We report the isolation of a cDNA encoding the third putative mammalian hyaluronan synthase, HAS3. Partial cDNAs and genomic fragments of mouse Has3 were obtained using a degenerate polymerase chain reaction approach. Partial clones facilitated the isolation of genomic and cDNA clones representing the mouse Has3 open reading frame. The open reading frame of 554 amino acids predicted a protein of 63.3 kDa with multiple transmembrane domains and several consenus HA binding motifs. Sequence comparisons indicated that mouse Has3 is most closely related to Has2 (71% amino acid identity) and also related to Has1 (57% identity), Xenopus laevis DG42 (56% identity), and Streptococcus pyogenes HasA (28% identity). Isolation of a genomic fragment of human HAS3 indicated high conservation between mouse and human sequences, similar to those observed for HAS1 and HAS2. Expression of the mouse Has3 open reading frame in transfected COS-1 cells led to high levels of hyaluronan synthesis, as determined through a classical particle exclusion assay, and by in vitro HA synthase assays. These results suggest that there are three putative mammalian hyaluronan synthases encoded by three separate but related genes which comprise a mammalian hyaluronan synthase (HAS) gene family.

Hyaluronan (HA) is a linear branched glycosaminoglycan (GAG) composed of repeating disaccharide units of D-glucuronic acid(β1→3)N-acetylglucosamine(β1→4). HA is a major constituent of the extracellular matrix of most tissues and organs, especially during embryonic development, where it has been proposed to play important roles in cell migration, proliferation, and the development of tissue architecture (1–3). In addition, HA has been implicated in tumorigenesis, and defects in HA metabolism are a hallmark of several important diseases including rheumatoid arthritis, Grave's ophthalmopathy, cirrhosis of the liver, and accelerated aging in Werner's syndrome (1–4). Unlike other GAGs, which are synthesized within the Golgi network and attached to protein, HA is synthesized at the inner face of the plasma membrane and is subsequently extruded to the outside of the cell (5, 6). Recently, we and others (7–11) have identified two mammalian genes, HAS1 and HAS2, encoding putative plasma membrane HA synthases related to the Streptococcus pyogenes HA synthase, HasA. Expression of either HAS1 or HAS2 by cells led to high levels of HA biosynthesis, consistent with both proteins playing critical roles in HA biosynthesis, possibly as the HA synthases themselves.

While attempting to isolate fragments of the human HAS2 gene using a degenerate PCR approach, we isolated fragments of an additional related gene in the mouse and human. We now report the molecular cloning and characterization of a cDNA encoding the third putative mammalian hyaluronan synthase, HAS3.

**EXPERIMENTAL PROCEDURES**

cDNA and Genomic Cloning and Expression—Previously described degenerate oligonucleotide primer pools (10), DEG 1 and DEG 5, were utilized in an attempt to amplify fragments of HAS genes from human and mouse genomic DNA. PCR buffer conditions were as recommended by the manufacturer (Boehringer Mannheim). The templates were 100 ng of human T47D mammary carcinoma cell line genomic DNA and 100 ng of mouse 129Sv/J genomic DNA, prepared by standard procedures. Cycling parameters were as follows: 35 cycles of 94 °C for 10 s, 50 °C for 30 s, and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. Amplified fragments of the expected size were identified through agarose gel electrophoresis, gel-purified, and cloned directly as described previously (10). Two additional degenerate oligonucleotide primer pools (DEG 10 and DEG 11) were designed, based upon the conserved amino acid sequences GWGTSGRK and RWLNQQTRW (Ref. 10 and Fig. 2). Similar PCR conditions were used to amplify fragments of the expected size from human and mouse genomic DNA using these primers.

Based upon the sequence of partial fragments obtained as described above, a single pair of oligonucleotide primers, forward 5′-TAC TGG ATG CTC TTT AAC GGT GAG-3′ (corresponding to nucleotides 790–813, Fig. 1B) and reverse 5′-GTC ATC CAG AGG TGG TGC TTG-3′ (corresponding to antisense complement of nucleotides 1142–1119, Fig. 1B), was designed to facilitate PCR screening of a mouse 129SvP1 genomic library (Genome Systems, St. Louis, MO). Three positive P1 clones were obtained, and restriction fragments spanning the entire mouse Has3 gene were identified and subcloned into pBlue-script (Stratagene, La Jolla, CA) based vectors using standard procedures. Sequence analyses, using synthetic oligonucleotides made to the mouse Has3 sequence and to vector sequence, permitted the identification of the predicted mouse Has3 open reading frame (ORF), based upon comparison with mouse Has1 and Has2 sequences (7, 10) and genomic structures. All sequences were determined from both DNA strands from multiple overlapping sequencing runs.

