

Demonstration of glycosaminoglycans in *Caenorhabditis elegans*

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Abstract A considerable amount (approximately 1.6 µg from 1 mg of dried nematode) of non-sulfated chondroitin, two orders of magnitude less yet an appreciable amount of heparan sulfate, and no hyaluronate were found in *Caenorhabditis elegans* nematodes. The chondroitin chains were heterogeneous in size, being shorter than that of whale cartilage chondroitin sulfate. The disaccharide composition analysis of heparan sulfate revealed diverse sulfation including glucosamine 2-*N*-sulfation, glucosamine 6-*O*-sulfation and uronate 2-*O*-sulfation. These results imply that chondroitin and heparan sulfate are involved in fundamental biological processes.

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Key words: Chondroitin; Heparan sulfate; Glycosaminoglycan; ¹H NMR; *Caenorhabditis elegans*

1. Introduction

Glycosaminoglycans (GAGs) have been implicated in the regulation and maintenance of cell adhesion, motility, proliferation, differentiation, tissue morphogenesis and embryogenesis [1–5]. The biological activities of GAGs depend on their ability to interact with a variety of proteins including growth factors, cytokines, enzymes, serine protease inhibitors and extracellular matrix proteins, and many such interactions are thought to be highly specific reflecting respective saccharide sequences embedded in GAG chains, which regulate the physiological functions of these proteins [6]. GAGs have a polysaccharide backbone which is a linear polymer composed of alternating amino sugar and hexuronic acid residues. This simple repeat structure of GAGs acquires a considerable degree of variability by extensive modifications involving sulfations and uronate epimerization [7]. Such structural variability of GAGs is the basis for the wide variety of domain structures with biological activities [2] and is generated by the elaborate, concerted actions of biosynthetic enzymes. Over the past several years, cDNAs of a number of the biosynthetic enzymes for GAGs have been cloned. Thus, the opportunity has arrived for modern GAG glycobiology to develop.

Investigation of the occurrence of GAGs in tissues of vertebrates and invertebrates has shown a wide distribution in the

animal kingdom [8], suggesting that GAGs are phylogenetically conserved through evolution, and play fundamental biological roles in animal development. The nematode *Caenorhabditis elegans* is an ideal experimental organism for studying a wide range of fundamental biological disciplines in development using modern techniques of molecular and cell biology since it is a small, rapidly growing, multicellular organism with characteristic nervous, muscle, digestive and reproductive systems. The progress made in the *C. elegans* genome project has revealed the existence of multiple genes that are related to GAG-synthesizing enzymes and appear to be ancestors of vertebrate counterparts. However, there is no direct evidence for the existence of GAGs in *C. elegans*. In this study we isolated and characterized a non-sulfated chondroitin and heparan sulfate (HS) in adult worms.

2. Materials and methods

2.1. Materials

Chondroitinase ABC (EC 4.2.2.4), chondroitinase AC-II (EC 4.2.2.5), chondroitinase B (EC 4.2.2.), heparinase (EC 4.2.2.7), heparitinase I (EC 4.2.2.8), heparitinase II (no EC number), *Streptomyces* hyaluronidase (EC 4.2.2.1), whale cartilage chondroitin sulfate (CS) A, human umbilical cord hyaluronic acid and standard unsaturated disaccharides derived from CS and HS were obtained from Seikagaku, Tokyo, Japan. Glycuronate 2-sulfatase was a gift from Dr. Keiichi Yoshida (Seikagaku). Sephadex gels and prepacked Superdex 75 and Superdex 200 columns (10×300 mm) were purchased from Amersham Pharmacia Biotech, Tokyo, Japan.

