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Developmental Biology

Homologs of the *Xenopus* developmental gene *DG42* are present in zebrafish and mouse and are involved in the synthesis of Nod-like chitin oligosaccharides during early embryogenesis

(gastrulation/nodulation)

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ABSTRACT **The *Xenopus* developmental gene *DG42* is expressed during early embryonic development, between the midblastula and neurulation stages. The deduced protein sequence of *Xenopus* DG42 shows similarity to *Rhizobium* Nod C, *Streptococcus* Has A, and fungal chitin synthases. Previously, we found that the DG42 protein made in an *in vitro* transcription/translation system catalyzed synthesis of an array of chitin oligosaccharides. Here we show that cell extracts from early *Xenopus* and zebrafish embryos also synthesize chitooligosaccharides. cDNA fragments homologous to *DG42* from zebrafish and mouse were also cloned and sequenced. Expression of these homologs was similar to that described for *Xenopus* based on Northern and Western blot analysis. The *Xenopus* anti-DG42 antibody recognized a 63-kDa protein in extracts from zebrafish embryos that followed a similar developmental expression pattern to that previously described for *Xenopus*. The chitin oligosaccharide synthase activity found in extracts was inactivated by a specific DG42 antibody; synthesis of hyaluronic acid (HA) was not affected under the conditions tested. Other experiments demonstrate that expression of DG42 under plasmid control in mouse 3T3 cells gives rise to chitooligosaccharide synthase activity without an increase in HA synthase level. A possible relationship between our results and those of other investigators, which show stimulation of HA synthesis by DG42 in mammalian cell culture systems, is provided by structural analyses to be published elsewhere that suggest that chitin oligosaccharides are present at the reducing ends of HA chains. Since in at least one vertebrate system hyaluronic acid formation can be inhibited by a pure chitinase, it seems possible that chitin oligosaccharides serve as primers for hyaluronic acid synthesis.**

The *Xenopus DG42* gene is expressed only during a short time in embryonic development, between midblastula and neurulation (1, 2). The *DG42* mRNA and protein move as a gradient through the embryo from the animal to vegetal pole (2). DG42 has sequence similarity to the *Rhizobium* Nod C protein, which synthesizes chitooligosaccharides (3–5), to the *Streptococcus* Has A protein, which has been reported to be a hyaluronate (HA) synthase (6), and also to fungal chitin synthases (7).

Bacteria of the genera *Rhizobium* establish a symbiotic relationship with legumes where the rhizobia are able to form nitrogen-fixing nodules in the roots (8). The nodulation, or "Nod" signaling system is activated by specific flavonoid compounds secreted by plant roots, which act as rhizobia chemoattractants (9) and elicit the induction of the nod genes present in rhizobia (10). The nodulation gene (nod) products are involved in the synthesis of lipooligosaccharides, modified chitin oligosaccharides (Nod factors) that function as plant morphogens by acting as signals that promote plant nodulation (11–13). The modifications found in the chitooligosaccharides include replacement of an acetyl residue at the nonreducing end of the oligosaccharide with a long chain acyl group, sulfation, acetylation, and glycosylation (11, 14–16). Several reviews have been published concerning the biology, genetics, and biochemistry of these signaling molecules (11–17).

The chitin oligosaccharides produced by the NodC enzyme vary in chain length from two to five residues of *N*-acetylglucosamine (3). Fungal chitin synthases produce a polymer chain length of 100 or more residues of *N*-acetylglucosamine (18). We found that the DG42 protein made in an *in vitro* transcription/translation system catalyzes the synthesis of chitin oligosaccharides that vary in chain length from three to six residues of *N*-acetylglucosamine (19). In the present work we demonstrate that it is possible to obtain synthesis of chitooligosaccharides only in the expected stage of development when DG42 is expressed (gastrulation–neurulation) with cell extracts from frogs and zebrafish embryos. This "chitin oligosaccharide synthase" activity can be inactivated with a specific anti-DG42 antibody.

MATERIALS AND METHODS

Animals. Adult zebrafish (*Brachydanio rerio*) and frogs (*Xenopus laevis*) were maintained under standard conditions (20, 21). Zebrafish embryos were obtained by natural spawning at 28°C, and when necessary they were dechorionated (20). *Xenopus* embryos were produced according to a previous report (21).

