Hyaluronan Biosynthesis by Class I Streptococcal Hyaluronan Synthases Occurs at the Reducing End

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Preceding studies reached different conclusions about whether class I hyaluronan synthases (HASs) elongate hyaluronic acid (HA) by addition to the reducing or the nonreducing end. Here we used two strategies to determine the direction of HA synthesis by purified class I HASs from Streptococcus equisimilis and Streptococcus pyogenes. In the first strategy we used each of the two UDP-sugar substrates separately to pulse label either the beginning or the end of HA chains. We then quantified the relative rates of radioactive HA degradation by treatment with β-glycosidases that act at the nonreducing end. The results with both purified HASs demonstrated that HA elongation occurred at the reducing end. In the second strategy, we used purified S. equisimilis HAS, UDP-glucuronic acid, and UDP[β-32P]-GlcNAc to radiolabel nascent HA chains. Under conditions of limiting substrate, the 32P-labeled products were separated from the substrates by paper chromatography and identified as HA-[32P]UDP saccharides based on their degradation by snake venom phosphodiesterase or hyaluronidase and by their binding to a specific HA-binding protein. The 32P radioactivity was chased (released) by incubation with unlabeled UDP-sugars, showing that the HA-UDP linkages turn over during HA biosynthesis. In contrast, HA-[32P]UDP products made by the purified class II Pasteurella multocida HAS were not released by adding unlabeled UDP-sugars, consistent with growth at the nonreducing end for this enzyme. The results demonstrate that the streptococcal class I HAS enzymes polymerize HA chains at the reducing end.

Since the first HAS was identified and cloned in 1993 (1, 2) we have learned much about the structure and function of these unusual glycosyltransferases (3–7). The molecular masses of the streptococcal (~49 kDa) or eukaryotic (~65 kDa) HASs are relatively small in view of the multiple functions mediated by these enzymes in order to synthesize HA (8). HAS binds UDP-glucuronic acid (UDP-GlcUA) and UDP-N-acetylglucosamine (UDP-GlcNAc) in the presence of MgCl2 and catalyzes two distinct intracellular glycosyltransferase reactions. HAS also binds and translocates the growing HA chain through the enzyme, thereby extruding the polymer through the cell membrane, and releases the HA chain extracellularly after up to 50,000 monosaccharides (~105 Da) have been assembled. Based on differences in protein structure and mechanism of action, the known HASs have been categorized into two classes (5). Class I members include HASs from Streptococcus, mammals, and other eukaryotes, whereas the bacterial HAS from Pasteurella multocida is the only class II member.

Despite great progress in our understanding of HAS structure and function, there is still controversy regarding the direction of HA synthesis. Stoolmiller and Dorfman (9) concluded in 1969 that the streptococcal HAS adds new sugars to the nonreducing end of HA. In conflict with this result, Prehm in 1983 (10) and Asplund et al. in 1998 (11) performed studies with membranes from eukaryotic cells and concluded that HA synthesis occurs at the reducing end. Although differences in the contributions of the three mammalian HAS isoenzymes to these latter results were not considered, it is highly likely that the mechanisms of HA chain elongation for all of the class I HAS members are the same (3). Recently, Hoshi et al. (12) reported that recombinant truncated variants of human HAS2 expressed in Escherichia coli were able to synthesize short HA oligosaccharides by addition to the nonreducing end. Because the crude membranes used in all the above studies contain multiple glycosyltransferases, some of these reported results might have alternate interpretations. To resolve these conflicting results about the direction of HA synthesis, which is a fundamental mechanistic feature of HAS function, we performed several types of experiments using two purified streptococcal HASs. Our results verify that addition of new saccharides does occur at the reducing end.

