Characterization and Molecular Evolution of a Vertebrate Hyaluronan Synthase Gene Family*

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The three mammalian hyaluronan synthase (HAS) genes and the related Xenopus laevis gene, DG42, belong to a larger evolutionarily conserved vertebrate HAS gene family. We have characterized additional vertebrate HAS genes from chicken (chas2 and chas3) and Xenopus (xhas2, xhas3, and a unique Xenopus HAS-related sequence, xHAS-rs). Genomic structure analyses demonstrated that all vertebrate HAS genes share at least one exon-intron boundary, suggesting that they evolved from a common ancestral gene. Furthermore, the Has2 and Has3 genes are identical in structure, suggesting that they arose by a gene duplication event early in vertebrate evolution. Significantly, similarities in the genomic structures of the mouse Has1 and Xenopus DG42 genes strongly suggest that they are orthologues. Northern analyses revealed a similar temporal expression pattern of HAS genes in developing mouse and Xenopus embryos. Expression of mouse Has2, Has3, and Xenopus Has1 (DG42) led to hyaluronan biosynthesis in transfected mammalian cells. However, only mouse Has2 and Has3 expressing cells formed significant hyaluronan-dependent pericellular coats in culture, implying both functional similarities and differences among vertebrate HAS enzymes. We propose that vertebrate hyaluronan biosynthesis is regulated by a comparatively ancient gene family that has arisen by sequential gene duplication and divergence.

Simple linear carbohydrate polymers make up the largest biomass on the Earth. These polymers include cellulose, chitin, and arguably their vertebrate equivalent, hyaluronan (HA). All of these polymers play protective or supporting roles in the organisms in which they are synthesized; cellulose makes up the plant cell wall, chitin makes up fungal cell walls and arthropod exoskeletons, and hyaluronan is an important component of skin, cartilage, and connective tissue. The hyaluronan capsule synthesized by group A streptococci also plays a protective role in this bacterium, shielding the organism from the host’s immune system (1−3). In addition to protective and supporting roles based upon physicochemical properties, simple carbohydrate polymers can act as signaling molecules. For example, in leguminous plants short lipochito-oligosaccharides produced by rhizobial species signal the formation of the specialized root nodule structure (4, 5). Moreover, in vertebrates hyaluronan can influence cell behavior including cell migration, proliferation, and differentiation through interactions with specific cell-surface HA receptors such as CD44 and RHAMM (6−9).

Recent studies are beginning to identify the genes encoding the enzymes that are responsible for the biosynthesis of these polymers (10−19). We have focused our efforts on the identification and study of putative eukaryotic HA synthases (13−18). HA is a high molecular weight glycosaminoglycan composed of a linear array of D-glucuronic acid β1−3N-acetylgalactosamine β1−4 disaccharide units. HA is synthesized at the inner face of the plasma membrane, and thus the enzymes responsible for HA biosynthesis are predicted to be plasma membrane proteins (20−23). We and others (13−18) have recently characterized three related mammalian genes (HAS1, HAS2, and HAS3) encoding putative plasma membrane hyaluronan synthases. The three gene products share 55−71% sequence identity. In addition, they display sequence homology with the Streptococcus pyogenes HA synthase, HasA, to the developmentally regulated Xenopus laevis DG42 protein and to other β-galactosaminyltransferases such as the rhizobial NodC proteins, yeast chitin synthases, and a recently reported family of putative plant cellulose synthases (18). Expression of any one mammalian HAS gene leads to HA biosynthesis by transfected mammalian cells, suggesting a critical role for these enzymes in the HA biosynthetic pathway. The three mammalian HAS genes are localized on separate autosomes (24), supporting the idea that this gene family may have arisen through comparatively ancient gene duplication events.

