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Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 7982–7986, July 1997
Developmental Biology

An important developmental role for oligosaccharides during early embryogenesis of cyprinid fish

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Contributed by Phillips W. Robbins, May 14, 1997

ABSTRACT **Derivatives of chitin oligosaccharides have been shown to play a role in plant organogenesis at nanomolar concentrations. Here we present data which indicate that chitin oligosaccharides are important for embryogenesis in vertebrates. We characterize chitin oligosaccharides synthesized *in vitro* by zebrafish and carp embryos in the late gastrulation stage by incorporation of radiolabeled *N*-acetyl-D-[U^{14}C]glucosamine and by HPLC in combination with enzymatic conversion using the *Bradyrhizobium* NodZ α-1,6-fucosyltransferase and chitinases. A rapid and sensitive bioassay for chitin oligosaccharides was also used employing suspension-cultured plant cells of *Catharanthus roseus*. We show that chitin oligosaccharide synthase activity is apparent only during late gastrulation and can be inhibited by antiserum raised against the *Xenopus* DG42 protein. The DG42 protein, a glycosyltransferase, is transiently expressed between midblastula and neurulation in *Xenopus* and zebrafish embryogenesis. Microinjection of the DG42 antiserum or the *Bradyrhizobium* NodZ enzyme in fertilized eggs of zebrafish led to severe defects in trunk and tail development.**

Lipo-chitin oligosaccharides (LCOs) are signal molecules that were discovered during study of the root nodulation process in leguminous plants. Nitrogen-fixing root nodules are the result of an association of plants with bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, commonly called rhizobia. LCOs produced by rhizobia are key factors in the specific recognition processes that underlie the formation of root nodules (1–3). The basic structure of these LCOs is a β-1,4-linked *N*-acetylglucosamine (GlcNAc) tetra- or pentasaccharide, which is *N*-acylated at the nonreducing glucosamine moiety (1, 2). The LCOs are synthesized and secreted by the *Rhizobium* nodulation (Nod) genes whose expression is induced by plant flavonoids. The NodC, NodB, and NodA proteins are involved in the synthesis of the core LCO structure and function as chitin oligosaccharide synthase, chitin oligosaccharide deacetylase, and acyl transferase, respectively. Other rhizobial enzymes function in the modification of the LCO core structure and are important for determining the host range of rhizobia. An example is the NodZ protein which transfers an α-1,6-linked fucose group to the C6 position of the reducing end glucosamine moiety (4; reviewed in ref. 5). The effects of LCOs are not restricted to leguminous plants since it was shown that they stimulate cell division in tobacco protoplasts (6) and that they can rescue a temperature-sensitive somatic embryogenic mutant of carrot (*Daucus*) (7).

It was suggested that chitin oligosaccharides might also play a role in animal embryogenesis since *Rhizobium* NodC is homologous to the developmentally regulated DG42 protein of *Xenopus laevis* (8). The DG42 gene also shows homology with hyaluronan synthases and fungal chitin synthases. Homologues of the *Xenopus* DG42 were identified in zebrafish and mouse (9). In *Xenopus*, DG42 is only expressed during embryonic development, between the gastrula and neurulation stages. The protein follows an anterior/posterior gradient followed by a stronger dorsal/ventral gradient (10). *In vitro* the *Xenopus* DG42 protein can direct the synthesis of chitin oligosaccharides and hyaluronan (9, 11–13). When the *Xenopus* DG42 protein is produced in an *in vitro* transcription/translation system, it can synthesize chitin oligosaccharides with a degree of polymerization of 4–6, from uridine 5′-diphospho-*N*-acetyl-D-glucosamine (UDP-GlcNAc) (14). By overexpressing the *Xenopus* DG42 gene in heterologous systems it can direct the synthesis of chitin oligosaccharides or the synthesis of hyaluronan, depending on the system and conditions used (9, 11, 12). It was suggested that DG42 might function to produce chitin oligosaccharides that act as templates for hyaluronan synthesis (9, 13). Here we describe the results of the characterization of chitin oligosaccharides synthesized by extracts of gastrulation stage embryos of cyprinid fishes: zebrafish and carp. A possible function for chitin oligosaccharides in embryogenesis is suggested by the developmental defects observed after microinjecting fertilized zebrafish eggs with anti-DG42 antiserum and the *Bradyrhizobium* NodZ α-1,6-fucosyltransferase.

