Three mammalian hyaluronan synthase genes, HAS1, HAS2, and HAS3, have recently been cloned. In this study, we characterized and compared the enzymatic properties of these three HAS proteins. Expression of any of these genes in COS-1 cells or rat 3Y1 fibroblasts yielded de novo formation of a hyaluronan coat. The pericellular coats formed by HAS1 transfectants were significantly smaller than those formed by HAS2 or HAS3 transfectants. Kinetic studies of these enzymes in the membrane fractions isolated from HAS transfectants demonstrated that HAS proteins are distinct from each other in enzyme stability, elongation rate of HA, and apparent $K_m$ values for the two substrates UDP-GlcNAc and UDP-GlcUA. Analysis of the size distributions of hyaluronan generated in vitro by the recombinant proteins demonstrated that HAS3 synthesized hyaluronan with a molecular mass of $1 \times 10^6$ to $1 \times 10^7$ Da, shorter than those synthesized by HAS1 and HAS2 which have molecular masses of $2 \times 10^5$ to $2 \times 10^6$ Da. Furthermore, comparisons of hyaluronan secreted into the culture media by stable HAS transfectants showed that HAS1 and HAS3 generated hyaluronan with broad size distributions (molecular masses of $2 \times 10^5$ to $2 \times 10^6$ Da), whereas HAS2 generated hyaluronan with a broad but extremely large size (average molecular mass of $>2 \times 10^6$ Da). The occurrence of three HAS isoforms with such distinct enzymatic characteristics may provide the cells with flexibility in the control of hyaluronan biosynthesis and functions.

Hyaluronan (HA) is a major component of most extracellular matrices, particularly in tissues with rapid cell proliferation and cell migration (1). The interaction of HA with various HA-binding proteins and cell-surface receptors plays important roles in regulating fundamental cell behaviors such as cell adhesion, migration, and differentiation (2, 3). Thus, HA has been greatly implicated in morphogenesis, regeneration, wound healing, tumor invasion, and cancer metastasis (4–6). In addition, HA is an important structural molecule required for the maintenance of various aspects of tissue architecture and function. The physical and biological properties of HA appear to be affected by many factors including HA concentration and chain length. Indeed, high molecular weight HA at high concentrations suppresses endothelial cell growth, whereas low molecular weight HA stimulated cell growth leading to induction of angiogenesis (7). In addition, viscosity of the HA gel and the ability to hydrate large amounts of water were shown to be dependent on the molecular size of the HA chain.

HA is a high molecular weight linear polymer composed of GlcUA $\beta$-1,3-GlcNAc $\beta$-1,4 disaccharide units and is synthesized by HA synthase at the inner face of the plasma membrane (8). Although a great deal of effort has been made to elucidate the mechanism of HA biosynthesis in mammalian cells, it has remained unclear due to difficulty in biochemical isolation of the active enzyme (9–11). Recently, three distinct yet highly conserved genes encoding mammalian HA synthases, HAS1, HAS2, and HAS3, have been cloned (12–19). The three gene products are similar in amino acid sequence and molecular structural characteristics. The existence of these distinct mammalian HA synthases raises additional questions regarding the potential differences in HA synthase activities and their biological significance. Transfection of HAS2 or HAS3 cDNAs into COS-1 cells led to de novo formation of HA coats as detected by particle exclusion assay, suggesting that these two enzymes are isozymes (20). However, the functional relationships between HAS1 and the others has remained to be studied to date. In addition, no information is available regarding the regulatory mechanisms of HA biosynthesis and the determination of HA chain length.