A controversy has emerged regarding the true biological function of the Xenopus DG42 protein (25). DG42 has been described as both a putative chito-oligosaccharide synthase (26, 27) and as a bona fide hyaluronan synthase (28, 29). Furthermore, some of these studies have proposed that there may be functional overlap in these two biosynthetic pathways, with chitin oligosaccharides possibly acting as primers that are required for and the limiting factor for hyaluronan biosynthesis (25, 27).

In this paper, we report the results of studies designed to elucidate the relationships between HAS family members and Xenopus DG42. These studies demonstrate that the Xenopus DG42 gene is a member of a larger vertebrate gene family that includes the mammalian HAS genes. The data presented also
show that the vertebrate hyaluronan synthases are differentially expressed and have related but distinct enzymatic properties.

EXPERIMENTAL PROCEDURES
dDNA and Genomic Cloning—Degenerate PCR primers specific for HAS genes (16, 18) were used to amplify HAS gene fragments from genomic DNAs of different animal species by the polymerase chain reaction (PCR). Genomic DNA samples included human (T47D mammary carcinoma cell line), 129SvJ mouse, chicken (Gallus gallus), and X. laevis genomic DNA. HAS gene-specific PCR fragments were amplified as described previously (18), and products were gel-purified and ligated directly into a pBluescript KS+ based T-vector (30) for subsequent analyses.

A. X. laevis Has1 (DG42) open reading frame (ORF) was amplified by reverse transcriptase-PCR from X. laevis embryonic stage 12 total RNA (kindly provided by Dr. Douglas DeSimone, University of Virginia, Charlottesville) with the following primers: forward, 5'-AAGCCCG-GGCAATAGGAGGAAAAAGCTGACACAAAC-3' and reverse, 5'-AACCGGGTCTAAAACCACTATAGCTAGTG-3', corresponding to nucleotides 53 to 81 and 1799 to 1829 (31). The amplified xHas1 (DG42) ORF was identical in sequence to the genomic variant xHas1 (DG42) that has been reported (accession number M22249). To obtain a full-length genomic DNA for the X. laevis HAS-related sequence (xHas-rs), the MARATHON cDNA cloning system (CLONTECH) was utilized with xHAS-rs-specific primers: 5' RACE, 5'-GCCAAAGATGCGTACAGAATACAAGGATACAG-3' and 3' RACE, 5'-CTCTATTGACCGTACGGCTAC-3' (corresponding to nucleotides 1390 to 1366 and 933 to 955, respectively) according to the manufacturer's recommendations. Amplified fragments representing overlapping 5' and 3' cDNAs for xHAS-rs were gel-purified and cloned as described previously (30). These cDNAs were subsequently ligated together to create a full-length xHAS-rs cDNA.

P1 clones (mouse strain 129Sv/J) containing all or part of the Has2 gene were also obtained (Genome Systems, St. Louis, MO) through a library screen with the following pair of Has2-specific PCR primers: forward, 5'-CTGCGCCAAAGCAATATGACAGAAGAG-3' and reverse, 5'-CCAGGATGCTTTCCAAC-3', corresponding to nucleotides 2343 to 2367 and 2680 to 2658, respectively (15). P1 clones (mouse strain 129Sv/J) containing the mouse Has3 gene were obtained using a similar strategy as described previously (18). Fragments of the mouse Has1 gene were amplified by high fidelity long PCR (32, 33) of 129Sv/J mouse genomic DNA. Amplified products were cloned as described previously. Conditions used for long PCR reactions were as follows: approximately 100 ng of genomic DNA, 10 units of polymerase (Boehringer Mannheim) and Vent DNA polymerase (New England Biolabs) at a ratio of 25:1 (1.25 units of Taq/0.05 units of Vent) in a total volume of 50 μl. A manual hot-start approach was used, and the reactions were amplified through 10 cycles of 94 °C 10 s, 65 °C 30 s, and 72 °C 5 min, followed by 22-25 cycles of 94 °C 10 s, 65 °C 30 s, 72 °C 5 min plus an extension of 15 s/cycle. Amplified products were cloned, sequenced, and restriction-mapped. All sequence was determined from both strands.

