Hyaluronan (HA), a functionally essential glycosaminoglycan in vertebrate tissues and a putative virulence factor in certain pathogenic bacteria, is an extended linear polymer composed of alternating units of glucuronic acid (GlcUA) and N-acetylg glucosamine (GlcNAc). Uncertainty regarding the mechanism of HA biosynthesis has included the directionalities of chain elongation, i.e. whether addition of monosaccharide units occurs at the reducing or non-reducing termini of nascent chains. We have investigated this problem using yeast-derived recombinant HA synthases from *Xenopus laevis* (xHAS1) and from *Streptococcus pyogenes* (spHAS). The enzymes were incubated with UDP-[3H]GlcUA and UDP-[14C]GlcNAc, under experimental conditions designed to yield HA chains with differentially labeled reducing-terminal and non-reducing terminal domains. Digestion of the products with a mixture of β-glucuronidase and β-N-acetylg glucosaminidase exoenzymes resulted in truncation of the HA chain strictly from the non-reducing end and release of labeled monosaccharides. The change in 3H/14C ratio of the monosaccharide fraction, during the course of exoglycosidase digestion, was interpreted to indicate whether sugar units had been added at the reducing or non-reducing end. The results demonstrate that the vertebrate xHAS1 and the bacterial spHAS extend HA in opposite directions. Chain elongation catalyzed by xHAS1 occurs at the non-reducing end of the HA chain, whereas elongation catalyzed by spHAS occurs at the reducing end. The spHAS is the first glycosyltransferase that has been unani mously demonstrated to function at the reducing end of a growing glycosaminoglycan chain.

Hyaluronan synthases (HAS(s)) are glycosyltransferases that catalyze the biosynthesis of hyaluronan (HA), a glycosami noglycan generated at the plasma membrane of bacterial and eukaryotic cells. HA is an important structural component of the extracellular matrix and is also involved in a wide variety of biological processes, such as tissue morphogenesis, cancer metastasis, wound healing, inflammation, and angiogenesis (1–5). This extended linear polymer is synthesized, apparently without any primer requirement, by alternate addition of d-glucuronic acid (GlcUA) and N-acetyl-d-glucosamine (GlcNAc) units from the corresponding UDP-sugar donors (UDP-GlcUA and UDP-GlcNAc). HASs have been tentatively classified based on primary structure, molecular size, predicted membrane topology, and enzymological characteristics (6). Class I thus includes the streptococcal, the vertebrate (3 isoforms, HAS1–3), and the viral enzymes, whereas Class II contains a single member, the *Pasteurella multocida* enzyme. However, without knowledge of the three-dimensional structures or the catalytic mechanism, the two-class nomenclature system remains an hypothesis.

Although biochemical properties and in vivo functional aspects of HAS proteins have been extensively studied, the mechanism of HA biosynthesis has remained unclear. The biosynthesis of chondroitin and heparan sulfates, and glycosaminoglycans with backbones of alternating hexuronic acid and hexosamine units, clearly occurs by a stepwise addition of monosaccharide units at the non-reducing terminal of nascent chains (7), and the same mechanism of elongation has been demonstrated for the *Escherichia coli* K4 and K5 capsular polysaccharides (8–9), as well as for chitin (10) and cellulose (11). In an early assessment of the directionality of HA chain elongation, Stoolmiller and Dorfman (12) used a particulate enzyme preparation from Group A *Streptococcus* (now known as HasA or spHAS). They identified products released by enzymatic digestion of metabolically labeled HA chains and concluded that chain elongation occurs at the non-reducing end, as in biosynthesis of other glycosaminoglycans (12). Prehm (13) approached the problem by digesting pulse-chase-labeled HA produced by a teratocarcinoma cell membrane fraction and proposed, by contrast, that chain elongation occurs at the reducing end. This notion has by and large prevailed (see e.g. Ref. 14). Both of these native enzyme systems failed to further extend preformed, exogenously added HA oligosaccharides. However, in more recent studies, recombinant HAS from *P. multocida* (15) as well as a recombinant truncated form of the vertebrate HAS2 isoform (16) were found to catalyze the addition of sugar units at the non-reducing ends of added oligosaccharide primers.

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Because of these contradictory results, we decided to reinvestigate the directionality of HA chain elongation catalyzed by a vertebrate and a bacterial HAS. Yeast recombinant HASs from the African clawed frog *Xenopus laevis* (xlHAS1) and from *Streptococcus pyogenes* (spHAS) were applied to experiments designed to yield HA samples with differentially radiolabeled reducing terminal and non-reducing terminal domains, which were subsequently degraded to monosaccharides by exoglycosidase digestion. Our results demonstrate, unexpectedly, that xlHAS1 and spHAS extend HA at opposite ends of the polysaccharide. The xlHAS1 adds new sugars at the non-reducing end of the growing HA chain, whereas spHAS extends HA at the reducing end.