MATERIALS AND METHODS

Embryos. Carp (*Cyprinus carpio*) embryos were obtained as described (15). Zebrafish (*Brachydanio rerio*) were maintained under standard conditions (16), and embryos were obtained by natural spawning at 28°C. After fertilization embryos were dichorionated (16).

Microinjection of Zebrafish Embryos. Zebrafish embryos at the one cell stage were injected with ≈5 nl of either DG42 antiserum (1:50 dilution), NodZ protein (0.1 mg/ml), or preimmune serum (1:50 dilution). After injection, embryos were allowed to develop to 24 h and were scored for the presence of abnormalities.

***In Vitro* Incubations.** At each developmental stage 150 carp embryos were dissociated using Ca^{2+}- and Mg^{2+}-free solution (16) and the cells were washed in PBS. The cells were homogenized in 100 μl of lysis buffer (0.125% Nonidet P-40/25 mM Tris·HCl pH 7.5/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride/20 μg/ml leupeptin). Where indicated, the cell extract was first incubated in the presence of the

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Abbreviations: GDP-[U-^{14}C]fucose, guanosine 5′-diphospho-β-[U-^{14}C]-L-fucose; GlcNAc, *N*-acetylglucosamine; LCO, lipo-chitin oligosaccharide; Nod, nodulation; UDP, uridine diphosphate.

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DG42 antiserum (1:50 dilution, described in ref. 10) for 60 min at 4°C, or a control preimmune serum was used. These cell extracts were incubated for 45 min at room temperature in the presence of 0.2 μCi (1 Ci = 37 GBq) UDP-[U^{14}C]GlcNAc, 5 mM GlcNAc, 12 mM $MgCl_2$, and 100 μM lognac (2-acetamido-2-deoxy-D-glucohydroximo-1,5-lactone, a competitive inhibitor of *N*-acetyl-β-D-glucosaminidases) (CarboGen, Zurich). For analysis of chitin oligosaccharides by NodZ transfucosylation or by alkalinization response of a *Catharanthus roseus* cell suspension, 0.5 mM of unlabeled UDP-GlcNAc was added instead of 0.2 μCi UDP-[U^{14}C]GlcNAc. The reactions were stopped by adding 150 μl water and 2-min boiling. The tubes were centrifugated and the precipitates washed twice with 100 μl water; the supernatants were combined. Similar conditions were used with zebrafish embryos as described (9).

Purification of Chitinase 63. Chitinase 63 was isolated from *Escherichia coli* expressing the chitinase 63 gene as described (17). This protein preparation (50% pure) was then used for further purification. The protein was dissolved in 20 mM Tris·HCl (pH 6.5) and loaded on a fast protein liquid chromatography anion exchange column (Resource^{Q}; Pharmacia LKB) pre-equilibrated with this buffer. After binding the sample, a gradient of 0–0.5 M NaCl was applied. Chitinase 63 was eluted from the column with 0.02–0.03 M NaCl.

HPLC Purification of Chitin Oligosaccharides. Chitin oligosaccharides were purified using an NH_2-silica HPLC column (Macherey-Nagel, Düren, Germany) as described (4). To prevent contamination, preparative runs were always preceded by a run in the absence of standards during which the fractions were checked for the absence of chitin oligosaccharides.

NodZ Transfucosylation Assay. The NodZ protein was purified as described (4). HPLC fractions were concentrated by vacuum evaporation and dissolved in water. For fucosyltransferase activity, these fractions were incubated at room temperature for 45 min with 1–2 μg of recombinant *Bradyrhizobium japonicum* NodZ protein in the presence of 20 nCi guanosine 5′-diphospho-β-[U-^{14}C]-L-fucose (GDP-[U-^{14}C]fucose) (285 mCi/mmol, Amersham), 1 mM ATP, 10 mM $MgCl_2$, and 20 mM phosphate buffer (pH 7.5). The samples were treated with Dowex ion exchange resin (Sigma) to remove the free GDP-[U-^{14}C]fucose and concentrated by vacuum evaporation. Thin layer chromatography (TLC) was performed using silica 60 TLC plates (Merck) with *N*-butanol/ethanol/water (5:3:2) as the mobile phase. After exposure with a Phosphor screen (Molecular Dynamics) the TLCs were visualized using a PhosphorImager and IMAGE QUANT software.