Restriction maps and genomic structures of the vertebrate HAS genes were derived through a combination of conventional restriction fragment subcloning and sequence characterization and through PCR amplification between various coding domains. Genomic structures for the Xenopus HAS-rs and xhas1 (DG42) genes were derived from products amplified from X. laevis genomic DNA through high fidelity long PCR using gene-specific oligonucleotides. In these instances, fragments representing the entire genes including 5'- and 3'-untranslated regions (UTRs) were amplified.

Northern Analyses—The expression of mouse, human, and Xenopus hyaluronan synthase genes were examined by Northern analysis. Northern blots of staged mouse embryo and human adult tissue poly(A)+ RNAs (CLONTECH) were hybridized sequentially with radiolabeled portions of mouse or human HAS cDNAs. 32P-Labeled probes were prepared by random priming (34). Total RNAs prepared from staged X. laevis embryos were kindly provided by Dr. Douglas DeSimone of the University of Virginia, Charlottesville. Approximately 20 μg of total RNA from each stage were loaded on a 1% agarose-formaldehyde gel, electrophoresed and transferred to Hybond N+ membranes (Amersham Corp.). Membranes were stained with methylene blue (35) prior to prehybridization to visualize ribosomal RNAs to determine sample loading and efficiency of transfer. Membranes were sequentially hybridized with partial cDNA probes representative of Xenopus Has1 (DG42), X. laevis Has2, X. laevis Has3, and X. laevis HAS-rs. Mem-
Degenerate PCR Cloning of Vertebrate HAS Gene Fragments—PCR using genomic DNA and degenerate HAS gene oligonucleotides was used to identify fragments of HAS genes from several vertebrate species. Specific fragments were amplified from human (Homo sapiens), mouse (Mus musculus domesticus), chicken (G. gallus), and the African clawed toad (X. laevis). Sequence analysis of cloned PCR fragments indicated that the human and mouse HAS fragments represented the HAS1, HAS2, and HAS3 genes. In the chicken, only clones representative of chas2 and chas3 were obtained. In X. laevis, clones representative of xhas1 (DG42), xhas2, xhas3, and a previously unidentified HAS-related sequence, designated xHAS-rs, were obtained. Extensive sequence conservation between species permitted the classification of HAS clones obtained from each animal (Fig. 1). In addition, sequence alignment indicated that a fragment of a zebrafish (Danio rerio) HAS gene previously reported as zebrafish DG42 (27) is clearly a fragment of zebrafish Has2 (chas2).

Multiple degenerate primer combinations failed to amplify fragments of additional HAS-related sequences in the species studied. On average, 20 independent clones were sequenced for each degenerate PCR product. Although clones representative of chicken Has1 were not obtained, we assume that this may be the result of primer bias. These results suggest that the HAS gene family consists of three members in human and mouse and four members in X. laevis, although we cannot formally rule out the possibility that additional family members as well as related genes with lower levels of sequence identity exist in these species.

cDNA Cloning of a Xenopus HAS-related Sequence (xHAS-rs)—To characterize further the HAS-related sequence (xHAS-rs) in Xenopus, 5’ and 3’ RACE (rapid amplification of cDNA ends) reactions were performed using Xenopus stage 12 total RNA. Overlapping products were directly cloned and sequenced. These clones spanned a total of 2.5 kb and contained an ORF of 1749 base pairs predicting a protein of 583 amino acids (Fig. 2). The amino acid sequence for Xenopus HAS-rs was most similar to Xenopus DG42 (now designated xHas1) (65% identity) and shared approximately 49% identity with the three mammalian HAS proteins (Fig. 3A) and 25% identity to Streptococcus HasA. Significantly, two residues critical for the activity of yeast chitin synthase 2 (39) and conserved among all other HAS proteins, chitin synthases, and cellulose synthases, are not conserved in xHAS-rs; these include an aspartate (Asp) residue in the motif DSST, which is NSDI in xHAS-rs, and an arginine (Arg) residue in the motif QXXXR, which is QPTPW in xHAS-rs (Fig. 3B). This might suggest that Xenopus HAS-rs is inactive as a processive glycosyltransferase (40) or may have diverged significantly to become functionally distinct from other HAS family members.

