

## Regulation of Hyaluronidase Activity by Alternative mRNA Splicing\*

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Hyaluronidase is a hyaluronic acid-degrading endoglycosidase that is present in many toxins and the levels of which are elevated in cancer. Increased concentration of HYAL1-type hyaluronidase correlates with tumor progression and is a marker for grade (G) 2 or 3 bladder cancer. Using bladder tissues and cells, prostate cancer cells, and kidney tissues and performing reverse transcription-PCR, cDNA cloning, DNA sequencing, and *in vitro* translation, we identified splice variants of HYAL1 and HYAL3. HYAL1v1 variant lacks a 30-amino acid (aa) sequence (301–330) present in HYAL1 protein. HYAL1v1, HYAL1v2 (aa 183–435 present in HYAL1 wild type), HYAL1v3 (aa 1–207), HYAL1v4 (aa 260–435), and HYAL1v5 (aa 340–435) are enzymatically inactive and are expressed in normal tissues/cells and G1 bladder tumor tissues. However, HYAL1 wild type is expressed in G2/G3 tumors and in invasive tumor cells. Stable transfection and HYAL1v1-specific antibody confirmed that the HYAL1 sequence from aa 301 to 330 is critical for hyaluronidase activity. All tumor cells and tissues mainly express HYAL3 variants. HYAL3v1 lacks a 30-aa sequence (299–328) present in HYAL3 protein, that is homologous to the 30-aa HYAL1 sequence. HYAL3v1, HYAL3v2 (aa 251–417 present in HYAL3 wild type), and HYAL3v3 (aa 251–417, but lacking aa 299–328), are enzymatically inactive. Although splicing of a single independent exon generates HYAL1v1 and HYAL3v1, internal exon splicing generates the other HYAL1/HYAL3 variants. These results demonstrate that alternative mRNA splicing controls cellular expression of enzymatically active hyaluronidase and may explain the elevated hyaluronidase levels in bladder/prostate cancer.

Hyaluronidases (HAases)<sup>1</sup> are a family of enzymes that are crucial for the spread of bacterial infections, toxins present in

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF502904 (HYAL1v1), AF502905 (HYAL1v2), AF502906 (HYAL1v3), AF502907 (HYAL1v4), AF502908 (HYAL1v5), AF502909 (HYAL3v1), AF502910 (HYAL3v2), AF502911 (HYAL3v3), and AF502912 (HYAL3wt).

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<sup>1</sup> The abbreviations used are: HAase, hyaluronidase; aa, amino acid(s); HA, hyaluronic acid; NED, no evidence of disease; BT, bladder

various venoms, and possibly, cancer progression (1–6). In humans, six HAase genes have been identified. These genes occur in clusters of three at two chromosomal locations (Ref. 7 and human genome blast search). HYAL1, HYAL2, and HYAL3 occur on chromosome 3p21.3, and PH20, HYAL4, and HYALP1 occur on chromosome 7q31.3. With the possible exception of HYAL4 and HYALP1, all other HAases degrade hyaluronic acid (HA) (7).

HA is a nonsulfated glycosaminoglycan made up of repeating disaccharide units, D-glucuronic acid, and N-acetyl-D-glucosamine. HA is present in body fluids, tissues, and the extracellular matrix (8–10). It keeps tissues hydrated and maintains osmotic balance and cartilage integrity (8, 10). HA also actively regulates cell adhesion, migration, and proliferation by interacting with specific cell surface receptors such as CD44 and RHAMM (11). The concentrations of HA are elevated in several inflammatory diseases and various carcinomas (*e.g.* bladder, prostate, lung, colon, and so forth; Refs. 9 and 12–19). For example, we have shown that urinary HA concentration is a highly sensitive and specific marker for detecting bladder cancer, regardless of its grade (18, 19). In tumor tissues, HA may promote tumor growth and metastasis probably by actively supporting tumor cell migration and offering protection against immune surveillance (20–22). Small fragments of HA, generated by HAases, stimulate angiogenesis (23–25). We recently showed that HA fragments of ~10–15 disaccharide units stimulate endothelial cell proliferation by acting through cell surface HA receptor, RHAMM, and activating the mitogen-activated protein kinase pathway (26). We have also shown that elevated levels of HYAL1-type HAase coincide with the presence of angiogenic HA fragments in prostate tumor tissues and in the urine of bladder cancer patients (17, 27).