Alkalinization Response of a *C. roseus* Plant Cell Suspension. Cell suspension cultures of *C. roseus* were grown as described (18). HPLC fractions were concentrated by vacuum evaporation and dissolved in water. At time zero 10% of the fraction was added to 3 ml of a 7-day-old *C. roseus* subculture and the pH of the medium was measured for 15 min. Where indicated, samples were incubated with chitinase 63 for 45 min at 37°C before being added to the plant cell suspension.

RESULTS AND DISCUSSION

Chitin Oligosaccharide Synthase Activity in Extracts of Zebrafish and Carp Embryos. We tested zebrafish and carp embryos at different developmental stages for their ability to synthesize chitin oligosaccharides. Previously it was shown that *in vitro* zebrafish embryos of the gastrulation stage incorporate radioactive uridine 5′-diphospho-*N*-acetyl-D-[U^{14}C]glucosamine (UDP-[U^{14}C]GlcNAc) into metabolites that in HPLC analysis have retention times similar to that of chitin pentaose (β-1,4-linked GlcNAc) (9). Using gastrulation stage embryos of carp we have obtained similar data (Fig. 1*A*). We also treated HPLC fractions with purified NodZ protein of *Bradyrhizobium.* This enzyme is an α-1,6-fucosyltransferase with substrate specificity for chitin oligosaccharides that can be used for *in vitro* transfucosylation (4). Metabolites synthesized by cell extracts of zebrafish and carp embryos from the gastrulation stage were purified by HPLC (see *Materials and Methods*). HPLC fractions having retention times similar to standards of chitin tetraose and chitin pentaose were used as substrates in the transfucosylation assay with radiolabeled GDP-[U-^{14}C]fucose (Fig. 1*B*). The results show that these fractions contain metabolites that can be fucosylated efficiently by the NodZ protein in the presence of GDP-[U-^{14}C]fucose. The fucosylated derivatives of the metabolites in HPLC fractions with retention times similar to chitin tetraose and chitin pentaose (Fig. 1*A*) migrate on TLC as fucosylated chitin tetraose (Fig. 1*B*, lane 3) or fucosylated chitin pentaose (Fig. 1 *B*, lane 5, and *C*, lanes 1 and 3), respectively. When these fucosylated materials were treated with a combination of chitinase and chitobiase, a degradation pattern typical for chitin oligosaccharides is found (Fig. 1*B*, lanes 2 and 4; and *D*, lanes 2 and 3). From these results we conclude that extracts of zebrafish and carp embryos of the gastrulation stage produce chitin oligosaccharides *in vitro*. A clear difference between the two is that carp embryos predominantly produce chitin tetrasaccharides (Fig. 1*B*, compare lanes 3 and 5), whereas zebrafish embryos produce both chitin tetrasaccharides and pentasaccharides (Figs. 1*A* and 2*A*).

Transient Production of Chitin Oligosaccharides During Zebrafish and Carp Embryogenesis. Suspension cultured tomato cells respond to chitin oligosaccharides by alkalinization of the growth medium (19). To establish a similar rapid test system for chitin oligosaccharides we used a *C. roseus* plant cell suspension, which can respond to chitin tetraose in quantities as low as 20 femtomols (data not shown). The chitin oligosaccharides synthesized by extracts of carp or zebrafish gastrulation stage embryos were used for the alkalinization assay (Fig. 2*A*). Samples that were first treated with a purified chitinase do not induce an alkalinization response. By comparing the pH shift induced by the zebrafish chitin oligosaccharides and a dilution series of chitin oligosaccharide standards, we can calculate that about $2 \pm 1 \times 10^{-11}$ mol of chitin oligosaccharide can be produced per 2,000 embryos in the incubation condition tested.