In this study, we examined the enzymatic characteristics of the mammalian HAS enzymes and their products both in vivo and in vitro. The results showed that HA biosynthesis and HA coat formation can be achieved by transfection of cells with any one of the three genes. In vitro studies demonstrated that HAS enzymes are distinct from each other in enzyme stability, elongation rate of HA, and $K_m$ values for UDP-GlcNAc and UDP-GlcUA. In addition, each HAS protein synthesized HA chains with different average chain lengths. The occurrence of such HAS isoforms is discussed with respect to their possible physiological roles.
Comparison of Mammalian Hyaluronan Synthases

EXPERIMENTAL PROCEDURES

Construction and Transfection of FLAG Epitope-tagged HAS Expression Vectors—A mouse HAS1 PCR fragment was amplified by 20 cycles using Fnu DNA polymerase (Stratagene) and the following primers: forward, 5'-GATAGTGTGACGCTACGCTGATAGGCATGTC-3' (this primer contained a Bgl II site and corresponded to amino acids 9-58; sequence of HAS1, Ref. 14); reverse, 5'-CAGGGCCTGCACATCCTCTCACCGGCTGGAC-3' (this primer contained a Bsp HI site and corresponded to amino acids 250-290). The resulting PCR fragment was cloned into the Bgl II and Bsp HI sites of the pFLAG-CMV2 vector (Eastman Kodak Co.) to generate pFLAG-HAS1. A HAS2 PCR fragment was amplified as described above using the following primers: forward, 5'-GATAGTGTGACGCTACGCTGATAGGCATGTC-3' (this primer contained a Bgl II site and corresponded to amino acids 9-58; sequence of HAS2, Ref. 18); reverse, 5'-CGACCCGTCGTCGACGCTGACATGTCATACTG-3' (this primer contained a Sma I site and corresponded to amino acids 549-598). The resulting PCR fragment was excised using the Eco RI and Sma I sites and subcloned into the Eco RI and Sma I sites of the pFLAG-CMV2 vector to generate pFLAG-HAS2.

A mouse HAS3 cDNA was cloned from mouse 17-day embryo cDNA (Marathon-Ready™ CDNA, CLONTECH Laboratories Inc.) as described previously (19). The mouse HAS3 PCR fragment was amplified as described above using the following primers: forward, 5'-CCGATCACTCTCTGTCGTCGACGCTGATAGGCATGTC-3' (this primer contained an Eco RI site and corresponded to amino acids 659-708); reverse, 5'-CGAATTCGTCGACGCTGACATGTCATACTG-3' (this primer contained an Eco RI site and corresponded to amino acids 250-290). The resulting PCR fragment was cloned into the Eco RI, gel-purified, and subcloned into the Eco RI site of the pFLAG-CMV2 vector to generate pFLAG-HAS3. Sequences of the resultant constructs were determined using an Applied Biosystems 310 automated DNA sequencer (Applied Biosystems, Foster City, CA).

The pE鑫eo2 bicistronic expression vector that contains an internal ribosome entry site has recently been developed to express proteins derived from inserted cDNA in transfected and cloned cells at high levels. The pE鑫eo-HAS1, -HAS2, and -HAS3 plasmids were generated from this vector and the pFLAG plasmids containing the mouse HAS1, HAS2, and HAS3 cDNA, respectively. The pFLAG-HAS1 and -HAS3 plasmids were digested with Sac I and Eco RV, and the cohesive ends were made blunt by incubation with T4 DNA polymerase (Roche Molecular Biochemicals). The pFLAG-HAS2 plasmid was digested with Sac I and Sma I, and the cohesive ends were made blunt by incubation with T4 DNA polymerase. The gel-purified HAS cDNA fragments were subcloned into the Eco RV site of the pE鑫eo vector to generate the pE鑫eo-HAS plasmids.

The pFLAG-HAS constructs were transfected into COS-1 cells by electroporation. For measurement of HAS activity in transfected COS-1 cells, the HAS expression vectors were co-transfected with a cytomegalovirus promoter (CMV) vector (pCMVneo) containing a hygromycin resistance cassette as a control. Rat 3Y1 fibroblasts were transfected with pCMVneo as a control. Electroporation was carried out according to the manufacturer's instructions using the Gene Pulser transfection apparatus (Bio-Rad). Transfected 3Y1 cells were cultured for 3 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37 °C. Cells were transfected with the pFLAG-CMV2 vector as control. Rat 3Y1 fibroblasts were transfected with pE鑫eo-HAS or with pE鑫eo control vector by the lipofection procedure and then selected in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 0.5 mg/ml G418 at 37 °C. The cells surviving during the selection period were cloned and used for the experiments.

