Molecular Basis of Anti-horseradish Peroxidase Staining in Caenorhabditis elegans*

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Cross-reactivity with anti-horseradish peroxidase antiserum is a feature of many glycoproteins from plants and invertebrates; indeed staining with this reagent has been used to track neurons in Drosophila melanogaster and Caenorhabditis elegans. Although in insects the evidence indicates that the cross-reaction results from the presence of core α,3-fucosylated N-glycans, the molecular basis for anti-horseradish peroxidase staining in nematodes has been unresolved to date. By using Western blots of wild-type and mutant C. elegans extracts in conjunction with specific inhibitors, we show that the cross-reaction is due to core α,3-fucosylation. Of the various mutants examined, one with a deletion of the fut-1 (K08F8.3) gene showed no reaction to anti-horseradish peroxidase; the molecular phenotype was rescued by injection of either the K08F8 cosmid or the fut-1 open reading frame under control of the let-638 promoter. Furthermore, expression of fut-1 cDNA in Pichia and insect cells in conjunction with antibody staining, high pressure liquid chromatography, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry analyses showed that FUT-1 is a core α,3-fucosyltransferase with an unusual substrate specificity. It is the only core fucosyltransferase in plants and animals described to date that does not require the prior action of N-acetylgalactosaminyltransferase I.

The structures of the oligosaccharides expressed by different organisms and tissues or on individual glycoproteins vary greatly. Over the years, it has become clear that the glycosylation machinery of any organism is ge-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ745071, AJ505020, AJ745072, AJ745073, and AJ745074.

1 The abbreviations used are: RP-HPLC, reverse phase-high pressure liquid chromatography; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; MS, mass spectrometry; MES, 4-morpholineethanesulfonic acid; BSA, bovine serum albumin; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; dabsyl, 4-(4-dimethylaminophenylazo)-benzolsulfonyl.

or phosphorylcholine groups, as well as the standard oligomannose structures (7). Multiantennary structures may also exist, but there are no sialylated complex glycans of the types found in vertebrates. Glycolipids of the arthro-series carrying phosphorylcholine (8) as well as unusual fucosylated O-glycans have also been described (9). Some of these features have already been found in parasitic nematodes and are a source of immunomodulatory, immunogenic, or allergenic activity (10).

Still, however, the exact structures of many of the N-glycans from the worm are unknown; in particular, the nature of the linkages of the multiple fucose residues is not resolved, although RP-HPLC evidence (2) indicates that both core α,1,3- and core α,1,6-fucosylation of N-glycans occur. The former is also a feature of plant glycoproteins and is the major epitope of many antibodies raised against plant glycoproteins, such as anti-horseradish peroxidase (anti-HRP) (11); this antibody also recognizes neuronal tissue in C. elegans (12, 13). Because of the known specificity of anti-HRP, this cross-reaction could be inferred to be due to the presence of core α,1,3-fucose; however, the actual molecular origin of the cross-reaction observed remained unresolved, particularly due to a lack of knowledge about the exact glycosylation capacity of C. elegans.

Because the glycosylation machinery of any organism is genetically determined, examination of genomes is useful in modern glyobiology. Indeed, a large number of glycosyltransferase homologues can be identified in the genome of C. elegans. However, in only a few cases has their activity been determined. In particular, N-acetylgalactosaminyltransferases with roles in O-glycan (14) and, potentially, N-glycan and glycolipid biosynthesis (specifically, the β1,4-N-acetylgalactosaminyltransferase BRE-4) (15) have been expressed in recombinant form. An α,1,2-fucosyltransferase with an unknown biological function (16), one N-acetylgalactosaminyltransferase V homologue (17), one glucosyltransferase that potentially modifies O-glycans (18), and three genes encoding proteins with N-acetylgalactosaminyltransferase I activity have also been described (19). The presence of the latter three is intriguing because plants, insects, and mammals would appear to only have one such gene. In mammals, N-acetylgalactosaminyltransferase I is necessary for the generation of complex N-glycans and is requisite for survival in utero (20, 21). Furthermore, the prior action of this enzyme is necessary in plants, insects, and mammals for both core α,1,3- and core α,1,6-fucosylation (22–24). However, the biosynthetic pathways required for the gen-
eration of the more unusual fucose modifications of *C. elegans* N-glycans have not been studied, although the initial assumption would be that core fucosylation would occur in a similar way as in other eukaryotes. Although there is putatively only one core 1,6-fucosyltransferase, there are five core 1,3-fucosyltransferases (25) which in theory could be responsible for the core 1,3-fucosylation and the anti-HRP binding observed.

Following our previous work on the anti-HRP epitope of *Drosophila* (26), we have now re-examined the binding of anti-HRP to *C. elegans* proteins. By using glycan analysis, Western blotting, and fucosyltransferase assays of extracts of both wild-type and mutant nematodes, we conclude that the cross-reaction is actually due to core 1,3-fucose. Furthermore, we have shown that one of the 1,3-fucosyltransferase homologues is indeed a core 1,3-fucosyltransferase responsible in vitro and in vivo for generation of the anti-herosradish peroxidase epitope.