EXPERIMENTAL PROCEDURES

Materials, Strains, and Plasmids—Reagents were supplied by Sigma unless stated otherwise. Media components were from Difco. The has gene from Streptococcus equisimilis or Streptococcus pyogenes was inserted into the pKK223-3 vector (Amersham Biosciences) and cloned into E. coli SURE™ cells (2, 13). Each HAS contained a C-terminal fusion of six His residues to facilitate purification (14). Streptavidin-coated 96-well plates were from BD Biosciences. The biotinylated HA-binding protein was from Seikagaku. UDP-[3H]GlcNAc (60 Ci/mmol) was from American Radiochemical, Inc., and UDP-[14C]GlcUA (285 mCi/mmol) was from Amersham Biosciences. Purified pmHAS (15) along with H-tetrasaccharides and H-octasaccharides of HA were generous gifts from Paul DeAngelis. UDP[32P]-GlcNAc (containing [32P]phosphate in the β position) was synthesized at a specific radioactivity of ~50 Ci/mmol as described by Reitman et al. (16). Bovine liver β-glucuronidase was from Roche Applied Science. N-Acetylgalcosamini-
dase was purified by the method of Li and Li (17) using jack beans obtained from a grocery store.

**Cell Growth and Membrane Preparation—** E. coli SURE™ cells containing the HAS-encoding plasmids were grown at 32 °C in Luria broth. HAS expression was induced, and membranes containing seHAS or spHAS were prepared as described recently (18). The membrane pellets were washed once with PBS containing 1.3 mM glycerol and protease inhibitors, sonicated briefly, aliquoted, and recentrifuged at 100,000 × g for 1 h. The final pellets were stored at −80 °C (14).

**HAS Extraction and Purification—** The extraction buffer, the procedure for solubilizing membranes, and affinity chromatography over a Ni²⁺-nitritolactic acid resin (Qiagen Inc.) have been described in detail (14, 18). HAS was eluted with 25 mM sodium and potassium phosphate, pH 7.0, 50 mM NaCl, 1 mM dithiothreitol, 2.7 mM glycerol, 1 mM dodecylmaltoside, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 46 µg/ml phenylmethylsulfonyl fluoride, and 200 mM histidine. HAS activity was determined using the standard assay conditions described previously (14, 17). Protein concentrations were determined with the Coomassie protein assay reagent (Ferree) using bovine serum albumin as the standard.

**Pulse Labeling of HA Chains and Direction of Synthesis Assay—** Purified seHAS or spHAS was prepared as noted above, except that the enzyme was not eluted from the Ni-NTA column after washing. Instead, the enzyme was incubated for two short successive periods designated to label HA chains early or late during one round of chain synthesis. There were four labeling situations for each HAS, namely early or late labeling with either UDP-[¹⁴C]GlcUA or UDP-[³H]GlcNAc. The first incubation was for 1.5 min at 22 °C with 0.08 µM UDP-GlcUA and 0.08 µM UDP-GlcNAc as well as 0.14 µCi of UDP-[¹⁴C]GlcUA, 0.2 µCi of UDP-[³H]GlcNAc, or no radiolabeled UDP-sugar. The HA-UDP-GlcNAc-Ni-NTA resin complex was then washed with 4 column volumes of wash buffer (50 mM Na₂KPO₄, pH 7.0, 150 mM NaCl, 0.5% dodecylmaltoside, and 2% glycerol), and the second labeling mixture was then added. After 1.5 min at 22 °C the resin was washed as above, and the radiolabeled HA was eluted with digestion buffer (25 mM sodium acetate, pH 5.2, containing 50 mM NaCl) at 37 °C for 1 h. Recovery of labeled HA was essentially complete as judged by the subtraction of bound HAS and any remaining HA with 1% trifluoroacetic acid. A 1-ml sample of labeled HA (∼50,000 dpm) was then incubated at 37 °C for the indicated times (Fig. 1) with 5 units of β-glucuronidase and 0.15 units of β-N-acetylglucosaminidase. The exoglycosidase digestions were terminated by the addition of SDS to a 2% (w/v) final concentration at room temperature. The amount of [¹⁴C]HA or [³H]HA remaining was determined by descending paper chromatog-
polymer chain is transferred intact, without cleavage of its UDP linkage.