2.2. Preparation of a GAG fraction from *C. elegans*

Wild-type *C. elegans* (strain N2, Bristol) were cultured on NGM plates seeded with *Escherichia coli* strain OP50, as described [9]. Freshly cultured nematodes were homogenized with a polytron and freeze-dried. The dried sample (60 mg) was extracted with acetone and then treated with 14 ml of 1.0 M NaBH₄/0.05 M NaOH at room temperature for 2 h. Acetic acid was added to stop the reaction. The sample was adjusted to 5% trichloroacetic acid and centrifuged. The soluble fraction was extracted with ether and the aqueous phase was adjusted to 80% ethanol. The resultant precipitate was dissolved in water and subjected to gel filtration chromatography on a column of Sephadex G-50 (fine) using 0.25 M NH₄HCO₃/7% 1-propanol as an eluent.

2.3. Enzymatic digestion

Digestion with chondroitinases ABC, AC-II or B, or a mixture of heparinase and heparitinases I and II was conducted as described previously [10,11]. Successive enzymatic digestion with heparitinases and then glycuronate 2-sulfatase was carried out as follows. Samples were first digested with heparitinases for 1 h at 37°C in a total volume of 30 µl and reactions were terminated by boiling for 1 min. The reaction mixture was mixed with 10 µl of water and 20 µl of 20 mM acetate-Na buffer, pH 6.5, containing 0.15% bovine serum albumin and 3 mIU of glycuronate 2-sulfatase, then incubated at 37°C for 1 h. *Streptomyces* hyaluronidase digestion was also carried out as follows. Samples were incubated with 2 mIU of the enzyme for 30 min at 60°C in a total volume of 20 µl of 100 mM acetate buffer, pH 5.0. Reactions were terminated by boiling for 1 min and the reaction

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Abbreviations: 2-AB, 2-aminobenzamide; ΔHexA, 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid; GlcA, D-glucuronic acid; IdceA, L-iduronic acid; CS, chondroitin sulfate; HS, heparan sulfate; GAG, glycosaminoglycan; HPLC, high-performance liquid chromatography

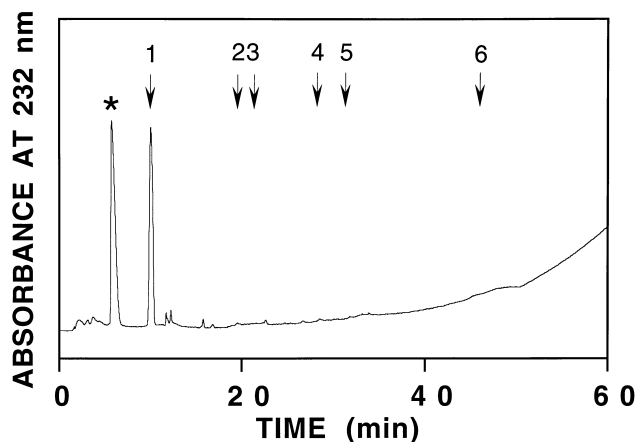


Fig. 1. HPLC analysis of the chondroitinase ABC digest of the *C. elegans* GAG fraction. The chondroitinase ABC digest of the *C. elegans* GAG fraction was analyzed by HPLC on an amine-bound silica column. The peak eluted at around 5 min and marked by an asterisk in the lower panel is an acetate ion derived from the incubation buffer. The elution positions of authentic unsaturated disaccharides are indicated by arrows. 1, $\Delta\text{HexA}\alpha 1\text{-3GalNAc}$; 2, $\Delta\text{HexA}\alpha 1\text{-3GalNAc(6-O-sulfate)}$; 3, $\Delta\text{HexA}\alpha 1\text{-3GalNAc(4-O-sulfate)}$; 4, $\Delta\text{HexA(2-O-sulfate)}\alpha 1\text{-3GalNAc(6-O-sulfate)}$; 5, $\Delta\text{HexA}\alpha 1\text{-3GalNAc(4,6-O-disulfate)}$; 6, $\Delta\text{HexA(2-O-sulfate)}\alpha 1\text{-3GalNAc(4,6-O-disulfate)}$.

mixture was analyzed by high-performance liquid chromatography (HPLC) on an amine-bound silica column as reported previously [12]. The heparitinase digests were derivatized with 2-aminobenzamide (2-AB) then analyzed by HPLC as reported previously [13].