**EXPERIMENTAL PROCEDURES**

**Materials**—Radiolabeled UDP-[14C]GlcNAc was obtained from Amersham Biosciences. UDP-[3H]GlcUA was obtained from PerkinElmer Life Sciences. Unlabeled UDP-GlcUA and UDP-GlcNAc were from Sigma. β-N-Acetyl-d-glucosaminidase from jack bean was purchased from Seikagaku Corporation, and D-glucuronidase from bovine liver was from Sigma. All separation media and columns were bought from Amersham Biosciences. Unless otherwise noted, additional chemicals and reagents were from Sigma-Aldrich Chemical Corporation.

**Expression and Purification of xlHAS1 and spHAS**—Recombinant yeast xlHAS1 and spHAS were prepared as described previously in (17). In brief, the *Saccharomyces cerevisiae* BJ5461 strain containing the pYES/DG plasmid (for xlHAS1 expression) or the pYES/HA plasmid (for spHAS expression) was grown in uracil-deficient medium containing 0.1% glucose and 5% glycerol, and expression was obtained by galactose induction to a final concentration of 1%. Upon induction, the enzyme expressed accumulates in the plasma membrane fraction. The cells were disrupted with silica/silicon beads (0.5 mm) in a MiniBead-Beater-8 (Biospec), and crude membranes were retrieved by ultracentrifugation. After harvesting, the membrane pellet was suspended in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 20 mM MgCl₂, and 0.1 mM EDTA, together with UDP-sugars as follows (see schemes in Fig. 1). In one approach (see Fig. 1, Protocol 1), the xlHAS1 or spHAS membranes were incubated with 150 μM UDP-[14C]GlcNAc and 50 μM UDP-GlcUA. After 1 min (xlHAS1) or 4 min (spHAS) of reaction, 1 μM UDP-[3H]GlcUA was added to the mixtures, and the reactions were allowed to continue for an additional 30 s (xlHAS1) or 6 min (spHAS). In a second approach (see Fig. 1, Protocol 2), the membranes were incubated with 25 μM UDP-GlcUA and 50 μM UDP-[3H]GlcUA. After 1 min (xlHAS1) or 5 min (spHAS) of reaction, 150 μM UDP-[14C]GlcNAc was added, and incubation was continued for 1.5 min (xlHAS1) or 10 min (spHAS). In a third approach (see Fig. 1, Protocol 3), the membranes were incubated simultaneously with both labeled UDP-sugars, 150 μM UDP-[14C]GlcNAc and 50 μM UDP-GlcUA. After 1 min (xlHAS1) or 4 min (spHAS) of reaction, 1 μM UDP-[3H]GlcUA was added to the mixtures, and the reactions were allowed to continue for an additional 30 s (xlHAS1) or 6 min (spHAS). In a second approach (see Fig. 1, Protocol 2), the membranes were incubated with 25 μM UDP-GlcUA and 50 μM UDP-[3H]GlcUA. After 1 min (xlHAS1) or 5 min (spHAS) of reaction, 150 μM UDP-[14C]GlcNAc was added, and incubation was continued for 1.5 min (xlHAS1) or 5 min (spHAS). In a third approach (see Fig. 1, Protocol 3), the membranes were incubated simultaneously with both labeled UDP-sugars, 150 μM UDP-[14C]GlcNAc and 50 μM UDP-GlcUA. After 1 min (xlHAS1) or 4 min (spHAS) of reaction, 1 μM UDP-[3H]GlcUA was added to the mixtures, and the reactions were allowed to continue for an additional 30 s (xlHAS1) or 6 min (spHAS). In a second approach (see Fig. 1, Protocol 2), the membranes were incubated with 25 μM UDP-GlcUA and 50 μM UDP-[3H]GlcUA. After 1 min (xlHAS1) or 5 min (spHAS) of reaction, 150 μM UDP-[14C]GlcNAc was added, and incubation was continued for 1.5 min (xlHAS1) or 5 min (spHAS). In a third approach (see Fig. 1, Protocol 3), the membranes were incubated simultaneously with both labeled UDP-sugars, 150 μM UDP-[14C]GlcNAc and 50 μM UDP-[3H]GlcUA, for 1.5 min (xlHAS1) or 10 min (spHAS). Reactions were terminated by addition of trichloroacetic acid (final concentration 1%), and the mixtures were neutralized with 4 M NaOH and then heated at 100 °C for 3 min. After centrifugation (16,000 × g, 5 min), the remaining UDP-sugars were separated from HA in the supernatants by gel chromatography on columns (1 × 13 cm) of Sephadex G-25 fine, equilibrated with 0.2 M NH₄HCO₃. Labeled products were quantified by scintillation counting.