Among the six HAases, HYAL1, HYAL2, and PH20 are well characterized. HYAL1 type HAase was originally purified from human plasma and urine (28, 29). However, we have shown that HYAL1 is the major tumor-derived HAase expressed in bladder and prostate cancer tissues (17, 30). It has an optimum pH range of 4.0–4.3, and the enzyme is 50–80% active at pH 4.5 (17). Triggs-Raine *et al.* (31) have shown that a lack of functional HYAL1 results in a disorder called mucopolysaccharidosis IX. In this study the authors identified that aa Glu<sup>268</sup> is crucial for HYAL1 activity. HYAL2 was originally designated as the lysosomal HAase, and it cleaves high molecular mass HA into ~20-kDa HA fragments (32). It has a pH optimum of ~4.0 and is possibly less active than other HAases. HYAL-2 may also be exposed to the cell surface through a GPI anchor (32). The third HAase gene in the 3p21.3 locus is HYAL3. Although

tumor; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay; TIM, triose phosphate isomerase.

HYAL3 transcripts have been detected in brain and liver tissues, its protein product is uncharacterized (31). Based on the sequence information deposited in GenBank™ HYAL3 is predicted to be made up of either 463 aa (accession number AF040710 (gi number 2935327)) or 417 aa (accession number BC012892 (gi number 15277616)).

Besides acidic HAases, PH20 (*i.e.* testicular HAase) is well characterized. PH20 is a sperm surface HAase that has a broad pH activity profile (pH 3.2–9.0) (33). In addition to being a HA-degrading enzyme, PH20 may also interact with HA to increase internal calcium, possibly by binding HA through aa 205–235 (34). This study also revealed that *N*-glycosylation and intrachain disulfide linkages are important for the HAase activity of PH20 (34).

Recently, the crystal structure of the bee venom HAase has been documented (35). The bee HAase shares ~30% sequence identity with human HAases. The crystal structure of bee HAase reveals a classical TIM barrel topology, where the catalytic site corresponds to Asp<sup>143</sup> and Glu<sup>145</sup> (aa numbering according to GenBank™ accession number AAA27730.1). Glu<sup>145</sup> is involved in the cleavage of the β-1,4 glycosidic bond between *N*-acetyl-D-glucosamine and D-glucuronic acid through an acid-base catalytic mechanism. These Asp and Glu residues are conserved in all mammalian HAases (32). For example, in HYAL1, the putative catalytic site residues are Asp<sup>131</sup> and Glu<sup>133</sup>, respectively, and in HYAL3, they are Asp<sup>127</sup> and Glu<sup>129</sup>, respectively (according to GenBank™ accession numbers AAD09137.2 and AAH12892.1). Based on the amino acid homology between bee HAase and mammalian HAases and conservation of several aa residues involved in both HA binding (*i.e.* substrate binding groove) and its catalysis (*i.e.* active site), a TIM barrel topology and a similar mechanism for the catalytic cleavage of HA are likely for the mammalian HAases (32).

In this study, we have identified several mRNA splice variants of HYAL1 and HYAL3 expressed in bladder and prostate cancer cells and in bladder and kidney tissues. These splice variants were characterized in terms of their protein product and HAase activity. We also identified a 30-aa region that is crucial for the HAase activity of HYAL1 and HYAL3 proteins.

#### EXPERIMENTAL PROCEDURES

**Tissue Specimens**—Normal bladder specimens were obtained from organ donors (20–50 years old), and the urothelial layer from these specimens was flash frozen after separation from the underlying muscle. Bladder tumor tissues (~1 g), bladder specimens with no evidence of disease (NED), and specimens of involved lymph node (*i.e.* local extension of tumor into lymph node) were obtained from bladder cancer patients undergoing cystectomy or transurethral resection of bladder tumor. The tissue specimens were split, and the mirror segment was examined by histology following fixation in formalin. In this study, we have included data from only those specimens that were histologically confirmed as normal and tumor (with grade information). All of the tissues were flash frozen and stored at –70 °C until use.

**Tissue Culture**—Established bladder cancer cell lines (*i.e.* HT1376, RT4, and UMUC-3) and prostate cancer cell lines (*i.e.* DU145 and LNCaP) were obtained from the American Type Culture Collection. Dr. Mark E. Stearns (Medical College of Pennsylvania, Philadelphia, PA) kindly provided the prostate cancer line PC3-ML (36). Bladder cancer cell lines 253J-Lung and 253J-parent were gifts from Dr. Colin Dinney (M. D. Anderson Cancer Center, University of Texas, Houston, TX) (37). All of these cell lines were cultured in RPMI 1640, 10% fetal bovine serum, and gentamicin. Primary normal bladder cell cultures were set up from the normal urothelium. The urothelial cells were gently scrapped from the bladder urothelium in RPMI 1640 containing 40% fetal bovine serum. The cell suspension (2 ml) was added to T-25 tissue culture flasks, and the cells were allowed to attach overnight. The following day, the nonadherent cells were washed off, and the cells were cultured in keratinocyte growth medium (Clonetics/BioWhittaker, San Diego, CA) until 80% confluence. The epithelial growth was confirmed by cytokeratin staining. Total RNA was extracted from the urothelial cultures on either the first or the second passage.

