Role of the Carbohydrate Binding Site of the \textit{Streptococcus pneumoniae} Capsular Polysaccharide Type 3 Synthase in the Transition from Oligosaccharide to Polysaccharide Synthesis*

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The type 3 synthase catalyzes the formation of the \textit{Streptococcus pneumoniae} type 3 capsular polysaccharide [\(\beta^{-3})\-\beta\-\text{D-GlcUA-(1,4)}\-\beta\-\text{D-Glc-(1-)}\]4. Synthesis is comprised of two distinct catalytic phases separated by a transition step whereby an oligosaccharyl-phosphatidylglycerol primer becomes tightly bound to the carbohydrate acceptor recognition site of the synthase. Using the recombinant synthase in \textit{Escherichia coli} membranes, we determined that a critical oligosaccharide length of \(\sim 8\) monosaccharides was required for recognition of the growing chain by the synthase. Upon binding of the oligosaccharide-lipid to the carbohydrate recognition site, the polymerization reaction entered a highly processive phase to produce polymer of high molecular weight. The initial oligosaccharide-synthetic phase also appeared to be processive, the duration of which was enhanced by the concentration of UDP-GlcUA and diminished by an increase in temperature. The overall reaction approached a steady state equilibrium between the polymer- and oligosaccharide-forming phases that was shifted toward the former by higher UDP-GlcUA levels or lower temperatures and toward the latter by lower concentrations of UDP-GlcUA or higher temperatures. The transition step between the two enzymatic phases demonstrated cooperative kinetics, which is predicted to reflect a possible reorientation of the oligosaccharide-lipid in conjunction with the formation of a tight binding complex.

The \textit{Streptococcus pneumoniae} type 3 capsule is a high molecular weight polysaccharide that grows at the nonreducing end by the alternative addition of Glc and GlcUA (1). Its synthesis is mediated by the type 3 synthase, which is a processive glycosyltransferase in the GT-2 family that includes other members that synthesize cellulose, hyaluronan, chitin, and related \(\beta\)-glycans (2–4). Similar to other members of this class, the type 3 synthase contains a carbohydrate recognition site that binds the growing polysaccharide and is an integral part of the complex catalytic mechanism (5). In both \textit{S. pneumoniae} type 3 and \textit{Escherichia coli} expressing the recombinant novel synthase, a novel lipid determined to be glucosylphosphatidylglycerol accumulates in the membranes and serves as a primer for polysaccharide synthesis (6). The first reaction catalyzed by the type 3 synthase is the transfer of Glc from UDP-Glc to phosphatidylglycerol. Glucosylphosphatidylglycerol is present at low concentrations in synthase-containing cells and serves as an acceptor for GlcUA from UDP-GlcUA to form the disaccharide lipid, glucuronyl-glucosyl-phosphatidylglycerol. When both UDP-sugars are present at very low concentrations, the synthase catalyzes a short lived processive reaction that results in the formation of a glycolipid fraction consisting of a mixture of short oligosaccharides (7). As the concentrations of the UDP-sugars increase, the reaction abruptly becomes highly processive, and the products are rapidly extended into polymers of dramatically increased chain length. Presumably, a critical step in the initiation and elongation of high molecular weight polymer is the binding of the oligosaccharide-lipid to the carbohydrate acceptor recognition site of the synthase.

Although much is known about carbohydrate acceptor recognition sequences among glycoside hydrolases, there is very little information on this aspect of catalysis by most polysaccharide biosynthetic systems. Several hundred glycoside hydrolases have been grouped into over 40 different families based on the sequences of their carbohydrate binding domains (8). In general, these binding domains are noncatalytic and serve to bring the biocatalytic domains into close proximity with their polysaccharide substrates (9). In contrast, structural studies of glycosyltransferases have not revealed significant sequence motifs that would provide for an interaction with carbohydrate acceptors. Based on hydrophobic cluster analysis, sequence homology, and x-ray crystallographic data, a fairly detailed picture is emerging of the binding and catalytic interaction of some glucosyltransferases with their nucleotide diphosphate sugar donor (10–12). This information has led to the development of a rather strong argument for the existence of only a single nucleotide-sugar binding site in polymersases, such as type 3 synthase, that require two distinct nucleotide sugar donors (11).

