ions are discussed here. The fragment at m/z 369.26 is consistent with phosphorylcholine-substituted GlcNAc. The fragment ion at m/z 897.50 is indicative of a sodiated adduct containing two Pc-substituted GlcNAc residues and the 2-AB terminus, and thus, support core substitution with Pc. Fragments at m/z 1061.36 and m/z 1222.92 represent similar fragment ions that, in addition, contain one or two Hex residues, respectively. Other fragments that contain two Pc substitutions, such as those present at m/z 1017.51 and m/z 1386.45, can be produced by compounds containing only core Pc or those with antennary GlcNAc substitution. The abundance of the Pc-GlcNAc fragment at m/z 369.26 suggests that a significant amount of antennary Pc is present. Thus, these data are consistent with the notion that Pc can substitute both core and terminally linked GlcNAc.

PCHex,HexNAc2-2AB, that eluted at 46–48 min in samples from adult nematodes (Fig. 1), was also analyzed by PSD (Fig. 7). The parent ion at m/z 1421.56 yielded fragments B1m, m/z 369.14 and B2m, m/z 530.94, whose presence strongly suggests that Pc substitution of antennary GlcNAc has occurred, as previously reported (9).

Detailed Analyses of C. elegans-specific Fucosyl and Methylated Oligosaccharides—C. elegans-specific fucosyl oligosaccharide ions were detected in all developmental stages with a general trend of the number and amounts being Dauer > L1 > adult ~ L4 > L3 ~ L2. The majority of these glycans eluted between 40 and 46 min, and their positions are marked with diamonds on the chromatogram shown in Fig. 8. Ions detected in all developmental stages include dHex,HexNAc2-2AB, [M+Na]+ m/z 1199.5 (Table III), dHex,HexNAc2-2AB, [M+Na]+ m/z 1361.8 (Fig. 8, A and B), and dHex,HexNAc2-2AB, [M+Na]+ m/z 1523.8, (Fig. 8, A, B, and D). Several native O-methyl-substituted species were also observed such as Me1dHex,HexNAc2-2AB, [M+Na]+ m/z 1537.7 (Fig. 8, C and D), Me1dHex,HexNAc2-2AB, [M+Na]+ m/z 1521.6 (Fig. 8, E and F), and Me1dHex,GlcNAc2-2AB, [M+Na]+ m/z 1375.6 (Fig. 8, C, D, and F). The C. elegans-specific fucosyl disaccharides detected in these studies are shown in Table III.

Table III

| Assignment | Observed | Calculated |
|-----------|----------|------------|
| m/z       |          |            |
| dHex,HexNAc2 | 1199.5   | 1199.4     |
| Me1dHex,HexNAc2 | 1213.5   | 1213.4     |
| Me2dHex,HexNAc2 | 1334.6   | 1334.5     |
| Me1dHex,HexNAc2 | 1375.6   | 1375.5     |
| dHex,HexNAc2   | 1361.8   | 1361.5     |
| Me1dHex,HexNAc2 | 1389.6   | 1389.5     |
| Me2dHex,HexNAc2 | 1521.6   | 1521.5     |
| dHex,HexNAc2   | 1523.6   | 1523.5     |
| Me1dHex,HexNAc2 | 1537.7   | 1537.7     |
| HexNAc2        | 1539.6   | 1539.6     |
| Me1dHex,HexNAc2 | 1683.8   | 1683.6     |
| dHex,HexNAc2   | 1685.9   | 1685.6     |

Table IV

| Assignment | Observed | Calculated |
|-----------|----------|------------|
| m/z       |          |            |
| Hex,HexNAc2 | 1053.5   | 1053.4     |
| Me1Hex,HexNAc2 | 1067.5   | 1067.4     |
| Me1Hex,HexNAc2 | 1215.5   | 1215.4     |
| Me1Hex,HexNAc2 | 1229.5   | 1229.4     |
| Me1Hex,HexNAc2 | 1377.6   | 1377.5     |
| Me1Hex,HexNAc2 | 1539.7   | 1539.5     |
| Me1Hex,HexNAc2 | 1701.8   | 1701.6     |
| Me1Hex,HexNAc2 | 1863.7   | 1863.5     |
| HexNAc2      | 2025.9   | 2025.7     |
| HexNAc2      | 2187.8   | 2187.7     |

