Molecular Cloning and Characterization of a Putative Mouse Hyaluronan Synthase*

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1 The abbreviations used are: HA, hyaluronan; RT-PCR, reverse transcriptase-polymerase chain reaction; ORF, open reading frame; CMV, cytomegalovirus promoter; PBS, phosphate-buffered saline; UTR, untranslated region; PKC, protein kinase C; PKA, protein kinase A; UDP-GlcNAc, UDP-N-acetylglucosamine; UDP-GlcUA, UDP-glucuronic acid; dpc, days postcoitum; bp, base pair(s); kb, kilobase(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

We report the isolation of a novel mouse gene which encodes a putative hyaluronan synthase. The cDNA was identified using degenerate reverse transcriptase-polymerase chain reaction. Degenerate primers were designed based on an alignment of the amino acid sequences of Streptococcus pyogenes HasA, Xenopus laevis DG42, and Rhizobium meliloti NodC. A mouse embryo cDNA library was screened with the resultant polymerase chain reaction product, and multiple cDNA clones spanning 3 kilobase pairs (kb) were isolated. The open reading frame predicted a 69-kDa protein with several transmembrane sequences, multiple consensus phosphorylation sites, and four putative hyaluronan binding motifs. The amino acid sequence displayed 55% identity to mouse HAS, 56% identity to Xenopus DG42, and 21% identity to Streptococcus HasA. Northern analysis identified transcripts of 4.8 kb and 3.2 kb, which were expressed highly in the developing mouse embryo and at lower levels in adult mouse heart, brain, spleen, lung, and skeletal muscle. Transfection experiments demonstrated that mouse Has2 could direct hyaluronan coat biosynthesis in transfected COS cells, as evidenced by a classical particle exclusion assay. These results suggest that mammalian HA synthase activity is regulated by at least two related genes. Accordingly, we propose the name Has2 for this gene.

Hyaluronan (HA) is a linear unbranched polymer made up of repeating disaccharide units of d-glucuronic acid(β1-3)N-acetylglucosamine(β1-4). More than 60 years after the isolation of hyaluronan from the vitreous humor (1), its synthetic pathway remains incompletely characterized. HA is synthesized as a free, linear polymer at the inner face of the plasma membrane of eukaryotic cells and is subsequently extruded to the outside of the cell (2-7). HA biosynthesis in mammalian cells may be regulated in part through signaling cascades (8, 9).

Certain bacteria can synthesize an HA polymer that is identical to the polymer synthesized by mammalian cells (10-12). Indeed, investigation of HA biosynthesis in the Group A Streptococcus, Streptococcus pyogenes, has recently led to the identification and cloning of several genes that encode enzymes critical for HA biosynthesis in this bacterium (11). However, until very recently, no genes have been identified that encode enzymes with similar activities in mammalian cells.

Degenerate reverse transcriptase-PCR has been a useful tool in the identification and cloning of many genes and gene families (13-15). This approach relies upon conserved sequences deduced from alignments of related gene or protein sequences. The HasA gene of S. pyogenes encodes hyaluronan synthase in this bacterium (11). Sequence analysis predicts that this protein is a membrane protein with a large intracellular loop encoding the active site of the enzyme (11). Similarly, in mammalian cells, the HA synthase has been localized to the plasma membrane, with the active site on the inner face of the membrane (4, 5). Data base searches have identified the Rhizobium sp. nodulation factor C (NodC) proteins, the Saccharomyces cerevisiae chitin synthase 2 (Chs2) proteins, and the Xenopus laevis DG42 protein as sharing sequence identity with HasA (16). This suggested to us that there might be HasA/DG42-related genes in mammals that play a role in HA biosynthesis. Accordingly, we utilized the aligned amino acid sequences of HasA, DG42, and NodC to design a degenerate RT-PCR strategy to successfully identify a HasA/DG42-related cDNA in the mouse. Surprisingly, the deduced sequence predicted from this cDNA is distinct from that of a recently reported mouse HAS cDNA (17), although the sequences are clearly related. Accordingly, we have designated this novel mouse gene, Has2, hyaluronan synthase 2. Transfection of mouse Has2 expression constructs into COS cells allowed them to synthesize HA, as determined through an HA coat assay. The identification and cloning of the second putative mammalian HA synthase gene will be instrumental in our future understanding of HA biosynthesis and function.

