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## **Biosynthesis of Hyaluronan**

### **Direction of Chain Elongation**

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## SYNOPSIS

The mechanism of hyaluronan biosynthesis in vertebrates had been proposed to occur at the reducing end of growing chains. This mechanism was questioned, because a recombinant synthase appeared to add new monosaccharides to the non-reducing end. I reinvestigated this problem with membranes from the eucaryotic B6 cell line. The membranes were incubated with UDP-[ $^3\text{H}$ ]GlcNAc and UDP-[ $^{14}\text{C}$ ]GlcA to yield differentially labelled reducing terminal and non-reducing terminal domains. Digestion of the product with a mixture of the exoglycosidases  $\beta$ -glucuronidase and  $\beta$ -*N*-acetylglucosaminidase truncated the hyaluronan chain strictly from the non-reducing end. The change in  $^3\text{H}/^{14}\text{C}$  ratio of the remaining hyaluronan fraction, during the course of exoglycosidase digestion, confirmed the original results that the native eucaryotic synthase extended hyaluronan at the reducing end. This mechanism demands that the UDP-hyaluronan terminus was bound to the active site within the synthase and should compete with the substrates for binding. Accordingly, increasing substrate concentrations enhanced hyaluronan release from the synthase. A model is proposed that explains the direction of chain elongation at the reducing end by the native synthase and at the non-reducing end by the recombinant synthase based on a loss of binding affinity of the synthase towards the growing UDP-hyaluronan chain.

## INTRODUCTION

Hyaluronan is synthesized, without any primer requirement, by alternate addition of D-glucuronic acid and *N*-acetyl-D-glucosamine units from the corresponding UDP-sugar donors (UDP-GlcA and UDP-GlcNAc) [1;2]. The growing chains are exported from streptococci and from eucaryotic cells by ABC transporters. Transport can be inhibited by inhibitors of multidrug resistance transporters [3-6]. Hyaluronan synthases have been tentatively classified based on primary structure, molecular size, predicted membrane topology, and enzymological characteristics [7]. Class I thus includes the streptococcal, the vertebrate and the viral enzymes, whereas Class II contains a single member, the *Pasteurella multocida* enzyme.

In 1983 I proposed that hyaluronan chains are elongated at the reducing end, because the digestion kinetics of pulse chase labelled chains with exoglycosidases immediately reduced the incorporated radioactivity that was introduced into the chains during the first pulse reaction [2]. These experiments were confirmed by Asplund et al. [8] with a human glioma cell line, and by Tlapak-Simmons et al. [9] with streptococci. Both of these native enzyme systems failed to further extend preformed, exogenously added hyaluronan oligosaccharides. In contrast, the structurally unrelated synthase from *Pasteurella multocida* synthesizes hyaluronan at the non-reducing end [10]. Uncertainty about the direction of hyaluronan synthesis in eucaryotic cells arose, because a recombinant hyaluronan synthase from *Xenopus laevis* expressed in yeast added new sugar residues to the non-reducing end [11]. In addition, a recombinant synthase was able to elongate hyaluronan oligosaccharides and this should not occur, if synthesis proceeded at the reducing end [12]. Because of these contradictory results, I decided to reinvestigate the directionality of hyaluronan chain elongation catalysed by native eucaryotic hyaluronan synthase applying the double labelling method of Bodevin-Authelet et al. [11]. The results confirmed that the native eucaryotic synthase added new sugars at the reducing end.

The results also shed light on the factors that determine chain length. I previously demonstrated that hyaluronan is liberated from the synthase by dissociation [13]. If nascent hyaluronan is degraded, the synthase elongates it to a size that is determined by thermodynamic factors [14;15]. It is an unexplained observation that the three eucaryotic hyaluronan synthases produce hyaluronan with different chain lengths [16]. My results

suggest that other determinants for chain length are the binding affinity of the synthase to the reducing hyaluronan chain terminus, and the concentrations of the nucleotide sugar precursors that compete with the growing hyaluronan chain for the active site in the enzyme.