**EXPERIMENTAL PROCEDURES**

Preparation of *C. elegans* Extracts—*C. elegans*, whether wild-type or mutant, were routinely maintained at 16 °C on a lawn of *Escherichia coli* OP50 from N2 plates or from bacteria and debris by 30% (w/v) sucrose gradient centrifugation. Pellets were stored at −80 °C before use. *C. elegans* extracts were prepared from a total of 1 g of nematodes (wet weight) homogenized in 3 ml of 50 mM MES, pH 7, containing protease inhibitor cocktail (EDTA-free, Roche Applied Science) and 1% (w/v) Triton X-100. After incubating for 30 min on ice, the debris was removed by centrifugation at 10,000 × g for 10 min at 4 °C. The supernatant was aliquoted and stored at −20 °C before use. These extracts were used for both enzyme assays and Western blotting.

Preparation of Transgenic Worms—Cosmids encoding fucosyltransferase homologues within their normal genetic environment were obtained from The Sanger Centre, Cambridge, UK. For constitutive expression, the put-1 gene, the entire open reading frame was obtained by reverse transcription-PCR using the primers K08F8.3/1/KpnI, GGGGTACCATGAGGGTTCGGCCAGC, and F59E12.13/2/XbaI, GCTCTAGACAACTACAAATATTTCGAAGC. In the case of the PCR product, re-amplification by using KOD Hot Start DNA polymerase was performed on 12.5% (v/v) sucrose.

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with *C. elegans* cDNA and the primers K08F8.3/3/PstI, AACTGCAGGAAAATCTGAGAAACAGGATTG, and K08F8.3/2/XbaI, GCTCTAGAC-TAATCTAACGGATAAATC. The PCR product was purified from the PCR mix using the GFX DNA purification kit (Amerham Biosciences). Both fragment and vector were digested with PstI and XbaI prior to ligation, transformation, selection, and sequencing. The expression vector was transformed into *Pichia pastoris* GS115, and colonies were selected and expression performed with methanol induction at 16 °C as described previously (28). Concentrated supernatants were assayed either directly or after Affi-Gel Blue-Sepharose chromatography as described previously (28). Concentrated supernatants were assayed either directly or after Affi-Gel Blue-Sepharose chromatography as described previously (28). Concentrated supernatants were assayed either directly or after Affi-Gel Blue-Sepharose chromatography as described previously (28). Concentrated supernatants were assayed either directly or after Affi-Gel Blue-Sepharose chromatography as described previously (28). Concentrated supernatants were assayed either directly or after Affi-Gel Blue-Sepharose chromatography as described previously (28).

**Analysis of the Enzyme Product**—Larger scale incubations of dansyl-MM and dansyl-MMF^6^ (2 nmol) with FUT-1 were subject to RP-HPLC. The purified products (putatively dansyl-MMF^6^ and dansyl-MMF^6^) were analyzed by MALDI-TOF MS and by methylation analysis. For the latter, the glycopeptides were permethylated, hydrolyzed, reduced, and peracetylated prior to analysis of the obtained partially methylated alditol acetates by capillary gas-liquid chromatography/mass spectrometry by using the instrumentation and microtechniques described elsewhere (32, 33).

**Expression in Insect Cells**—*Drosophila* S2 cells grown at 27 °C were transiently transfected using TransFectin Lipid Reagent (Bio-Rad), according to the manufacturer’s protocol for adherent cells, with either empty pIZT/V5-His or pIZT/V5-His constructs containing complete open reading frames of either one of the five *C. elegans* fucosyltransferase homologues or *Arabidopsis thaliana* FucTA. Transfected cells were lysed 3 days post-transfection, and equal amounts of extracted proteins were resolved by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-HRP antibodies as described above. Successful transfection was confirmed by the fluorescence of the gfp^zeo^ gene product encoded by the vector.

**Incubation of Fucosyltransferase with a Neoglycoconjugate**—A neoglycoconjugate, BSA-MM, consisting of a glycopeptide from bovine fibrin modified to remove sialic acid, galactose, and N-acetylgalactosaminyl residues cross-linked to bovine serum albumin using dinitrodifluorobenzene (11), was incubated with FUT-1 overnight in the absence and presence of GDP-Fuc prior to SDS-PAGE and Western blotting as described above. In parallel, the band corresponding to BSA-MM was excised from a Coomassie-stained gel and subjected to trypsin and peptide-N-glycosidase A digestion as described (30).

### RESULTS

**Antibody Binding Studies**—In initial studies we screened antibodies that recognize core α1,3-fucose with *C. elegans* extracts. Whereas anti-HRP is a polyclonal antibody shown previously to bind both core α1,3-fucose and β1,2-xylose (11, 34–36), anti-bee venom antisera contain a proportion of antibodies recognizing core α1,3-fucose on insect and plant glycoproteins (37), and the monoclonal antibody YZ1/2.23 has been shown to bind core α1,3-fucose (11, 34, 38). In the present study, previous Western blotting results (39) were confirmed that showed that anti-horseradish peroxidase binds wild-type *C. elegans* glycoproteins; furthermore, anti-bee venom and YZ1/2.23 also showed cross-reactivity (Fig. 1A). The Reactivities of both anti-

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**Scheme 1. Structures of N-glycans referred to in this study.** Abbreviations are based on the system of Schachter; see also Refs. 26 and 28.

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[^1]: K. Paschinger and I. B. H. Wilson, unpublished data.
horseradish peroxidase and anti-bee venom were greatly reduced in the presence of 0.5 μM of a neoglycoconjugate consisting of bromelain glycopeptides attached to bovine serum albumin (BSA-MUX/F3) (Fig. 1A). Even 10 times more of the corresponding defucosylated conjugate (BSA-MUX) did not generate the same inhibitory effect; this is in contrast to the results with *Schistosoma mansoni* egg extracts with which the defucosylated conjugate also showed significant inhibitory effect (data not shown). On this basis we concluded that the cross-reaction of anti-horseradish peroxidase to *C. elegans* is due to core α1,3-fucose, whereas in the case of *S. mansoni* both xylose and fucose would appear to play a role. Indeed, to date, no xylose has been detected on the *N*-glycans of any nematode, whereas this monosaccharide is a known component of trematode *N*-glycans (40).