HAS Activity Assays—Fixed sheep erythrocytes (Inter-Cell Technologies, Inc.) were reconstituted in phosphate-buffered saline (PBS) to a density of 5 x 10⁶ cells/ml and used for the particle exclusion assay as described previously (21). HA matrices were visualized by adding 1 x 10⁶ erythrocytes to the growth medium and viewing under an Olympus IMT-2 inverted phase-contrast microscope. The HA pericellular coat to cell area ratios were determined by image analysis using NIH Image (version 1.57) software on a Macintosh computer (Apple Computer Inc., Cupertino, CA). All measurements were made by trac-
Comparison of Mammalian Hyaluronan Synthases

RESULTS

HA Matrix Formation, HA Production, and HAS Expression of Various HAS Transfectants—We previously showed that introduction of mouse HAS1 cDNA into a mouse mammary carcinoma mutant cell line defective in HA biosynthesis led to the recovery of HA biosynthesis and pericellular coat formation (14, 26). Human HAS1 cDNA also induced HA biosynthesis in Chinese hamster ovary cells (16). However, when the mouse HAS1 cDNA was transfected into COS-1 cells, the recombinant protein did not show detectable HA synthase activity in the membrane fractions, despite sufficient levels of protein expression (20). This controversial observation regarding the true biological function of HAS1 has led to the suggestion that HAS1 may be only one of the components of the HA synthase complex as reported previously (9) and that this and other HAS proteins may not be functionally equivalent. We therefore reexamined the ability of HAS1, HAS2, and HAS3 cDNAs to induce HA synthesis and coat formation in COS-1 cells. The in vivo expression of any one of the HAS cDNAs resulted in the formation of pericellular coats in COS-1 cells but not in the control mock transfectants (Fig. 2). Thus, HAS1, HAS2, and HAS3 constituting a family are functionally complementary to each other with respect to the ability to induce coat formation. However, the HA coat formed in HAS1 transfectants was significantly smaller than those of HAS2 and HAS3 transfectants.

To examine further the differences in HA coat formation among transfectants expressing the distinct HAS proteins, rat 3Y1 fibroblasts were transfected with mouse HAS cDNAs in the pEXneo bicistronic expression vector containing an internal ribosome entry site. Several neomycin-resistant colonies arising from single cells transfected with each pEXneo-HAS plasmid were grown to subconfluence and screened for HA synthetic activity. Three independent clones producing different levels of HA were selected for each HAS transfectant and characterized. As observed with the COS-1 transfectants, expression of any one of the HAS cDNAs resulted in the de novo formation of pericellular coats in 3Y1 cells (Fig. 2). These HAS transfectants also showed significant elevation of synthase activity (data not shown). Parental untransfected 3Y1 cells, mock

Fig. 1. Estimation of the amount of FLAG epitope-tagged HAS protein. A, Western blotting analyses of FLAG-tagged BAP protein and serial dilution of membrane fraction isolated from COS-1 transfec-
tants expressing FLAG-tagged HAS1 protein. The intensity of the 49 (BAP protein) and 60-kDa (HAS1 protein) bands increased with increasing content of FLAG-tagged BAP protein and membrane protein, respectively. B, curve depicting the relationship between the content of BAP protein and the band density; a linear correlation was obtained ($R^2 = 0.991$). C, curve depicting the relationship between the content of the membrane proteins and the content of HAS1 protein estimated from the BAP standard curve; a linear correlation was also obtained ($R^2 = 0.995$). The amount of recombinant HAS protein was expressed in arbitrary units with the intensity of 1 ng of FLAG-tagged BAP protein.