**Enzymatic Degradation of Hyaluronan**—Labeled HA was desalted on PD-10 columns and lyophilized under speed vacuum overnight. The

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**FIG. 1.** Scheme of experimental strategies used to assess the directionality of HA chain elongation. **RE**, reducing end; **NRE**, non-reducing end. For further information see the text.
samples were then dissolved in 3–4 ml of MES-NaOH buffer pH 5.5 containing 0.1% of bovine serum albumin, 0.5 units of β-N-acetylgalactosaminidase, and 10,400 units of β-glucuronidase and incubated at 37 °C. Aliquots of 0.6–0.8 ml were withdrawn after different periods of time, and digestion was stopped by the addition of 20 µl of 20% SDS. The samples were then boiled for 3 min at 100 °C and clarified by centrifugation. Digests and control samples were analyzed by gel chromatography on a FPLC Superose 6 column (1 × 30 cm) equilibrated with 0.5 M NH₄HCO₃. Effluent fractions were analyzed for radioactivity by scintillation counting. Labeled monosaccharides released from HA emerged as a single distinct peak at the elution position of standard monosaccharides. The amounts of radioactivity in the monosaccharide peak were calculated and expressed as percent of total label retrieved after gel chromatography.

RESULTS

Experimental Strategy—Recombinant xHAS1 or spHAS was incubated with UDP-[3H]GlcUA and UDP-[14C]GlcNAc, under conditions designed to yield HA chains with differentially labeled reducing terminal and non-reducing terminal domains. Predictions as to the distribution of the label in relation to the two alternative modes of chain elongation are based on the schemes in Fig. 1. The actual distribution of the label was assessed by digestion of the products with a mixture of β-glucuronidase and β-N-acetylgalactosaminidase, resulting in truncation of the HA chain strictly from the non-reducing end and release of monosaccharide products (19). A HA chain initiated in the presence of UDP-[14C]GlcNAc (and unlabeled UDP-GlcUA) and further elongated by incorporation also of [3H]-GlcUA units, would be 14C-labeled uniformly throughout, whereas the distribution of 3H label would depend on the mode of chain elongation (Fig. 1, Protocol 1). Elongation at the reducing terminus would yield a HA chain preferentially 3H-labeled in the reducing terminal domain, such that the monosaccharides released by exoglycosidase digestion would initially be exclusively 14C-labeled, then subsequently 3H as well as 14C-labeled. A progressive increase in the 3H/14C ratio of monosaccharide digestion products thus would be an indication of reducing terminal chain elongation in HA biosynthesis. Conversely, HA elongated at the non-reducing end would carry the 3H label primarily toward the non-reducing end of the chain, and yield monosaccharides of progressively decreasing 3H/14C ratio upon exolytic cleavage. Changing the experimental conditions to achieve continuous 3H labeling, and 14C incorporation only toward the end of incubation with UDP-sugar precursors (Fig. 1, Protocol 2), reverses all predictions from Protocol 1. A control with continuous incorporation of both isotopes was undertaken to ensure that under these conditions the 3H/14C ratio of all monosaccharide products would remain unchanged (Fig. 1, Protocol 3).

Kinetics of HA Chain Elongation—Pilot experiments were performed to establish optimal conditions for the dual labeling protocol. At saturating substrate concentrations (Kₘ for UDP-GlcUA = 190 ± 40 µM; Kₘ for UDP-GlcNAc = 400 ± 100 µM), xHAS1 incorporates ~3 monosaccharide units/s into a nascent HA chain (20). spHAS displays substrate affinities of the same order (Kₘ for UDP-GlcUA = 40 ± 4 µM; Kₘ for UDP-GlcNAc = 149 ± 3 µM (21)). Prerequisite to our analytical approach, HA chains should be initiated and progressively elongated during the incubation period, with neither formation of unduly long chains nor significant initiation of additional new chains. The protocol finally selected involved subsaturating substrate concentrations (150 µM UDP-GlcNAc and 50 µM UDP-GlcUA). Gel chromatography of xHAS1 polymer products generated under these conditions (label introduced through UDP-[3H]GlcUA) showed reasonably homogeneous populations of HA chains, growing from <20 kDa after 30 s of incubation to ~40 kDa (~200 monosaccharide units) within 2 min (Fig. 2A). HA produced by spHAS, at the same substrate concentrations, showed similar progressive elongation albeit at a lower rate, chains growing from <20 kDa after 4 min of incubation to ~40 kDa after 10 min (Fig. 2B). The samples isolated after the longer incubation periods showed no significant initiation of new chains under these reaction conditions, irrespective of enzyme system.