In order to determine the genetic basis of this cross-reaction, we examined homozygous mutants that have deletions in genes encoding proteins with homology to α1,3-fucosyltransferases. It was obvious that only the VC378 strain, which carries a deletion in the *fut-1* (K08F8.3) gene, did not display anti-horseradish peroxidase staining in Western blots (Fig. 1B). Most interestingly, a strain in which all three *N*-acyetylglucosaminyltransferase I genes are mutated still displayed anti-horseradish peroxidase staining (Fig. 1B, lane 7). Indeed, extracts of the wild-type nematode show no *N*-acyetylglucosaminyltransferase I activity, and MS analysis of its *N*-glycans generates data compatible with the absence of its activity (41). The blotting data with this mutant was, therefore, a first indication that the core α1,3-fucosyltransferase of the nematode does not require the presence of 0.5 μM of a neoglycoconjugate consisting of bromelain glycopeptides attached to bovine serum albumin (BSA-MUX/F3) (Fig. 1A). Even 10 times more of the corresponding defucosylated conjugate (BSA-MUX) did not generate the same inhibitory effect; this is in contrast to the results with *Schistosoma mansoni* egg extracts with which the defucosylated conjugate also showed significant inhibitory effect (data not shown). On this basis we concluded that the cross-reaction of anti-horseradish peroxidase to *C. elegans* is due to core α1,3-fucose, whereas in the case of *S. mansoni* both xylose and fucose would appear to play a role. Indeed, to date, no xylose has been detected on the *N*-glycans of any nematode, whereas this monosaccharide is a known component of trematode *N*-glycans (40).

**N-Glycan Analyses—**As a follow up to these data, we examined the *N*-glycans of wild-type and mutant nematodes and found that the VC378 strain lacks a subset of *N*-glycans (compare Fig. 2, A and B; see also Table II), in particular a number of putative tri- and tetrafucosylated glycans with the following predicted compositions: FucHexHexNAc (monoisotopic m/z 1371), FucHexHexNAcHex (m/z 1533), FucHexHexNAcHexNac (m/z 1679), FucHexHexNacHexNacMe (m/z 1693), FucHexHexNacHexNacMe2 (m/z 1695), FucHexHexNacHexNacMe3 (m/z 1841), FucHexHexNacHexNacMe4 (m/z 1855), FucHexHexNacHexNacMe5 (m/z 1857), and FucHexHexNacHexNacMe6 (m/z 2017). Furthermore, in the monoisotopic spectra of VC378 glycans there appear to be nine mono- and dimethylated species that do not seem to occur in wild-type nematodes: FucHexHexNacHexNacMe2 (m/z 1269), FucHexHexNacHexNacMe3 (m/z 1415), FucHexHexNacHexNacMe4 (m/z 1431), FucHexHexNacHexNacMe5 (m/z 1561), FucHexHexNacHexNacMe6 (m/z 1577), FucHexHexNacHexNacMe7 (m/z 1591), FucHexHexNacHexNacMe8 (m/z 1723), FucHexHexNacHexNacMe9 (m/z 1725), and FucHexHexNacHexNacMe10 (m/z 1871). There is also, qualitatively at least, a large increase in the intensity of the FucHexHexNacHexNacMe10 peaks (m/z 1403, 1549, and 1711) and FucHexHexHexNacHexNacMe10 (m/z 1563) and a decrease in the intensity of FucHexHexNacHexNacMe (m/z 1225) as compared with neighboring ones. Some of these differences are only revealed with the monoisotopic spectra.

Of the other four fucosyltransferase mutants examined, only RB706 (with a deletion in the *fut-6* gene) appeared to lack some species with the same m/z as those absent in VC378 (Table II), specifically those of m/z 1679, 1693, 1695, 1841, 1855, and 1717, and 2017. On the other hand, no obvious non-wild-type species were present in RB706. The other three fucosyltransferase mutants gave spectra considered more or less equivalent to wild type (Table II). Our own data (not shown) with the triple knock-out gly-14 gly-12 gly-13 showed the presence of fucosyl-
**Core α1,3-Fucosylation in C. elegans**

**Table II**

Composition of N-glycans from wild-type and mutant C. elegans

| Composition                      | m/z   | N2 wild-type | VC378 fut-1 | VC182 fut-3 | VC212 fut-4 | RB511 fut-5 | RB706 fut-6 |
|----------------------------------|-------|--------------|-------------|-------------|-------------|-------------|-------------|
| Fuc,Hex,HexNAc                  | 917.27| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 931.31| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 933.39| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1063.44| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1077.27| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1079.47| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1093.27| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1095.46| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1136.54| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc                  | 1225.59| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1239.61| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc                  | 1241.56| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1255.62| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1257.59| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1269.42| -            | +           | -           | -           | -           | -           |
| Fuc,Hex,HexNAc                  | 1282.61| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1301.45| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1339.62| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc                  | 1371.66| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1385.67| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc                  | 1387.66| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1401.63| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1403.55| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1415.53| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1417.58| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1419.62| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1431.48| -            | +           | -           | -           | -           | -           |
| Fuc,Hex,HexNAc,PC               | 1447.54| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1483.52| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc,PC                   | 1504.56| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc                  | 1533.69| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1547.40| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc                  | 1549.75| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1561.48| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1563.60| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1577.56| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc                  | 1581.68| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1589.60| -            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc                  | 1679.79| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1693.65| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1695.61| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc,Me                   | 1709.33| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1711.53| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc,Me                   | 1723.58| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1725.58| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1743.68| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc                  | 1811.70| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 1855.61| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1857.54| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc                  | 1871.65| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc                  | 1903.42| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 1905.63| +            | +           | +           | +           | +           | +           |
| Fuc,Hex,HexNAc,Me               | 2017.65| +            | +           | +           | +           | +           | +           |
| Hex,HexNAc                      | 2067.54| +            | +           | +           | +           | +           | +           |