1.3% potassium acetate. The precipitated materials were washed with 80% ethanol and further incubated at 55°C for 3 h in a solution containing 500 μg/ml protease K, 100 mM NaCl, 100 mM EDTA, 0.2% SDS, and 50 mM Tris-HCl, pH 8.0. The digests were then precipitated in the same way as described above. The HA-containing fractions were washed with 80% ethanol, lightly dried, and dissolved in water. The HA-containing fractions thus obtained were further incubated at 55°C for 1 h with or without 10 TRU Streptomyces hyaluronidase and fractionated by agarose gel electrophoresis (24).

Electrotransfer of HA onto nylon membranes was performed as described previously (24). After electrotransfer, the nylon membranes were covered with PBS containing 10% skim milk (Diffco) and 20 μg/ml denatured salmon sperm DNA (Sigma) and incubated at 37°C overnight to block nonspecific binding sites. The membranes were washed three times with TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.4) and then covered with 10 ml of PBS containing biotinylated HABR (100 μg) and 1% bovine serum albumin for 1 h at room temperature. The membranes were washed three times with TBS containing 0.05% Tween 20 and then covered with 10 ml of TBS containing peroxidase-conjugated streptavidin and 1% bovine serum albumin for 30 min at room temperature. After washing the membrane, the HABR-streptavidin complexes were detected by the ECL detection system (Amersham Pharmacia Biotech). HA with average mass of 21.3, 14.1, 9.9, 6.4, 4.6, and 105 Da (determined by light scattering analysis) was used as standard.

Determination of HA Molecular Weight by Gel Filtration—The conditioned medium collected from the cultures of the 3Y1-HAS1-16, 3Y1-HAS2-5, and 3Y1-HAS3-4 transfectants were lyophilized and dissolved in 0.5 ml of cold water. The samples (400 μl) containing 10 μg of HA were applied to a column of Tosoh TSK-GEL G6000SW (inner diameter, 7.5 mm × 30 cm) connected to Tosoh TSK-GEL G5000PWxl (inner diameter, 7.5 mm × 30 cm) equilibrated with 0.2 mM NaCl, 0.1 mM Tris-HCl, pH 8.5. The column was eluted at a flow rate of 0.5 ml/min, and fractions of 0.5 ml were collected. HA with average mass of 14.6, 5.2, 2.5, and 0.6 × 105 Da (determined by light scattering analysis) was used as standard. Amounts of HA in the fractions were measured by quantitative high pressure liquid chromatography analysis of unsaturated disaccharide (Di-HA) derivated from HA after digestion with CHase ABC and ACII. To 100 μl of the each fraction, 20 μl of 5 units/ml CHase ABC (Seikagaku Co., Tokyo, Japan) was added, mixed gently and then digested at 37°C for 2 h. After CHase digestion, 20 μl of 5 units/ml CHase ACII (Seikagaku Co., Tokyo, Japan) and 20 μl of 1 mM sodium acetate buffer, pH 6.0, were added to the reaction mixture. The mixture was incubated at 37°C for 2 h to generate Di-HA (disaccharide). The digest was ultrafiltered using an Ultrafave C3GC system (molecular size cut-off 10,000; Japan Millipore Ltd., Tokyo, Japan), and the filtrate obtained was analyzed by high pressure liquid chromatography. Microdetermination of the unsaturated disaccharide derived from HA was performed according to the method of Toyoda et al. (25).
FIG. 2. Visualization of HA matrices around cells expressing mammalian HAS proteins. Particle exclusion assay was used to detect HA coats surrounding cells (arrowheads). COS-1 cells were transiently transfected with pFLAG-CMV2 expression vector (A), pFLAG-HAS1 (C), pFLAG-HAS2 (E), or pFLAG-HAS3 (G), respectively. Rat 3Y1 fibroblasts were transfected with control vector or with the respective pEXneo-HAS expression vector, and clonal transfectants were established. HA coat formation of 3Y1-Mock9 cells (B), 3Y1-HAS1-16 cells (D), 3Y1-HAS2-5 cells (F), or 3Y1-HAS3-4 cells (H). Photomicrographs were taken under an OLYMPUS IMT-2 inverted phase-contrast microscope at × 200 magnification.