Table V

| Assignment | Observed | Calculated |
|-----------|----------|------------|
| m/z       |          |            |
| Hex,HexNAc2 | 1053.5   | 1053.4     |
| Me1Hex,HexNAc2 | 1067.5   | 1067.4     |
| Me1Hex,HexNAc2 | 1215.5   | 1215.4     |
| Me1Hex,HexNAc2 | 1229.5   | 1229.4     |
| Me1Hex,HexNAc2 | 1377.6   | 1377.5     |
| Me1Hex,HexNAc2 | 1539.7   | 1539.5     |
| Me1Hex,HexNAc2 | 1701.8   | 1701.6     |
| Me1Hex,HexNAc2 | 1863.7   | 1863.5     |
| HexNAc2      | 2025.9   | 2025.7     |
| HexNAc2      | 2187.8   | 2187.7     |

[+M+Na]+ m/z 1970.8 (Fig. 4, A–D, filled triangles). Higher molecular weight Pc glycans (Fig. 5, filled triangles), including those with Fuc, were most abundant in L1 and Dauer larvae. Difucosyl Pc glycans such as PcHex,HexNAc2-2AB, [M+Na]+ m/z 2646.8 (Fig. 5B), were also observed in the above stages. To our knowledge, these have not been previously observed in any organism. The relatively high molecular weight compounds observed in Dauer larvae (Fig. 5B) such as PcHex,HexNAc2-2AB, [M+H]+ m/z 2598.6, are highly substituted with HexNAc, suggesting that Lact-Nac, chito-, and/or GlcNAc trisubstituted Man may be present. As shown in L1, Fig. 5D, ions consistent with Pc glycans lacking Golgi added GlcNAc were also observed such as PcHex,HexNAc2-2AB, [M+H]+ m/z 1364.7. Table I shows the occurrence of the previous compounds, as well as others containing more Pc than Golgi GlcNAc substitutions, and thereby suggests that Pc may substitute residues other than antennary GlcNAc.

L1, L4, and Dauer larvae contained native methylated glycans without fucose such as Me1Hex,HexNAc2-2AB, [M+Na]+ m/z 1067.50, whereas L1 and L4 larvae contained Me1Hex,HexNAc2-2AB, [M+Na]+ m/z 1229.50. The former was analyzed by MALDI-QoTOF MS/MS (Fig. 10). Key fragments observed were B2m, m/z 509.15, C2m, m/z 527.16, and B3m, m/z 712.20. These indicate that the methyl is neither on Man nor on the second GlcNAc. Y1 m/z 378.15 and Z1 m/z 360.14
fragments show that the methyl is at the reducing end GlcNAc.

Other glycans detected as 2-AB derivatives could be assigned as having high mannose structures and are seen in Table IV. It is possible that shorter glycans, such as Hex₄HexNAc₂-2AB, may contain solely Man or Gal and Man additions to the chitobiose core, because both compositions can be predicted from glycan precursors previously described in C. elegans.

**Fig. 3.** QqTOF MS/MS analysis of C. elegans Hex₄HexNAc₂-2AB, [M+Na]⁺ m/z 1621.61. Fragmentation indicates that two isomers are present. The fragmentation pattern is consistent with the presence of LacNAc and core bisecting GlcNAc in Structures I (1) and II (2), respectively.

**Fig. 4.** C. elegans Pc oligosaccharides. MALDI-TOF MS spectra produced from fractions collected from 52–54 min are consistent with Pc₃Hex₃HexNAc₄-2AB, [M+H]⁺ m/z 1602.6, and Pc₃Hex₃HexNAc₄-2AB, [M+H]⁺ m/z 1970.8. These were observed in L1, L4, adult, and Dauer larvae glycan pools as shown in Fig. 3 (A–D), respectively. The sodiated form of the above, Pc₃Hex₃HexNAc₄-2AB, [M+Na]⁺ m/z 1624.6, and Pc₃Hex₃HexNAc₄-2AB, [M+Na]⁺ m/z 1790.0, were also observed. Pc oligosaccharides are indicated with ▲.
DISCUSSION

This study has shown major differences in the N-glycans of C. elegans developmental stages. Among the principal findings are: (a) the N-glycan profile of each developmental stage appears to be unique (Fig. 1). (b) the pattern of complex N-glycans was stage-specific (Fig. 2). The general trend of the number and...
abundance of glycans was Dauer \( \sim L1 > \) adult \( \sim L4 > L3 > L2 \).