***In Vitro* Incubations.** At each developmental stage, 100 zebrafish embryos were dissociated using Ca^{2+}- and Mg^{2+}-free solution (20), and the cells were washed twice with the same solution. They were resuspended and homogenized in 100 μl of lysis buffer [0.125% Nonidet P-40/25 mM Tris·HCl (pH 7.4)/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride/5 μM E-64] and centrifuged for 10 min at 14,000 rpm. The supernatants were used as enzyme preparations. For fast protein liquid chromatography (FPLC) fractionation of the hyaluronate complex, the lysis buffer was prepared with 1% digitonin, 25 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM NaCl, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 5 μM E-64. *Xenopus* embryos (40 at each developmental stage) were also dissociated with Ca^{2+}- and Mg^{2+}-free solution and prepared as above.

Standard incubations were carried out with 100 μl of the cell extract, supernatant of immunoprecipitation, or FPLC frac-

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Abbreviations: HA, hyaluronic acid; PCR, polymerase chain reaction; Nod, nodulation; E-64 cysteine protease inhibitor; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U53222 and U53223).

tion in 12 mM $MgCl_2$, 5 mM *N*-acetylglucosamine, 20 μM 2-acetamido-2-deoxy-D-glucohydroximo-1,5-lactone, a competitive inhibitor of *N*-acetyl-β-D-glucosaminidases (Carbo Gen), 25 mM Tris·HCl (pH 7.4), and either 0.2 μCi (7.4 KBq) of UDP-*N*-acetyl-D-[^{14}C]glucosamine (307 mCi/mmol) (DuPont NEN), 2 μCi (74 KBq) of UDP-*N*-acetyl-D-[6-^{3}H]-glucosamine (25 Ci/mmol) (DuPont NEN), or 0.2 μCi (7.37 KBq) of UDP-[^{14}C]glucuronic acid (320.2 mCi/mmol) (DuPont NEN). Where indicated, UDP-glucuronic acid (200 μM) or UDP-*N*-acetyl-D-glucosamine (150 μM) were also added. The final volume was adjusted to 150 μl. Incubations were carried out at room temperature for 60 min and were stopped by the addition of 200 μl of water and boiling for 2 min. The tubes were centrifuged, and the precipitates were washed twice with 100 μl of water. The combined supernatants were 1) filtered through a DEAE-Sephacel (Sigma) column equilibrated in water, and the products in the effluent were fractionated by high performance liquid chromatography (HPLC) for analysis of chitin oligosaccharides or 2) filtered through a Bio-Gel A-5 m column for analysis of hyaluronic acid (see Table 1).

DG42p Immunoprecipitation. Embryonic zebrafish or frog cells obtained after dissociation of 100 or 40 embryos, respectively, with Ca^{2+}- and Mg^{2+}-free saline solution were resuspended and homogenized in 100 μl of lysis buffer as above (see *In Vitro Incubations*). After centrifugation (10 min at 14,000 rpm), the supernatant was used for immunoprecipitation. First, 100 μl of supernatant was incubated for 30 min at 0°C in the presence of 10 μl of 10% *Staphylococcus aureus* suspension, preincubated with 2 μl of normal rabbit serum, and then centrifuged for 10 min at 14,000 rpm (preclear). The pellet was washed with 100 μl of the same lysis buffer, and the wash was combined with the first supernatant. In the second stage, 200 μl of supernatant was incubated with 20 μl of the same 10% *S. aureus* suspension, preincubated with 2 μl of normal rabbit serum or 2 μl of anti-DG42 antibody (a gift from Dr. Igor Dawid) during 60 min at 0°C. The tubes were then centrifuged, and the precipitates were washed as above, yielding about 300 μl of final supernatant. This material was used for *in vitro* incubations as above (see *In Vitro Incubations*).

Western Blot Analysis. Dechorinated zebrafish embryos (10) from each developmental stage or a sample from FPLC fractions were resuspended in 1 × SDS gel loading buffer, boiled for 2 min, and centrifuged for 10 min at 14,000 rpm. The supernatant samples were then loaded on a 10% SDS-polyacrylamide gel. Kaleidoscope prestained proteins (Bio-Rad) were used as standards. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (BA85, Schleicher & Schuell), during 4 h at 4°C using 160–200 mA. The filters were blocked overnight in TBST [10 mM Tris·HCl (pH 8.0)/150 mM NaCl/0.05% Tween 20] with 5% milk and were then treated with a rabbit anti-DG42 antibody at 1:500 dilution followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Pierce) at 1:1000 dilution. The anti-DG42 binding reaction was visualized using a chemiluminescent substrate system (LumiGLO, Kirkegaard & Perry Laboratories, Inc.). Autoradiography was performed during the first 5–10 min of chemiluminescent reaction with Kodak x-ray film (X-Omat AR).