The UDP released during each transfer step comes from the HA-UDP intermediate formed by the addition of the previous sugar. Thus, of the two net UDP groups released when a disaccharide unit is assembled at the reducing end, only one UDP (set as \(\text{UDP}^\text{boldface}\) in this example) comes from the last sugar added prior to addition of the new disaccharide unit. Scheme 2, shown here,

(i) \(\text{N-UDP} + A - \text{UDP} \rightarrow A - \text{N-UDP} + \text{UDP}\)

(ii) \(\text{A-N-UDP} + A - \text{UDP} \rightarrow A - \text{A-N-UDP} + \text{UDP}\)

(iii) \(\text{A-N-A-UDP} + N - \text{UDP} \rightarrow A - \text{A-N-A-UDP} + \text{UDP}\)

**Scheme 2**

illustrates the individual steps in this reaction mechanism with GlcUA and GlcNAc indicated by A and N, respectively, and the UDP groups for these sugars indicated, respectively, by italic or boldface font.

Because a key feature of synthesis at the reducing end is the rapid turnover of the UDP groups on growing HA chains, we sought to demonstrate this feature for class I HASs. Using UDP\(^{32}\text{P}\)-GlcNAc and limiting substrate concentrations, we established conditions in which seHAS makes a very large number of shorter HA chains rather than fewer longer chains (Fig. 2). These conditions favor detection of seHAS products that are end-labeled with \(^{32}\text{P}\). After descending paper chromatography, UDP\(^{32}\text{P}\)-GlcNAc and a variety of smaller \(^{32}\text{P}\)-labeled breakdowns (such as UDP\(^{32}\text{P}\), UMP\(^{32}\text{P}\), \(^{32}\text{P}\)phosphate and \(^{32}\text{P}\)pyrophosphate) migrated in a broad region \(17–27\) cm from the origin (Fig. 2A). In the presence of purified seHAS most of the \(^{32}\text{P}\) products were found at the origin, although this varied from experiment to experiment, but some products also migrated as a broad peak between \(7\) and \(13\) cm. However, in the absence of seHAS, essentially background radioactivity was detected between the origin and the large peak starting at \(17\) cm (Fig. 2C). When samples were treated with snake venom phosphodiesterase or hyaluronidase prior to chromatography, the radioactivity at the origin and in the \(7–13\) cm region was substantially reduced, close to that of the no-HAS controls (Fig. 2B and D). In multiple experiments, the amount of larger \(^{32}\text{P}\) products remaining at the origin after chromatography was reduced by \(80\%\) after treatment with either hyaluronidase or phosphodiesterase (Fig. 2D), supporting the conclusion that these products are UDP\(^{32}\text{P}\)-HA oligomers. As chromatography references, we used reduced HA-alditol oligomers containing 4 or 8 sugars; these migrated, respectively, at 10–15 cm and 0–2 cm (Fig. 2B).

In a separate experiment (Fig. 3), similar samples were incubated after the labeling period and prior to chromatography with unlabeled substrates. The “chase” with UDP-sugars eliminated almost all of the \(^{32}\text{P}\) products. The results are consistent with the conclusion that seHAS synthesizes HA saccharides that are still linked to UDP and that the HA-\(^{32}\text{P}\)/UDP

![Fig. 1. Degradation of pulse-labeled HA chains. Purified spHAS (A and B) or seHAS (C and D) were first incubated with nonradioactive UDP-sugars for 1.5 min and then washed and radiolabeled with UDP-\(^{14}\text{C}\)GlcUA or UDP-\(^{3}\text{H}\)GlcNAc (B and D) for the second 1.5-min period (open symbols). Other samples were first incubated with UDP-\(^{14}\text{C}\)GlcUA or UDP-\(^{3}\text{H}\)GlcNAc for 1.5 min and then washed and incubated with nonradioactive UDP-sugars for the second 1.5 min period (filled symbols). The radiolabeled HA samples were then collected, treated with \(\beta\)-N-acetylglucosaminidase and \(\beta\)-glucuronidase for the indicated times, and the amount of radiolabeled HA remaining was determined by paper chromatography. All data were compared with a control (set at 100%), which was the amount of \(^3\text{H}\) or \(^{14}\text{C}\)-radioactivity recovered in untreated HA samples.](image-url)
linkage is dynamic. The experiment in Fig. 3 also shows that, at a fixed UDP-sugar concentration, the amount of $^{32}$P products first increases and then decreases as the seHAS concentration is increased. A maximum occurred at $\frac{1}{H1011} \times 0.4 M$ enzyme; above and below this value $^{32}$P incorporation decreased by $\frac{1}{H1102} \times 90\%$ to near control levels. This biphasic behavior is expected because, as the enzyme concentration decreases, fewer but longer end-labeled HA chains are made (i.e. the maximum number of HA chains is equal to the number of seHAS molecules). As the enzyme concentration increases, shorter and shorter oligosaccharides are made until a point is reached at which, theoretically, only disaccharides or no products can be made.