transfectants, and β-galactosidase-expressing cells showed no detectable coat-forming ability on particle exclusion assay (Table I). The amounts of HA secreted into the media by these transfectants and the expression levels of respective HAS recombinant proteins were assayed and compared as described under “Experimental Procedures.” All of these HAS transfectants had the tendency to increase in the HA production and HAS expression almost at the same level although the values varied from clone to clone (Table I). However, HAS1 transfectants had a significantly smaller pericellular coat than those formed by HAS2 or HAS3 transfectants. These observation suggested that intrinsic enzymatic properties of three HAS proteins may regulate their coat sizes.

Characterization of Enzymatic Properties of Three Different Mammalian HAS Proteins—We then examined the differences in enzyme properties of each HAS protein which may have been due to the observed differences in HA coat size by investigating the molecular stability, steady-state kinetics, and relative activity of each recombinant HAS protein. First, incorporation rates of radiolabeled sugars from precursor sugar nucleotides into HA were compared among the three recombinant HAS isozymes using the membrane fractions isolated from 3Y1-HAS1-16, HAS2-5, and HAS3-4 transfectants. They all showed high expression of the recombinant HAS proteins as shown in Table I. Recombinant HAS3 showed an almost linear increase in radiolabel incorporation with incubation up to 8 h (Fig. 3). On the other hand, HAS1 and HAS2 proteins showed the linear increases with the incubation times up to 1 and 4 h, respectively, but after these times there were no further increases in incorporation indicating that the stability of the HAS proteins differs from each other under our incubation conditions. Considering these results, we determined the reaction conditions for kinetic analyses as described under “Experimental Procedures.” Then, we compared the differences in kinetic behavior among the three recombinant HAS proteins in the membrane fractions isolated from 3Y1-HAS1-16, HAS2-5, and HAS3-4 transfectants. Apparent Km values of HAS proteins for the substrates UDP-GlcNAc and UDP-GlcUA were determined by measuring synthase activity as a function of UDP-sugar concentration (Figs. 4 and 5). The recombinant membrane-bound HAS1 protein exhibited higher Km values for both UDP-GlcNAc and UDP-GlcUA than those of HAS2 and HAS3 (Table II).
The molecular masses of recombinant HAS1 and HAS2 proteins migrated with average concentrations of UDP-sugars, both the products synthesized by known molecular weights. In the presence of saturating concentrations of UDP-GlcNAc and UDP-GlcUA, the three mammalian enzymes even after long incubation periods and had an average molecular mass of $2.5 \times 10^6$ Da. This observation suggested that the major population of HAS3 is rapidly released from the growing HA chain and may start the next round of elongation. On the other hand, as estimated in the time course experiment (Fig. 3), the elongation of HA synthesized by the membrane-bound HAS1 had almost completely ceased within 1 h. All the products were degraded by *Streptomyces* hyaluronidase and identified as HA (data not shown).

We then examined the HA elongation rate by determining the HA product sizes at early time points. At 1 min, large HA molecules ($>3 \times 10^5$ Da) were already synthesized by HAS1 and HAS2, whereas HAS3 synthesized HA in the size range of 0.4 to $1.0 \times 10^5$ Da (Fig. 7). Since the molecular weights of radiolabeled HA synthesized by the three HAS isoforms increased linearly until 5 min, the synthetic rates could be calculated by dividing the increase in chain length by the duration of the reaction. Based on the HA sizes at various time points obtained by agarose gel analysis, we estimated that HAS1, HAS2, and HAS3 produced HA at average rates of $1256 \pm 251$, $1014 \pm 338$, and $174 \pm 42$ monosaccharides/min, respectively. The HAS1 and HAS2 enzymes, therefore, showed inherently faster elongation rates than HAS3.