For testing chitin oligosaccharide synthase activity in embryos of different developmental stages we used cell extracts of carp embryos (Fig. 2*B*). The results show that embryos at the end of gastrulation stage produce chitin oligosaccharides *in vitro* that induce an alkalinization response in the *C. roseus* plant cell suspension. In contrast, embryos of early gastrulation or segmentation stages do not produce sufficient chitin oligosaccharides to induce an alkalinization response. The synthesis of the chitin oligosaccharides by the extracts of carp embryos of late gastrulation stage is inhibited by antibodies raised against the *Xenopus* DG42 protein (Fig. 2*B*), suggesting that a protein similar to DG42 is involved in their synthesis. A question still remaining is whether the chitin oligosaccharides synthesized by zebrafish and carp embryos remain as such *in vivo* or are subject to modifications. For the function of the *Rhizobium* LCOs, it is essential that the chitin oligosaccharides are acylated at the nonreducing terminus. However, there are indications that the fatty acyl moiety is only important for transport of the LCO inside the plant tissue (20). When chitin oligosaccharides are introduced directly in the plant root cells by microballistic targeting they can induce cell division similar to that seen when LCOs are applied outside of the root (H. Schlaman, A. A. Gisel, N. Quaedvlieg, G. Bloemberg, B. Lugtenberg, J. Kijne, I. Potrykus, H.P.S., and C. Sautter, unpublished data).

Microinjection of Anti-DG42 Serum or the *Bradyrhizobium* NodZ Enzyme in Fertilized Zebrafish Eggs Leads to Malformations in Trunk and Tail. Immunolocalization of the DG42

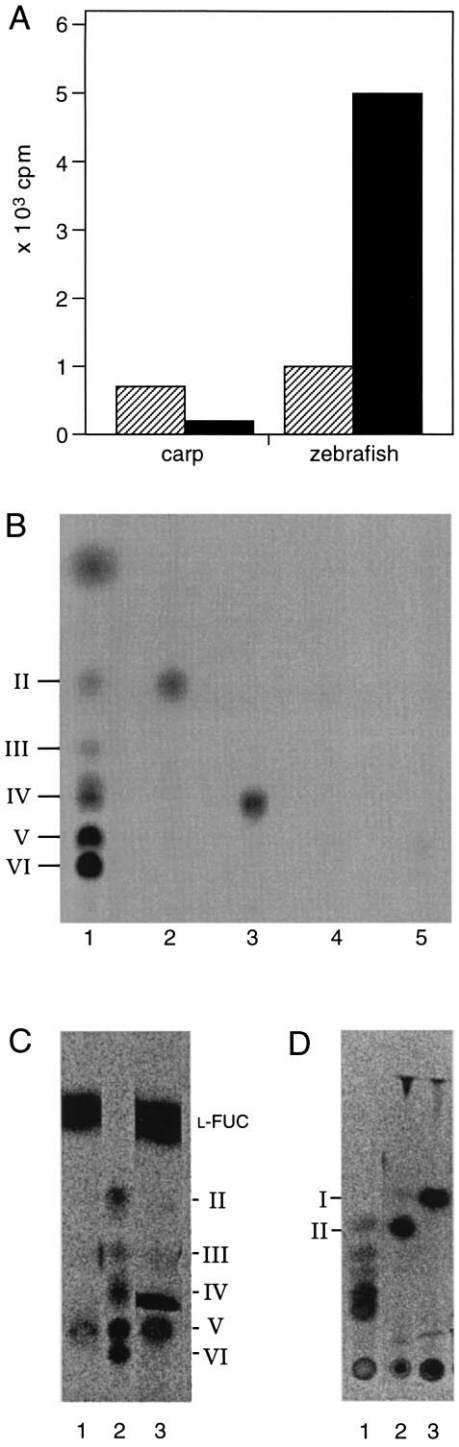
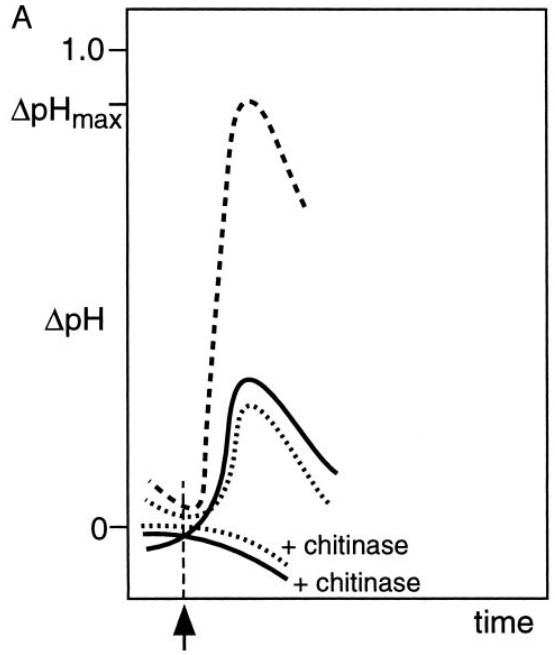