Genomic Structure Analysis of Vertebrate HAS Genes—The
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...genomic structure for Xenopus DG42 (now designated xhas1) has been previously reported (31). Similarly, we have previously reported the locations of exon-intron boundaries within the mouse Has3 gene (18). We determined the complete genomic structures for mouse Has1, Has2, Has3, and Xenopus HAS-rs to understand potential evolutionary relationships among all known vertebrate HAS genes. Restriction maps were derived for each gene, and the locations of all exon-intron boundaries were determined (Fig. 4 and Table I). Mouse Has2 represents the largest of the HAS genes, spanning approximately 30 kb. Xenopus has1 (DG42) represents the smallest of the HAS genes, spanning only 4 kb. The mouse Has1, Xenopus has1 (DG42), and Xenopus HAS-rs genes are made up of 5 exons, whereas the mouse Has2 and Has3 genes are made up of only 4 exons.

Pairwise comparisons of exon-intron boundaries revealed important evolutionary relationships among vertebrate HAS genes (Fig. 4B and Table I). The genes segregated into two classes: Xenopus has1 (DG42) xHAS-rs and mouse Has1 comprising one class, and mouse Has2 and Has3 comprising the second class. Mouse Has1 and Xenopus has1 (DG42) are essentially identical in genomic structure. In both genes, the predicted start codon (ATG) is located at the end of the first exon (Fig. 4B and Table I). In contrast, although Xenopus HAS-rs shares three out of four exon-intron boundaries with Xenopus has1 (DG42) and mouse Has1, the predicted start codon of xHAS-rs is located within the second exon (Fig. 4B and Table I). Mouse Has1, Xenopus has1 (DG42), and xHAS-rs also share relatively short 5'- and 3'-UTRs. Furthermore, Xenopus has1 (DG42) and mouse and human HAS1 have multiple copies of the sequence AUUUA within their 3'-UTRs which may play a role in the turnover of these mRNAs (41). Interestingly, the sequence at the start of the mouse Has1 gene first intron contains 27 copies of a CA repeat (GT), which includes the consensus GT of the splice donor sequence (Table I).

Mouse Has2 and Has3 share all exon-intron boundaries. The start codon of these genes is located at the beginning of exon 2, with the entire 5'-UTR, including the first three base pairs (−3) of the Kozak consensus (42), contained within exon 1 (Fig. 4B and Table I). Both genes, particularly Has3, have comparatively long 3'-UTRs. Only a single exon-intron boundary of the Has2 and Has3 genes is shared with Xenopus has1 (DG42), xHAS-rs, and mouse Has1 (Fig. 4B and Table I).

Northern Analysis of Vertebrate HAS Expression—We have previously reported the temporal expression of mouse Has2 and Has3 in the developing mouse embryo (16, 18). These Northern studies were extended by analyzing the expression of mouse Has1 in staged mouse embryos and the expression of Xenopus has1 (DG42), xHas2, xHas3, and xHAS-rs in staged Xenopus embryos (Fig. 5A). In addition, we analyzed the expression of human HAS1, HAS2, and HAS3 in a panel of poly(A)+ RNAs isolated from adult tissues (Fig. 5B).

The expression of mouse Has1, like its orthologue xHas1 (DG42) (31), is restricted during embryonic development. Of the four stages analyzed, only the 7.5-day postcoitum gastrulating mouse embryo expressed a single Has1 transcript of approximately 2.2 kb (Fig. 5A). In contrast to xHas1 (DG42), Xenopus Has3 was expressed from relatively early in development, but its expression increased up to early tadpole (stage 25). Xenopus Has3 was not expressed between stages 1 through 25. Similarly, in the mouse embryo, Has3 expression is barely detectable in the midgestation embryo but is expressed at significant levels in the late gestation embryo (equivalent to Xenopus development > stage 25). Xenopus HAS-rs was expressed in a pattern almost identical to Xenopus Has1 (DG42).