To confirm that seHAS synthesizes HA-$^{32}$P, we also developed an HA capture assay using streptavidin-coated wells loaded with biotin-HABP. If the $^{32}$P products made by seHAS are HA oligosaccharides, then they should be bound by this highly specific HABP, which is purified from bovine cartilage (20). In particular, most of the larger HA saccharides remaining at the origin are likely longer than a dodecamer, which is the minimum size needed to occupy the HABP binding site with high affinity (21). These larger HA products are preferentially represented in this assay, because only a few percent of the total radiolabeled products is captured by the biotin-HABP. When increasing volumes of seHAS reaction mix were incubated per well, the amount of bound $^{32}$P progressively increased 4–5-fold (Fig. 4). However, when the streptavidin-coated wells were treated with either free biotin or unlabeled HA during the initial incubation with biotin-HABP, the amount of bound $^{32}$P was decreased by 92 and 88%, respectively. These controls demonstrate that the capture of HA-$^{32}$P in this assay is specifically mediated by the biotin-HABP. Consistent with the conclusion that the $^{32}$P products are HA-UDP, virtually all of the captured radioactivity was released by hyaluronidase treatment.

![FIG. 2. SeHAS synthesizes HA containing $^{32}$P-phosphate.](image)

**Panel A**, replicate samples of purified seHAS were incubated as described under “Experimental Procedures” with 25 $\mu$M UDP-GlcUA and 25 $\mu$M UDP-$^{32}$P-GlcNAc for 1.5 min or 1 h (○) at room temperature. The reactions were stopped by the addition of 1 mM UDP, and 1.5-min reaction samples were then treated for 2 h with nothing (●), hyaluronidase (□), or snake venom phosphodiesterase (■). The samples were then subjected to paper chromatography, strips were cut into 1-cm pieces, and radioactivity was determined.

**Panel B**, a blowup of the region from 0–16 cm shown in panel A. The migration positions of standard HA oligosaccharide alditols of four or eight sugars are indicated by lines at the top. Panel C, an independent experiment was performed as in panel A with a no seHAS control (▲), and treatment after the reaction was done with nothing (●), hyaluronidase (□), or snake venom phosphodiesterase (■). Note that the amount of $^{32}$P-labeled products remaining at the origin was much greater in this second experiment.

**Panel D**, the degradation of the $^{32}$P-labeled products remaining at the origin by treatment with hyaluronidase (HAase) or phosphodiesterase (PDase) is summarized. The values are the mean ± S.D. ($n = 5$) expressed as a percent relative to untreated samples (100%).

![FIG. 3. Effect of enzyme concentration on the synthesis of HA-$^{32}$P/UDP by seHAS.](image)

Purified seHAS (0.1–4 $\mu$g) was incubated for 1.5 min at 25 °C in 50 $\mu$L of HAS assay buffer as described under “Experimental Procedures” with 25 $\mu$M UDP-GlcUA and 5 $\mu$M UDP-$^{32}$P-GlcNAc. Incorporation of $^{32}$P into HA was measured by paper chromatography (●). The minus-seHAS background control (~700 cpm) was subtracted. Parallel samples of seHAS (1 $\mu$g) were incubated with either 1.0 mM of each unlabeled UDP-sugar (■) or with $10 \mu$g of snake venom phosphodiesterase (▲).
The biochemical reactions involved in glycoside bond formation were first described these differences for hyaluronic acid in 1967. (19). Robbins et al. (19) may have been due to other glycosyltransferases in the crude membrane preparations used, whose products may have confounded the analysis. There are at least two possible explanations for the report (12) that a recombinant HAS2 fragment, expressed in E. coli, was able to synthesize short oligosaccharides by addition to the nonreducing end. First, because only indirect evidence was obtained in the latter study, the authors did not rule out that another cellular glycosyltransferase, whose specificity was altered by expression of the HAS fragment, was responsible for the observed synthesis. Direct evidence would have been provided if a purified HAS fragment was shown to possess this activity. However, if the results are valid, they indicate a second possibility, i.e. that the normal mode of synthesis by intact HAS may be dramatically altered by elimination of multiple protein domains and disruption of the protein’s normal topological organization (3, 23). Further studies will be needed to determine whether this intriguing latter possibility is correct.