EXPERIMENTAL PROCEDURES

Degenerate Reverse Transcriptase-PCR (RT-PCR)—Degenerate oligonucleotide primer pools were designed based upon an alignment of the X. laevis DG42 amino acid sequence with the S. pyogenes HasA and the Rhizobium meliloti NodC amino acid sequence (16). Three degenerate pools were designed, two of which were predicted to anneal on the antisense strand and one on the sense strand. The oligonucleotides were made corresponding to the peptide sequences AFYNVERACQ, GDDRHLTN, and QQTRWTKSYF and had the following degenerate sequences: DEG 1 primer, 5’-GCN TTY AAY GTN GAR MGN GCM TGY CA 3’ (sense strand), DEG 3 primer, 5’-RTT NGT NAR RTG NCK RTK RTC NCC-3’ (antisense strand), and DEG 5 primer, 5’-RAA RTA NWW YTT NGT NCC NCK NGT YTT YTG-3’ (antisense strand). RNA was isolated using Trizol™ reagent (Life Technologies, Inc.) according to the manufacturer’s directions. Reverse transcription reactions were performed on total RNA isolated from 10.5 and 14.5 days postcoitum (dpc) C57BL/6J mouse embryos. Briefly, 5 μg of total RNA were heat-denatured at 95 °C, then split into two separate reactions. One reaction
served as a control and amplified a fragment of 28 S ribosomal RNA. The reverse transcriptase was carried out at 42 °C using 10 units of Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim) in a total volume of 25 μL. Five microliters of each resulting first-strand cDNA were amplified in separate 100-μL PCR reactions using combinations of degenerate primer pools. Amplification conditions were as follows: 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, followed by a final extension for 72 °C for 10 min. Primer pools were used at a final concentration of 1 μM. Twenty microliters of each PCR reaction was separated through a 2.0% agarose gel. Amplified products (Fig. 1A) were gel-purified and ligated directly into a pBluescript KS II + (Stratagene Cloning Systems, La Jolla, CA) T-vector prepared as described (18). Resultant plasmids were sequenced by dideoxy sequencing of double-stranded plasmid DNA using a Sequenase version 2.0 sequencing kit (United States Biochemical Corp.).

cDNA Library Screening and Cloning—A 300-bp cDNA fragment, MHas300, isolated through degenerate RT-PCR, was used as a probe to screen a primary agt10 cDNA library constructed from 8.5-dpc C57BL/6J poly(A)-RNA (kindly provided by Dr. J. L. Lee, Mayo Clinic Scottsdale). The probe was labeled to high specific activity using random priming in the presence of [α-32]PdCTP (19). Approximately 1.5 × 10⁶ plaque-forming units were screened using standard procedures (20). Double-positive plaques were identified and taken through two additional rounds of plaque purification. In addition, a portion of each primary plaque was screened by PCR to determine insert size relative to the MHas300 fragment. This was carried out through a combination of primers that flanked the agt10 cloning site and MHas2 specific primers. Fourteen positive clones were obtained and analyzed. EcoRI restriction fragments were subcloned into pBluescript KS II+ for sequence analysis. Sequence was determined from both strands using synthetic oligonucleotide primers made to the mouse Has2 sequence and to the vector.

Northern and Southern Analysis—Mouse tissue Northern blots (CLONTECH) were hybridized to a 0.5-μg competitor DNA probe corresponding to the 1.65-kb open reading frame (ORF) of the mouse Has2 gene. Blots were hybridized at 42 °C and washed to high stringency according to the manufacturer’s recommendations. Blots were exposed for 1–70 h to BioMax MR film (Eastman Kodak Co.) with intensifying screens. To control for variation in loading, blots were stripped and rehybridized with a 32P-labeled probe for the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Mouse 129Sv/j genomic DNA was prepared from tail snips using standard procedures. Approximately 15-μg samples of genomic DNA were digested overnight with restriction endonucleases, size-separated through 0.8% agarose gels, and transferred to Hybond N membranes (Amersham). Membranes were hybridized to a 0.5-μg competitor DNA probe corresponding to the 1.65-kb ORF of mouse Has2. Hybridization conditions were performed as recommended by the manufacture. Membranes were washed to low (1 × SSC and 0.1% SDS at 37 °C) and high (0.1 × SSC and 0.1% SDS at 55 °C) stringency (1 × SSC (saline sodium citrate) is 150 mM NaCl, 15 mM sodium citrate), and autoradiography was performed as described above.