Results obtained from Northern analysis of human adult tissue mRNAs indicated that HAS genes are differentially expressed in adult tissues. HAS1 was the most widely expressed, with a major transcript of 4.6 kb expressed most highly in heart, liver, and skeletal muscle and a smaller transcript of 2.4 kb that was mainly expressed in prostate and ovary. HAS3 was also widely expressed, with transcripts of 4.3, 3.0, and 2.4 kb being detected. HAS2 transcripts were prevalent in the heart and submucosa of the small intestine, with lower levels in other tissues (Fig. 5B). Two transcripts of approximately 3.2 and 4.4 kb were detected.

Functional Assays—A simple but classical particle exclusion assay (43) was used to investigate the ability of vertebrate HAS enzymes to generate a HA-dependent pericellular coat in cultured mammalian cells. These assays demonstrated that only Has2 and Has3 expression led to the formation of significant pericellular coats that were specifically destroyed by the action of Streptomyces hyaluronidase in both COS-1 (Fig. 6, top) and 293 cells (data not shown).

In addition to HA-coat assays, we isolated crude membranes from COS-1 cells transfected with vertebrate HAS expression vectors and investigated the ability of these preparations to
synthesize HA in vitro. Results from these experiments indicated that membranes isolated from mouse Has2, Has3, and Xenopus Has1 (DG42) expressing cells possessed significant HA synthase activity in vitro, whereas membranes isolated from mouse HAS1 and Xenopus HAS-rs expressing cells did not have HA synthase activity that was significantly above that seen in control experiments.
observed in membranes isolated from control pCIneo-transfected cells (Table II). This is a striking, yet puzzling, result since xHas1 (DG42)-transfected cells failed to form HA-dependent pericellular coats in culture (Fig. 6, top). Overall, it should be noted that very little high molecular weight product was synthesized by any membrane preparation in the absence of UDP-GlcNAc. Treatment of radiolabeled products, synthesized in the presence of 1 mM UDP-GlcNAc, with 1 TRU Streptomyces hyaluronidase resulted in a typical reduction of between 75 and 100% of the counts remaining at the origin of the paper chromatography strips.

We investigated the molecular mass of the in vitro synthesized products by agarose gel electrophoresis. Results from these experiments indicated that, under our assay conditions, mouse Has2 expression leads to the synthesis of a significantly larger hyaluronidase-sensitive polymer than that synthesized by mouse Has3 or Xenopus Has1 (DG42), a molecular mass of $3 \times 10^5$ Da to $>2 \times 10^6$ Da versus a molecular mass of $<2 \times 10^5$ Da to $3 \times 10^5$ Da (Fig. 6, bottom).

Characterization of HAS-reactive Antisera by Immunoblotting—Rabbit polyclonal antisera were raised against Has2-derived synthetic peptides to create reagents to detect HAS proteins. Immunoblotting revealed that antisera raised against peptide 1 (MC285) were specific for mouse Has2 (Fig. 7). This antisera detected a major 52-kDa species and several smaller species only in crude membrane preparations of mouse Has2-transfected COS-1 cells. Antisera raised against peptide 3 (MC288) were also specific for mouse Has2 (data not shown). In contrast, antisera raised against peptide 2 (MC287) detected all HAS proteins, including Xenopus Has1 (DG42), but excluding Xenopus HAS-rs (Fig. 7). Proteins of the following estimated molecular masses were detected: Xenopus Has1 (DG42), 52 kDa; mouse Has1, 58 kDa; mouse Has2, 52 kDa; and mouse Has3, 52 kDa. Proteins were expressed at similar levels. Under our conditions, all vertebrate HAS enzymes migrated faster than their predicted molecular masses. A similar phenomenon
has been previously reported for \textit{S. pyogenes} HasA (12) and \textit{Xenopus} Has1 (DG42). Cross-reacting proteins were not detected in samples prepared from xHAS-rs or pCIneo (control)-transfected cells. We assume that the observed lack of cross-reactivity with xHAS-rs is sequence-based and not due to lack of expression of this protein in COS-1 cells. Significantly, an antisera raised against an N-terminal portion of \textit{Xenopus} Has1 (DG42) was completely specific for \textit{Xenopus} Has1 (DG42) protein (65% identity) and was identified in \textit{Xenopus} Has1 (DG42) protein of 583 amino acids that was similar in structure to other HAS members and most closely related to the xHas1 (DG42) protein (65% identity).