We further examined whether the molecular size of HA was affected by the reaction rates of the enzymes. The membrane fraction of each HAS transfectant was incubated with various concentrations (0.1, 0.25, 0.5, 1.0, 1.5, and 2.0 mM) of UDP-GlcNAc. Even at low concentrations of UDP-GlcNAc, the distributions of HA produced after the incubation period of 1 h did...
not change significantly at least under these conditions (data not shown).

For the determination of molecular weights of steady-state products, the molecular sizes of HA secreted into the conditioned media of 3Y1-HAS1-16, HAS2-5, and HAS3-4 transfectants were analyzed by gel-filtration and agarose gel electrophoresis. The elution profiles of all HA species produced by HAS transfectants had similar profiles on gel-filtration analysis (data not shown). A peak consisting of large HA was eluted in the void volume (more than $2.5 \times 10^6$ Da), and a second peak was eluted at a more retarded position (from $1 \times 10^5$ to $2.5 \times 10^6$ Da). HA size distributions in culture medium containing 10% fetal calf serum and the conditioned medium from 3Y1-Mock9 cells were also as-

**TABLE II**

Michaelis-Menten constants for membrane-bound HAS1, HAS2, and HAS3

Membranes containing HAS1, HAS2, and HAS3 were assayed for synthase activity as described under "Experimental Procedures." Results are the means ± S.E. from three experiments performed as described in the legends to Figs. 4 and 5.

<table>
<thead>
<tr>
<th>HAS</th>
<th>UDP-GlcUA</th>
<th>$K_m$ for UDP-GlcNAc</th>
<th>$V_{max}$</th>
<th>UDP-GlcNAc</th>
<th>$K_m$ for UDP-GlcUA</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAS1</td>
<td>0.05</td>
<td>799.0 ± 65.6</td>
<td>84.1 ± 30.4</td>
<td>0.1</td>
<td>73.2 ± 0.7</td>
<td>32.5 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>500.9 ± 32.3</td>
<td>197.7 ± 43.2</td>
<td>0.5</td>
<td>53.3 ± 5.9</td>
<td>133.7 ± 14.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1,011.4 ± 37.6</td>
<td>330.5 ± 81.0</td>
<td>2.0</td>
<td>72.9 ± 4.3</td>
<td>283.8 ± 18.2</td>
</tr>
<tr>
<td>HAS2</td>
<td>0.05</td>
<td>107.6 ± 27.2</td>
<td>123.5 ± 23.5</td>
<td>0.1</td>
<td>32.9 ± 3.3</td>
<td>56.1 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>330.6 ± 37.7</td>
<td>320.8 ± 78.7</td>
<td>0.5</td>
<td>29.2 ± 4.3</td>
<td>196.6 ± 16.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>348.2 ± 15.1</td>
<td>398.5 ± 50.6</td>
<td>2.0</td>
<td>30.0 ± 3.4</td>
<td>336.9 ± 21.1</td>
</tr>
<tr>
<td>HAS3</td>
<td>0.05</td>
<td>82.1 ± 19.1</td>
<td>115.5 ± 16.3</td>
<td>0.1</td>
<td>34.2 ± 4.4</td>
<td>40.5 ± 3.4</td>
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<tr>
<td></td>
<td>0.2</td>
<td>223.3 ± 11.3</td>
<td>255.9 ± 22.8</td>
<td>0.5</td>
<td>35.1 ± 5.3</td>
<td>180.7 ± 18.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>247.2 ± 12.8</td>
<td>340.6 ± 33.9</td>
<td>2.0</td>
<td>34.6 ± 2.0</td>
<td>308.2 ± 12.4</td>
</tr>
</tbody>
</table>

**FIG. 5.** Double-reciprocal plot estimation of $K_m$ for UDP-sugar precursors. The specific incorporation data used to generate Fig. 4, A-F, were plotted as $1/v$ versus $1/[S]$. A, C, and E, 0.05 (○), 0.2 (□), or 1.0 mM (△) UDP-GlcUA. B, D, and F, 0.1 (●), 0.5 (■), or 2.0 mM (▲) UDP-GlcNAc. The $x$ axis intercept signifies $-1/K_m$. 