The sequence obtained from analysis of genomic clones was confirmed from cDNA sequence through the reverse transcriptase-polymerase chain reaction amplification, cloning, and sequencing of a mouse Has3 ORF cDNA from late gestation (17.5 days postcoitum) mouse C57BL/6J embryotal RNA. Oligonucleotides possessed EcoRI restriction endonuclease sites (underlined) at their 5′ termini to facilitate

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‡ The abbreviations used are: HA, hyaluronan; GAG, glycosaminoglycan; HAS, hyaluronan synthase; HAS1, hyaluronan synthase 1; HAS2, hyaluronan synthase 2; HAS3, hyaluronan synthase 3; PCR, polymerase chain reaction; ORF, open reading frame; kb, kilobase(s).

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subsequent cloning steps and had the following sequence: forward, 5'-CCGAAATTCAAG ATG GCG GTG CAG CTG ACT ACA GCC-3', corresponding to nucleotides 1–24, Fig. 1B) and reverse, 5'-CCGAAATTCTCA CTC TGT CGG AAA AGC CAG GC-3', corresponding to the antisense complement of nucleotides 1665–1643, Fig. 1B). First-strand cDNA synthesis was performed as described (10) using the mouse Has3 reverse oligonucleotide primer. First-strand cDNAs were PCR-amplified using standard PCR buffer conditions supplemented with 2% deionized formamide, through 35 cycles of 94 °C for 10 s, 65 °C for 30 s, and 72 °C for 2 min, followed by a final extension step of 72 °C for 10 min. Amplified cDNAs of the expected size were gel-purified and cloned as described previously. All sequence analyses were performed using the Genetics Computer Group (GCG) package and MacVector programs.

To determine the temporal expression pattern in the developing mouse embryo, the mouse Has3 ORF cDNA was labeled with [α-32P]dCTP by random priming (12) and hybridized to a Northern blot of mouse embryo messenger RNA (CLONTECH, Palo Alto, CA) under conditions recommended by the manufacturer.

Transfections, HA Coat, and HA Synthase Assays—The mouse Has3 ORF, amplified and cloned as described above, was cloned into the EcoRI site of the expression vector pCIneo (Promega, Madison, WI). The mouse Has3 expression vector was co-transfected with a pCMV-β-gal vector into COS-1 (SV40-transformed African green monkey kidney) cells using the Life Technologies™ LifefectAMINE™ transfection reagent according to the manufacturer’s instructions. Positive control transfections utilized the mouse Has2 expression vector previously described (10). HA coat assays (13) and detection of β-galactosidase activity were performed as described (10).

Crude cell membrane preparations were isolated from COS-1 cells transfected with the mouse Has3 expression vector, the mouse Has2 expression vector (10), and the pCIneo vector (control), essentially as described (14), except the final membrane pellets were resuspended in 50 μl of lysis buffer (LB) consisting of 10 mM KCl, 15 mM MgCl2, 2.5 mM HEPES, pH 7.1, 1 mM UDP-GlcNAc, 0.4 μM UDP-GlcUA, 0.5 μM aprotinin, and 0.05 mM phenylmethylsulfonyl fluoride (LB+) . Protein content of crude membrane preparations was determined by a BCA assay (Pierce). To detect HA synthase activity, duplicate samples of approximately 100 μg of crude membrane protein were incubated overnight at 37 °C in a total reaction volume of 200 μl under the following conditions: 5 mM dithiothreitol, 15 mM MgCl2, 25 mM HEPES, pH 7.1, 1 mM UDP-GlcNAc, 0.05 mM UDP-GlcUA, 0.4 μg of aprotinin, 0.4 μg of leupeptin, 0.5 μ Ci of UDP-[14C]GlcUA (ICN, Costa Mesa, CA). An additional specificity control reaction was set up in which UDP-GlcNAc was omitted. After overnight incubation, samples were boiled for 10 min and subsequently divided in two. Streptomyces hyaluronidase (1 turbidity reducing unit) was added to one half and incubated for an additional hour at 37 °C. SDS was added to a final concentration of 1%, and samples were boiled and analyzed by descending paper chromatography essentially as described (15).