(c) Dauer larvae contained complex glycans with higher molecular mass than those observed in other stages (Fig. 5B). MALDI-QqTOF MS/MS of Hex_{\text{HexNAc}}\_2AB, \([M+Na]^+ \) \(m/z\) 1621.7, observed mainly in L1, showed a lacNAc substitution not previously described in \( C.\ egans \) (Fig. 3). (d) Pc-containing glycans also appear to be stage-specific (Figs. 4 and 5). (e) higher molecular weight Pc-containing glycans, including those that contain fucose, were most abundant in L1 and Dauer larvae (Fig. 5). Difucoyl Pc glycans such as Pc\_dHex\_Hex\_Hex\_HexNAc\_\_2AB, \([M+Na]^+ \) \(m/z\) 2646.8, seen in Dauer larvae (Fig. 5B) had thus far not been observed in any organism. When the species Pc\_Hex\_Hex\_Hex\_Hex\_HexNAc\_\_2AB, \([M+H]^+ \) \(m/z\) 1748.5, from Dauer larvae was subjected to PSD analysis, the results showed that Pc may substitute both core and terminally linked GlcNAc: no such structures have been previously reported in any organism. (f) \( C.\ egans \)-specific fucosyl and native methylated glycans were found in all developmental stages.

The methodology used in this study, consisting of reductive amination of PNGase F-released oligosaccharides followed by separation on reversed phase high-performance liquid chromatography and offline MS analysis allowed us to detect higher order complex and intact Pc-containing glycans. Fragmentation analyses of some of the latter structures showed that Pc can substitute not only antennary but also core N-acetylgalcosamines or both. The latter had not been reported previously. In some Pc oligosaccharides up to two Fuc may be present. Although the amounts available were insufficient for further analysis, we hypothesize that Fuc may be antennary or in a novel linkage to the core GlcNAc, as has been found for glycans in \( S.\ tortus \) (29, 30). We can exclude the possibility that Fuc is linked \( \alpha 1,3 \) to the reducing end GlcNAc, because PNGase F is inactive toward \( \alpha 1,3 \)-substituted structures. The majority of the Pc oligosaccharides have the composition previously described for other nematodes in which \( N\)-acetylgalcosamines in GlcNAc\_\_2(GlcNAc\_\_6Man- and GlcNAc\_\_2(GlcNAc\_\_4Man- are substituted with Pc. This result is consistent with our recent observation that the phosphatidylcholine:oligosaccharide phosphorylcholine transferase of \( C.\ egans \) preferentially uses as its \textit{in vitro} substrate oligosaccharides containing Man disubstituted with GlcNAc (7). Rare Pc oligosaccharides with up to five phosphorylcholine substitutions have been reported in some nematodes (31). We did not observe any of these structures in the present study. However, we cannot rule out the possibility that these compounds are present in very low quantities.

In the present study, it was determined that higher order complex glycans, including those containing up to three Fuc, are most abundant in the L1 and Dauer stages. Because PNGase F was used successfully in the workup, it is likely that the Fuc is antennary; in this turn suggests that one of the glycans is fucosylated LacdiNAc. Previous studies showed that \( C.\ egans \) has a \( \beta\)-GlcNAc transferase, which catalyzes the addition of GalNAc to terminal GlcNAc to form the LacdiNAc structure GalNAc\_\_1,4GlcNAc\_\_1,R in membrane preparations and in Lec 82 and Lec 8 cells \textit{in vivo} (14). In addition, a \( C.\ egans \) \( \alpha 1,3\)-fucosyltransferase, CEFT-I, has been shown to synthesize the fucosylated LacdiNAc, GalNAc\_\_1,4(Fuca1,3)-GlcNAc\_\_1,R (15). These enzymatic activities are consistent with some oligosaccharide compositions detected in this study and also with species previously reported in \textit{Hemonchus contortus} (32, 33) but not in \( C.\ egans \). We have also demonstrated that \( C.\ egans \) produces LacNAc structures, a feature that had not been previously reported. The \( C.\ egans \) genome has been shown to possess three homologues of UDP-Gal: \( \beta\)-GlcNAc\_\_1,4-galactosyltransferase II, and this further supports the hypothesis that LacNAc structures exist in this nematode. \textit{In vitro}, the \( C.\ egans \) fucosyltransferase CEFT-I, \( \alpha 1,3\)-fucosyltransferase, catalyzes fucose addition to GalNAc\_\_1,4GlcNAc\_\_1,R to form Gal\_\_1,4(Fuca1,3)GlcNAc\_\_1,R (fucosylated LacNAc), the Lewis X (LeX) epitope. From the compositions seen here, it is possible that LeX structures exist, even though antibodies to LeX that have been tested so far fail to bind to \( C.\ egans \) extracts. In the future, antibodies raised against the glycans described here will help to pinpoint their specific tissue location.