The mechanisms for polysaccharide biosynthesis are fundamentally different depending on whether the chain grows from the reducing or the nonreducing end (19, 24). Robbins et al. (19) first described these differences for hyaluronic acid in 1967. The biochemical reactions involved in glycoside bond formation determine the nature of donor and acceptor relationships among the substrates. For the class II pmHAS (25), UDP is released from a precursor UDP-sugar (which is the donor) when this sugar is added to the nonreducing end of an HA.

Finally, treatment of the seHAS/UDP-[32P]-GlcNAc reaction mixes with unlabeled UDP-sugars, hyaluronidase, or phosphodiesterase decreased the [32P] radioactivity captured by the biotin-HABP by ~90% (Fig. 5, black bars). Reaction mixes using the class II pmHAS and UDP-[32P]-GlcNAc also produced HA-[32P]UDP products that were captured by the biotin-HABP-coated well assay (Fig. 5, gray bars), as indicated by ~90% decreases in bound [32P]-radioactivity after hyaluronidase or phosphodiesterase treatment. Unlike the results with seHAS, however, the UDP-sugar chase did not decrease the amount of HA-[32P]UDP recovered from pmHAS reactions.

**DISCUSSION**

With the exception of mouse HAS1 (22), the mammalian HASs have been very difficult to solubilize and purify. In contrast, we have readily been able to purify large amounts of the recombinant streptococcal HASs (14, 17). Consequently, the streptococcal HASs have been an excellent experimental model in which to address the molecular details of how the class I HAS enzymes function (7). To determine the direction of synthesis by purified seHAS and spHAS, we pulse-labeled HA either at the beginning or at the end of chains during one round of chain synthesis. We then quantified the rate of radioactivity released from the labeled HA by β-glucuronidase and β-N-acetylgalcosaminidase, both of which act only at the nonreducing end. The results showed that the first sugars added during HA biosynthesis were preferentially removed by the later glycosidase treatment, i.e. the first sugars added become closer to the nonreducing end as HA chains elongate. Thus, both of the purified class I enzymes extend HA chains by addition to the reducing end and, therefore, the direction of HA chain growth is from the nonreducing to the reducing end of the polysaccharide.

Our second strategy to confirm that these class I HASs catalyze HA chain growth at the reducing end used purified seHAS and UDP-GlcNAc, with [32P] in the β position, to radiolabel nascent HA chains. The results showed that purified seHAS synthesizes HA with a UDP group remaining at the reducing end of the growing HA chain. HA-[32P]UDP products, made using a high ratio of enzyme-to-substrate, could be separated from the substrates by paper chromatography and were destroyed by treatment with hyaluronidase or phosphodiesterase. Importantly, the [32P] could also be removed by “chasing” the enzyme reaction mixtures with excess unlabeled UDP-sugars. These results demonstrate that the [32P]UDP group is present at the reducing end and turns over when a new UDP-sugar is added. For the class II pmHAS, because the sugar addition is at the nonreducing end, a chase had no effect, and the [32P]UDP was not removed from the HA chain.