## **EXPERIMENTAL**

### **Materials**

Radiolabelled UDP-[<sup>14</sup>C]GlcA (specific activity 0.196 Ci/mMole) was obtained from Amersham Biosciences and UDP-[<sup>3</sup>H]GlcNAc (specific activity 38.5 Ci/mMole) from PerkinElmer Life Sciences. For preparation of the substrate solution, 50 µCi of UDP-[<sup>3</sup>H]GlcNAc was mixed with 132 µg of unlabelled UDP-GlcNAc and diluted to 20 ml for the substrate solution to obtain a final concentration of 10 µM and a specific activity of 0.1 Ci/mMole. Additional chemicals were from Sigma-Aldrich Chemical Corporation.

### **Isolation of plasma membranes**

B6 cells, a hybrid cell line derived from mouse mammary carcinoma and a Chinese hamster lung cell line which produced large amounts of hyaluronan [17;18] were grown in suspension culture in Dulbecco's modified Eagles medium supplemented with streptomycin/penicillin (100 units of each/ml), kanamycin (100 units/ml) and 10 % foetal calf serum. A suspension culture was grown to a density of  $2 \times 10^5$ /ml in 4 culture flasks (180 cm<sup>2</sup> surface area). Four hours before cell harvest, hyaluronan synthesis was stimulated by supplementation of additional foetal calf serum (5 %). The cells were sedimented at 1500 g for 5 min, washed with phosphate buffered saline (PBS) and suspended in 30 ml of ice-cold PBS [3]. The cells were transferred into a Parr-bomb, exposed to a nitrogen pressure of 900 psi for 15 min and disrupted by nitrogen cavitation [19]. Nuclei were sedimented at 2000 g for 10 min and the particulate fraction was obtained by centrifugation at 20.000 g for 20 min.

### **Hyaluronan Synthesis and Isolation**

For synthesis of [<sup>3</sup>H]hyaluronan, the membrane fraction was suspended in 1 ml of prewarmed substrate for hyaluronan synthesis that contained 8 µM UDP-GlcA, 10 µM UDP-[<sup>3</sup>H]GlcNAc, 4 mM dithiothreitol, 20 mM MgCl<sub>2</sub> in 50 mM TRIS-malonate pH 7.0 and incubated again at 37°C for 30 min. The membranes were sedimented by centrifugation for 5 min at 14.000 g. An aliquot (1/3) was solubilized in 1 % SDS and heating to 90°C for 5 min. Undissolved material was sedimented by centrifugation for 5 min at 14.000 g and the supernatant was applied to gel filtration on Sephacryl S-1000 (1.4x 64 cm) with 10 mM NH<sub>4</sub>HCO<sub>3</sub> as eluant at a flow rate of 14 ml/h. Fractions of 2 ml were collected and the radioactivity of 100 µl was determined.

For synthesis of dual labelled hyaluronan, the rest of the membrane fraction (2/3) was sedimented and suspended in 1 ml of prewarmed substrate for hyaluronan synthesis that contained 8  $\mu$ M UDP- $^{14}$ C]GlcA, 150  $\mu$ M UDP-GlcNAc, 4 mM dithiothreitol, 20 mM  $\text{MgCl}_2$  in 50 mM TRIS-malonate pH 7.0 and incubated at 37°C for 15 min. The membrane fraction was again sedimented, solubilized in SDS and applied to gel filtration on Sephacryl S-1000 as described above.

### **Enzymatic Degradation of Hyaluronan**

The dual labelled hyaluronan fractions from the Sephacryl S-1000 column (fractions 25 to 35) were collected, lyophilized to dryness and dissolved in 4 ml of 0.1 M sodium acetate pH 5.2. The enzymes  $\beta$ -N-acetylglucosaminidase (0.8 U) and  $\beta$ -glucuronidase (10000 U) were added and incubated in four aliquots in dialysis bags at 37°C and simultaneously dialyzed against 5 l of 10 mM sodium acetate pH 5.2. After different time periods, the radioactivity of the remaining [ $^3\text{H}/^{14}\text{C}$ ]hyaluronan in the dialysis bags was determined.