Formation and Degradation of Differentially Labeled HA Chains—The xHAS1 membranes were first incubated according to Protocol 1 (Fig. 1), with incorporation of [14C]GlcNAc throughout the incubation period, whereas [3H]GlcUA was available only during the second phase of HA chain elongation. The resulting products were isolated and subjected to graded exoglycosidase (β-glucuronidase and β-N-acetylgalactosaminidase) digestion, as described under “Experimental Procedures.” The digestion products were identified as [14C]GlcNAc and [3H]GlcUA monosaccharides by gel chromatography (Fig. 3).
dicted. After 4 h of incubation with the exoglycosidases ~25% of the total $^{14}$C and ~32% of the $^3$H occurred in the monosaccharide peak. The progressive exoglycosidase action yielded monosaccharides of continuously decreasing $^3$H/$^{14}$C ratios, from 2.1 after 30 min to 1.3 after 4 h of digestion (Table I and Fig. 4). Similar results were obtained in another independent experiment (see supporting information, Table II). These data indicate that xHAS1 adds sugar residues at the non-reducing end of HA chains (Fig. 1). Conversely, HA chains were generated according to Protocol 2, i.e. with continuous $^3$H labeling and $^{14}$C incorporation only during the final stage of chain elongation. The monosaccharides obtained upon enzymatic degradation of this sample showed increasing $^3$H/$^{14}$C ratio with increasing exoglycosidase digestion time (Table I and Fig. 4). Again, a repeat experiment gave similar results (see supporting information, Table II). This finding, the reverse of that obtained with Protocol 1, again implicates the non-reducing terminus as the site of HA chain elongation (Fig. 1). The degradation products of a control experiment with the continuous presence of both labeled sugar precursors throughout the incubation with xHAS1 (Protocol 3) yielded monosaccharides of a constant $^3$H/$^{14}$C ratio during exoglycosidase digestion (Table I and Fig. 4A), in accord with prediction (Fig. 1).

Similar experiments were performed with spHAS enzyme, but in this case, the initial exoglycosidase degradation products of HA obtained according to Protocol 1 (Fig. 1) were predominantly $^{14}$C-labeled. After 30 min of exoglycosidase incubation ~23% of the total $^{14}$C but only ~4% of the $^3$H occurred in the monosaccharide peak (Table I and Fig. 4B). After 5 h of digestion ~38% of the total $^{14}$C and ~28% of the $^3$H were released as monosaccharides. Gradual exoglycosidase action yielded monosaccharides of a continuously increasing $^3$H/$^{14}$C ratio, from 0.2 after 30 min to 0.7 after 6 h of digestion (Table I and Fig. 4B). This change in ratio is opposite to that recorded in the corresponding experiment with xHAS1 (Table I and Fig. 4A). Conversely, exoglycosidase digestion of HA generated according to Protocol 2 yielded monosaccharides of a progressively decreasing $^3$H/$^{14}$C ratio (Table I and Fig. 4B). Similar results were obtained in two independent series of experiments (see supporting information, Table II). Again, exoglycosidase degradation of HA isolated after continuous incorporation of both radioisotopes (Protocol 3) released monosaccharides of constant $^3$H/$^{14}$C ratio. Taken together, these results strongly implicate the reducing end of HA as the site of chain elongation catalyzed by spHAS. We conclude that xHAS1 and spHAS operate at the opposite ends of the polymeric product.