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FIG. 6. Size distribution of HA synthesized by mammalian HAS proteins. The membrane fractions isolated from 3Y1-HAS1-16 (A), HAS2-5 (B), and HAS3-4 transfectants (C) were incubated for the indicated times with UDP-sugar precursors at saturating concentrations in the presence of UDP-[14C]GlcUA. Radioactive HA samples were separated on one gel by 0.5% agarose gel electrophoresis and detected by BAS2000II. The incubation times were 10 min (lane 1), 20 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), and 8 h (lane 7). HA with average mass (21.3, 14.1, 9.9, 6.4, 4.6, and 1.0 × 10^5 Da) were used to estimate molecular sizes of the products.

FIG. 7. Size distribution of HA synthesized at early incubation times by mammalian HAS proteins. The membrane fractions isolated from 3Y1-HAS1-16, HAS2-5, and HAS3-4 stable transfectants were incubated for the indicated times with UDP-sugar precursors at saturating concentrations in the presence of UDP-[14C]GlcUA. Radioactive HA samples taken at 1, 2.5, and 5 min were quenched with SDS, separated by 0.5% agarose gel electrophoresis, and detected by BAS2000II. HA with average mass (21.3, 14.1, 9.9, 6.4, 4.6, and 1.0 × 10^5 Da) were used to estimate molecular sizes of the products.

FIG. 8. Size distribution of HA from cultures of HAS transfectants. HA-containing fractions were isolated from culture medium or the conditioned medium of 3Y1-Mock9, 3Y1-HAS1-16, HAS2-5 or HAS3-4 transfectants as described under “Experimental Procedures.” The HA fractions were incubated with (+) or without (−) 10 μg/mL Streptomyces hyaluronidase at 55 °C for 3 h and fractionated by 0.5% agarose gel electrophoresis. HA was detected using the ECL detection system with biotinylated HABR and peroxidase-conjugated streptavidin. HA with average mass (21.3, 14.1, 9.9, 6.4, 4.6, and 1.0 × 10^5 Da) were used to estimate molecular sizes of products.

Since the HA pericellular coat has been suggested to be involved as the cellular microenvironment in a variety of important biological events, its formation is probably strictly controlled (5). Previous studies have shown that cell surface receptors and various HA-binding molecules contribute to stabilization of the HA pericellular coat (27–30). Indeed, the HA coat formation in COS cells expressing a high affinity HA receptor CD44 has been reported to require supplementation with HA-binding proteoglycan in addition to HA (31). In contrast, our current and also previous studies demonstrated that expression of three HAS isoforms in COS cells was at least sufficient for HA coat formation without HA receptor expression and proteoglycan supplementation (18, 19). We showed here that the HA pericellular coats of the HAS2 and HAS3 transfectants were obviously larger in size than that of the HAS1 transfectants. These results suggested that the differences in the ability to form the HA coat among these three HAS transfectants may reflect the differences in the intrinsic properties of these HAS enzymes.