## RESULTS

### Synthesis of dual labelled hyaluronan

The eukaryotic hyaluronan synthase has a rather high  $K_M$  value for UDP-GlcNAc (about 60  $\mu\text{M}$ ) and thus high substrate concentrations are required to obtain sufficient chain elongation. On the other hand, large amounts of UDP- $^3\text{H}$ GlcNAc with high specific radioactivity impede the purification of  $^3\text{H}/^{14}\text{C}$ hyaluronan. Therefore a series of preliminary experiments were performed to evaluate the optimal experimental conditions to produce dual labelled hyaluronan chains by incubation of membranes from the eukaryotic B6 cell line with different substrate concentrations, specific radioactivities, and synthesis periods. A compromise was found that coped with both a sufficient chain elongation rate and a measurable  $^3\text{H}$ hyaluronan radioactivity. Hyaluronan was first labelled by incubation of membranes with UDP- $^3\text{H}$ GlcNAc at a concentration of 10  $\mu\text{M}$  and unlabelled 8  $\mu\text{M}$  UDP-GlcA for 30 min. The  $^3\text{H}$ hyaluronan was analysed by gel filtration using Sephacryl S-1000. Fig. 1a shows that most of the  $^3\text{H}$ hyaluronan was included in the middle of the column eluate. In a second experiment, the membranes were incubated with a substrate solution containing 8  $\mu\text{M}$  UDP- $^{14}\text{C}$ GlcA and unlabelled 150  $\mu\text{M}$  UDP-GlcNAc for 15 min and subjected again to gelfiltration. Fig. 1b shows that this labelling condition yielded a size of hyaluronan that was also included in the column eluate. In the third experiment, the two incubation conditions were utilized sequentially and the size of  $^3\text{H}/^{14}\text{C}$ hyaluronan was again analysed by gelfiltration. Fig. 1c shows that  $^3\text{H}$ hyaluronan was further elongated by  $^{14}\text{C}$ hyaluronan. For the subsequent exoglycosidase digestion, the  $^3\text{H}/^{14}\text{C}$ hyaluronan containing fractions were isolated.

### Exoglycosidase digestion of dual labelled hyaluronan

The dual labelled hyaluronan of high molecular weight was subjected to graded exoglycosidase ( $\beta$ -N-acetylglucosaminidase and  $\beta$ -glucuronidase) digestion as described in the Methods section. The digestion was performed with simultaneous dialysis. Aliquots in the dialysis bags were withdrawn at various time periods to determine the residual radioactivity. The results are shown in Table 1. The radioactivity of the dual labelled hyaluronan decreased continuously. The ratio of  $^3\text{H}/^{14}\text{C}$  decreased during digestion. This result was verified by two independent experiments. It indicated that the  $^3\text{H}$ hyaluronan introduced first into the growing chain was digested faster. Consequently, most of the hyaluronan chains must have



been elongated at the reducing end.

### **Effect of substrate concentration on hyaluronan release from the synthase**

If the terminal UDP-hyaluronan is bound to the synthase during elongation at the sites where the substrates also bind, the concentration of the substrates should influence not only the rate of elongation, but also the rate of chain release. Therefore membranes from B6 cells were incubated with different concentrations of substrates and the release of hyaluronan from the membranes into the soluble fractions was determined. Fig. 2a shows that hyaluronan was first incorporated into the membrane fraction and then released. The proportion of soluble hyaluronan increased with the substrate concentrations (Fig. 2b). Similar results were obtained, when only the concentration of substrate was altered (data not shown). Thus the substrates appeared to compete with UDP-hyaluronan for binding to the synthase.