PCR. Fragments of DNA were amplified by the PCR. A mouse 7-day embryonic cDNA library (Clonetech) and a zebrafish, gastrula–neurula stage (9–16 h) cDNA library in λ ZAP (gift of Dr. R. Riggleman) were amplified in the appropriate *Escherichia coli* host strains, and phage DNA was purified from each. Degenerate primers: CCAAGCTTGTICA(A/G)G-TITG(T/C)GA(T/C)TCIGA and (T/C)TTIGTCCAICGI-GT(T/C)TG(T/C)TG(A/G)TT, 5′ and 3′, respectively, were synthesized by the MIT Biopolymer Lab. Standard methods were used for PCR reactions, cloning, selection of recombinant clones, plasmid DNA purification, and analysis of products by restriction enzyme digestion (22).

DNA Sequence Analysis. Plasmids with inserts of about 450 bp were sequenced by cycle sequencing (Epicentre Technologies) using forward and reverse primers. The sequences were compared with the GenBank protein database using BLAST (National Center for Biotechnology Information). Multiple sequence alignments were compiled using MEGALIGN software (DNAStar).

Southern and Northern Blot Analysis. The cloned fragments identified as homologs of *DG42* from mouse and zebrafish were used as probes in Southern and Northern blot hybridizations using standard methods (22). Total RNAs from zebrafish embryos were isolated by the guanidinium-isothiocyanate method (23). Filters were exposed to Reflection X-ray film (Amersham).

Chromatographic Techniques. HPLC was carried out with an analytical microsorb 5-μm amino (-NH_2) column (46 × 250 mm; Rainin Instruments) under isocratic conditions (water/acetonitrile, 25:75, vol/vol) run at 40°C with a flow rate of 2 ml/min with fractions of 1 ml. Pure chitooligosaccharides (Seikagaku America, St. Petersburg, FL) from 2 to 6 units of *N*-acetylglucosamine residues were used as internal standards and detected by UV absorption at 210 nm.

FPLC was carried out with a Superose 6 HR 10/30 column (Pharmacia), equilibrated in Tris·HCl 25 mM buffer (pH 7.4), 50 mM NaCl, 5% glycerol, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 5 μM E-64, and 0.1% digitonin, at a flow rate of 0.25 ml/min with fractions of 0.5 ml.

Bio-Gel A-5 m (200–400 mesh) column (12 × 250 mm) (Bio Rad), was eluted with 0.1 M pyridinium acetate buffer (pH 5.0), 50 mM NaCl at a flow rate of 0.1 ml/min, and 0.25-ml fractions were collected. Blue dextran and $CoCl_2$ were used as indicators of total exclusion and inclusion volumes, respectively.

Thin layer chromatography (TLC) was performed on silica gel G plates (5 × 20 and 10 × 20 cm, Merck) in *n*-butanol/ethanol/water, 5:4:3 (vol/vol).

Radioactivity was measured in Ecolite scintillation solution in a liquid scintillation counter (Beckman LS 3801). TLC plates were exposed to Kodak x-ray film (X-Omat AR) for 1–4 weeks.

Enzymatic Degradations. *Streptomyces plicatus* chitinase-63 (24) and *Serratia marcescens* chitinase (Sigma) were used in 25 mM phosphate buffer (pH 6.5) at 37°C for 2-h incubations at a final concentration of 0.1 mg/ml. Four-hour incubations with hyaluronate lyase (10 units) from *Streptomyces hyalurolyticus* (Sigma) and hyaluronidase (25 units) from bovine testes type IV (Sigma) were carried out in 25 mM sodium acetate buffer (pH 5.0).

RESULTS

***DG42* Genes of Zebrafish and Mouse and Their Expression.** To determine whether the *DG42* gene is present in other vertebrates, we designed a combination of degenerate PCR primers (see *Materials and Methods*) using conserved regions present in *DG42*, *Nod C*, and a fungal chitin synthase DNA sequences (Fig. 1). In the *Xenopus DG42* gene there are three introns in the region being amplified (see Fig. 1). Therefore, the DNAs of embryonic cDNA libraries were used as templates. The expected 450-bp fragment was obtained for both zebrafish and mouse. These fragments were each cloned, and their DNA sequence was determined. Comparisons of the cDNA sequences (data not shown) and the deduced amino acid sequences (Fig. 1) for the three species show that the *DG42* gene is highly conserved. The zebrafish and mouse cDNA and amino acid sequences are 79 and 93% identical, respectively, and the cDNA and amino acid sequences have 67 and 70% identity, respectively, with the *Xenopus* sequences. Southern blot analysis showed hybridization of fragments to