FIG. 1. Radiolabeling of gastrulation-specific metabolites. Extracts of zebrafish or carp embryos from the gastrulation stage were incubated in the presence of radiolabeled or unlabeled UDP-GlcNAc (see *Materials and Methods*). (*A*) Incorporation of UDP-[U^{14}C]GlcNAc into HPLC fractions with retention times similar to chitin tetraose (striped box) and chitin pentaose (filled box). In the incubations where unlabeled UDP-GlcNAc was used, the equivalent fractions were incubated with the NodZ protein in the presence of GDP-[U-^{14}C]fucose. By using this assay it is possible to specifically detect chitin oligosaccharides at concentrations as low as 1 picomol (data not shown). The pooled fractions were used for chitinase and chitobiase treatments and separated on TLC (*B–D*). (*B*) Radiolabeling of metabolites obtained from carp embryos and separated by HPLC, using the NodZ transfucosylation assay. Lanes: 1, fucosylated chitin oligosaccharide standards (as described in ref. 4); 2 and 3, HPLC fractions with retention times similar to chitin tetraose after transfucosylation, incubated with chitinase (lane 2) or without chitinase (lane 3); 4 and 5, HPLC fractions with retention times similar to chitin pentaose after transfucosylation, incubated with chitinase (lane 4) or without chitinase (lane 5). (*C* and *D*) Radiolabeling of metabolites obtained from zebrafish embryos using the NodZ transfucosylation assay. Fucosylated chitin oligosaccharide standards (*C*, lane 2; and *D*, lane 1); HPLC fractions with retention times similar to chitin pentaose after transfucosylation {*C*, lanes 1 and 3 (without removing free GDP-[U-^{14}C]fucose)} incubated with chitinase (*D*, lane 2), or incubated with chitinase and chitobiase (from *Streptomyces griseus*, Sigma) (*D*, lane 3).



B

Metabolite	% ΔpH$_{max}$
Zebrafish	
HPLC fraction chitin tetraose	57
HPLC fraction chitin pentaose	30
Carp (total extract)	
gastrulation stage (5 h)	No response
late gastrulation stage (10h)	35
plus pre-immune serum	13
plus DG42 antiserum	No response
segmentation stage (15h)	0.1

FIG. 2. Induction of an alkalinization response of a *C. roseus* plant cell suspension by *in vitro* synthesized metabolites of zebrafish and carp embryos (see *Material and Methods*). (*A*) Typical response curve obtained with metabolites from gastrulation stage zebrafish embryos (arrow, the time point of addition; dotted line, fractions with retention times similar to chitin tetraose; solid line, fractions with retention times similar to chitin pentaose; dashed line, maximal pH shift obtained by adding 0.3 μg of chitin tetraose). (*B*) Alkalinization response of metabolites synthesized *in vitro* by embryo extracts at early gastrulation stage, late gastrulation stage, and segmentation stage, indicated as a percentage of the maximal pH shift (%ΔpH$_{max}$). Results shown are from experiments independent of those shown in *A*. This indicates a variation of approximately 20% in the alkalinization assay. Indicated also are the results obtained with extracts of late gastrulation stage embryos that were pre-incubated with DG42 antiserum or preimmune serum.