2.4. 500 MHz ^1H NMR spectroscopy

Samples for NMR analysis were repeatedly exchanged in $^2\text{H}_2\text{O}$ with intermediate lyophilization. The 500-MHz ^1H NMR spectra were measured on a Varian-500 at a probe temperature of 26°C using a Nano-NMR probe containing 40 μl of the sample solution. The Nano-NMR probe spins samples rapidly at the magic angle to remove the magnetic-susceptibility contributions to the ^1H NMR line widths [14]. The spin rate was typically about 2 kHz. Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly relative to acetone (δ 2.225) in $^2\text{H}_2\text{O}$ [15].

2.5. Gel filtration chromatography of *C. elegans* chondroitin

The *C. elegans* GAG fraction was analyzed by gel filtration chromatography on a column (10×300 mm) of Superdex 75 or Superdex 200 eluted with 50 mM pyridine-acetate buffer, pH 5.0, at a flow rate of 1 ml/min in a Pharmacia FPLC system (Amersham Pharmacia Biotech, Tokyo, Japan). Fractions were collected at 1.0-min intervals, lyophilized and digested with chondroitinase ABC. The digests were derivatized with 2-AB then analyzed by HPLC on an amine-bound silica column [13].

3. Results

To investigate the existence of GAGs in *C. elegans*, a GAG fraction was prepared from the acetone powder of the crude homogenate of the worms. The acetone powder was treated with alkaline NaBH_4 to release GAGs from putative core proteins and a GAG fraction was recovered by ethanol precipitation followed by gel filtration on a Sephadex G-50 column. An analysis was carried out on this fraction by HPLC to identify the disaccharides produced by digestion with specific GAG lyases.

Upon digestion with chondroitinase ABC, a single major peak was detected at the elution position of the authentic non-sulfated chondroitin disaccharide, $\Delta\text{HexA}\alpha 1\text{-3GalNAc}$, and no other peaks were detected at the sulfated disaccharide positions (Fig. 1). Chondroitinase ABC acts not only on chondroitin yielding $\Delta\text{HexA}\alpha 1\text{-3GalNAc}$ but also on hyaluronic acid producing $\Delta\text{HexA}\alpha 1\text{-3GlcNAc}$ [16]. Since both disaccharides are eluted at the same position on HPLC under the conditions used [12], the peak was identified as $\Delta\text{HexA}\alpha 1\text{-3GalNAc}$ based on the resistant nature of the GAG fraction to the action of *Streptomyces* hyaluronidase (data not shown), which is specific for hyaluronic acid but not for chondroitin [17]. Approximately 250 nmol of the unsaturated disaccharide was recovered by chondroitinase ABC digestion from 60 mg of dried worms.

To further elucidate the structure of $\Delta\text{HexA}\alpha 1\text{-3GalNAc}$, the chondroitinase ABC digestion product was analyzed by 500 MHz ^1H NMR spectroscopy (data not shown). Proton NMR spectroscopy is a powerful tool in obtaining structural information about complex carbohydrates [15]. Advanced ^1H -

Table 1
 ^1H -Chemical shifts of the oligosaccharide prepared by chondroitinase ABC digestion

Reporter groups		Reference compound ^a		Chondroitinase ABC digest	
		α	β	α	β
GalNAc	H-1	5.215 (4.0 ^b)	4.710 (8.5)	5.216 (4.0)	4.737 (8.0)
	H-2	4.285	3.989	4.269	3.944
	H-3	4.103	3.927	4.09	3.91
	H-4	4.176	4.106	4.14	4.08
	H-5	4.142	ND ^c	ND	3.72
	H-6	ND	ND	3.69 - 3.72	3.62 - 3.68
	H-6'	ND	ND	3.69 - 3.72	3.62 - 3.68
	NAc	2.055	2.055	2.056	2.059
ΔHexA	H-1	5.245 (4.5)	5.197 (4.5)	5.261 (4.0)	5.228 (4.5)
	H-2	3.810	3.807	3.821	
	H-3	4.099	4.099	4.09	
	H-4	5.904	5.904	5.924	

Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly in reference to acetone (δ 2.225 ppm) in $^2\text{H}_2\text{O}$ at 26°C . The estimated error for the values to two decimal places was only ± 0.01 ppm because of partial overlap of signals. That for the values to three decimal places was ± 0.002 ppm.

^aReference compound is the non-sulfated disaccharide derived from chondroitin, $\Delta\text{HexA}\alpha 1\text{-3GalNAc}$ [18].

^bCoupling constants $J_{1,2}$ (in Hz) are given in parentheses.

^cND, not determined.

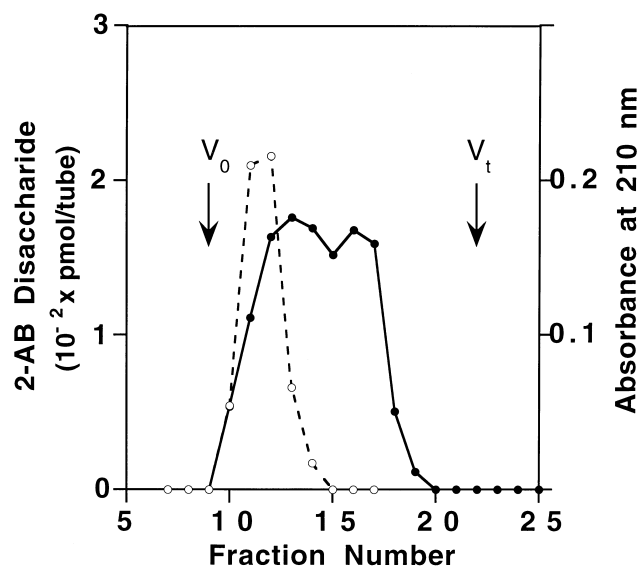


Fig. 2. Molecular size analysis of the *C. elegans* chondroitin on gel filtration chromatography. The *C. elegans* GAG fraction (corresponding to 1.2 nmol of chondroitin) was subjected to gel filtration chromatography on a Superdex 200 column. The chondroitinase ABC digests of individual fractions were derivatized with 2-AB then analyzed by HPLC. The amount of the 2-AB derivative of $\Delta\text{HexA}\alpha 1\text{-3GalNAc}$ was calculated based on fluorescence intensity (solid line). V_0 and V_t were determined using human umbilical cord hyaluronic acid and NaCl, respectively. The broken line indicates the elution profile of the whale cartilage CS-A, the molecular mass of which was reported to be 25–50 kDa by the manufacturer. It was monitored by measurement of the absorbance at 210 nm after each fraction was dried and resolved in distilled water.

NMR methods are suitable for determining the proton chemical shifts, which indicate the presence, type and location of substituents, such as acetyl, methyl, phosphate and sulfate groups [15]. The proton chemical shifts were assigned using two-dimensional homonuclear Hartmann-Hahn and correlation spectroscopy (data not shown) and the NMR data are summarized in Table 1. The data were virtually identical with those reported previously for the authentic $\Delta\text{HexA}\alpha 1\text{-3GalNAc}$ [18], indicating that the disaccharide is derived from chondroitin, but not from hyaluronic acid and that no structural modifications by any uncharged groups or sulfate are present.

The *C. elegans* GAG fraction was resistant to the action of chondroitinase B but sensitive to that of chondroitinase AC-II, indicating that the non-sulfated unsaturated disaccharide is derived from chondroitin, which is composed of $\text{-4GlcA}\beta 1\text{-3GalNAc}\beta 1\text{-}$ units, rather than dermatan composed of $\text{-4IdcA}\alpha 1\text{-3GalNAc}\beta 1\text{-}$ units [16].