aded N-glycans of the form Fuc_{2-4}Hex_{4-9}HexNAc_{2-9} in this strain even in the absence of N-acetylglucosaminyltransferase I activity (41). Based on the anti-HRP binding evidence presented in Fig. 2, we assume some of the fucosylated glycans in the latter mutant are core α1,3-fucosylated.

**Assays of Fucosyltransferases in Nematode Extracts**—The data from Western blotting and N-glycan analysis suggested that the VC378 strain lacks core α1,3-fucosylase activity toward dabsyl-MM as shown in Fig. 3. We followed up this latter result by using MALDI-TOF MS and RP-HPLC assays of wild-type and mutant C. elegans. Wild-type extracts displayed activity toward dabsyl-MM as shown by an increase, in the presence of GDP-Fuc, in the m/z corresponding to the mass of one fucose. In contrast, VC378 extract did not display this activity (Fig. 3), although control experiments with dabsyl-GnGn demonstrated that this extract did contain a putative core α1,6-fucosyltransferase activity.

4 K. Paschinger, E. Staudacher, G. Fabini, and I. B. H. Wilson, submitted for publication.
In order to gain evidence that the fucose transferred by *C. elegans* extracts to MM is indeed core α1,3-linked, the assays were also performed using dansyl-MM and dansyl-MMF6 (derived from human IgG). As shown previously with *Drosophila* and mung bean core α1,3-fucosyltransferases with dansyl-GnGn and dansyl-GnGnF6, core α1,3-fucosylation results in a reduction in retention time of dansylated IgG glycopeptides upon RP-HPLC (23, 26, 29), whereas α1,6-fucosylation results in an increase in retention time. The former effect was apparent for dansylated MM and MMF6 upon incubation with *C. elegans* N2 extract in the presence of GDP-Fuc, whereas no shift was apparent with VC378 extract (Fig. 4), indicating a lack of core α1,3-fucosyltransferase activity in the mutant. This result is consistent with the absence of anti-agaroside staining in this strain.

**Cloning of α1,3-Fucosyltransferase Homologues**—The five α1,3-fucosyltransferase homologue cDNAs were cloned to generate the full-length reading frames. 5' and 3'-primers for isolation of the fut-1, fut-3, and fut-6 cDNAs were designed on the basis of EST information. In the case of *fut-1* and *fut-6*, EST entries with SL1 spliced leaders are present in the GenBankTM/EMBL data base, whereas one *fut-3* EST has an SL2 spliced leader. For *fut-4* and *fut-5*, the exact location of the 5'-end was unknown from previous cDNA sequences. Indeed for *fut-5*, there are no corresponding ESTs, and the initially predicted T05A7.5 reading frame included both the *fut-5* and *fut-6* sequences (the two genes being in fact separated by ~10 kb), whereas the initially predicted K12H6.3 (*fut-4*) reading frame encoded no transmembrane domain. Thus, homology searching and visual inspection of the translated genomic sequences to search for conserved motifs and potential transmembrane domains was performed prior to primer design for both *fut-4* and *fut-5*. The lengths of the predicted proteins range from 378 to 433 residues. For *fut-3*, *fut-5*, and *fut-6*, the respective predicted protein sequence lengths are the same as given for CEFT-3, CEFT-2A, and CEFT-2B as indicated in Ref. 42. As judged from the EST data bases, FUT-1 is expressed in mixed populations, in L1 and L2 larvae, and in hermaphroditic embryos. The genes encoding all five α1,3-fucosyltransferase homologues are present on chromosome II.

The predicted FUT-1 protein sequence is the longest of the five α1,3-fucosyltransferase homologues, the major difference compared with the others being in the putative stem region (Fig. 5). FUT-1 shares 33–35% identity with the other four α1,3-fucosyltransferases over 260–310 residues, and it shares 78% identity over the whole sequence with the *Caenorhabditis briggsae* predicted protein CBP14511. Ignoring CBP14511, the closest hits on performing a BLAST search are *Drosophila melanogaster* FucTC (identities 101/291), *S. mansoni* FucTA (102/302), *Glossinia mortisana* FucTC (99/296), *Xenopus laevis* Lewis 2 α1,3/4-fucosyltransferase homologue (110/337), and *D. melanogaster* FucTA (103/341), all of these being more highly ranked matches than any of the other *C. elegans* fucosyltransferases; however, of these homologues, only the *D. melanogaster* core α1,3-fucosyltransferase FucTA has proven enzymatic activity. In the case of *C. elegans* FUT-3, the potential N-glycosylation site residue 152, conserved in all five *C. elegans* and in many animal α1,3-fucosyltransferases (e.g. Asn-194 of FUT-1), has been shown as part of a survey of glycoproteins to be actually glycosylated (43). On the other hand, the DXD or SXD motif present in many α1,3/4-fucosyltransferases, claimed in many glycosyltransferases to have a role in metal co-factor or donor substrate binding, is changed to DTP (residues 202–204) in the case of FUT-1.