protein shows a gradient pattern throughout the *Xenopus* embryo suggesting that the protein could be involved in the execution of a polarity-determining program (10). The DG42 antiserum used in those studies can inhibit *in vitro* chitin synthase activity observed during late gastrulation in carp

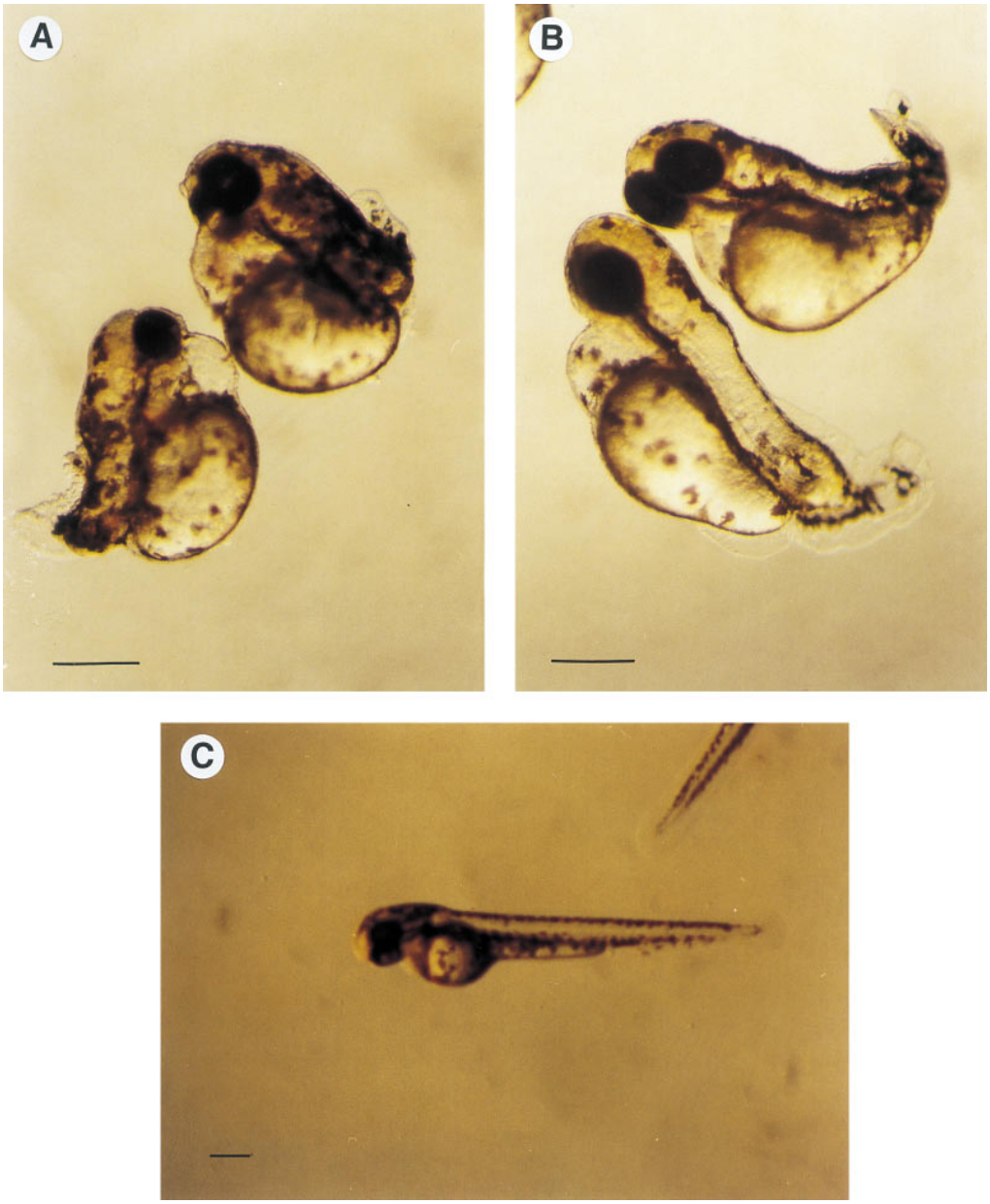


FIG. 3. Results of microinjection experiments in zebrafish. All embryos shown are 48 h old and represent typical examples. (*A*) Embryos injected with DG42 antiserum. Sixty-one percent of the embryos (n = 288) were consistently and reproducibly affected in the formation of the trunk and tail. (*B*) Embryos injected with NodZ protein. Sixty-nine percent of the injected embryos (n = 106) show defects similar to those observed after injection of the DG42 antiserum. As a control, embryos were injected with an identical preparation of NodZ protein inactivated prior to injection by boiling for 5 min. Sixteen percent of these controls (n = 98) were affected, although the defects observed are nonspecific and do not resemble those seen when injecting the active protein (data not shown). (*C*) Control embryos injected with rabbit preimmune serum. Five percent of control embryos (n = 116) were affected by the injection procedure, but the observed defects were not specific. (Bars = 250 μm.)

embryos (this study) and zebrafish embryos (9). To study the effect of this inhibition on embryogenesis, zebrafish embryos at the one cell stage were injected with the DG42 antiserum or, as a control, with rabbit preimmune serum. Injected embryos developed normally up to gastrulation. However, beginning at the tailbud stage, the embryos injected with DG42 antiserum showed defects in the formation of the tail, in particular the somites. At 24 h postfertilization, a large percentage of embryos injected with the DG42 antiserum are reduced in length and show severe malformations in the trunk and tail (Fig. 3*A*), an effect that was not observed for control injected embryos (Fig. 3*C*). We also tested the effect of injection of the NodZ fucosyltransferase. Injection of the NodZ protein led to the same developmental defects as the injection of the DG42 antiserum; all or most of tail development is affected (Fig. 3*B*). Similar results were obtained when a plasmid with the *Bradyrhizobium* NodZ gene under control of the human cytomegalovirus (CMV) enhancer-promoter was injected in the developing embryo (22). As reported previously, the NodZ enzyme can fucosylate oligosaccharides with at least two GlcNAc residues at the reducing end. Chitin oligosaccharides are the preferred substrates of the NodZ protein with a K_m value of 0.12 mM (4). Related compounds such as chitosan or hyaluronan are not used as substrates (data not shown). Thus, the observed developmental defects of NodZ injection is probably the result of modification of an oligosaccharide with at least two GlcNAc residues at the reducing terminus.

In conclusion, our results show that oligosaccharides play a major developmental role during early embryogenesis of fish. This may also be true in other vertebrate embryos because a

close homolog (97% identity) of DG42 has been found in the mouse (9). The results of the *in vitro* studies suggest that chitin oligosaccharides could be the active compounds. The effects of the microinjection of the DG42 antiserum indicate that the synthesis of embryogenesis-related oligosaccharides probably depends on DG42. The same antiserum was used for the immunolocalisation of DG42 in *Xenopus* embryos and showed that the protein follows an anterior-posterior gradient followed by a stronger dorsal-ventral gradient (10). At the tailbud stage the protein is last detectable in the posterior ventral mesoderm (10). A number of zebrafish mutations described recently present phenotypes reminiscent of that observed in this study (21, 23). It will be of interest to see whether the spatial distribution as well as presence or absence of the DG42 protein is altered in these zebrafish mutants.

We thank Drs. M. Allende and N. Hopkins (Massachusetts Institute of Technology) for help with the zebrafish embryos and discussion of the manuscript. We thank Drs. F. Menke and A. H. M. Wijfjes (Leiden University) for their help with part of the experiments and Prof. Dr. B. J. J. Lugtenberg (Leiden University) for stimulating discussions. The research was supported by the Netherlands Organization of Scientific Research (NWO-PIONIER grant awarded to H.P.S.) and by National Institutes of Health Grants GM31318 (to P.W.R.) and CA14051 (to R. O. Hynes).

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