The molecular size of the *C. elegans* chondroitin was analyzed by gel filtration chromatography. It was eluted in the flow-through fraction of a Superdex 75 column but included in a Superdex 200 column. To monitor small amounts of chondroitin, aliquots of individual fractions collected at 1.0-min intervals were lyophilized and digested with chondroitinase ABC, then the products were derivatized with a fluorophore 2-AB [13] and analyzed by HPLC. The amount of the 2-AB derivatized disaccharide product $\Delta\text{HexA}\alpha 1\text{-3GalNAc}$ was calculated based on its fluorescence intensity. Since molecular weight standards of chondroitin oligosaccharides with

appropriate sizes were not available, the molecular mass of the *C. elegans* chondroitin was compared with commercial whale cartilage CS-A (25–50 kDa). As shown in Fig. 2, a broad peak of the *C. elegans* chondroitin was observed, indicating the heterogeneous sizes, and the average chain length was shorter than that of commercial CS-A.

The *C. elegans* GAG fraction was also subjected to HS disaccharide composition analysis after digestion with a mixture of heparinase, heparitinases I and II. Since the amount of the disaccharide products obtained was far less than that of chondroitin, the resulting disaccharides were labeled with a fluorophore 2-AB and analyzed by HPLC on an amine-bound silica column. The chromatogram obtained after removal of the reagent (2-AB) by paper chromatography is shown in Fig. 3. Based on the fluorescence intensity, the yield of each disaccharide was calculated and the data are summarized in Table 2. Most of the non-sulfated disaccharide product $\Delta\text{HexA}\alpha 1\text{-3GlcNAc}$ is lost during the paper chromatographic purification of 2-AB derivatives as reported previously [13] and peaks of some contaminants derived from the paper strip used for removing the reagent were detected at around the elution position of the non-sulfated disaccharide as can be seen in Fig. 3B. Hence, the non-sulfated disaccharide was quantified by injecting another aliquot before removing the reagent (data not shown), which gave a sharp distinguishable peak of the non-sulfated disaccharide as reported [13]. The total amount of HS disaccharides produced by digestion with heparitinases was only 1.7 nmol per 60 mg of dried worms, two orders of magnitude less than that of chondroitin. The identity of the disaccharide peaks eluted at the positions of $\Delta\text{HexA}(2\text{-O-sulfate})\alpha 1\text{-4GlcN}(2\text{-N-sulfate})$ and $\Delta\text{HexA}(2\text{-O-sulfate})\alpha 1\text{-4GlcN}(2\text{-N},6\text{-O-disulfate})$ were established based on the sensitivity to glycuronate 2-sulfatase, which hydrolyzes the sulfate ester bond of the 4,5-unsaturated uronic acid 2-O-sulfate structure at the non-reducing end of disaccharides [19]. Upon the digestion, these peaks were shifted indeed to the positions of $\Delta\text{HexA}\alpha 1\text{-4GlcN}(2\text{-N-sulfate})$ and $\Delta\text{HexA}\alpha 1\text{-4GlcN}(2\text{-N},6\text{-O-disulfate})$, respectively (data not shown). Due to a limited availability of these disaccharides, it was not feasible to measure ^1H NMR spectra of the disaccharides.