**FUT-1 Generates the Anti-HRP Epitope in Vivo**—The constructs encoding full-length forms of all five α1,3-fucosyltransferases were transfected into Schneider cells. As Schneider cells do not express the anti-agaroside peroxidase epitope, they represent a suitable null background for expression of core α1,3-fucosyltransferases. As controls, constructs carrying either a known core α1,3-fucosyltransferase, from *A. thaliana* (44), or no insert were used. Of the five *C. elegans* α1,3-fucosyltransferases, only cells transfected with *fut-1* gained anti-agaroside peroxidase reactivity (Fig. 6A), a result that is in accordance with the Western blotting and glycan and enzymatic data from the corresponding mutant VC378 strain. The
Core α1,3-Fucosylation in *C. elegans*

Gain of the epitope was indeed even more pronounced with the *C. elegans* than with the *A. thaliana* enzyme.

In order to show that *fut-1* was capable of “rescuing” the anti-HRP defect of the VC378 strain, complementation was performed by co-injection into VC378 mutant worms of either the cosmid K08F8 or the pPD118.25/rol-6 vector, three rolling lines were isolated, which all displayed anti-HRP binding (Fig. 6B, lanes 6–8). Injection of pRF4 alone did not rescue staining (Fig. 6B, lane 5). All complemented lines have a similar anti-HRP staining pattern as wild-type worms, with constitutive expression resulting in a higher intensity of staining as compared with wild-type and cosmid-rescued lines. As both experiments upon transfection of insect cells and *C. elegans* were successful, we concluded that FUT-1 is the core α1,3-fucosyltransferase necessary for the acquisition of the anti-HRP epitope in wild-type *C. elegans*. For the subsequent experiments it was therefore decided to focus on the properties of FUT-1.

**Assays of Recombinant FUT-1**—Constructs encoding soluble forms (i.e. lacking the cytoplasmic and transmembrane regions) of *C. elegans* FUT-1, with or without an N-terminal FLAG tag, were transformed into *P. pastoris*. The culture supernatants were assayed for fucosyltransferase activity toward dabasylated MM, GnGn, GalGal, and βGnβGN. Supernatants from yeast transformed with both forms of the *fut-1* construct only displayed activity toward dabasyl-MM (Fig. 7A), an activity absent from control transformants. Furthermore, FUT-1 was also tested with glycans from the corresponding mutant, VC378; a reduction in the Hex$_3$HexNAc$_2$ (m/z 934) peak and an increase in the Fuc$_2$Hex$_3$HexNAc$_2$ (m/z 1226) species was observed, compatible with the conversion of putative MM and MMF$^6$ to MMF$^3$ and MMF$^3$F$^6$ respectively, assuming that the species with m/z 1080 consists of MMF$^3$ after incubation with FUT-1, rather than MMF$^6$, as it would before such treatment (compare Fig. 7, C and D). Tests were also performed using pyridylaminated forms of lacto-N-tetraose and lacto-N-neo-tetraose at the same concentration as used with the glycopeptide substrates (i.e. 50 μM) and at 400 μM; however, no shifts in retention time indicative of conversion to fucosylated forms of these tetrasaccharides were observed at either concentration. Furthermore, whereas a dansylated peptide carrying Man$_5$GlcNAc$_2$ was not a substrate for recombinant FUT-1, a dabasylated peptide carrying GnM was an acceptor (data not shown). The latter result would suggest that some substitution of the α1,6-arm may be tolerated by FUT-1, although certainly the α1,3-linked mannose must be free.

The activity of recombinant FUT-1 was also confirmed in an HPLC-based assay. As in the case of wild-type *C. elegans* extracts, a shift to lower retention time was observed when either dansyl-MM or dansyl-MMF$^6$ was used as a substrate, with a higher conversion being apparent with the latter substrate (Fig. 8). The HPLC-purified products were confirmed by MALDI-TOF MS to have m/z values compatible with fucosylation; furthermore, methylation analysis of the enzymatic products demonstrated that the two fucose residues of the putative MMF$^3$F$^6$ were indeed on the same GlcNAc residue, because a GlcNAc with a nonsubstituted 6-hydroxyl, a feature absent from control transformants. Furthermore, FUT-1 was also tested with glycans from the corresponding mutant, VC378; a reduction in the Hex$_3$HexNAc$_2$ (m/z 934) peak and an increase in the Fuc$_2$Hex$_3$HexNAc$_2$ (m/z 1226) species was observed, compatible with the conversion of putative MM and MMF$^6$ to MMF$^3$ and MMF$^3$F$^6$ respectively, assuming that the species with m/z 1080 consists of MMF$^3$ after incubation with FUT-1, rather than MMF$^6$, as it would before such treatment (compare Fig. 7, C and D). Tests were also performed using pyridylaminated forms of lacto-N-tetraose and lacto-N-neo-tetraose at the same concentration as used with the glycopeptide substrates (i.e. 50 μM) and at 400 μM; however, no shifts in retention time indicative of conversion to fucosylated forms of these tetrasaccharides were observed at either concentration. Furthermore, whereas a dansylated peptide carrying Man$_5$GlcNAc$_2$ was not a substrate for recombinant FUT-1, a dabasylated peptide carrying GnM was an acceptor (data not shown). The latter result would suggest that some substitution of the α1,6-arm may be tolerated by FUT-1, although certainly the α1,3-linked mannose must be free.