RESULTS

While cloning fragments of human HAS2, we isolated a fragment of an additional gene that was related to but distinct from human HAS1 and HAS2. We isolated this fragment through PCR amplification with previously described HAS-specific degenerate oligonucleotide primers DEG 1 and DEG 5 (10). In contrast to our previous studies in which we amplified off a cDNA template, in this instance we used a genomic DNA template. HAS fragments were amplified from human and mouse genomic DNA. Subsequent cloning and sequence analyses revealed that all the human and mouse clones fell into two categories. The first category represented clones of human and mouse Has3, while the second category of clones was highly conserved between human and mouse, and represented fragments derived from a related gene that was not HAS1 or HAS2. Subsequently, we used additional combinations of degenerate primers to amplify and clone additional fragments of this novel gene, which we have designated HAS3 in humans and Has3 in

6.0–6.5 kb and a minor transcript of approximately 4.0 kb, were observed. Mouse Has3 expression appears to be highest in the late gestation embryo (17.5 days postcoitum).

![Table of Days of Gestation](https://example.com/table.png)

| Days of Gestation | 7.5 | 11.5 | 15.0 | 17.5 |
|------------------|-----|------|------|------|
| 9.5              | 54.4|
| 7.5              |     |
| 4.4              |     |
| 2.4              |     |
| 1.35             |     |
the mouse (Fig. 1A). Alignment of the partial sequence of human HAS3 and mouse Has3 indicated a very high level of sequence conservation (99%) (Fig. 1A). This is similar to the high level of conservation observed for human and mouse HAS1 (96%) and HAS2 (99%) (7–11).

Through genomic cloning and sequence analyses and confirmation of this sequence from cDNA-derived clones, we identified an ORF of 1662 base pairs for mouse Has3 (Fig. 1B). This ORF encodes a polypeptide of 554 amino acids with a predicted molecular mass of 63.3 kDa. This polypeptide is only 2 amino acids longer than the mouse Has2 polypeptide (10). Sequence alignments indicated that mouse Has3 is 71, 57, 56, and 28% identical to mouse Has2 (10), mouse Has1 (HAS protein) (7), Xenopus DG42 (16), and S. pyogenes HasA (17), respectively (Fig. 2A). Like Has1 and Has2, residues demonstrated to be critical for N-acetylglucosaminyltransferase activity of yeast chitin synthase 2 (18) are completely conserved. In addition, these motifs are located at similar positions within the Has3 polypeptide. In contrast to mouse Has2, which is highly expressed from as early as day 7.5 postcoitum through late gestation in the developing mouse embryo (10), mouse Has3 is expressed predominately in the late gestation embryo (Fig. 1C). One major transcript of approximately 6.0–6.5 kb and a minor transcript of approximately 4.0 kb were observed (Fig. 1C).

To test the enzyme activity of mouse Has3, we transfected an expression vector carrying the Has3 ORF into COS-1 cells. We tested mouse Has3 alongside mouse Has2 and a negative control.
FIG. 3. Mouse Has3-transfected COS-1 cells generate HA-dependent pericellular coats. COS-1 cells were transfected with mouse Has2 pCIneo (A), pCIneo (B), and mouse Has3 pCIneo (C–F) vectors. Addition of fixed horse erythrocytes to culture dishes permitted the visualization of pericellular coats. Pericellular coats were produced by mouse Has2-transfected cells (A) as described previously (10) and by mouse Has3-transfected cells (C and E). pCIneo (vector only control) transfected cells failed to produce coats (B). Mouse Has3-transfected cells produced pericellular coats that were destroyed by treatment with a specific hyaluronidase from Streptomyces (5 turbidity reducing units/ml for 1 h at 37 °C) (compare panels E, before hyaluronidase treatment, and F, after hyaluronidase treatment). In contrast, pericellular coats remained on mock hyaluronidase-treated cells (compare panels C, before, and D, after mock hyaluronidase treatment).

DISCUSSION

Three mammalian putative hyaluronan synthases, HAS1, HAS2, and HAS3, have now been identified. The three proteins are encoded by three separate but related genes, which constitute a mammalian HAS gene family. Sequence comparisons and structural predictions suggest that the mammalian HAS proteins are very similar in structure. They are predicted to have one or two N-terminal transmembrane domains and a cluster of C-terminal transmembrane domains separated by a large cytoplasmic loop. This topology is extraordinarily similar to that predicted for the bacterial HA synthase, HasA (21), and to that recently reported for the Rhizobium meliloti nodulation factor, NodC (22). In addition, the mammalian HAS sequences, the Xenopus DG42 sequence, HasA sequence, NodC sequence, and the recently reported putative plant cellulose synthases share critical residues shown to be required for N-acetylglucosaminyltransferase activity of yeast chitin synthase 2, making it highly likely that all these proteins are functionally related processive β-glycosyltransferases. It has been suggested that three similar regions containing highly conserved aspartate (Asp) residues will be present in all such processive glycosyltransferases (23) and Fig. 2B). These highly conserved residues may represent sites such as cation binding sites that in turn may coordinate nucleotide-sugar interaction with the enzyme.