The conserved genomic structures of vertebrate HAS genes, including \textit{xhas1} (DG42) and \textit{xHAS-rs}, suggest that they represent a single gene family arising from a common ancestral gene. All HAS genes share a common exon-intron boundary, between exons 2 and 3. Based upon shared exon-intron boundaries, the HAS genes could be split into two subfamilies (Fig. 4 and Table I). Our data support a model of three sequential gene duplication events; the first event generating two related genes that diverged to form the HAS1 and HAS2 lineages, followed by two additional events to generate HAS1 and HAS2 and HAS3 and HAS2, respectively. Our future experiments will attempt to trace the origin of the vertebrate HAS gene family.

Northern analyses demonstrated that the three mammalian HAS genes have distinct patterns of expression in the developing embryo and in the adult, suggesting that the regulatory elements that control transcription of these genes have diverged substantially. However, the embryonic expression patterns of mouse Has genes were very similar to those of their orthologues in the developing \textit{Xenopus} embryo (Fig. 5A). This suggests that although the transcriptional regulatory elements of HAS genes have diverged substantially between genes, the regulatory elements may have been essentially conserved between species. Significantly, the embryonic pattern of expression of mouse Has1 was the most restricted and corresponded closely to the pattern observed for \textit{Xenopus} Has1 (DG42). Thus, our genomic structure and expression data strongly suggest.
that mouse Has1 represents the orthologue of Xenopus DG42. Thus, we propose a re-evaluation of the nomenclature for the X. laevis DG42 gene and its encoded product and suggest that this gene be named xhas1.

The functions of the mammalian HAS proteins and Xenopus Has1 (DG42) have been previously assessed by ourselves and other investigators (13–18, 26–29). These studies have employed in vitro transcription/translation, expression in bakers' yeast, and expression in a number of established mammalian cell lines. We have extended these studies to a functional comparison of vertebrate HAS enzymes in a single mammalian cell line, COS-1. Results from our studies suggest that vertebrate HAS enzymes are not functionally equivalent. Thus, mouse Has1, although previously shown to be active in other mammalian cell lines (13, 15), was not significantly active in COS-1 cells. In contrast, mouse Has2 and mouse Has3 expression led to the formation of substantial HA-dependent pericellular coats. However, the size of the HA synthesized in vitro by Has2 membranes was substantially larger than that synthesized by Has3 membranes. Interestingly, membrane preparations of Xenopus Has1 (DG42) expressing COS-1 cells possessed substantial HA synthase activity in vitro and synthesized a comparatively small hyaluronidase-sensitive polymer, yet COS-1 and 293 cells failed to form HA-dependent pericellular coats in culture. Thus, it appears that polymer size and subsequent fate of the newly synthesized HA may be regulated by expression of a specific HAS enzyme in a given cell type.