## DISCUSSION

It would be desirable to obtain a homogeneous preparation of double labelled hyaluronan for an experimental proof of the direction of hyaluronan chain elongation by exoglycosidase digestion. However, I have shown previously that continuous new chain initiation occurred during hyaluronan synthesis [1] and thus conclusions can only be derived from enriched hyaluronan preparations in our study on the native enzyme as well as on the recombinant enzyme by Bodevin-Authelet et al. [11]. Therefore, the double labelled hyaluronan preparation used for exoglycosidase digestion was heterogeneous and its size overlapped with the single labelled hyaluronan. But most of the first labelled [ $^3\text{H}$ ]hyaluronan increased in molecular weight during the second incubation and coeluted with the second [ $^{14}\text{C}$ ]labelled hyaluronan, indicating that it had been further elongated to some extent. This heterogeneity is reflected in the simultaneous release of both labels during exoglycosidase digestion, and conclusions are only based on differences of release rates. In contrast to the study of Bodevin-Authelet et al. [11] on recombinant hyaluronan synthase, I found that hyaluronan was elongated at the reducing end by the native synthase confirming the original mechanism [1;2].

It is surprising that a recombinant eucaryotic hyaluronan synthase expressed in yeast synthesizes hyaluronan at the non-reducing end [11], because the structural homology between streptococcal and vertebrate synthases should reflect similar mechanisms, as opposed to the synthase from *Pasteurella*. This discrepancy calls for an explanation. It can be found in a detailed consideration of the experimental conditions and the probable mechanism of enzymatic reactions.

It is obvious that the two substrates UDP-GlcA and UDP-GlcNAc must bind to two active sites within the enzyme. Since no other molecule is required for chain initiation and elongation, the growing UDP-hyaluronan shuttles from one substrate to the other during elongation as illustrated in Fig. 3. Polymerisation at the reducing end is therefore dependent on a high affinity of the growing UDP-hyaluronan to both active sites. It is likely that the binding affinities of UDP-hyaluronan to these sites is proportional to those for the two substrates. The binding affinity of UDP-GlcA and UDP-GlcNAc is reflected by the  $K_M$ -values which have been measured for both the native and the recombinant synthases. Table 2

demonstrates that the recombinant synthase has dramatically reduced affinities for both substrates. Even detergent solubilization appeared to decrease binding affinity. It is thus likely that UDP-hyaluronan that binds to the same sites has also reduced affinity to the synthase. An additional component that holds the growing hyaluronan chain close to the synthase in the native membrane is the hyaluronan exporter, because it has been demonstrated that hyaluronan export and synthesis are closely associated in eucaryotic plasma membranes [5] and that growing hyaluronan chains exert feedback inhibition on the synthase [14;15]. In addition, the exporter is also absent from the recombinant enzyme preparations.

The possible scenario for synthesis at the non-reducing end by the recombinant synthase is illustrated in Fig. 3. At high substrate concentrations that were used for assessment of growth direction of the recombinant synthase (150  $\mu$ M UDP-GlcNAc and 50  $\mu$ M UDP-GlcA) [11], the active sites will be constantly occupied by substrates. Any initially formed UDP-hyaluronan will be ousted from the synthase. However, the enzymatic activities for the transferase reactions remain the same or are even increased, as indicated by the increased  $v_{\max}$  values. Thus the synthase tries to find targets for transfer of monosaccharides from the nucleotide sugars. The only abundant targets are soluble nucleotides sugars. It is thus likely that the synthase transfers the monosaccharides to soluble substrate, instead of transferring bound UDP-hyaluronan. In fact, Bodevin-Authelet et al. only analysed soluble hyaluronan after precipitation of membrane proteins with trichloroacetic acid and unfortunately, did not indicate whether there existed any membrane bound hyaluronan [11]. Such a trichloroacetic acid precipitation has previously been shown to precipitate most of the newly synthesized hyaluronan [20], especially after the short periods of chain growth that were used for determination of direction of elongation .