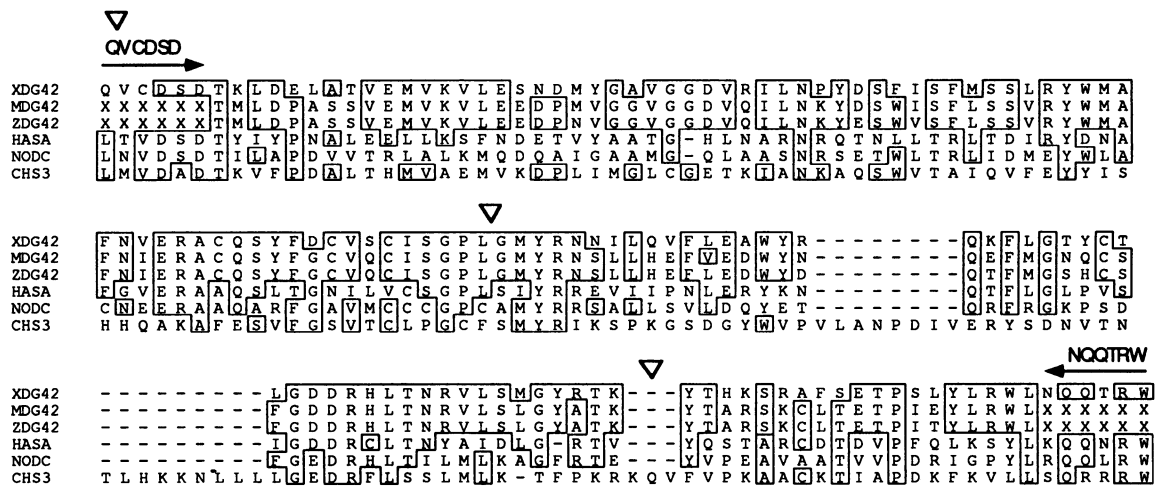

FIG. 1. Amino acid alignment of DG42 homologs and related proteins. Comparison was made between the region defined by the primers (indicated by arrows) of *Xenopus* DG42 [XDG42, amino acids 236–381 (2); mouse DG42 homolog (MDG42); zebrafish DG42 homolog (ZDG42); HasA (amino acids 131–274 (25); NodC (amino acids 137–281 (26); and a yeast chitin synthase (CHS3, amino acids 832–996 (27)]. Open vertical triangles indicate the position of each of three introns in the *Xenopus* genomic sequence. Within the sequences, X indicates that the amino acid is not known, and dashes indicate gaps. Amino acids that are boxed are in common in at least three of six sequences.

their respective genomic DNAs (data not shown). One to three fragments hybridized depending on the restriction enzyme used. The results suggest that the *DG42* homologs of both fish and mouse are each a single copy gene with introns.

To define the window of development in which the homologous *DG42* gene is expressed in zebrafish, we carried out Northern blot analysis with RNA samples from postfertilization to adult stages (Fig. 2) and Western blot analysis with protein samples from 4 h (blastula stage) to 28 h (postsomitogenesis stage) and an anti-*DG42* antibody (data not shown). Two transcripts about 5.5 and 3.2 kb in length hybridized with the zebrafish cDNA probe as shown in Fig. 2. The 3.2-kb RNA is probably the mature mRNA. Peak expression of both transcripts was between 4 and 7 h or during the blastula and gastrula stages of development. Neither transcript was present in the 1 h sample, confirming that the *DG42* mRNA is an early embryonic and not a maternally derived message. The transcripts are less prevalent in postgastrula stages of development and are absent from mature fish (120 h sample). The *Xenopus* anti-DG42 antibody bound a 63-kDa protein from zebrafish during the gastrula–early neurula stages (5–13 h) (data not shown). Detection of this protein lags the presence of transcript shown in Fig. 2 by 1–2 h. The transcription and translation of the *DG42* gene during embryonic development of zebrafish embryos are consistent with the results obtained previously in *Xenopus* (1, 2).

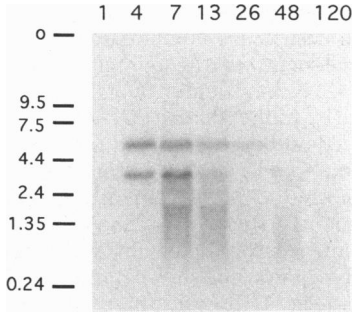

FIG. 2. Northern blot hybridization. Zebrafish total RNA was extracted from embryos at the times indicated by the top row (hours after fertilization). The probe was DNA of the cloned PCR fragment of the zebrafish *DG42* homolog. The origin (O) and size standards (in kilobases) are designated by bars.