Our results confirm the earlier studies by Prehm (10) and Asplund et al. (11) and demonstrate that class I HASs can elongate at the reducing end. The conflicting results of Stoolmiller and Dorfman (9) may have been due to other glycosyltransferases in the crude membrane preparations used, whose products may have confounded the analysis. There are at least two possible explanations for the report (12) that a recombinant HAS2 fragment, expressed in E. coli, was able to synthesize short oligosaccharides by addition to the nonreducing end. First, because only indirect evidence was obtained in the latter study, the authors did not rule out that another cellular glycosyltransferase, whose specificity was altered by expression of the HAS fragment, was responsible for the observed synthesis. Direct evidence would have been provided if a purified HAS fragment was shown to possess this activity. However, if the results are valid, they indicate a second possibility, i.e. that the normal mode of synthesis by intact HAS may be dramatically altered by elimination of multiple protein domains and disruption of the protein’s normal topological organization (3, 23). Further studies will be needed to determine whether this intriguing latter possibility is correct.
polymer (which is the acceptor). Therefore, when one disaccharide unit is added, the two UDP groups that are released come from the two new sugars added, and the HA-UDP linkage is not involved. The chase experiment (Fig. 5) confirms that the HA-UDP made by pmHAS does not turn over during HA synthesis. Our results also show for the first time that both seHAS and pmHAS can initiate HA synthesis by performing the reaction labeled (i) in Scheme 2 to make the first disaccharide, GlcUA-GlcNAc-UDP. It remains to be determined whether pmHAS or seHAS can also synthesize the alternative first disaccharide, GlcNAC-GlcUA-UDP.

Because pmHAS elongates at the nonreducing end, the disaccharide-UDP it creates is stable. The situation, however, is very different for chain elongation at the reducing end, because the seHAS cleaves this disaccharide-UDP linkage when the third sugar is added as in the reaction labeled (ii) in Scheme 2. During chain elongation at the reducing end the UDP-sugars are not the donors, but rather they are the acceptors (3, 19, 24). The donors are the hyaluronyl chains, which contain either GlcNAc or GlcUA at the reducing end and are activated by their attachment to UDP. The new HA-UDP product becomes the donor in the next transferase reaction. Therefore, a class I HA synthase transferase activity that utilizes UDP-GlcNAc actually creates the GlcUA(1,3)GlcNAc linkage. In contrast the class II pmHAS activity that utilizes UDP-GlcNAc creates the GlcNAc(1,4)GlcUA linkage (25). In each cycle of monosaccharide addition at the reducing end, the released UDP is derived from the previously added monosaccharide, and the growing HA chain is always attached to UDP, which is derived from the last sugar added. Unlike the class II pmHAS, an HA chain cannot be extended further by a class I Streptococcus HAS without the UDP present at the reducing end.

To synthesize HA, these membrane-bound class I HASs must perform the following multiple functions (7, 8): 1) binding of acceptor UDP-GlcNAc; 2) binding of acceptor UDP-GlcUA; 3) binding of donor HA-GlcUA-UDP; 4) binding of donor HA-GlcNAc-UDP; 5) HA-GlcUA-UDP:UDP-GlcNAc, β1,3(HA)-GlcUA transferase activity; 6) HA-GlcNAc-UDP:UDP-GlcUA, β1,4(HA)-GlcNAc transferase activity; and 7) translocation of HA through the protein and the cell membrane. The glycosyltransferase names associated with functions 5 and 6 follow the guidelines for naming transferases (i.e. donor-acceptor, group transfer). Thus, the activity that adds a GlcUA residue to a GlcNAc at the reducing end of the growing HA chain is a (HA)-GlcNAc-UDP:UDP-GlucUA, β1,4-hyaluronyltransferase. Similarly, a (HA)-GlcUA-UDP:UDP-GlcNAc, β1,3-hyaluronyltransferase is the activity that adds a GlcNAc to a HA-GlcUA-UDP chain. These two glycosyltransferase activities combine a donor HA-UDP and an acceptor UDP-sugar to add sugars continually and release UDP that was formerly linked to HA.

Other polysaccharides assembled by addition to the reducing end are xanthan (26) and probably succinoglycan (27), although the activated precursors in these cases are oligosaccharide-P-P-polyprenols. These polysaccharides are elongated by transfer of the growing polymer-P-P-polyprenol to a new pentasaccharide-P-P-polyprenol unit (26). In contrast, most other polysaccharides (e.g. glycogen, starch, xyloolitran, chondroitin, heparin, and other glycosaminoglycans) are elongated by addition at the nonreducing end. The cellulose synthase from Cladophora is also reported to elongate cellulose by addition to the nonreducing end (28). However, many different cellulose synthases occur in many species, so it is too early to conclude that they all act by addition to the nonreducing end. The type 3 capsular polysaccharide syntheses of Streptococcus pneumoniae also elongates at the nonreducing end (29).