As previously reported in studies where oligosaccharide release was performed using either PNGase A or hydrazinolysis, a novel group of \( C.\ egans \)-specific, Fuc-substituted, glycans occur with both \( \alpha 1,3\)- and \( 1,6\)-Fuc core substitutions (5, 6, 9, 12, 13, 16). Fuc may also be terminal, with an \( \alpha 1,2\)-linkage with Man and Gal as the penultimate sugar. Evidence for this was supported by the observation that, in \( C.\ egans \) srf\_3 mutants, which are defective in UDP-Gal transport, the highly fucosylated structures released by PNGase F and A treatments were diminished. This strongly suggests that most of these glycans contain internal Gal. In the same study we identified Gal\_\_1,Man\_\_2GlcNAc\_\_2, which may be an intermediate in the biosynthesis of the fucosylated species.

This and previous studies document the occurrence of natural \( O\)-methyl substitutions in glycans from \( C.\ egans \) (5, 9). The 3-O-methyl GlcNAc had also been previously reported in the cellulosome of \textit{Clostridium thermocellum} (34), as well as in as the Great pond snail \textit{Lymnaea stagnalis} and the Roman snail \textit{Helix pomatia} wherein glycans from hemocyanin contain Fuc with up to three \( O\)-methyls (35). In \textit{Rhizobium etli} CE3, repeating \( O\)-trisaccharide chains contain mono- and \( O\)-methyl substitutions, in addition to the capping sequence containing \( O\)-methyl Fuc. Methylation is hypothesized to prevent further elongation of \( O\)-chains (36).

Why is the N-glycan pattern more abundant and elaborate in the L1 and Dauer stage of \( C.\ egans \)? Although we do not yet have a definitive answer to this question, we speculate that both stages occur in conjunction with significant lifestyle changes in the worm. L1 larvae emerge at a time of development when the worm has exited embryonic development and enters vegetative growth while, at the Dauer stage the worm leaves vegetative growth to pass into a developmentally ar-
rested stage. Changes associated with glycans may be related to changes in the development of the nematode, innate immunity or processes in the secretory system required for the stage status. In this context, it has been reported that the unfolded protein response is highly active in the L1 through L2 stage, which would suggest a high rate of glycoprotein biosynthesis. Ire-1/pek-2 mutants of *C. elegans* are deficient in the unfolded protein response and arrest in the L2 stage (37).

In summary, we have presented the first account of stage-specific N-glycan expression in *C. elegans*. Developmental...
C. elegans Life Cycle and N-Glycosylation

stages that are under-represented in mixed stage preparations were shown here to contain novel N-glycans. The stage-specific glycan expression profiles here should facilitate efficient selection of stages to examine for further study of the C. elegans glycosynthetic pathways.

Acknowledgments—We thank the members of the Boston University School of Medicine Mass Spectrometry Resource. We give special thanks to Shui-Yung Chan for her helpful advice for optimizing chemistries used in this study.