DG42 Enzymatic Activity in Zebrafish and Frog Embryos. Previous studies that demonstrated the chitin oligosaccharide synthase activity of DG42 protein in an *in vitro* transcription/translation system (19) were adapted to analyze cell extracts prepared from zebrafish embryos at varying stages of development (from early blastula to late neurula) (see *Materials and Methods*). We detected *in vitro* chitin oligosaccharide synthase activity in the extracts with a peak in the late gastrula–early neurula stage (Fig. 3). This activity has the same pattern during development as expression of the DG42 protein detected on Western blots (data not shown). The chain lengths of the oligosaccharides are from 3 to 6 units of *N*-acetylglucosamine, confirming the result obtained previously with the *in vitro* expression system (19). With the embryo system, we obtained the pentasaccharide as the major product (Fig. 3). All products ran on TLC at the positions expected of chitooligosaccharides, confirming the result obtained by HPLC. Moreover, incubation of the zebrafish chitooligosaccharides with chitinase gave rise to the expected mono- and disaccharide products (data not shown).

Previous studies (2) defined the expression pattern of DG42 protein during frog development (*Xenopus*). We therefore repeated the *in vitro* incubation experiments described above with frog extracts and found the same pattern of chitin oligosaccharide synthase activity as observed in zebrafish. The frog chitooligosaccharides obtained from late gastrula–early neurula stage were purified by HPLC and run on TLC. They were also digested with chitinase. The same profile of degradation was obtained in all cases (data not shown).

In another experiment, we incubated a zebrafish cell extract from late gastrulation stage (9 h) embryos with UDP-[^{14}C]GlcA and UDP-GlcNAc in the presence or absence of chitinase 63 to determine whether degradation of chitooligosaccharides (premade or *de novo*) would affect the synthesis of the HA. We detected 87% inhibition of synthesis of [^{14}C]HA in presence of the chitinase (data not shown).

Immunoprecipitation of DG42 Activity in Zebrafish and Frog Embryos. In order to determine whether the chitin oligosaccharide synthase activity detected in the zebrafish embryos is catalyzed by the DG42-like protein, we carried out an experiment with cell extract preparations from different stages after immunoprecipitation with anti-*DG42* antibody. We were also interested in studying the possible relationship between DG42 expression and HA formation since the DG42 protein shows homology with Has A (Fig. 1), a protein from