Comparison of the enzymatic properties among the three mammalian hyaluronan synthases HAS1, HAS2, and HAS3 may help to clarify their respective roles in HA biosynthesis and address their functional relationships. Recently, Weigel and co-workers (32–34) demonstrated that two bacterial hyaluronan synthases from Streptococcus pyogenes and Streptococcus equisimilis have distinct enzyme activities and kinetic behaviors. In the present study, we also demonstrated that the mammalian HAS proteins are functionally equivalent in HA biosynthesis, since the expression of any one of three HAS proteins led to significant levels of HA production in COS and 3Y1 cells, but molecular stability, kinetic characteristics, and molecular sizes of HA were significantly different among the three mammalian HAS isozymes. As the product may be secreted through the plasma membrane, it may associate with the cell surface via the synthase to which HA can still attach. It is too speculative but interesting to imagine that the destabilization of synthase may result in rapid release of HA from the plasma membrane and in turn lead to a decrease in the size of the HA coat. Considering this possibility, it should be noted that HAS1 protein easily lost its activity (Fig. 3). Furthermore, based on the results of kinetic analysis, the V_{max} values of recombinant HAS1, HAS2, and HAS3 proteins were not significantly different in the presence of saturating concentrations of UDP-sugars, whereas the K_{m} value of HAS1 was higher than those of HAS2 and HAS3. Since the cellular concentrations of UDP-sugars have been estimated to be in the range of 10^{-4}–10^{-5} M (35–37), our results suggested that the smaller HA coat observed in HAS1 transfectants may also be attributable to its higher K_{m} value which may cause the lower synthetic rate at cellular concentrations of sugar nucleotides. These possibilities suggested that the pool sizes of the cellular sugar nucleotides as well as the intrinsic properties of the synthases are important factors for regulating HA coat formation.
It is possible that the physiological function of HA varies depending on its chain length as well as its concentration. Indeed, HA with high molecular weights at high concentrations inhibits cell growth, whereas HA with low molecular weights at low concentrations acts in the opposite manner (38, 39). In addition, viscosity of the HA gel and the ability to hydrate large amounts of water were dependent on the molecular size of the HA chain. However, little evidence is available regarding the mechanism by which HA chain length is controlled. The present study showed that HA chain length synthesized by three HAS isoforms varied. The results of the present in vitro and in vivo studies supported the idea that HA size may be modulated at least in part by HA elongation rate, enzyme stability of HAS, and its capacity to bind to growing HA products. It is also interesting to note that HA molecules synthesized in vitro and in vivo by HAS3 are significantly different in size. This suggested that additional mechanisms, for example, intracellular environment and accessory proteins of HAS, may participate in the multiple regulation of HA chain length. Previous studies showed that the size of HA synthesized by recombinant DG42 (frog HAS) differed markedly between expression in yeast cells and that in COS-1 cells (20). Therefore, HA chain length may also be controlled in a cell type-dependent manner. Future studies are required to resolve these points.

The three HAS proteins are considered to be structurally similar and can be divided into three domains composed of an N-terminal region, central cytoplasmic region, and C-terminal hydrophobic region. The central cytoplasmic regions are conserved among HAS proteins, and the levels of sequence identity in these domains are 75–87%. Despite sequence similarities, the three HAS proteins showed differences in their enzymatic properties, prompting us to study the mechanisms by which the HA synthetic rate and chain length are controlled. It will be of biologically great benefit to identify the specific regions and/or amino acid residues in the proteins responsible for the differences in characteristics of the various HAS proteins.

Differences in the spatial and temporal regulation of transcription among the three HAS isoforms during development and differentiation, if they exist, could be important for understanding the physiological roles of the HAS proteins in these events. Previous studies have shown that the mRNA expression patterns of these HAS genes are distinct (15, 16, 20). In fact, the mechanism of regulation in response to transforming growth factor-β differs between HAS1 and HAS2 genes in keratinocytes and dermal fibroblasts (41). Thus, the existence of three HA synthases with different characteristics may provide the cell with greater flexibility with respect to HA functions. A number of reports have suggested that the functions of HA may vary considerably during embryonic development and in different tissues (42, 43). Therefore, regulation of HA function should be different, depending on differences in developmental stage and tissues, which may be possible by controlling the expression levels of HAS proteins with different enzyme properties.

In conclusion, our results demonstrated that the three mammalian HA synthases are related to each other but have distinct enzymatic properties, which suggest different physiological roles of each synthase. In future studies, we will assess the in vitro physiological roles of the respective HAS proteins by modulating expression of each by gene manipulations.

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Three Isoforms of Mammalian Hyaluronan Synthases Have Distinct Enzymatic Properties
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