Transfection Studies—Expression constructs were created in the mammalian expression vector, pCIneo (Promega Corp.). Mouse Has2 ORFs were amplified by PCR, off a template of mouse Has cDNA clone λ11.1 (Fig. 1B). PCR primers were designed to create a mouse Has2 cDNA with an optimized Kozak consensus, A—ATGG, and to contain the SmaI/Xmal sites at each end suitable for cloning. Primers were as follows: 5′-CCCCGGGCAA ATG TAT GGT GAG AGG TTA TCA TGT GTC GTG CTC-3′ (bp 504 to 537, Fig. 2) and 5′-CCCCGGG TCA GAC CAT ATC GTA ATC GTG CTC-3′ (bp 2163 to 2153, Fig. 2). Gel-purified PCR products were cloned directly into a pBluescript KS II+ T-vector for sequence verification, prior to subcloning into the Xmal I site of pCIneo.

The mouse Has2 expression vector was co-transfected with a cytomegalovirus promoter (CMV)-driven β-gal expression vector into COS-1 (SV40-transformed African green monkey kidney) cells (21) using Li-pofectAMINE™ (Life Technologies, Inc.) according to the manufacturer’s instructions. The β-gal expression plasmid was used in all transfections to permit the visual identification of cells that had been successfully transfected. Control co-transfections were pCIneo (vector control) and LacZ vector. Cells were analyzed 36 h after lipofection (transient transfection). The COS-1 cell line and the mouse 3T6 (Swiss embryonic fibroblast) cell line (22) were routinely maintained at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM glutamine, in a humidified chamber at 5% CO2.

HA Coat Assays—Glutaraldehyde fixed horse erythrocytes (Sigma) were reconstituted in phosphate-buffered saline (PBS), washed several times to remove traces of sodium azide, and finally resuspended in PBS plus 1 mg/ml bovine serum albumin to a density of 5 × 10⁸ cells/ml. HA coats were visualized around live cells growing in individual wells of a 24-well plate or 6-well plate by adding 1 × 10⁶ or 5 × 10⁶ red blood cells, respectively, to the growth medium. Red cells were allowed to settle for 15 min at 22°C for HA coats were scored. To confirm the coats as being composed of HA, red cells were removed by extensive washing with PBS, and one well of each experimental sample was treated with 10 units/ml bovine testicular hyaluronidase (Calbiochem) or 5 units/ml Streptomyces hyaluronidase (Calbiochem) in Dulbecco’s modified Eagle’s medium plus 0.5% fetal bovine serum for 1 h at 37 °C. Equivalent wells were incubated under the same conditions in the absence of hyaluronidase. After incubation, red cells were added to the wells, as described previously, and coats were again scored. HA coats were imaged at >200 magnification. After imaging, red cells were removed by extensive washing with PBS. Cells were stained to detect β-galactosidase (LacZ) activity (23) and imaged as described.

RESULTS
cDNA Cloning of the Putative Mouse Hyaluronan Synthase— Utilizing degenerate RT-PCR, we successfully amplified partial cDNAs corresponding to a novel mouse gene, Has2 (HA synthase 2) (Fig. 1A), similar in sequence to X. laevis DG42 (24) and S. pyogenes hasa (11). Mouse λ-cDNA library screening yielded multiple overlapping clones, which collectively spanned approximately 3 kb (Fig. 1B). Sequence analyses identified an open reading frame (ORF) of 1656 bp, flanked by 5′- and 3′-untranslated regions (UTRs) of 507 and 772 bp, respectively (Fig. 2). The predicted translation initiation site conformed to the Kozak consensus for initiation (25). Although there were four additional upstream ATGs within the 5′-UTR, none of these fitted the Kozak consensus and all were followed closely in-frame stop codons. The presence of several upstream ATGs has, however, been more commonly described in oncogenic sequences (26). The 3′-UTR contained two consensus sequences for polyadenylation, a CA repeat and a TA repeat (Fig. 2).