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The recombinant FUT-1 could be eluted with 0.6 M NaCl from Affi-Gel-Sepharose and subsequent SDS-PAGE indicated the presence of a protein of Mr 55,000 (as compared with a theoretical value of 46,000 for the soluble form of FUT-1). Tryptic digestion of the nontagged form indicated the presence of 10 peptides compatible with the theoretical map of FUT-1, whereas Western blotting of the FLAG-tagged form showed strong reactivity also around Mr 55,000 (data not shown). Subsequent characterization of the activity of FUT-1 was per-

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**Fig. 5. Alignment of *C. elegans* α1,3-fucosyltransferase homologues.** The sequences of all five homologues were aligned using the Multalin program with subsequent manual adjustment. Residues identical in two or more sequences are white on black, and conserved residues in other sequences at these positions are black on gray. Putative transmembrane and N-glycosylation sites are underlined; numbering refers to the residues of FUT-1.

| Residue | FUT-1 | FUT-2 | FUT-3 | FUT-4 | FUT-5 |
|---------|-------|-------|-------|-------|-------|
| 47      | Y     | Y     | Y     | Y     | Y     |
| 102     | F     | F     | F     | F     | F     |
| 142     | F     | F     | F     | F     | F     |
| 248     | Y     | Y     | Y     | Y     | Y     |
| 491     | Y     | Y     | Y     | Y     | Y     |

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[^1]: [Insert reference](#)

[^2]: [Insert reference](#)
formed on these preparations of the enzyme. The optimal pH was found to be between pH 6.5 and 8, whereas at least 2-fold higher activity was found in the presence of Mg(II) ions as compared with Co(II), Mn(II), Ca(II), Fe(II) or EDTA; no activity was detected in the presence of either Cu(II), Ni(II), Zn(II), or Fe(III) ions.

Finally, in order to show that FUT-1 indeed generates the anti-HRP epitope in vitro, a neoglycoconjugate of bovine serum albumin carrying an MM-glycopeptide was incubated with purified FUT-1 in the absence and presence of GDP-Fuc. As predicted from the other substrate specificity experiments, the thereby modified BSA-MM conjugate was recognized by anti-HRP (Fig. 9A, lane 2). MALDI-TOF MS analysis of the glycans of the conjugate incubated in the presence of GDP-Fuc showed the presence of a peak with a composition compatible with an MMF3 structure (Fig. 9C), whereas this peak was absent from the control (Fig. 9B).

DISCUSSION

The fucosylation of C. elegans glycoconjugates is potentially highly complex due to the presence of around 20 α1,2-fucosyltransferases, five α1,3-fucosyltransferases, and one α1,6-fucosyltransferase homologue. To date the exact structures of only some of the fucosylated N- and O-glycans are known (2, 9), whereas no fucosylated glycolipids have as yet been detected. There is also only fragmentary data on the fucosylation of other nematodes: up to three fucose residues on the core of N-glycans (45, 46) and the presence of a Lewis-type fucosyltransferase (47) in Haemonchus, fucosylation of artho-series glycolipids from Ascaris suum (48), and the presence of Lewis epitopes in Dictyocaulus viviparus (49). In C. elegans, the single obvious α1,6-fucosyltransferase homologue (FUT-8) has been expressed

![Fig. 6. C. elegans FUT-1 creates the anti-HRP epitope in vitro. A. expression in insect cells: extracts of nontransfected Drosophila S2 cells (lane 1) or of Drosophila S2 cells transfected with pIZT-V5-His construct containing either no insert (lane 2) or complete reading frames of A. thaliana FucTA (positive control; lane 3), C. elegans fut-1 (lane 4), C. elegans fut-3 (lane 5), C. elegans fut-4 (lane 6), C. elegans fut-5 (lane 7), and C. elegans fut-6 (lane 8) were subjected to Western blotting by using rabbit anti-HRP. B, complementation of the VC378 mutant: extracts of wild-type N2 (lane 1), VC378 (lane 2), or rolling lines of VC378 injected with pRF4 and cosm id K08F8 (lane 3, line 1.7; lane 4, line 1.3), pRF4 alone (lane 5) or pRF4 and pPD118.25/fut-1 (lane 6, line 5.1; lane 7, line 5.2; lane 8, line 5.3) were subjected to Western blotting with rabbit anti-HRP (1:12,500).](image)

![Fig. 7. MALDI-based fucosyltransferase assays of recombinant C. elegans FUT-1. Dabsyl-MM (A and B) or N-glycans from VC378 (C and D) were incubated overnight at room temperature in the presence (A and C) and absence (B and D) of GDP-Fuc together with culture supernatant of yeast expressing FUT-1.](image)

![Fig. 8. HPLC-based fucosyltransferase assays of recombinant C. elegans FUT-1. Dansyl-MM (A and B) or dansyl-MMF6 (C and D) were incubated overnight at room temperature in the absence (A and C) or presence (B and D) of GDP-Fuc together with culture supernatant of yeast expressing FUT-1.](image)
in an active recombinant form, and the determination of the enzymatic activity of only one α1,2-fucosyltransferase (FUT-2 or CE2FT1) has been published (16) but left its biological substrate unrevealed. However, the activity of most of the α1,2-fucosyltransferase homologues is still unknown, whereas published and preliminary data suggested that the α1,3-fucosyltransferase homologues were Lewis-type enzymes (42, 50, 51). Thus even if Lewis-type glycans and fucosylated glycoproteins have not been found in C. elegans, the presence of enzymes capable of generating such structures in vitro suggests there is the potential that Lewis or related epitopes occur in small amounts in the worm, depending on the presence of suitable in vivo precursors, even though there are data indicating no binding of Lewis* or Lewis* antibodies in Western blots (52). The presence of core α1,3-fucose, on the other hand, was not accounted for at all at the genetic level.