**DISCUSSION**

The current notion on HA biosynthesis, favoring elongation at the reducing end of the chain, is based on experiments performed more than 20 years ago (13). In that previous study, teratocarcinoma cell membranes containing an endogenous HAS enzyme were pulse-labeled by incubation with UDP-$[^{14}$C]GlcNAc and UDP-$^3$H-GlcUA for 15 min, followed by a 15-min chase with unlabeled UDP-GlcUA. Conclusions regarding the elongation mechanism were based primarily on the rapid loss of label from the macromolecular HA product upon digestion with exoglycosidases. However, this observation would be compatible also with non-reducing terminal chain elongation, if the membrane-bound enzyme contained preformed, nascent HA chains that were completed already during the pulse period. Moreover, using the recombinant, but presumably related xHAS1 enzyme, we were
unable to establish appropriate chase conditions, i.e. to quench the incorporation of radiolabel into HA, by adding excess unlabeled UDP-GlcUA after the pulse-labeling period (data not shown). Similar objections may apply also to more recent experiments with HAS in a membrane fraction from a human glioma cell line (14). In our present study these obstacles have been overcome by selective dual labeling of de novo initiated HA chains, generated by recombinant xHAS1 or spHAS, using differentially radiolabeled UDP-sugar precursors. No significant initiation of novel chains occurred after the initial phase of incubation (Fig. 2). The problem of inefficient chasing was avoided by maintaining constant incorporation of one label throughout the incubation. The lack of endogenous HA chains in the enzyme preparation was ascertained by the use of S. cerevisiae, a yeast lacking the UDP-GlcUA precursor (17), to produce both recombinant synthases.

The distribution of the label in HA synthesized by xHAS1, as reflected by the $^3$H/$^14$C ratio of monosaccharides released by exoglycosidases, clearly implicates a mechanism of chain elongation occurring at the non-reducing end of the polysaccharide. The generation of HA chains by xHAS1 thus appears similar to the process catalyzed by the HA synthase from P. multocida (15) and to the biosynthesis of proteoglycan constituents such as chondroitin sulfate and heparan sulfate (7). Our conclusion is in agreement with recent studies of a truncated form of recombinant mammalian HAS2. This enzyme, contrary to the intact protein, was found to use exogenously supplied HA oligosaccharides as sugar acceptors in chain elongation reactions (16). Acceptor capacity thus was demonstrated for a tetrasaccharide with an intact non-reducing terminal GlcUA residue, generated by the digestion of HA with testicular hyaluronidase. By contrast, a tetrasaccharide containing a 4,5-unsaturated hexuronic acid residue, generated by microbial lyase digestion, showed no acceptor activity. It was suggested that monosaccharide units were added at the non-reducing end of oligosaccharide acceptors.

On the other hand, the application of the dual labeling protocols to HA biosynthesis catalyzed by the spHAS enzyme revealed an elongation mechanism involving the reducing terminus of the nascent chain. The mechanism of HA chain formation by spHAS thus appears similar to those described for a growing number of polysaccharides such as Salmonella O-antigen polysaccharide (22), bacterial cell murein (23), and dextran (24). This finding concords also with the demonstration of the formation of a dynamic hyaluronan-UDP linkage during the synthesis of HA by HAS from Streptococcus equisimilis.
(25). This synthase incubated with β-32P-labeled UDP-GlcNAc and cold UDP-GlcUA incorporated 32P into HA. When the synthase was further incubated in presence of excess unlabeled UDP-sugars the radiolabel was removed from HA implicating a mechanism of chain growth at the reducing end of the UDP-sugar acceptor.

It is interesting that two HAS enzymes belonging to the same putative class, xiHAS1 and spHAS, differ in their mechanism of chain elongation, whereas the only member of class II, P. multocida HAS, adopts the same chain elongation process as two members of class I, xiHAS1, and human HAS2 (15). Could this difference in polymerization mechanism be the result of independent evolution of the eukaryotic and streptococcal HAS genes? The central regions (~200 residues) of the eukaryotic hyaluronan synthases are ~25% completely identical in sequence to the spHAS. If this similarity is ascribed to horizontal gene transfer from mammals to bacteria, it implies that extensive modification of the HA synthase occurred to optimize its function in the bacterial cell. Alternatively, our results may argue more in favor of an independent but convergent functional evolution of the eukaryotic and the streptococcal HAS genes. Indeed, the existence of spHAS in an operon, which is similar to the biosynthetic operons of other bacterial species that produce other extracellular polysaccharides is another strong argument against the horizontal gene transfer from mammals to bacteria (26). The elucidation of the eukaryotic and streptococcal HAS structures and the identification of the active site(s) will help unravel the evolutionary relationship among the HAS enzymes and will shed more light on this intriguing chain elongation process. The simple two-class HAS system probably needs to be revised.

In conclusion, we demonstrate that HA synthesis catalyzed by X. laevis HAS1 occurs by elongation at the non-reducing end of the growing chain. Presumably this deduction applies also to the other vertebrate HAS enzymes which are ~70% completely identical at the protein level. In addition, we clearly showed that the streptococcal HAS adopts an opposite mode of elongation, i.e. at the reducing end of the growing chain. To date it is the only glycosyltransferase that has been unanimously demonstrated to function at the reducing end of a growing glycosaminoglycan chain.

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