The chemical reaction is virtually identical to the initial step of polymerisation that results in the first disaccharide unit for elongation at the reducing end. But now elongation proceeds at the non-reducing end. The only difference is the transferred moiety: UDP-hyaluronan to the nucleotide sugars for the synthesis at the reducing end and the nucleotide sugars to hyaluronan for the synthesis at the non-reducing end. This may also explain the failure of Bodevin-Authelet et al. [11] to chase a pulse labelled hyaluronan chain. In contrast, a chase

was demonstrated previously for the natural eucaryotic synthase [21] and again in this paper (Fig. 1).

In accord with hyaluronan chain elongation at the reducing end were results that nucleotide sugars should compete with UDP-hyaluronan for binding to the enzyme and high concentrations increased shedding and reduced the chain length. Thus intracellular concentrations of the nucleotide sugars and the affinity of the growing UDP-hyaluronan chain to the enzyme determine the dissociation rate.

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## LEGENDS

### Fig.1 Synthesis of dual labelled hyaluronan

(A) A membrane fraction of B6 cells was incubated with UDP-[ $^3\text{H}$ ]GlcNAc and unlabelled UDP-GlcA for 30 min. The membranes were solubilized in 1% SDS and subjected to gel filtration on a Sephacryl S-1000 column (1.4 x 64 cm). Fractions of 2 ml were collected and analysed for  $^3\text{H}$  ( $\square$ ) radioactivity. The scale of the fractions from 51 to 65 containing the substrate was reduced by the factor of 10. (B) The membranes were incubated with UDP-[ $^{14}\text{C}$ ]GlcA and unlabelled UDP-GlcNAc for 15 min and analysed as above for  $^{14}\text{C}$  ( $\blacksquare$ ) radioactivity. (C) The membranes were first incubated with UDP-[ $^3\text{H}$ ]GlcNAc and unlabelled UDP-GlcA for 30 min and then with UDP-[ $^{14}\text{C}$ ]GlcA and unlabelled UDP-GlcNAc for 15 min and analysed as above for  $^3\text{H}$  ( $\square$ ) and  $^{14}\text{C}$  ( $\blacksquare$ ) radioactivity. Fractions 23 to 36 were combined for subsequent digestion.

### Fig. 2 Release of hyaluronan from the membrane bound synthase

Hyaluronan was synthesized by B6 membranes in the presence of substrate containing 8  $\mu\text{M}$  UDP-[ $^{14}\text{C}$ ]GlcA and unlabelled 166  $\mu\text{M}$  UDP-GlcNAc ( $\blacksquare$ ,  $\square$ ), or at substrate diluted 1:2 ( $\bullet$ ,  $\circ$ ) or 1:3 ( $\blacktriangle$ ,  $\triangle$ ) with buffer. At the times indicated the membranes were sedimented for 3 min at 14,000 g, dissolved in 1% SDS and applied to descending paper chromatography (solid symbols). The supernatants (open symbols) were applied directly [1]. The origins containing hyaluronan were excised and the radioactivities were determined (A). From these data the ratios of released and membrane bound hyaluronan were calculated (B).

### Fig. 3 Transfer reactions for hyaluronan synthesis at the reducing and non-reducing end

After disaccharide formation, high affinity binding of UDP-hyaluronan to the active sites of the native enzyme **E** ensures that elongation proceeds at the reducing end. Reduced binding affinity to the recombinant enzyme **F** leads to transfer of monosaccharides to soluble substrates at the non-reducing end.