Considering that we had determined previously that core α1,3-fucosylation is the probable basis for anti-horseradish peroxidase binding in D. melanogaster (26) and that xylose had not been found to be a constituent of C. elegans N-glycans, we hypothesized that one or more core α1,3-fucosyltransferases were responsible for generation of the epitope in the nematode. Initial results with inhibitors suggested that core α1,3-fucose was responsible for the cross-reaction, but we failed to detect core α1,3-fucosylation by C. elegans extracts in vitro when using dabsyl- or dansyl-MM as substrates. These substrates are utilized by core α1,3-fucosyltransferases from plants and insects (26, 44), as well as by the core α1,6-fucosyltransferase of C. elegans. It was only when we wished to rule out the native core α1,6-fucosyltransferase could utilize MM that we detected a core fucosyltransferase in wild-type C. elegans (4). Indeed, this enzyme preferentially transfers fucose at nonreducing ends and thus the core α1,6-fucosyltransferase structure.

In parallel to the inhibition Western blots and enzyme assays with wild-type worm extracts, we surveyed the anti-horseradish peroxidase binding characteristics and N-glycan spectra of the five publicly available α1,3-fucosyltransferase knockout mutants. Of these, only VC378 lacked the epitope (Fig. 1B). This was a surprising result, considering that VC378 has a 597-bp deletion in the fut-1 gene, resulting in the absence of residues 46–190 of the predicted protein sequence. Previously, mammalian cells transfected with fut-1 cDNA were found to express a Lewis-type enzyme activity (50).

However, encouraged by the results with the VC378 strain, we initiated our own studies on the expression of fut-1; indeed, compatible with the various results with the mutant, transfection of fut-1 into yeast, C. elegans, or insect cells resulted in expression of an enzyme with the ability to generate the anti-horseradish peroxidase epitope (Figs. 6 and 9). In studies with the yeast-produced enzyme only MM-fucosylating activity was detected (Figs. 7 and 8). Also, blotting analysis of the relevant mutants and transfection into insect cells indicated that the other four α1,3-fucosyltransferases are not involved in expression of the anti-HRP epitope (Figs. 1B and 6A). Thus, there is a number of key results (lack of epitope, enzyme activity, and certain glycans in the VC378 strain, complementation of the molecular phenotype with either the cosmid or the open reading frame, generation of the epitope in insect cells, and, in vitro, demonstration of fucosyltransferase activity in transformed yeast and linkage analysis of the enzymatic product) that demonstrate that FUT-1 is the core α1,3-fucosyltransferase that synthesizes the anti-HRP epitope in C. elegans.

The different conclusions from our work and the earlier study on the enzymology of FUT-1 have a number of potential origins. First, the previous study did not apparently involve testing of N-glycan substrates. Second, a relatively high lacto-N-neo-tetraose concentration (5 mM) was used in the earlier study (50), which gave rise to less than 0.003% conversion of acceptor to product/h. Third, a different expression system could theoretically affect the substrate specificity. In our experiments with dabsyl- and dansyl-MM, we used 50 μM acceptor substrate and attained 5–10% conversion/h. We also examined whether FUT-1 displayed activity to dabsyl-GalGal and lacto-N-neo-tetraose at 50 μM (the latter also at 400 μM), but we observed no transfer. Thus, it may be that the Lewis-type activity is indeed a property of FUT-1, but only at very high substrate concentrations. Furthermore, all three expression systems we used (C. elegans, insect, and yeast) showed production of the anti-HRP epitope upon transfection into a null background, and thus the core α1,3-fucosylating property is reproduced independent of the expression system used.

The newly defined properties of FUT-1, particularly its preference for MM as opposed to GnGn, indicate that the biosynthesis of core difucosylated N-glycans differs between the nematode and insects. This may mean that during evolution either the core α1,3-fucosyltransferase of the nematode has changed its properties or that a functional convergence has occurred that the same glycan product can be generated by a different series of biosynthetic steps. Indeed, it would appear that most, if not all, glycans that carry core α1,3-fucose in D. melanogaster arc core α1,6-fucosylated (26), and this may be true also in C. elegans (2). However, there are more solely core α1,6-fucosylated N-glycans in both species. This would suggest that the core α1,6-fucosyltransferase acts first, after N-acetylgalactosaminyltransferase I. In insects, however, the core α1,3-fucosyltransferase still requires the presence of nonreducing terminal N-acetylgalactosamine residues, and we assume that the action of core α1,6-fucosyltransferase acts first, after N-acetylgalactosaminyltransferase I. In insects, however, the core α1,3-fucosyltransferase still requires the presence of nonreducing terminal GlcNAc residues, and we assume that the action of core α1,6-fucosyltransferase is followed by that of core α1,3-fucosyltransferase (FucTA, in the case of D. melanogaster), and then a Golgi β-hexosaminidase, such as that present in SF9 cells (53), can act to generate MMF*F*F*. In C. elegans, however, the core α1,3-fucosyltransferase does not act when a nonreducing terminal GlcNAc substitution is present on the α1,3-arm, and so one may hypothesize that in wild-type C. elegans, the action of the N-acetylgalactosaminyltransferase I isoforms is followed by those of core α1,6-fucosyltransferase, α-mannosidase II, and by a Golgi β-hexosaminidase (the latter having already been described in C. elegans (54)). The thereby generated