Data base searches indicated that the predicted amino acid sequence of mouse Has2 aligned most significantly with Xenopus DG42 (56% identity, 70% similarity) (24), streptococcal HasA (21% identity, 28% similarity) (11), Rhizobium sp. NodC (27, 28), and S. cerevisiae chitin synthase 2 (Chs2) (29) (Fig. 3). A partial cDNA sequence that has been reported recently to encode a mouse chitin oligosaccharide synthase (30) was identical to the central area of the mouse Has2 open reading frame. In addition, mouse Has2 displayed 55% identity and 73% similarity to a recently reported mouse Has gene (17) and the human homolog of this gene (31). We have recently isolated clones for a second human Has gene, which shares greater than 95% amino acid identity to mouse Has2 and thus is predicted to represent the human HAS2 gene (2). This suggests that there are at least two related Has genes in both mouse and humans.

Predicted Characteristics of Mouse Has2—Investigation of the primary amino acid sequence of mouse Has2 identified several potential transmembrane sequences (Fig. 4), four potential HA binding motifs fitting the BXBX consensus (32), and numerous consensus sequences for phosphorylation by protein kinase C (PKC) and cyclic AMP-dependent kinases, such as protein kinase A (PKA) (33). Has2 was predicted to be a multiple membrane-spanning protein with a large cytoplasmic loop, similar to the predicted structure of Streptococcus Hasa and mouse HAS (Has1) (Fig. 1). Sequence alignment with S. cerevisiae chitin synthase 2 (Chs2) demonstrated that the residues recently shown to be required for catalytic activity

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in this molecule (34) are conserved within the large predicted cytoplasmic loop of mouse Has2 (Fig. 3B). It has been suggested that these residues may be generally conserved within glycosyltransferases that catalyze the synthesis of oligosaccharides with β1-34 linkages (34). Significantly, the predicted cytoplasmic loop of the Has2 molecule is the most highly conserved across species, and thus we predict this part of the protein to form the catalytic domain.

MRNA Expression of Mouse Hyaluronan Synthase 2—Northern analyses detected two transcripts of approximately 3.2 kb and 4.8 kb, respectively (Fig. 5). The 4.8-kb transcript was expressed at levels approximately 20-fold higher than the 3.2-kb transcript. High levels of expression were observed in the developing mouse embryo, in addition to lower levels in adult mouse heart, brain, spleen, lung, and skeletal muscle (Fig. 5). All of the isolated cDNA clones were predicted to form an identical ORF. Thus, rather than being the result of alternative splicing, the 4.8-kb transcript most probably corresponds to a mouse Has2 mRNA with an alternate poly(A) signal, generating a 3'-UTR with approximately 1.8 kb of sequence, in addition to that reported herein.

Mouse Has2 Is a Single Copy Gene—The pattern of hybridizing restriction fragments that was observed through Southern analyses was consistent with mouse Has2 being a single copy gene within the mouse genome (Fig. 6). In addition, the pattern observed in digests of total mouse genomic DNA was identical to that observed in equivalent digests of recently isolated mouse Has2 genomic clones. Low stringency wash conditions failed to identify any further hybridizing fragments including those fragments corresponding to the related mouse HAS (17) hasA, DG42, nodC, and the 180-bp and 300-bp PCR products and, thus, probably arose through nonspecific PCR amplification. This suggests that the level of sequence identity (55%) between mouse Has2 and mouse HAS, and possibly other Has-related genes, is not sufficient to permit detection through Southern hybridization. Thus, while these results preclude the existence of a mouse Has2 pseudogene, they do not preclude the existence of other genes related to mouse Has2 and mouse HAS (Has1).
able coat-forming ability in HA pericellular coat-forming assays (Fig. 7B). In contrast, untransfected 3T3 mouse embryonic fibroblast cells had well-developed HA coats (Fig. 7A). Transient co-transfection of mouse Has2 and LacZ expression constructs enabled transfected COS-1 cells to produce large HA coats (Fig. 7, D–I). Cells acquiring an HA coat also stained positively for β-gal activity (Fig. 7, D–I). β-gal activity was utilized as a marker to confirm that cells that generated coats had successfully taken up DNA. HA coats were destroyed by treatment with Streptomyces hyaluronidase (Fig. 7H) or bovine testicular hyaluronidase. Control pCIneo transfected cells produced no coats (Fig. 7C) and were indistinguishable from parental untransfected COS-1 cells. Equivalent numbers of LacZ positive cells were observed in experimental and control transfections (data not shown). These results indicate that parental COS-1 cells express all other factors required for HA biosynthesis and pericellular coat formation, but presumably lack HA synthase activity. Expression of Has2 in COS-1 cells is sufficient for HA coat formation.