The polymerization reaction catalyzed by many processive glycosyltransferases is thought to involve a cyclical translocation process wherein the polymer chain detaches, slides forward, and then reattaches to the enzyme to allow another round of sugar additions. Enzymes similar to the type 3 synthase have been postulated to undergo a translocation step after the addition of every two sugars to avoid the necessity of rotating either the polymer or the synthase (2, 13). Previous work from this laboratory has shown that polysaccharide chains are ejected from the type 3 synthase when one UDP-sugar is present at high concentration and the other is limiting or absent, suggesting that an abortive translocation process might serve to terminate chain growth (5). The current investigation indicates that the carbohydrate recognition site may form a complex with an approximate eight-sugar segment of the growing polysaccharide chain, and that this interaction may provide for the high degree of processivity of chain assembly as well as for controlling the ultimate size of the polymer.

**EXPERIMENTAL PROCEDURES**

**Materials**—Econo-safe scintillation mixture was obtained from Research Products International Corp. and Biogel P-4 was from Bio-Rad Laboratories. Lysozyme, UDP-Glc, UDP-GlcUA, and \textit{Streptomyces
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chromofuscus phospholipase D (PLD) were from Sigma. Chromatography paper (3MM Chr) and DEAE-cellulose (DE-52) were from Whatman. UDP-[14C]GlcUA (175 mCi/mmol) was purchased from ICN, and Nonidet P-40 was from Calbiochem. Todd Hewitt broth, yeast extract, and tryptone were from Difco.

Preparation of Type 3 Oligosaccharide Standards—[14C]GlcUA-labeled type 3 polymer was digested with Bacillus cereus type 3-specific depolymerase as previously described (5, 14, 15). The released oligosaccharides were fractionated and purified by DEAE-cellulose chromatography and gel filtration on Biogel P-4, as described below.

Growth Conditions and Membrane Preparation—Recombinant E. coli strain JD424 containing the type 3 synthase (Cps3S) has been described (16). The clone contains a 2.1-kb Sau3AI fragment from S. pneumoniae WU2 consisting of the 3’-end of cps3D and the complete cps3S. The growth of strain JD424, induction of synthase expression, and harvesting of the cells was performed as described previously (5). Spheroplasts and membranes were obtained following lysozyme digestion and sonication, as described previously except that leupeptin and pepstatin were omitted from the spheroplasting reaction (7).

Assay of Synthase Activity—Type 3 synthase activity was determined in 100-μl reaction mixtures consisting of 100 mM Hepes (pH 7.5), 10 mM MgCl2, 1 mM UDP-Glc, 0.01 μCi of UDP-[14C]GlcUA, and E. coli membranes (3–15 μg of protein). Standard reactions were incubated at 35 °C for 10 min and terminated by the addition of 5 μl of 12.5 mM acetic acid. The reaction components were separated by ascending paper chromatography on Whatman 3MM paper with a solvent mixture of butanol:acetic acid:water (44:16:40). The chromatograms were cut into 1-cm strips, and polymer (origin product) and oligo-lipid (migrating slightly faster than Glc) were quantified by liquid scintillation counting. Enzyme activity was determined by calculating the nmol of hexuronic acid incorporated per mg of protein. All experiments were repeated multiple times and/or under varying conditions. The results shown are representative of these experiments.

Preparation of Preloaded Synthase—The carbohydrate acceptor binding site of the type 3 synthase was preloaded with unlabeled growing polymer in a 3-ml reaction mixture consisting of E. coli membranes (2 mg of protein), 100 mM Hepes (pH 7.5), 10 mM MgCl2, 0.1 mM UDP-Glc, and 0.1 mM UDP-GlcUA. The reaction was incubated for 2 min at 25 °C and terminated by placing on ice. The membrane-polymer complex was collected by centrifugation at 10,000 g for 5 min. The precipitate was removed by centrifugation at 10,000 × g for 5 min.