The most recently identified HAS family member, Xenopus HAS-rs, has been inactive in all functional assays performed to date. Genomic structure and expression analyses support the hypothesis that the xHAS-rs gene duplicated from the xhas1 (DG42) gene. All of our efforts to identify a mammalian orthologue have failed. Significantly, expressed sequence tag (EST) clones for all three mammalian HAS genes have been identified through homology searches of public domain data bases. No other HAS-related sequences have been identified. It is possible, therefore, that the xHAS-rs gene arose by a duplication event that occurred after the divergence of the amphibian lineage. Alternatively, this gene may have arisen before the amphibian divergence and subsequently been lost from some vertebrate lineages. It is entirely possible that the open reading frame for the xHAS-rs gene is not stably expressed and that this gene is thus non-functional. The sequence around the predicted translation initiation codon (ATG) of xHAS-rs does not conform to the most favorable Kozak initiation signal of A or G at −3 base pairs.

The vertebrate HAS enzymes are related to other eukaryotic β-glycosaminyltransferases, including the chitin and cellulose synthases, at the primary amino acid sequence level. These gene families may have evolved from independent origins, their sequence identities being the result of functional convergence. There are many examples of enzymes that catalyze the same reaction evolving independently. These include the superoxide

with methylene blue prior to hybridization to detect ribosomal RNAs (rRNA) to determine efficiency of transfer and variation in sample loading. All probes were labeled to similar specific activities, and membranes were exposed to autoradiographic film for identical lengths of time; mouse poly(A)+ RNA blot, 16 h at −80 °C; Xenopus total RNA blot, 3 days at −80 °C. B, expression of HAS genes in a panel of human adult tissues. Membranes were sequentially hybridized with partial cDNA probes for the respective human HAS genes. To determine variation in sample loading membranes were hybridized with a human β-actin probe. Membranes were exposed to autoradiographic film for an average of 5 days at −80 °C. Sk., skeletal; Sm., small. PBL, peripheral blood leukocyte.
dismutases, alcohol dehydrogenases, and serine proteases (reviewed in Ref. 47). Alternatively, it is possible that the eukaryotic chitin, cellulose, and HA synthases have evolved through divergent evolution from a common ancestral eukaryotic β-galactosaminyltransferase and thus constitute a super family of genes.

The *S. pyogenes* hyaluronan synthase, HasA, is encoded within the HAS operon, which encodes three polypeptides including HasA, HasB (a UDP-glucose dehydrogenase), and HasC (a UDP-glucose pyrophosphorylase) (48). Similar operons occur in other Gram-positive bacteria; for instance the operon controlling type 3 polysaccharide capsule biosynthesis in *Streptococcus pneumoniae*. Significantly, the Cap3B glycosyltransferase enzyme encoded within this operon is also flanked by ORFs encoding a UDP-glucose dehydrogenase and a UDP-glucose pyrophosphorylase (49). Furthermore, Cap3B is 26% identical to HasA and creates a similar polysaccharide, consisting...
ing of β1–3-linked disaccharide units of cellubiose acid (GlcUAβ1–4Glc) (50). It is likely, therefore, that the HAS operon of group A streptococci evolved in the context of other prokaryotic capsule polysaccharide synthase operons, rather than by horizontal gene transfer (51) from eukaryotes as has been recently proposed (52). This would suggest that HAS enzymes have evolved independently on at least two occasions, to generate the prokaryotic and eukaryotic enzymes, respectively. As genes encoding additional related β-glicosaminyltransferases are cloned, the evolution of these enzymes can be more fully investigated. In particular, the cloning of invertebrate (Drosophila or crab for instance) or vertebrate (Paralipophrys trigloides, the blennie, a teleost fish, one of the rare vertebrates that appears to synthesize chitin within its pectoral fins (53)) chitin synthase genes would represent a major advance in this area.

In conclusion, we have attempted to define genetic, transcriptional, and functional relationships among vertebrate HA synthases. Differences in mRNA expression patterns in concert with apparent functional differences suggest that there are multiple levels of regulation of HA biosynthesis in vertebrates. Future experiments will continue investigation into the molecular evolution of this gene family and will consider the respective role and functional relationships of HA synthases in vivo using the mouse as a model.

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