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5 Details of the deletion are given under the on-line address: aceserver.biotec.ubc.ca/cgi-bin/generic/allele?class=Allele,name=gk183.
MMF<sup>6</sup>-carrying glycoproteins would then be hypothesized to act as substrates for FUT-1, which synthesizes MMF<sup>Fl</sup>. It is probable, however, that the in vitro situation is more complicated. First, there are low amounts of glycans with the same composition as MMF<sup>Fl</sup> (i.e. Fuc<sub>2</sub>Hex<sub>2</sub>HexNAc<sub>2</sub>) still present in VC378. Second, there is a series of tri- and tetrafucosylated N-glycans present in the wild-type absent from the VC378 mutant as well as mono- and difucosylated species in the gly-14 gly-12 gly-13 triple knock-out.

With respect to the first point, a number of isomers of Fuc<sub>2</sub>Hex<sub>2</sub>HexNAc<sub>2</sub> are conceivable. In addition to MMF<sup>Fl</sup>, which is probably present in the wild-type as judged from previous chromatographic evidence (2), the second residue of the core chitobiose could also be modified by α,1,3-fucose, such as observed in Haemonchus contortus (45, 46). Considering the findings with the RB706 mutant, which also lacks some of the more highly fucosylated structures but not the anti-HRP epitope, C. elegans FUT-6 is certainly an interesting candidate for future studies on nematode N-glycosylation pathways.

The second point, based on the presence of tri- and tetrafucosylated structures, raises the question whether MM is the only substrate of FUT-1 in vivo. On the other hand, in vitro FUT-1 only obviously fucosylates glycans that are probably MM and MMF<sup>6</sup> among a mixture of VC378 N-glycans (Fig. 7C). Thus, it may be that some larger glycans cannot function as substrates for FUT-1 because of subsequent glycosylation events resulting in “NOGO” signals; this would explain why adding FUT-1 to VC378 glycans does not generate the full series of wild-type glycans. Further complicating the picture is the finding that worms lacking GlcNAc-TI possess fucosylated glycans (Fuc<sub>2</sub>Hex<sub>2</sub>HexNAc<sub>2</sub>) not found in wild-type worms, while lacking MM, as judged by the absence of Hex<sub>2</sub>GlcNAc<sub>2</sub> (41), proteins from these worms bind anti-HRP and so therefore can be assumed to contain N-glycans resulting from the action of FUT-1. Thus, one can surmise that further substrates, yet to be defined (other than MM, which is more or less absent from the gly-14 gly-12 gly-13 triple knock-out), may be used by FUT-1 in vivo and/or that unknown hexosyl- and fucosyltransferases can act before and after FUT-1. Further characterization of potential substrates, as well as of the tri- and tetrafucosylated N-glycans in Caenorhabditis, is therefore still required.

As part of our studies, we performed various N-glycan analyses and confirmed the complexity we and others have reported previously. Our data agree well with those of Zhu et al. (41) in terms of the high degree of fucosylation of wild-type glycans as well as the presence of mono- and difucosylated glycans in the gly-14; gly-12 gly-13 triple knock-out. However, the results with wild-type worms differ in that Zhu et al. (41) detected glycans of the form Me<sub>2</sub>GlcNAc<sub>2</sub>Fuc<sub>2</sub>Hex<sub>2</sub>HexNAc<sub>2</sub>, which we did not, but they did not find phosphorylcholine, which we did detect. This may be because of the fact that they released their glycans by hydrazinolysis, which may remove phosphorylcholine, while enabling the release of that proportion of glycans hypothesized to have a high degree of galactosylation on the core fucose residues (55). Some of the structures with four or more hexose residues, three or more fucose residues, and 2-O-methylation of fucose, observed by us and others, are still unique to C. elegans and have yet to be discovered in other nematodes; however, the presence of galactose attached to core fucose residues has already been observed on squid rhodopsin (56).

Although the VC378 mutant appears to be viable under normal laboratory conditions, we assume that core α1,3-fucosylation has some, possibly subtle, function in nematode biology. In C. elegans, expression of this epitope appears to be restricted to 10% of the neurons (12), and indeed fut-1 is apparently only expressed in specific neural cells (16). However, from the viewpoint of the wider importance of nematodes as parasites, more significant is that core α1,3-fucose is a known IgE epitope (39, 57), and so the presence of this epitope on excretory-secretory antigens, for example (as shown previously by antibody binding for H. contortus (39)), may have a role in the interactions of parasitic nematodes with their hosts. C. elegans also shares carbohydrate epitopes with H. contortus that induce protective immunity (58). Other tests on protective immunity to H. contortus conferred by an antibody specific for excretory-secretory antigen suggest α1,3-fucosylated LaedlNAc has a role (59), although structural evidence for this epitope in C. elegans and H. contortus is lacking. On the other hand, the H. contortus H11 glycoprotein, which also confers protection, is known to be core α1,3-fucosylated (46). Furthermore, glycans of parasitic nematodes generate Th2 responses, a fact that also has been found to hold for C. elegans glycans (even though this is not of direct pathogenic significance, because C. elegans is not an animal parasite); and even more significant is preliminary data indicating that fucosylated C. elegans glycans are responsible for the observed Th2 response (60). Therefore, C. elegans fucosylation mutants should be interesting models, not just for biosynthetic and glycomics studies but also for future immunological investigations.

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