Analytical Methods—The length of the type 3 polysaccharide was determined using the lipid anchor as a marker for the reducing end oligosaccharide fragment following hydrolysis of the polymer with type 3-specific depolymerase.3 The size of the residual hydrophobic oligosaccharide was determined, and polymer size was calculated based on the proportions of radioisotope in the hydrophilic and hydrophobic oligosaccharide fractions. Protein was determined as described with a Coomassie Blue dye using bovine serum albumin as the standard (17). Ion exchange chromatography was carried out on 0.6 × 2.5-cm columns of DEAE-cellulose (OH−) following removal of the lipid component from samples by PLD digestion. The columns were equilibrated with 5 ml of 20 mM ammonium acetate (pH 7.4) and washed with 3 volumes of H2O prior to application of the sample in 2 ml of H2O. The columns were washed with 2 ml of HzO, and the products were eluted by one of two ion exchange methods. Optimum oligosaccharide separation was achieved with a 40-ml linear gradient of 0–0.2 M ammonium acetate (pH 7.4) at a flow rate of 0.8 ml/min. Fractions (1.2 ml) were collected and monitored for radioactivity by liquid scintillation counting and for ion strength by conductivity. Oligosaccharide and polymer mixtures were fractionated with a 2-step gradient at a flow rate of 0.4 ml/min. The first step consisted of a 30-ml gradient of 0–0.3 M ammonium acetate (pH 7.4) and was followed by a second step of 0.3–1.5 M ammonium acetate (pH 7.4). Fractions (0.65 ml) were monitored as above.

Gel filtration chromatography was carried out on 1.5 × 88-cm columns of Biogel P-4 irrigated with a solution consisting of 0.2 M NaCl 0.1 M ammonium acetate (pH 7.4), 0.04% Nonidet P-40, and 0.02% sodium azide at a flow rate of 10 ml/h. Prior to application to the column, samples were digested with PLD as described above. Column fractions of 0.9 ml were collected and monitored for radioactivity.

RESULTS

Effect of UDP-sugar Concentrations and Temperature on the Progressivity of the Synthase Reaction—When equimolar concentrations of UDP-Glc and UDP-GlcUA are present in reactions with E. coli membranes containing type 3 synthase, a rapid transition from oligosaccharide-lipid to polymer synthesis occurs in the UDP-sugar concentration range of 1–10 μM (7). By maintaining the UDP-Glc at the more physiological concentration of 1000 μM (18) and varying the UDP-GlcUA concentration, the rate of formation of oligosaccharide-lipids was found to be hyperbolic, whereas the corresponding values of polymer formation generated a concave curve at low concentrations of UDP-GlcUA (Fig. 1A). As the temperature was increased from 40 to 54 °C, the concavity of the rate curve for polymer formation was strikingly accentuated (Fig. 1B). The synthase was stable at these temperatures, and the Vmax at 54 °C was greater than twice that at 35 °C (data not shown).

We previously suggested that these concave rate curves might be due to polysaccharide ejection from the carbohydrate acceptor binding site of the synthase (5), since the highest rates of ejection occur at low concentrations of UDP-GlcUA and high levels of UDP-Glc. However, a similar effect was observed when the concentration of UDP-Glc was reduced 10-fold (data not shown), and under these conditions, the rate of ejection would be greatly reduced. Likewise, ejection is relatively low under conditions of high UDP-GlcUA and low UDP-Glc (5), but temperature enhancement of concavity was also observed when UDP-GlcUA was maintained at 1 mM while varying the UDP-Glc concentration (Fig. 1C). We therefore further explored the basis for this sigmoid-like behavior.

At equimolar concentrations of the two UDP-sugars, polymer synthesis occurs subsequent to that of the oligosaccharide-lipid precursor, indicating a precursor-product relationship (7). A time lag in the onset

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of polymer formation was also apparent at 35 °C in reactions containing 1000 μM UDP-Glc and 1.5 μM UDP-GlcUA, whereas the oligosaccharide-lipid began to accumulate immediately (Fig. 2A). Even if this lag were taken into account, the concavity shown in Fig. 1A would be only marginally reduced. At 45 °C and at a UDP-Glc-UA concentration of 7.5 μM, which is well within the concave region of the 45 °C rate curve in Fig. 1B, there was only a slight lag in the initiation of polymer formation (Fig. 2B). Accentuation of the concave rate curves with increasing temperature as seen in Fig. 1B is therefore not explained by the time lag in polymer formation.