**Table 1**

**Digestion dual labelled hyaluronan with exoglycosidases**

Experiment 1

Digestion period (h)	$^3\text{H}$ (cpm)	$^{14}\text{C}$ (cpm)	$^3\text{H}/^{14}\text{C}$ ratio
0	7162	14216	0,50
3	4281	10157	0,42
9	2076	6498	0,32
27	1606	5780	0,28

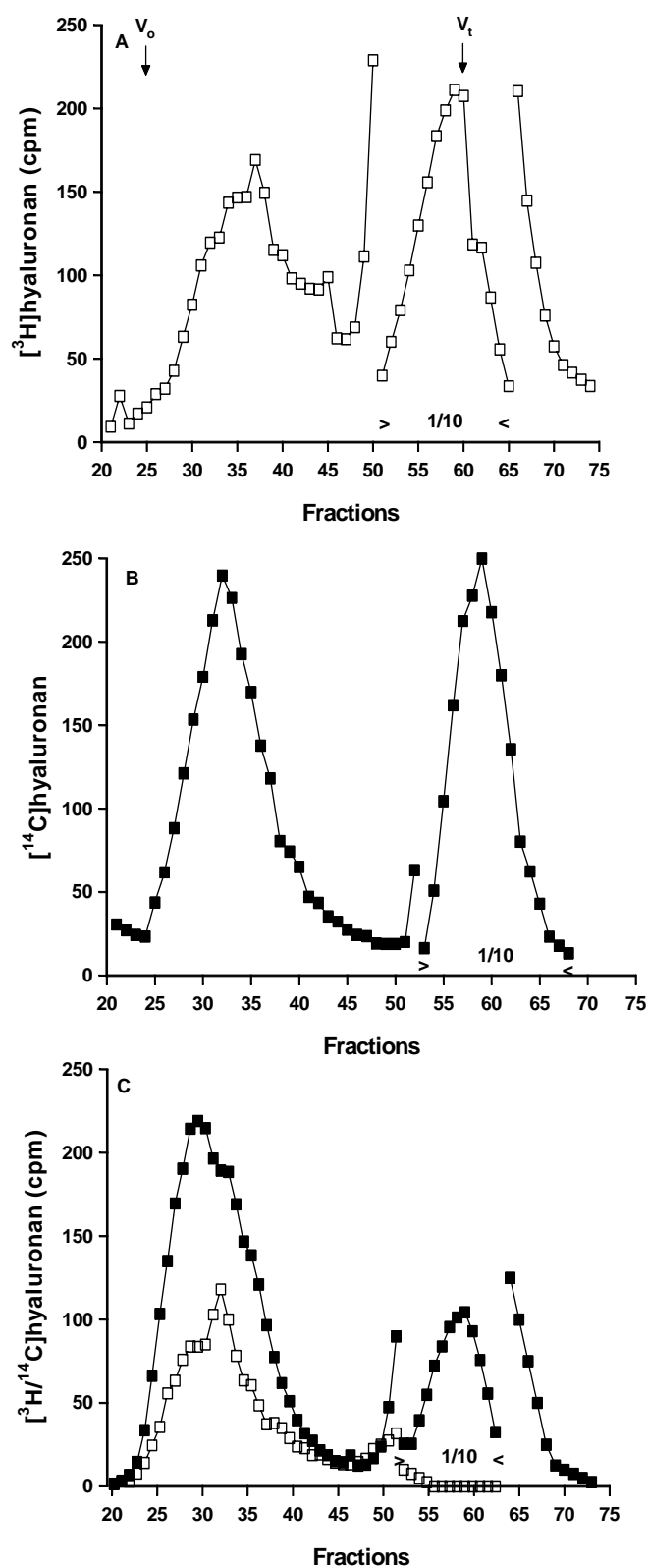
Experiment 2

Digestion period (h)	$^3\text{H}$ (cpm)	$^{14}\text{C}$ (cpm)	$^3\text{H}/^{14}\text{C}$ ratio
0	3725	10560	0,35
3	2799	9512	0,29
8	1434	5264	0,27
24	1461	5616	0,26