4. Discussion

The complete genome sequence of *C. elegans* (1×10^8 bp) has been determined, and a number of developmentally important genes have been revealed. However, the glycobiology and conventional biochemistry of *C. elegans* have advanced less than the genetics, anatomy, neurobiology and develop-

Table 2
Disaccharide composition analysis of *C. elegans* HS

Disaccharides	Proportion (mol%)
$\Delta\text{HexA}\alpha 1\text{-4GlcNAc}$	47
$\Delta\text{HexA}\alpha 1\text{-4GlcNAc}(6\text{S}^a)$	6
$\Delta\text{HexA}\alpha 1\text{-4GlcN}(\text{NS})$	14
$\Delta\text{HexA}\alpha 1\text{-4GlcN}(\text{NS},6\text{S})$	ND ^b
$\Delta\text{HexA}(2\text{S})\alpha 1\text{-4GlcN}(\text{NS})$	18
$\Delta\text{HexA}(2\text{S})\alpha 1\text{-4GlcN}(\text{NS},6\text{S})$	15

^a6S, NS or 2S represents 6-O-sulfate, 2-N-sulfate, or 6-O-sulfate, respectively.

^bND, not detected.

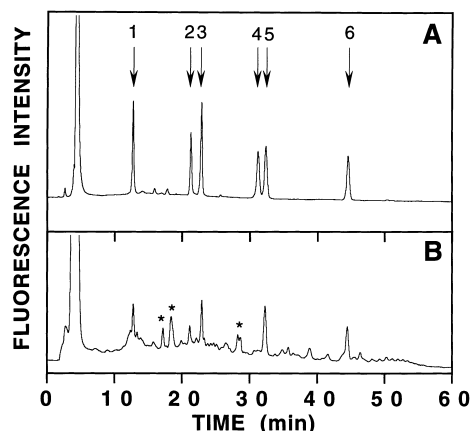


Fig. 3. HPLC analysis of the digest of the *C. elegans* GAG fraction with a mixture of heparinase and heparitinases I and II. Upper panel (A), the 2-AB derivatives of standard unsaturated HS disaccharides. 1, $\Delta\text{HexA}\alpha 1\text{-4GlcNAc}$; 2, $\Delta\text{HexA}\alpha 1\text{-4GlcNAc(6-O-sulfate)}$; 3, $\Delta\text{HexA}\alpha 1\text{-4GlcN(2-N-sulfate)}$; 4, $\Delta\text{HexA}\alpha 1\text{-4GlcN(2-N,6-O-disulfate)}$; 5, $\Delta\text{HexA(2-O-sulfate)}\alpha 1\text{-4GlcNAc(2-N-sulfate)}$; 6, $\Delta\text{HexA(2-O-sulfate)}\alpha 1\text{-4GlcNAc(2-N,6-O-disulfate)}$. Lower panel (B), the 2-AB derivative of the digest of the *C. elegans* GAG fraction with a mixture of heparinase and heparitinases I and II. The peaks eluted at around 4 min is due to the reagent (2-AB), which was carried over from the purification step of 2-AB derivatives. The peaks marked by asterisks in the lower panel are due to unknown substances derived from the GAG preparation, the enzyme preparation, or the column resin upon high sensitivity analysis.

mental biology. Although many genes that encode the putative GAG-synthesizing enzymes have been identified, no direct evidence has been presented for the existence of GAGs in *C. elegans* so far. In the present study, we isolated and characterized GAGs from adult worms of *C. elegans* and demonstrated the existence of chondroitin and HS. A considerable amount (approximately 1.6 μg from 1 mg of dried nematode) of non-sulfated chondroitin, a far less yet appreciable amount of HS and no hyaluronate were detected under the conditions used. *E. coli* OP50, which was supplied as food for *C. elegans*, contained no GAGs (data not shown), confirming that the nematode chondroitin and HS were not derived from the bacteria but synthesized by the worms themselves.