Effect of UDP-sugar Concentrations on Oligosaccharide-lipid Formation—When S. pneumoniae membranes are incubated with labeled UDP-Glc-UA in the absence of UDP-Glc, the reaction catalyzed by the type 3 synthase is completely nonprocessive and only a single glucuronosyl unit is transferred to the oligosaccharide-lipid. Digestion of this product with PLD yields a water-soluble product slightly larger than a disaccharide (presumably disaccharyl-glycerol) (6). Incubation of the recombinant E. coli membranes in the presence of labeled UDP-Glc-UA (1 μM) but in the absence of UDP-Glc similarly resulted in the addition of only a single glucuronosyl unit to the oligosaccharide-lipid. The PLD-digested product of this reaction eluted from DEAE-cellulose at the same ionic strength as a disaccharide Glc-β1,3-Glc-UA standard (Fig. 3A, left). Increasing the concentration of UDP-Glc to 0.3 μM resulted in two additional oligosaccharide components that eluted at positions suggesting charges similar to the di- and triuronosyl standards (Fig. 3B, left). When both UDP-sugars were present at 1 μM, the major oligosaccharide corresponded to the charge of the triuronosyl elution position, and an additional minor peak corresponding to the expected charge of a tetrauronosyl constituent was also visible (Fig. 3C, left). At 1 mM UDP-Glc, the triuronosyl oligosaccharide was the predominant constituent, and a small but significant tetrauronosyl oligosaccharide was again evident (Fig. 3D, left).

The single anionic product in reaction A (Fig. 3A, left) was resolved into two components by gel filtration on Biogel P-4 (Fig. 3A, right). Based on charge and size, the more rapidly migrating product appears to be glucuronosyl-glycerol, whereas the slower moving product appears to be consistent with Glc-UA-Glc-glycerol. The mono-, di-, and triuronosyl components shown in Fig. 3, B and C (right), eluted from the Biogel P-4 column slightly later than the respective type 3 di-, tetra-, and hexasaccharide standards (Glc-UA-Glc-UA), which were previously characterized (14). These positions are consistent with the retention of glycerol at their reducing termini following PLD hydrolysis of the respective oligosaccharide-lipids, suggestive of the homologous series (Glc-UA-Glc-UA), glycerol. The oligosaccharides represented by the peaks in the right panel of Fig. 3D were synthesized at a very high concentration of UDP-Glc, and they appear to be slightly larger than their respective homologs in Fig. 3, B and C, which were synthesized at a 1000-fold lower concentration of UDP-Glc. Presumably, the products formed at the high UDP-Glc concentration would have Glc at their nonreducing termini, resulting in the formation of the alternate series, Glc-UA-Glc-UA-glycerol. At the 1 μM UDP-Glc concentration, 5–10% of the incorporated label was in polymer, as demonstrated by gel filtration (Fig. 3C, right), indicating that a low percentage of the oligosaccharide-lipids had attained a size sufficient for recognition by the carbohydrate binding site and had been extended into a high molecular weight polysaccharide. At 1 mM UDP-Glc, ~20% of the incorporated label was in polymer (Fig. 3D, right).
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When the concentration of UDP-Glc was maintained at 1 mM and UDP-GlcUA was increased to 5 mM, there was a dramatic shift from oligo-lipid formation to polymer formation, with 82% of the incorporation occurring in polymer (data not shown). Coincident to this shift was an increase in polymer size from ~200 sugars at 1 mM UDP-GlcUA to ~4000 sugars at 5 mM UDP-GlcUA. The rapid transition to polymer formation was comparable to that observed at equimolar concentrations of the UDP-sugars (7). Particularly significant under these conditions, as seen in the left and right panels of Fig. 3D, are the very prominent size of the triuronosyl oligosaccharide, a very small amount of a tetraurinosyl oligosaccharide, and then a very large gap lacking any larger intermediate size components prior to high molecular weight polysaccharide. These data are consistent with the carbohydrate binding site of the type 3 synthase requiring an approximate octasaccharide (i.e. tetraurinosyl)-lipid primer for recognition and the ensuing highly processive polymer formation reaction.