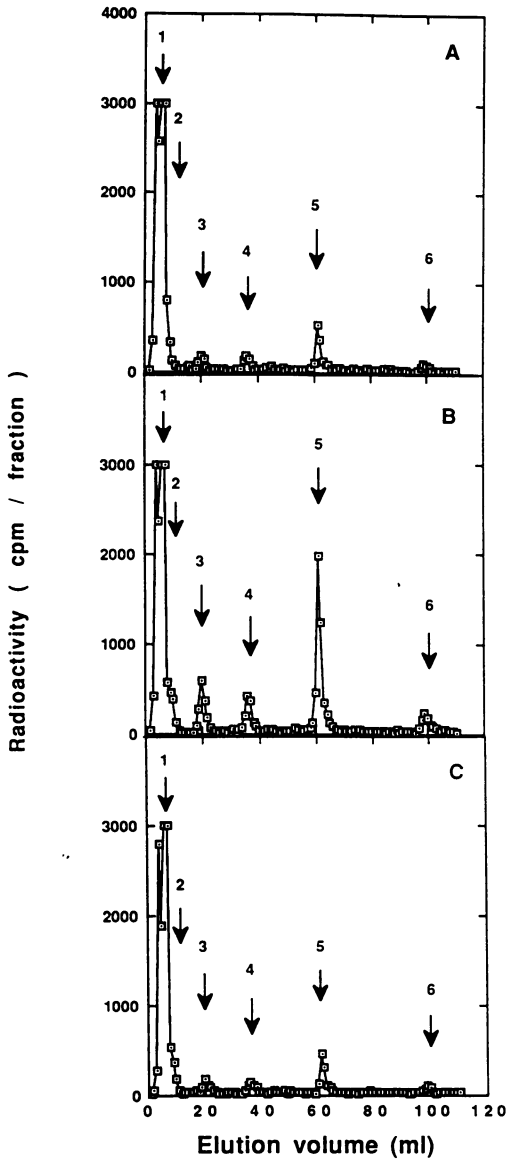

FIG. 3. HPLC separation of chitin oligosaccharides made by zebrafish cell extract preparations. Zebrafish embryos were used to obtain cell extracts from different developmental times and were incubated in the presence of UDP-[^{3}H]GlcNAc (see *Materials and Methods*). *A*, early gastrula (5 h); *B*, late gastrula (9 h); and *C*, late neurula (14 h). The unbound fraction from the DEAE-Sephacel column (see *Materials and Methods*) was concentrated and chromatographed on a Hewlett-Packard 1050 HPLC instrument with a microsorb-NH_2 column. Isocratic elution was carried out with water/acetonitrile, 25:75 (vol/vol). Arrows indicate the elution position of *N*-acetylglucosamine, 1, and chitin oligosaccharides from 2–6 residues of *N*-acetylglucosamine, 2–6, used as internal standards.

Streptococcus that is believed to catalyze synthesis of hyaluronic acid (6). The results (Table 1) indicate that we can immunoprecipitate the chitin oligosaccharide synthase activity with the anti-DG42 antibody. HA synthase activity is not precipitated or inhibited by the anti-DG42 antibody at any stage of embryonic development tested. Furthermore, the level of hyaluronic acid synthase activity detected does not appear to change during development, in contrast to the pattern of DG42 protein expression (Table 1). Finally, in the presence of high concentrations of UDP-GlcA (200 μM), we still detect chitooligosaccharides (Table 1), demonstrating that DG42 chitin oligosaccharide synthase activity is not inhibited under conditions that permit HA formation. The same results

Table 1. Immunoprecipitation of hyaluronate and chitin oligosaccharide synthase activity in zebrafish embryos

Tube	Embryo stage	UDP–GlcA (200 μM)	Antibody used: anti-DG42	Antibody used: nrs	HA	Chitin-oligosaccharides
1	early gastrula	+	+	−	2,697	<40
2	early gastrula	−	+	−	1,990	<40
3	early gastrula	+	−	+	2,640	210
4	early gastrula	−	−	+	1,870	286
5	early neurula	+	+	−	2,566	<40
6	early neurula	−	+	−	1,831	<40
7	early neurula	+	−	+	2,710	390
8	early neurula	−	−	+	1,980	586
9	late neurula	+	+	−	2,680	<40
10	late neurula	−	+	−	2,010	<40
11	late neurula	+	−	+	2,730	110
12	late neurula	−	−	+	1,956	155

Embryos (200) from three developmental times (early gastrula, early neurula, and late neurula stages) were used to prepare cell extract as described in *Materials and Methods*. The cell extract from each developmental time was separated into two tubes. One was immunoprecipitated with anti-DG42 antibody (anti-DG42) and the other with normal serum rabbit (nrs) as control, as described in *Materials and Methods*. The supernatants of immunoprecipitation were incubated in the presence of UDP-[^{3}H]GlcNAc, with (+) or without (−) adding UDP–GlcA (see *Materials and Methods*). The incubations were stopped by adding 200 μl of water and boiling for 2 min and then were centrifuged. The precipitates were washed twice with 100 μl of water. The combined supernatants (~500 μl) were tested as follows: 1) 100 μl was used for detection of HA (see *Materials and Methods*). The labeled material in the exclusion volume of the Bio-Gel A-5m column (2–4 × 10^3 kDa) was pooled and concentrated. An aliquot of each sample was counted, and the remaining material was incubated with hyaluronidase from bovine testes or hyaluronate lyase from *S. hyalurolyticus* (see *Materials and Methods*). The material was then rechromatographed in the same system, and the compounds in the exclusion as well as the inclusion volume were counted. The amount of radioactive material present in the inclusion volume was used to calculate the total hyaluronic acid, indicated in the table as HA. In all cases, using both enzymes, the percentage of material remaining in the exclusion volume was 20–25%. 2) The remaining 400 μl was used to detect chitin oligosaccharides as described in *Materials and Methods*. The total amount of labeled chitin oligosaccharides (3, 4, 5, and 6 residues) present in each supernatant is indicated in the table.

were obtained when cell extract preparations from *Xenopus* embryos were used (Table 2). The lack of complete dependence of HA synthesis on addition of unlabeled UDP-GlcA probably reflects the presence of nucleotide sugars in the crude extracts used for enzymatic incubations. In all cases, the HA incorporation reported represents material sensitive to hyaluronidase and hyaluronate lyase.