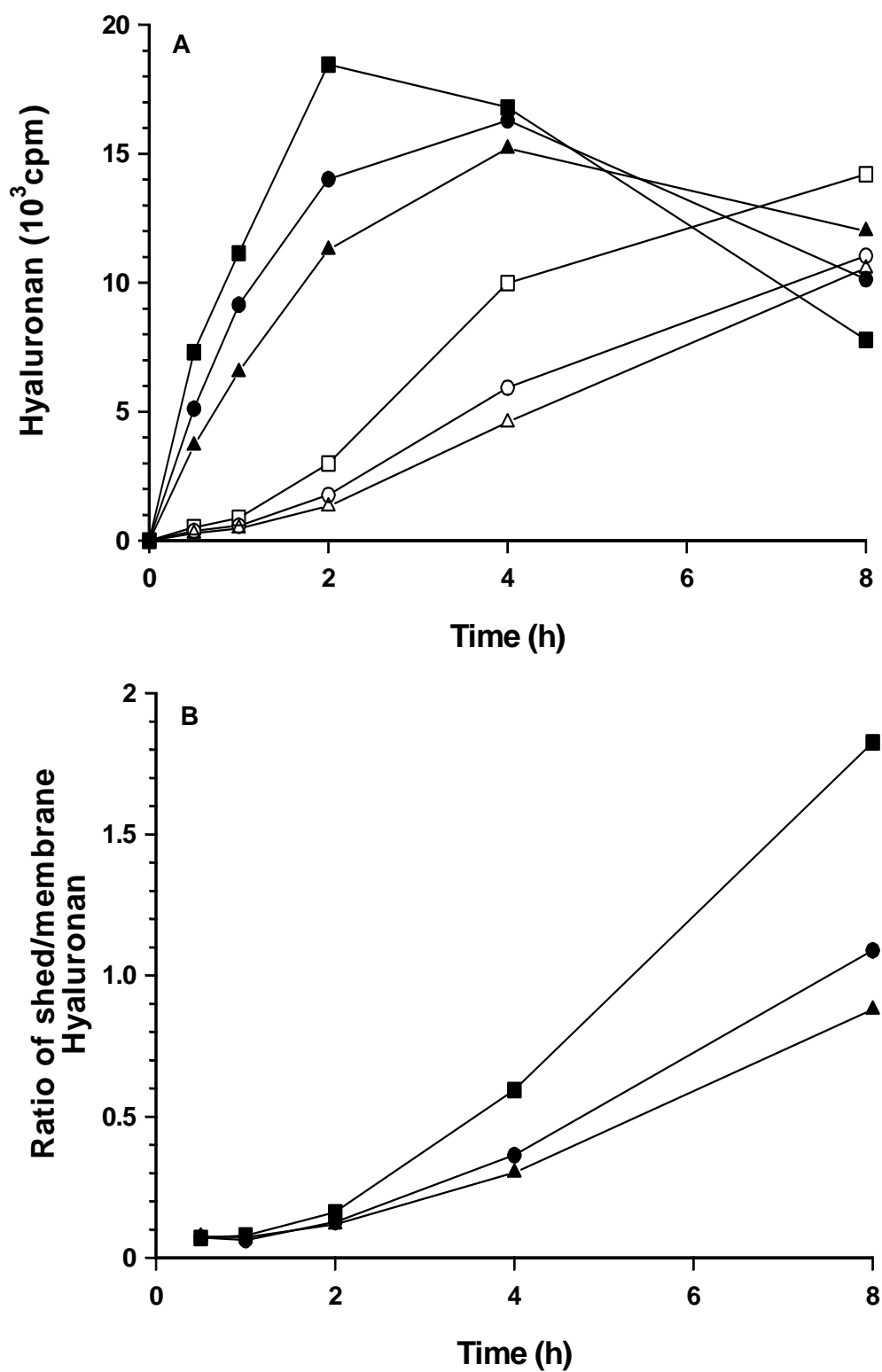
**TABLE 2**

**Comparison of published Michaelis Menten constants for native and recombinant eucaryotic hyaluronan synthases**

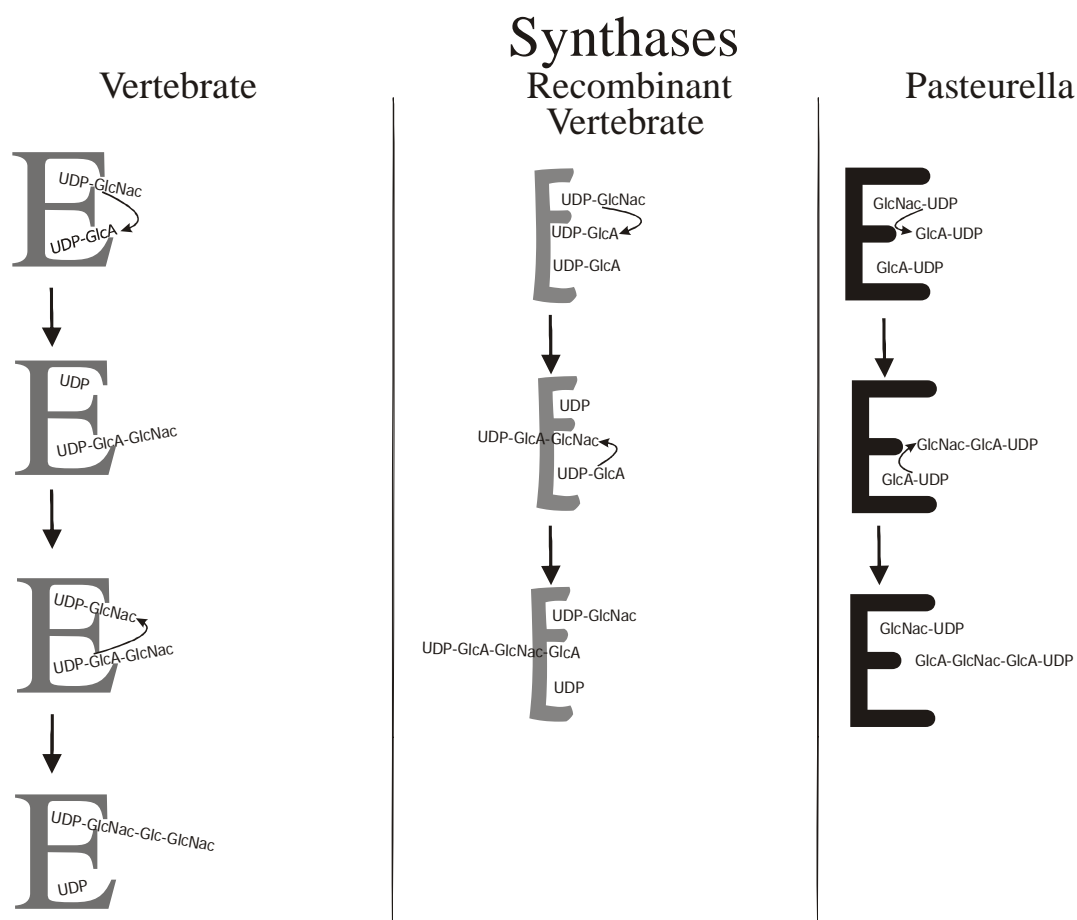
	K <sub>m</sub> (μM) for UDP-GlcA	K <sub>m</sub> (μM) for UDP-GlcNAc	V <sub>max</sub> (nmol/h/mg) for UDP-GlcA	V <sub>max</sub> (nmol/h/mg) for UDP-GlcNAc	Reference
native chick fibroblasts	20	50	10	13	[22]
native human fibroblasts	3	21	70	70	[23]
native human fibroblasts	1	12	6	7	[24]
native mouse fibroblasts	10.8	58.4	7	1	[25]
detergent-solubilized, native mouse oligodendroglioma	50	100			[26]
recombinant xenopus DG42	60	235			[27]
recombinant mouse HAS1	60	950	2772	3090	[28]



Prehm, Fig. 1



Prehm, Fig. 2



Prehm, Fig. 3