REFERENCES

1. Haltiwanger, R. S., and Lowe, J. B. (2004) Annu. Rev. Biochem. 73, 491–537
2. Lowe, J. B. (2003) Curr. Opin. Cell Biol. 15, 531–538
3. Gorelik, E., Galili, U., and Raz, A. (2001) Cancer Metastasis Rev. 20, 245–277
4. Harris, T. W., Chen, N., Cunningham, F., Tello-Ruiz, M., Antoshechkin, I., Altmann, F., Fabini, G., Ahorn, H., and Wilson, I. B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 18851–18863
5. Kawar, Z. S., Van Die, I., and Cummings, R. D. (2002) J. Biol. Chem. 277, 22793–22803
6. DeBose-Boyd, R. A., Nyame, A. K., and Cummings, R. D. (1998) Glycobiology 8, 905–917
7. Zhu, S., Hanneman, A., Reinhold, V. N., Spence, A. M., and Schachter, H. (2004) Biochem. J. 382, 995–1001
8. Chen, S., Tan, J., Reinhold, V. N., Spence, A. M., and Schachter, H. (2002) Biochim Biophys. Acta 1573, 271–279
9. Chen, S., Zhou, S., Sarkar, M., Spence, A. M., and Schachter, H. (1999) J. Biol. Chem. 274, 288–297
10. Warren, C. E., Križus, A., Roy, F. J., Culotti, J. G., and Dennis, J. W. (2002) J. Biol. Chem. 277, 22793–22795
11. Natsuka, S., Adachi, J., Kawaguchi, M., Nakakita, S., Hase, S., Ichikawa, A., Kawar, Z. S., Van Die, I., and Cummings, R. D. (2002) J. Biol. Chem. 277, 53, 64
12. Schachter, H., Chen, S., Zhou, S., Sarkar, M., Spence, A. M., Zhu, S., Staudacher, E., and Schachter, H. (2003) Biochem. J. 372, 53–64
13. Cipollo, J. F., Awad, A. M., Costello, C. E., and Hirschberg, C. B. (2004) Glycobiology 14, 34924–34932
14. Kawar, Z. S., Van Die, I., and Cummings, R. D. (2002) J. Biol. Chem. 277, 34924–34932
15. DeBose-Boyd, R. A., Nyame, A. K., and Cummings, R. D. (1998) Glycobiology 8, 905–917
16. Zhu, S., Hanneman, A., Reinhold, V. N., Spence, A. M., and Schachter, H. (2004) Biochem. J. 382, 995–1001
17. Chen, S., Tan, J., Reinhold, V. N., Spence, A. M., and Schachter, H. (2002) Biochim Biophys. Acta 1573, 271–279
18. Chen, S., Zhou, S., Sarkar, M., Spence, A. M., and Schachter, H. (1999) J. Biol. Chem. 274, 288–297
19. Warren, C. E., Križus, A., Roy, F. J., Culotti, J. G., and Dennis, J. W. (2002) J. Biol. Chem. 277, 22793–22795
20. Zhu, S., Hanneman, A., Reinhold, V. N., Spence, A. M., Zhu, S., Staudacher, E., and Schachter, H. (2003) Biochem. J. 372, 53–64
21. Tomiya, N., Narang, S., Lee, Y. C., and Betenbaugh, M. J. (2004) Glycoconjug. J. 21, 343–360
22. Epstein, H. F., and Shakes, D. C. (1995) Caenorhabditis elegans: Modern Biological Analysis of an Organism, Academic Press, San Diego, CA
23. Veronese, M. F., Lubowski, C., and Trimble, R. B. (2000) Anal. Biochem. 278, 111–122
24. Dubuis, M., Gilles, K. A., Hamilton, J. K., Rebers P.A., and Smith, F. (1956) Anal. Biochem. 28, 350–356
25. Cucinotta, I., and Kerek, F. (1984) Carbohydr. Res. 131, 209–217
26. Routier, F. H., Hunselli, E. R., Rudd, P. M., Takahashi, N., Boud, A., Hay, F. C., Alavi, A., Axford, J. S., and Jeffries, R. (1998) J. Immunol. Methods 213, 113–130
27. Watanabe, T., Inoue, N., Kutsuzake, T., Matsuki, S., and Takeuchi, M. (2000) Bioll. Pharm. Bull. 23, 269–273
28. Harvey, D. J. (1999) Mass Spectrom. Rev. 18, 349–450
29. Khoo, K. H., Huang, H. H., and Lee, K. M. (2001) Glycobiology 11, 149–163
30. Nymay, A. K., DeBose-Boyd, R., Long, T. D., Tsang, V. C., Spence, A. M., and Cummings, R. D. (1999) Glycobiology 8, 615–624
31. Haaslem, S. M., Houston, K. M., Harnett, W., Reasson, A. J., Morris, H. R., and Dell, A. (1999) J. Biol. Chem. 274, 20853–20860
32. Haaslem, S. M., Coles, G. C., Reasson, A. J., Morris, H. R., and Dell, A. (1998) Mol. Biochem. Parasitol. 93, 143–147
33. Haaslem, S. M., Coles, G. C., Munn, E. A., Smith, T. S., Smith, H. F., Morris, H. R., and Dell, A. (1998) Mol. Biochem. Parasitol. 93, 143–147
34. Gerwig, G. J., de Waard, P., Kamerling, J. P., Vliegenthart, J. F., Morgenstern, E., Lamed, R., and Bayer, E. A. (1989) J. Biol. Chem. 264, 1027–1035
35. Van Kuik, J. A., Sijbesma, R. P., Kamerling, J. P., Vliegenthart, J. F., and Wood, E. J. (1987) Eur. J. Biochem. 169, 399–411
36. Forsberg, L. S., Bhat, U. R., and Carlson, R. W. (2000) J. Biol. Chem. 275, 18851–18863
37. Shen, X., Ellis, E. R., Lee, K., Liu, C. Y., Yang, K., Solomon, A., Yoshida, H., Morimoto, K., Kurnit, D. M., Mert, K., and Kaufman, R. J. (2001) Cell 107, 893–903
N-Glycans of Caenorhabditis elegans Are Specific to Developmental Stages
John F. Cipollo, Antoine M. Awad, Catherine E. Costello and Carlos B. Hirschberg

J. Biol. Chem. 2005, 280:26063-26072.
doi: 10.1074/jbc.M503828200 originally published online May 17, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M503828200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 11 of which can be accessed free at
http://www.jbc.org/content/280/28/26063.full.html#ref-list-1