Effect of Temperature on the Transition from Oligosaccharide-lipid to Polymer Formation—In reactions containing 2 mM UDP-GlcUA and 1 mM UDP-Glc, incubation at 25 °C resulted in ~50% of the incorporated label in polymer and 50% in oligosaccharides (Fig. 4A). At 45 °C, the formation of polymer was reduced to ~15% (Fig. 4B), and only negligible polymer was synthesized in the 54 °C reaction (Fig. 4C). There was also a marked effect on the size profile of the oligosaccharides, with a steady reduction in size occurring with increasing temperature. Thus, binding of the oligosaccharide-lipid at the carbohydrate recognition site and the cooperativity of the series of reactions preceding the recognition step are quite sensitive to changes in temperature.

Effect of Preformed Polysaccharide on the Rate of Polymer Formation—When the type 3 synthase contained preformed polysaccharide, the rate curves for polymer formation were no longer concave but were typically hyperbolic (compare Fig. 5 with Fig. 1B). These data clearly demonstrate that the cooperativity occurred either before or during the binding of the oligosaccharide-lipid primer to the carbohydrate binding site of the synthase. It should also be noted that polymer formation rates in Fig. 5 were greater at the higher temperature, as expected, and in contrast to Fig. 1B.

In reactions conducted with 2.5 mM UDP-GlcUA and 1 mM UDP-Glc, the activity with enzyme preloaded with polysaccharide was linear for only a short duration, particularly at the higher temperature (Fig. 6). These data are in agreement with previous observations (5) demonstrating that the rate of polymer ejection increases with increasing temperature and with decreasing UDP-GlcUA concentrations. These data also illustrate why the rate of polymer formation shown in Fig. 1B is lower at the higher temperature, which is contrary to the expected norm. When insufficient UDP-GlcUA is present to saturate the synthase, the percent of enzyme engaged in polymer synthesis decreases as the temperature increases. The high and low processive states of the synthase appear to exist in a reciprocal steady state, with polymer formation favored by higher UDP-GlcUA concentrations and lower temperature, although oligosaccharide-lipid formation is favored by lower nucleotide sugar levels and higher temperatures (see Fig. 8 and "Discussion"). In agreement with this, polymer formation with preloaded synthase was increasingly linear with time as the concentration of UDP-GlcUA was increased (data not shown). This is consistent with the decreased rate of ejection from S. pneumoniae membranes as the concentration of the conjugate UDP-sugar was increased (5). As would be expected, the initial rate of oligosaccharide-lipid formation by preloaded membranes was greatly reduced as compared with polymer formation (compare Fig. 7 with Fig. 2).

DISCUSSION

Carbohydrate acceptor binding sites are thought to play an important role in the biosynthesis of numerous polysaccharides, but very little data is available on this part of the assembly process. Type 3 synthase in S. pneumoniae membranes is tightly bound to preformed nascent
polysaccharide chains (5). Under most conditions the polysaccharide chain remains tightly associated with the enzyme, and virtually no dissociation occurs during various experimental manipulations, including centrifugation, freezing, and thawing, and incubation at room temperature. These isolated complexes are able to catalyze the addition of GlcUA to extend the length of the preformed chains, indicating that the nascent chain was rapidly extended by a highly processive reaction subsequent to binding.

We previously reported on the tendency of the type 3 synthase to generate sigmoid-like kinetics at low concentrations of the nucleotide sugar substrates, but also noted that the enhanced rate of chain ejection under these same conditions complicated further evaluation (5). The marked concavity observed here occurs in the absence of bound polysaccharide and is primarily related to an event(s) during the initial sequence of reactions leading to the binding of the polysaccharide-lipid primer to the carbohydrate recognition site of the synthase. Sigmoid kinetic behavior is also displayed by hyaluronan synthase in Streptococcus pyogenes (19), and indirect evidence of a possible conformational change in this enzyme in Streptococcus equisimilis has been presented (20). Based on these studies a "pendulum model" of chain elongation at the reducing end has been proposed in which two functional synthase domains would each contain a separate binding site for the two distinct UDP-sugars required for synthesis (21).