**DISCUSSION**

Hyaluronan is a major constituent of the extracellular matrix of most tissues and organs, especially during embryonic development. Within the developing embryo, HA accumulates at sites of cell migration and proliferation and has been proposed to play important roles in craniofacial, limb, heart, and neural tube development (35–44). Over the last 10 years, HA has received considerable attention through the identification of specific cell surface receptors and binding proteins for HA (hyaladherins). These proteins appear to mediate the effects of HA upon cell behavior (reviewed in Refs. 45–47). The study of...
Fig. 5. Northern analyses of mouse Has2 expression. Multiple tissue Northern blots of poly(A)⁺ RNA isolated from mouse embryos and adult tissues were hybridized with a mouse Has2 ORF cDNA probe as described under “Experimental Procedures.” Blots were washed to high stringency. The mouse embryo blot was exposed overnight at −70 °C with two intensifying screens, whereas the adult tissue blot was exposed for 6 days at −70 °C with two screens. Both blots were stripped and rehybridized with a mouse GAPDH probe to control for variation in loading. Both GAPDH hybridized blots were exposed for 1 h at −70 °C with two screens. Mouse Has2 transcripts of approximately 4.8 kb and 3.2 kb were observed in the embryonic RNA samples, whereas only the 4.8-kb message was observed in RNA from adult tissues. The relative positions of RNA molecular weight markers are indicated at the left of each blot.

Fig. 6. Southern analysis of mouse Has2. Total 129Sv/J mouse genomic DNA was digested with the restriction enzymes EcoRI (E), BamHI (B), HindIII (H), and SacI (S). Digested DNAs were separated by electrophoresis through 0.8% agarose gels, transferred to nylon membranes by Southern blotting, and hybridized to a radiolabeled mouse Has2 cDNA probe corresponding to the complete ORF, as described. Membranes were washed at low, intermediate, and high stringency, and the hybridizing bands were compared in each case. There was no difference in the pattern obtained at all three stringencies. This indicates that the mouse Has2 probe, although related to mouse HAS (Has1), failed to identify restriction fragments derived from this gene even at low stringency. M indicates 1 kilobase pair ladder.

HA itself, however, has not been easy as no eukaryotic genes that encode proteins involved in the HA biosynthetic pathway have been identified until recently (17).

In the bacterium, *Streptococcus pyogenes*, the ability to synthesize an HA capsule segregates as a virulence factor (10). A major advance in our understanding of HA biosynthesis has come through the characterization of the genes required for HA biosynthesis in *S. pyogenes*. Polymerization of HA occurs through the action of a single enzyme, HA synthase, encoded by the hasA gene (11). This protein is localized to the membrane and is predicted to have several transmembrane domains and a large intracellular loop encompassing the active site of the enzyme. Transfer of the hasA gene and a second gene, hasB, into heterologous bacterial species allows them to synthesize an HA capsule (11). The hasB gene encodes a UDP-glucose dehydrogenase, which converts UDP-glucose to UDP-glucuronic acid (UDP-GlcUA), a subunit of HA. Furthermore, purified, immobilized HasA has been shown to be sufficient for HA polymerization in vitro (12).