FPLC Fractionation of the Hyaluronic Acid Synthase Complex and Chitin Oligosaccharide Synthase from Zebrafish Embryos. Previous work has shown that in eukaryotic cells active hyaluronate synthase is a high molecular weight enzyme complex (28–30). Schwartz *et al.* (28) developed a successful fractionation of functional hyaluronate synthase that involves extraction of membranes with digitonin followed by gel filtration. We used this technique to obtain membrane extracts of zebrafish embryos from the late gastrula stage (9 h). The extract was filtered through a Superose 6 column, and each fraction was assayed for hyaluronate and chitin oligosaccharide synthase activity as shown in Fig. 4. The results show that the hyaluronate synthase activity is associated with high molecular weight molecules and chitin oligosaccharide synthase with a lower molecular weight fraction, which follows the elution profile of the DG42 protein. The results also show that the DG42 protein is not associated with the high molecular weight hyaluronate synthase enzyme complex. However, a very

Table 2. Immunoprecipitation of hyaluronate and chitin oligosaccharide synthase activity in *Xenopus* embryos

	Crude extract	Antibody used	
		nrs	anti-DG42
HA	998	880	919
Chitin-oligosaccharides	768	644	<100

Xenopus embryos (120) from late gastrula stage were used to prepare cell extracts as described in *Materials and Methods*. In this case, the cell extract was separated into three portions. One part was used for immunoprecipitation with anti-DG42 antibody (anti-DG42), the second with normal rabbit serum (nrs), and the third was not immunoprecipitated (crude extract). The supernatant of each tube was incubated in the presence of UDP-[^{3}H]GlcNAc and UDP-GlcA (see *Materials and Methods*). The same methods used in Table 1 for analysis of HA and chitin oligosaccharide were used here.

small amount of a protein smaller than the major DG42 band can be seen in the HA synthase regions of the Western blot in Fig. 4.

Synthesis of Chitin Oligosaccharides in Extracts of Mouse 3T3 Cells Carrying Plasmid-Expressed DG42. DG42 cDNA was incorporated into a pLENneo plasmid and used to transform mouse 3T3 cells. As shown in Table 3, extracts of transformants carrying DG42 synthesize chitooligosaccharides *in vitro*. In contrast, control transformants carrying an α integrin cDNA were inactive.

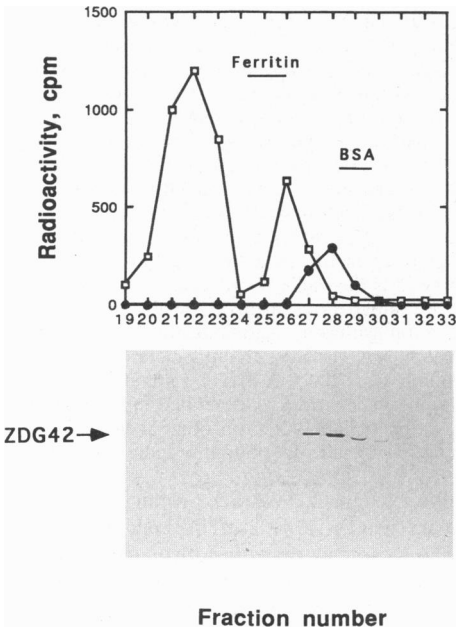

FIG. 4. Distribution of the zebrafish DG42-like protein in a size exclusion Superose 6 column and fractionation of hyaluronate and chitin oligosaccharide synthase activity. A sample of 300 zebrafish embryos from late gastrula stage were lysed in 200 μl of 1% digitonin buffer and injected into a Superose 6 column equilibrated with 0.1% digitonin buffer as described in *Materials and Methods*. Fractions 19–33 were used to detect activity of hyaluronate synthase (□) (using labeled UDP-[^{14}C]GlcA and unlabeled UDP-GlcNAc) or chitin oligosaccharide synthase activity (●) (using labeled UDP-[^{3}H]GlcNAc and unlabeled UDP–GlcA) (see *Material and Methods*). The same methodology used in Table 1 for analysis of HA and chitin oligosaccharides was used here. An aliquot (100 μl) of each fraction was concentrated, resuspended in 1% SDS loading buffer, boiled for 2 min, and applied to a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane and used to develop a Western blot with the anti-DG42 antibody as described in *Materials and Methods*. The figure at the bottom indicates with an arrow the fractions where zebrafish DG42-like protein was detected (ZDG42). Superose 6 has an exclusion size of 5 × 10^3 kDa. Blue dextran (2 × 10^3 kDa), ferritin (440 kDa), and bovine serum albumin (66 kDa) were used to calibrate the column.