Another model has also been proposed to explain how a processive β-glycosyltransferase with only a single catalytic center could provide for the elaboration of a polysaccharide composed of alternating sugars (22). It was suggested that the last sugar residue added to the nonreducing end of the bound acceptor in the catalytic center might redefine the affinity of the site for the alternate nucleotide donor. We have previously noted that the type 3 synthase ejects any bound polysaccharide from the carbohydrate binding site when only one UDP-sugar is present, and this ejection is inhibited as the concentration of the second UDP-sugar increases (5). Although the available information concerning the complexity of these interactions is scant, the best evidence suggests that ejection of the polysaccharide occurs during an abortive attempt to translocate the chain. Of special interest were the ejection of the nascent chain remains tightly associated with the enzyme, and virtually no dissociation occurs during various experimental manipulations, including centrifugation, freezing, and thawing, and incubation at room temperature. These isolated complexes are able to catalyze the addition of Glc and GlcUA to extend the length of the preformed chains, indicating that the nascent chain was rapidly extended by a highly processive reaction subsequent to binding.

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Additional evidence that the primary catalytic barrier is situated at this point in the overall reaction sequence is provided in Fig. 4B, which shows that a moderate reduction in the level of the triuronosyl oligosaccharide due to increasing the temperature to 45 °C was accompanied by a dramatic reduction in polysaccharide synthesis. It would also appear that the heightened concavity in polymer formation observed at the higher temperatures reflected, at least in part, the cooperative action of an elevated nucleotide-sugar level required to counter the temperature-induced higher rate of dissociation of oligosaccharide-lipid from the synthase.

The polymer formation rate curves presented here represent a composite of a series of reactions and do not lend themselves to standard kinetic analysis. If the transition step could be studied in isolation from the hyperbolic reactions on either side, it can be speculated that purely sigmoidal kinetics would be observed that could be further analyzed. Based on the current knowledge of the biosynthesis of type 3 polysaccharide, the most probable explanation of these results may be a conformational change in the synthase. Because the type 3 synthase and related β-glycosyltransferases are known to simultaneously transport their polysaccharides during synthesis, the initiation of polymerization by the type 3 synthase may involve a reorientation of the growing oligosaccharide and possibly the transport of the lipid anchor from the inner to the outer membrane leaflet. Here, it serves as an anchor for the capsule following ejection of the completed chain from the enzyme catalytic site (6).

The transition event is also thought to lock the oligosaccharide-lipid in a tight binding complex with the synthase, whereupon the highly processive polymerization phase ensues. In theory, the addition of each sugar would now fine tune the affinity of the binding site for the appropriate nucleotide sugar to donate the succeeding glycosidic addition (22), providing for a stable alternating catalytic center enabling the repetitive addition of Glc and GlcUA to the growing polymer. Just how the synthase provides for the integrity of glycosidic addition in the initial reaction sequence to produce the oligosaccharide-lipid in Fig. 3A, right. This latter product appeared primarily in the absence of UDP-Glc and may not occur in vivo at greatly elevated levels of UDP-Glc. In the absence of a bound oligosaccharide substrate to act as a cofactor, the synthase demonstrates a decreased fidelity in recognizing the appropriate acceptor substrate.

It is significant that polymer formation rate curves were concave as a function of the concentrations of both UDP-Glc and UDP-GlcUA and that the concavity of both of these curves was thermally enhanced. This suggests a possible chain of protein-mediated catalytic events initiating at the nucleotide-sugar binding site and translated through the carbohydrate binding site to modulate binding, translocation, and ejection of the elongating polysaccharide chain, resulting in a steady state equilibrium among the various modes of synthase activity, as depicted in Fig. 8. The presence of high concentrations of UDP-GlcUA or low temperature favors a steady state with a high proportion of the synthase in the polymer-forming, highly processive state. At lower concentrations of UDP-GlcUA or higher temperatures, the steady state concentration of synthase in the polymer-forming mode is reduced because of an increased rate of ejection of polysaccharide from the carbohydrate binding site. In the latter case, there is also an increased rate of dissociation of the oligosaccharide-lipid from the synthase resulting in the accumulation of oligosaccharide-lipid consisting of mostly three or less repeating disaccharides. It is through this intricate sequence of interactions that slight fluctuations in the concentration of UDP-GlcUA are able to modulate the chain length of type 3 polymer over a large range. The size of the polymer is a function of both the rate of the polymerization reaction, and the length of time the growing chain remains bound to the synthase. Both of these parameters behave in a convex curvilinear manner with respect to the concentration of UDP-GlcUA, providing for a large increase in polymer size with only a minimal increase in UDP-GlcUA.3

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