A second protein, originally identified in *Streptococcus equisimilis* as the HA synthase (48), has no sequence similarity to *S. pyogenes* HasA. However, this protein has significant sequence similarity to bacterial proteins involved in oligopeptide binding and transport. Although the total amount of HA synthesized by bacterial cells overexpressing this protein increased, the length of the resultant HA chains was significantly shorter, suggesting that the increase may be a function of an elevation in the rate of HA transport from the cell (49). Thus, rather than being directly involved in HA biosynthesis, this protein may play a role in the transport of HA (49). Antibodies raised against the *S. equisimilis* protein cross-reacted with a 52-kDa protein present in the membrane of mouse B6 cells (50). This mammalian protein associates with the HA receptor, RHAMM, and was proposed to represent the eukaryotic hyaluronan synthase (50). It is more likely, however, that this protein may play a role in the transport of HA, or may participate in HA synthesis as an accessory molecule, rather than as the synthase itself.

Using degenerate RT-PCR, we identified a novel mouse gene, Has2, that encoded a protein with significant sequence identity to DG42, HasA, NodC, and Chs2 (Fig. 3). In addition, mouse Has2 is related to but distinct from a recently reported mouse DG42 homolog with chitin oligosaccharide synthase activity (30). Based upon the identification of two related putative mammalian hyaluronan synthase (Has) genes, we propose the nomenclature Has1, Has2, and so on. According to this nomen-
culation, the recently reported mouse HAS gene would be designated mouse Has1.

Residues demonstrated to be critical in terms of the (β1→4)glycosyltransferase activity of yeast Chs2 were conserved in mouse Has2, mouse Has1, Streptococcal HasA, *Xenopus* DG42, and *Rhizobium* NodC (Fig. 3B). Furthermore, although overall sequence identity between mouse Has2 and *S. pyogenes* HasA was only 21%, a 180-amino acid region within the predicted intracellular loop (residues 182 to 361) was highly conserved. This region exhibited 54% similarity between mouse Has2 and bacterial HasA, and greater than 80% similarity between mouse Has2, mouse Has1, and *Xenopus* DG42. This level of sequence conservation suggests that these proteins are functionally related. Experiments are currently in progress to investigate the effects of mutation of the conserved residues on mouse Has2 function.

Sequence analyses predicted that mouse Has2 encodes a membrane protein with multiple transmembrane domains, similar to the predicted structures for bacterial HasA protein and mouse Has1 (Fig. 4). This prediction fits well with the results obtained from previous studies, which have localized mammalian HA synthase to the plasma membrane (2–7). Significantly, four consensus binding sites for HA were identified, three of which were predicted to be intracellular. These sites may thus represent areas of potential binding of HA chains during elongation and/or may represent sites at which the newly synthesized HA polymer remains attached prior to release from the cell. In addition to putative HA binding sites, numerous consensus sequences for phosphorylation by PKC and cAMP-dependent kinases were identified within the predicted intracellular loop of the molecule. This is significant, as mammalian HA biosynthesis has been shown to be dependent on activation by PKC (8, 9), and suggests that the PKC dependency may partly involve direct activation of Has2 through phosphorylation. Experiments to investigate the role of potential PKC phosphorylation sites on Has2 function are currently starting.

Localization of HA has been described in some detail in the developing mouse embryo (51, 52). HA is present at significant levels starting as early as the egg cylinder stage (5.5 dpc) (51), when it is secreted into the expanding yolk cavity. Based upon the expression pattern of HA in the early postimplantation embryo, HA has been proposed to play a role in the formation and expansion of embryonic cavities (51). From 9.5 dpc, synthesis increases, and the HA assumes a pericellular distribution, rather than being primarily associated with fluid-filled spaces (52). HA is present at high levels within the developing vertebral column, the neural crest-derived mesenchyme of the craniofacial region, and the heart and smooth muscle throughout the midgestation embryo (52). In adult tissues, HA expression has been detected in tissues including brain, central nervous system, cartilage, skin, cardiac and skeletal muscle, lung, and lymph node (45, 53–58).