Semino et al. (24) have postulated that DG42 and its related mammalian homologues, rather than being bona fide HA synthases, may stimulate HA production through synthesizing chitin oligosaccharide primers, which are required and rate-limiting for eukaryotic HA biosynthesis. However, cell membranes isolated from bakers’ yeast, Saccharomyces cerevisiae, engineered to express DG42 have HA synthesis activity in vitro when supplied with the required UDP precursors (25). This is highly significant as S. cerevisiae is deficient in UDP-glucuronic acid production and is thus incapable of HA biosynthesis. This result and ours suggest that DG42 and its related mammalian counterparts are bona fide eukaryotic hyaluronan synthases. Clearly, however, this is an area that must be thoroughly examined in future experiments by, for instance, purifying the enzyme activities.

Expression of any one of the mammalian HAS proteins in transfected mammalian cells leads to a dramatic increase in HA biosynthesis. This would suggest that the proteins have similar activities. However, the high degree of sequence conservation (96–99% identity) between human and mouse HA synthases contrasts with the lower level of identity between synthases within a species (Has1/Has2, 55% identity; Has1/Has3, 57% identity; Has2/Has3, 71% identity), arguing for evolutionary conservation of functionally important residues and for some differences in the mode of action of the three proteins. Potential differences in function of the proteins could relate to the length of the HA chain synthesized, the rate of HA synthesis, the ability to interact with cell-type specific accessory proteins, and whether or not the HA is preferentially secreted by the cell or alternatively retained by the cell in the form of a pericellular coat.

In conclusion, a small gene family encoding putative plasma membrane hyaluronan synthases is present in mammals. We have recently determined that the mouse and human HAS genes are localized on three separate autosomes (26). Our data suggest that a primitive ancestral HA synthase gene duplicated comparatively early in vertebrate evolution, and that the HAS genes have subsequently diverged with respect to the regulatory sequences controlling their expression and possibly with respect to their mode of action. This in turn would suggest

| Vector          | + UDP-GlcNAc | − UDP-GlcNAc | Hyaluronidase |
|-----------------|--------------|--------------|---------------|
| Mouse Has3 pCIneo | 204.2⁺ | 1.9⁺ | − |
| Mouse Has2 pCIneo | 26.9  | 2.5  | − |
| pCIneo (control) | 11.0 ND⁺ | 10.3 ND⁺ | − |

a Plus and minus symbols indicate whether or not UDP-GlcNAc was included in these reactions.

b Plus and minus symbols indicate whether or not a reaction was subsequently treated for 1 h at 37 °C with 1 TRU Streptomyces hyaluronidase prior to paper chromatography.

Numbers represent picomoles of radiolabeled product formed and were calculated taking into account the specific activity of the UDP-[¹⁴C]GlcUA used, the amount of cold UDP-GlcUA per reaction, and assumed a scintillation counting efficiency of >95%. Based upon these calculations, 1 pmol of radiolabeled product is represented by 384 dpm, i.e. 204.2 pmol of product was calculated from 78,413 dpm. Numbers represent the mean calculated from duplicate reactions.

d Number represents the result of a single reaction in each instance.

Not determined.

Mouse Hyaluronan Synthase 3

TABLE I

Hyaluronan synthase activity of transfected COS-1 cells

| Vector          | + UDP-GlcNAc | − UDP-GlcNAc | Hyaluronidase |
|-----------------|--------------|--------------|---------------|
| Mouse Has3 pCIneo | 204.2⁺ | 1.9⁺ | − |
| Mouse Has2 pCIneo | 26.9  | 2.5  | − |
| pCIneo (control) | 11.0 ND⁺ | 10.3 ND⁺ | − |

a Plus and minus symbols indicate whether or not UDP-GlcNAc was included in these reactions.

b Plus and minus symbols indicate whether or not a reaction was subsequently treated for 1 h at 37 °C with 1 TRU Streptomyces hyaluronidase prior to paper chromatography.

Numbers represent picomoles of radiolabeled product formed and were calculated taking into account the specific activity of the UDP-[¹⁴C]GlcUA used, the amount of cold UDP-GlcUA per reaction, and assumed a scintillation counting efficiency of >95%. Based upon these calculations, 1 pmol of radiolabeled product is represented by 384 dpm, i.e. 204.2 pmol of product was calculated from 78,413 dpm. Numbers represent the mean calculated from duplicate reactions.

d Number represents the result of a single reaction in each instance.

Not determined.
that HA biosynthesis is regulated at many levels within the vertebrate organism.

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