Based on hydrophobic cluster analysis (30), the known glycosyltransferases have been classified into ~60 enzyme families (31) (afmb.cnrs-mrs.fr/CAZY/). The hypothesis in this effort was that there would be a high degree of structural and functional conservation among family members. Presently, all the HA, cellulose, and chitin synthases, as well as the glycosyltransferases that transfer a single sugar, are members of family 2. These family members catalyze an inverting mechanism, which makes them β-glycosyltransferases, although they share only a few small amino acid motifs involved in the sugar addition reactions. Many family members, such as the HA and cellulose synthases, show no significant homology and will likely not have identical structure-function relationships or mechanisms of catalysis. Although this classification system has been useful, some users assume that family members must share a common mechanism for synthesis, which has not been broadly tested. The finding that the directions of synthesis for the class I and class II HASs are different indicates that the assumptions about family groupings in this classification system should be made with caution.

The requirement of HA-UDP as the donor provides a possible mechanism to explain chain termination during the biosynthesis of large HA chains by class I HASs, because random hydrolysis of the HA-UDP linkage and generation of a free reducing end would stop further sugar addition. If this occurs, HAS might more readily release the free HA chain, thus freeing up the enzyme to initiate a new HA chain. Also, the probability that hydrolysis of a growing HA-UDP chain will occur increases with increasing chain length (i.e. the increasing length of time the UDP linkage exists for the growing chain). If loss of the -UDP group is not a significant mechanism regulating chain release, then the released HA products will have UDP attached at the reducing end, from the last sugar unit added. Because both types of HA-UDP linkage are less stable (as the α-ankomers) under physiological conditions than either type of β-glycoside bond in HA, the UDP will be susceptible to hydrolysis, even at near neutral pH. Therefore, commercial HA that has been processed in various ways will probably not contain UDP at the reducing ends. In addition, it is very intriguing to consider that the presence of a novel HA-UDP structural element at the reducing end of newly released HA chains could provide a specific recognition group for a class of binding proteins or enzymes designed to recognize this linkage, e.g. a hyaluronyl transferase that could covalently attach the activated HA chain to another molecule. Such novel intracellular or extracellular interactions or hyaluronyl modifications could be very important physiologically and will be investigated in future studies.

Finally, elongation of HA at the reducing end provided a rationale for proposing a mechanism to explain how the integral membrane streptococcal HAS proteins could simultaneously couple biosynthesis with the translocation of growing polysaccharide chains through the protein and the membrane to the cell exterior. This “pendulum hypothesis” for polysaccharide synthesis was described briefly in a preliminary report (32) with an animation illustrating the hypothesis and will be presented in detail elsewhere. In this model we propose that the streptococcal class I HASs have two functional domains that act as “arms”; each arm contains a binding site for one of the UDP-sugars, an active site for one of the hyaluronyltransferase functions, and a binding site for the donor HA-UDP. Each arm can “swing” to different positions in which its transferase is either inactive or active. Only one arm can be active at a time, and the activities are reciprocal so that when one arm is active as a transferase the other is binding the UDP-sugar acceptor. As HA is assembled, the alternating arm movement would...
drive extrusion of the bound growing HA chain through the protein and across the membrane.

Note Added in Proof—The results presented here were reported in preliminary form by Tlapak-Simmons et al. (33). A study by Bodevin-Authelet et al. (34), which was processed by the Journal in parallel with the present report, supports the conclusion that HA synthesis by the membrane-bound streptococcal spHAS occurs at the reducing end. Interestingly, the authors found that elongation by Xenopus laevis HAS1 in yeast membranes is at the nonreducing end. Because X. laevis HAS1 is the least conserved member of the vertebrate HAS family, however, other class I HA synthases may elongate at the reducing end.

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