Table 3. Hyaluronic acid and chitin oligosaccharide synthase activity from pLENneo transfected 3T3 cells

Construction	HA	Chitin-oligosaccharides mainly $(GlcNAc)_5$	Protein detected with specific antibody	
			DG42	α Integrin
3T3-pLENneo-α 3 A	789	<50	−	+
3T3-pLENneo-DG42	744	435	+	−

pLENneo is a derivative of pLEN made available to us by Dipersio (32). Three different constructions of pLENneo plasmid were used to transfect 3T3 cells: 1) pLENneo itself; 2) pLENneo-α3A, carrying an α3-subunit of chicken integrin; and 3) pLENneo-DG42, carrying the 1,780-bp *Eco*RI-*Xho*I DG42 coding region. After transfection, cells were selected for resistance to Geneticin (G418 sulfate) GIBCO/BRL. The resistant colonies that appeared after 2 weeks were tested for expression of α3-integrin or DG42 by immunohistochemistry using specific antibodies. The cells were resuspended in lysis buffer. The incubation supernatants were tested for HA and chitin oligosaccharide synthesis as described in *Materials and Methods* and Table 1. The results are expressed in total cpm.

DISCUSSION

The *DG42* gene appears to be conserved among vertebrates and is expressed during early development. *In vitro*, the *Xenopus* DG42 protein catalyzes the synthesis of chitin oligosaccharides (19). We have shown here that the *Xenopus* enzyme as well as the zebrafish and mouse homologs synthesize chitin oligosaccharides in extracts from embryos of appropriate age. Our previous paper (19), the present work, and the accompanying paper by Meyer and Kreil (31) clearly establish the following facts.

1) The DG42 protein made in an *in vitro* transcription/translation system is able to catalyze synthesis of chitin oligosaccharides, but not hyaluronic acid, under the conditions tested.

2) Zebrafish, *Xenopus*, and mouse extracts from appropriate developmental stages are able to synthesize chitin oligosaccharides. This synthesis, but not the synthesis of hyaluronic acid, can be inhibited by an antibody to the *DG42* protein under the conditions tested.

3) The chitin oligosaccharide synthase activity as well as most of the DG42 protein in a digitonin extract of embryos can be separated from the hyaluronate synthase activity by chromatography on a Superose 6 sizing column.

4) As shown in the accompanying paper by Meyer and Kreil (31), expression of DG42 with a vaccinia vector in mammalian RK13 cells under certain conditions leads to large increases in the levels of hyaluronic acid synthesis.

Several interpretations of the facts above are possible. One simple explanation is that the DG42 enzyme by itself is able to catalyze the synthesis of either chitin oligosaccharides or hyaluronic acid depending on the conditions of incubation. Both products do contain β-1,4-linked acetylglucosamine residues. To the present time, however, there are no data to support this hypothesis, but it obviously deserves further exploration. As far as we are aware, the pure DG42 protein has not yet been isolated.

A second possibility is that chitin oligosaccharides serve as primers for hyaluronic acid synthesis and that chains grow at the nonreducing ends by the addition of monosaccharides. In experiments to be presented elsewhere, we have shown that borotritide reduction of pure hyaluronic acid followed by exhaustive hyaluronidase digestion gives rise to labeled chitin oligosaccharides, an observation consistent with this priming concept.

The simple fact that DG42 is a developmental gene expressed between the late blastula and neurulation stages (1, 2), while hyaluronic acid synthesis occurs throughout the life of the organism, means that HA synthesis must usually occur in the absence of DG42 expression. Since mammalian hyaluronic acid synthase activity is normally found associated with a high molecular weight complex (28), it is entirely possible that a homolog of DG42, or another chitin oligosaccharide synthase, is part of the complex. If this is the case, DG42 itself could have a completely independent function *in vivo* such as generation of signaling oligosaccharides during a narrow window of early development. It is also possible that hyaluronic acid synthesis occurs with or without chitin oligosaccharide priming depending on the nature of the complex formed in specific tissues. Obviously the nature of HA synthase complexes and their regulation need further exploration.

The major focus of our research continues to be the question of whether the chitin oligosaccharides made by the DG42 enzyme have a signaling function in the embryo and whether these oligosaccharides do or do not play a role in embryonic hyaluronic acid synthesis.

Note Added in Proof. We have recently found that plasmid-based expression of two proteins unrelated to HA synthesis, an integrin subunit and p34 (a membrane protein involved in recognition of MHC I), also leads to increased hyaluronic acid formation in 3T3 cells.

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