The observed expression pattern of mouse Has2, based upon our Northern analyses, correlates well with the previously described expression pattern of HA. We detected expression of Has2 in the primitive streak stage embryo (7.5 dpc) and an increase in Has2 expression in the later embryo. This is in contrast to the reported expression patterns for *Xenopus* DG42 (24) and a recently reported zebrafish DG42 homolog (30), which are expressed during a narrow window of embryonic development corresponding to gastrulation and neurulation. In the adult mouse, Has2 expression was detected in heart, brain, spleen, lung, and skeletal muscle, but not in liver or kidney (Fig. 5). The level of expression of Has2 was markedly reduced in adult tissues as compared to the embryo. Certain cells surround themselves in culture with a pericellular matrix or coat, which has been shown to depend upon the presence of hyaluronan (59, 60). The HA coat can be visualized through a simple particle exclusion assay (59). Fixed red blood cells are added to the culture medium and, upon settling, are excluded from a region surrounding each cell by the HA coat, as a consequence of the size and charge of the HA. Treatment of cells with hyaluronidase removes the coat from the cells. HA-dependent pericellular coats have been proposed to form through two alternate mechanisms. The first mechanism is HA receptor-dependent and HA synthesis independent. This type of coat can form through association of HA with cell surface HA receptors, and stabilization of the coat by association of HA binding proteoglycans, such as aggrecan and link protein (60, 61). Presumably, this permits cells expressing HA receptors to enter an environment rich in HA and to organize an HA matrix around themselves that is independent of the ability to synthesize HA. The second mechanism is HA receptor independent and requires the synthesis and extrusion of HA through the plasma membrane. It has been proposed that the extruded HA associates with the membrane through continued attachment to the synthesize, and that this coat is stabilized by HA-HA and HA-protein bridges (62).

Expression of mouse Has2 by COS-1 cells enabled them to form large well-pronounced HA coats, as determined by the particle exclusion assay (Fig. 7). Previous studies in COS cells have shown that transfection of the HA receptor, CD44, and the presence of exogenous HA (15 μg/ml) and proteoglycans to the medium was required for HA-dependent pericellular matrix formation (61). In contrast, our studies demonstrate that expression of mouse Has2 in COS cells, in the absence of HA receptor expression, exogenously added HA, or proteoglycans, was sufficient for HA coat formation. This suggests that Has2 expression leads to the synthesis of HA, which is extruded through the plasma membrane and may associate with the cell surface to form an HA coat through continued attachment to the synthesize. In this respect, the consensus HA binding motifs predicted within mouse Has2 may play an important role.

HA biosynthesis requires two enzyme activities: to transfer UDP-N-acetylgalactosamine (UDP-GlcNAc) and UDP-glucuronic acid (UDP-GlcUA), respectively, to the growing HA chain (63). In *S. pyogenes*, a single enzyme, HasA, carries out both activities. Based upon our sequence analyses, it is clear that mouse Has2 and the recently reported mouse HAS (now designated Has1 according to our nomenclature) (17) are related to streptococcal HasA and to *Xenopus* DG42. It is possible, therefore, that DG42 encodes a *Xenopus* HA synthase. Indeed, a recent report has demonstrated that expression of *Xenopus* DG42 in mammalian cells leads to the synthesis of hyaluronan (64). In contrast, it has been shown that recombinant DG42 protein could synthesize short chitin oligomers from UDP-GlcNAc in vitro, but could not synthesize a hyaluronan chain in the presence of UDP-GlcNAc and UDP-GlcUA (65). It is conceivable, however, that recombinant DG42 may not have the same enzyme activity as DG42 translated in vivo. More recently, studies have suggested that *Xenopus* DG42 and its related vertebrate homologs are chitin oligosaccharide synthases active in early embryogenesis (30) and thus may not represent true HA synthases. Significantly, the same group reported that treatment of embryonic extracts with chitinase almost completely inhibited HA synthase activity (30), suggesting that chitin may play an important role in vertebrate HA synthesis, possibly as a primer.

Collectively, our results and others demonstrate that expression of *Xenopus* DG42 and related mammalian Has genes leads to HA synthesis in mammalian cells. This may proceed through
direct HA synthase activity of the enzymes or through the synthesis of chitin oligosaccharides that act as primers that are required for and the limiting factor for HA synthesis. The identification of two mammalian genes related to Xenopus DG42 leads to obvious questions. Do both encoded proteins function in the same manner? What is the expression pattern of the two genes? Based upon the level of sequence identity between the two mouse Has genes, it is likely that both enzymes at least have β1→4glycosyltransferase activity. Future studies will clearly need to address the relationship between Has1 and Has2 and their respective roles in chitin and hyaluronan synthesis. This will lead to important insights into the biosynthesis of hyaluronan in mammalian cells.

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