The putative genes encoding a few HS glycosyltransferases as well as several HS sulfotransferases have been identified in the *C. elegans* genome. The former include HS polymerase [20] and glucuronyltransferase I for the synthesis of the GAG-protein linkage region tetrasaccharide [21]. The latter include glucosaminyl *N*-deacetylase/*N*-sulfotransferase [22,23], uronyl 2-*O*-sulfotransferase [24], glucosaminyl 3-*O*-sulfotransferase [25] and glucosaminyl 6-*O*-sulfotransferase [26]. Although none of the above enzyme activities in the worm homogenate have been demonstrated and no evidence of the in vitro expression of these enzyme activities has been presented so far, the existence of these putative genes appears to be consistent with our observations that HS existed in the worm and was extensively sulfated. The wide distribution of HS demonstrated to date in the animal kingdom and the existence of extensively sulfated HS in *C. elegans* demonstrated in this study strongly support the notion that the highly sulfated structural characteristics of HS have been maintained throughout evolution and HS chains specifically modified by epimerization and sulfation reactions are required

for fundamental biological events. In fact, the important role of HS proteoglycans in fibroblast growth factor signaling has been particularly well documented [27]. The biological functions of fibroblast growth factors include the regulation of cell proliferation, differentiation and tissue patterning, where HS has been implicated in stabilizing or inducing the formation of fibroblast growth factor dimers or a ternary complex composed of ligand plus high and low affinity receptors [6]. In addition, recent studies have demonstrated a critical role for HS in Wingless signaling which is required for dorsal-ventral patterning during *Drosophila* development [28]. In addition, *Drosophila* homologues of a putative HS polymerase EXT1 and HS 2-*O*-sulfotransferase (encoded by *ttv* and *pipe*, respectively) were recently implicated in the Hedgehog diffusion and the formation of embryonic dorsal-ventral polarity, respectively [29,30].

In contrast to the advanced development of the molecular cloning of the biosynthetic enzymes involved in HS, the number of cloned enzymes involved in CS biosynthesis is much limited. No cDNA cloning of glycosyltransferases for the synthesis of the repeating disaccharide unit of the chondroitin backbone has been reported. Chondro-6-sulfotransferase [31,32] is the only cloned biosynthetic enzyme for CS. No homologue of this sulfotransferase gene is found in the *C. elegans* genome, being consistent with our observation of non-sulfated chondroitin in the worm. Although a *C. elegans* homologue of the recently reported chondroitin/dermatan uronyl 2-*O*-sulfotransferase [33] is present in the *C. elegans* genome, its homology is higher to HS uronyl 2-*O*-sulfotransferase than chondroitin/dermatan uronyl 2-*O*-sulfotransferase [33]. Thus, it may be that sulfotransferases for CS evolved later than those for HS. Several lines of evidence have suggested the involvement of CS proteoglycans in the development and maintenance of the neural network in mammalian brain and peripheral nerves [34–36] and sulfation of chondroitin may be required for the functions of this class of polysaccharides in evolutionary higher animals. It remains to be determined whether HS and chondroitin chains are covalently bound to proteins as in vertebrates. In this regard, it should be noted that the *sqv-8* gene homologous to the glucuronyltransferase I gene [21] for the synthesis of the GAG-protein linkage region common to HS and CS is present in the *C. elegans* genome, although it is also homologous to the glucuronyltransferase genes involved in the synthesis of the HNK-1 carbohydrate epitope. Interestingly, mutations of *sqv-8* perturb vulval invagination in *C. elegans* [37], which may suggest the involvement of GAGs in the tissue morphogenesis.

Although a homologous gene (accession number: Z72516) of human and murine hyaluronan synthase is found in the *C. elegans* genome [38] despite that hyaluronan was not detected in the present study, it may be associated with chitin synthesis as assumed from its homology to chitin synthase [39]. Alternatively, hyaluronan may be present below the detection limit of the present method. Since the detection limit of the microanalysis using 2-AB was a subpicomolar level (~ 0.5 pmol), the amount of hyaluronan, if any, is less than 5 ng per 1 mg of dried nematode. Future systematic functional analyses of multiple genes associated with GAG biosynthesis along with the *Drosophila* and human genome projects should provide us with enough new information to elucidate the biological functions and evolutionary significance of the non-sulfated and sulfated GAGs.

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