**RT-PCR, cDNA Cloning, and Sequence Analyses**—Total RNA was extracted from bladder/prostate cells, bladder tissues, and lymph node specimens using a RNA extraction kit (Qiagen). Total RNA from kidney tissues was purchased from BD Biosciences/CLONTECH (Palo Alto, CA). Total RNA (~1 μg) was subjected to first strand cDNA synthesis using a Superscript™ preamplification system and oligo(dT) primers (Invitrogen). The entire HYAL1 coding sequence was amplified from the first strand cDNA using a HYAL1-L3/HYAL1-R2 primer pair that we have used previously (17). HYAL1-L3 primer sequence is as follows: 5'-CTTCTCCAGGAGTCTCTGGT-3'. The HYAL1-R2 primer sequence is as follows: 5'-ATCACCACATGCTCTTCCGC-3'. This primer pair should amplify a 1926-bp HYAL1 cDNA that contains the entire coding region of HYAL1 (618–1925). When compared with the HYAL1 clone HSU03056 that is deposited in GenBank™, HYAL1 cDNA amplified by the HYAL1-L3/HYAL1-R2 primer pair will contain an extra C at the 5' terminus. This C is present in the human LUCA13 cosmid clone from the chromosome region 3p21.3 (GenBank™ accession number ACC002425). Thus, in this study, as compared with the HYAL1 clone HSU03056, the numbering of nucleotides in HYAL1wt and HYAL1 variant cDNAs is offset by 1.

To amplify HYAL3 cDNA, we used the following primer pair: 1) HYAL3-L2: The sequence of this primer corresponds to nucleotides 157–176 in the HYAL3 clone AF040710. This sequence is not present in the HYAL3 clone BC012892. HYAL3-L2 primer sequence is as follows: 5'-CCAGAGGCCAGCATCAACAT-3'. 2) HYAL3-R2: The sequence of this primer (5'-GACTCACATGATCTCAGAGG-3') is reverse complementary to the sequence between nucleotides 1629 and 1648 in the HYAL3 clone AF502912, nucleotides 1628 and 1647 in the clone AF040710, and nucleotides between 1464 and 1483 in the clone BC012892. The HYAL3-L2/HYAL3-R2 primer pair should amplify a 1491-bp HYAL3 product. The amplification of HYAL1 and HYAL3 cDNAs was carried out by PCR as described previously (17). PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

The PCR products were directly cloned into the eukaryotic expression vector pcDNA3.1/v5/His-TOPO using a TOPO cloning kit (Invitrogen). This expression vector contains a strong cytomegalovirus promoter for eukaryotic expression, a neomycin resistance gene for selecting stable mammalian cell transfectants, and an ampicillin resistance gene for bacterial selection. All of the cloned HYAL1 and HYAL3 cDNAs were sequenced in an automated DNA sequencer in the DNA core facility at the University of Miami.

**Generation of RT4 Stable Transfectants**—RT4 cells (2 × 10<sup>5</sup> cells/6-cm dish) were transfected with 5 μg of either HYAL1 wild type (HYAL1wt) or HYAL1v1 (one of the HYAL1 variants) or pcDNA3.1/v5/His-TOPO vector using the Effectene™ transfection agent (Qiagen). Following 48 h of incubation in the growth medium (RPMI 1640, 10% fetal bovine serum, gentamicin), the transfectants were selected in neomycin (100 μg/ml)-containing growth medium. The RT4 clones resistant to neomycin were expanded and tested for HAase activity and HYAL1 protein expression using an HAase activity ELISA-like assay and immunoblot analysis, respectively, as described below.

**In Vitro Translation**—HYAL1 and HYAL3 cDNAs were *in vitro* translated using the TNT® Quick coupled transcription/translation system (Promega, Madison, WI). For generating [<sup>35</sup>S]methionine-labeled product, 1 μg of cDNA (vector, HYAL1, or HYAL3) was mixed with TNT® Quick master mix and 20 μCi of Amersham Biosciences Redivue™ L-[<sup>35</sup>S]methionine. The reaction was carried out at 30 °C for 90 min. Following incubation, 5-μl aliquots of each sample were mixed with 20 μg of bovine serum albumin and precipitated with equal volume of ice-cold 20% trichloroacetic acid. The precipitates were centrifuged, washed twice in 10% trichloroacetic acid, and washed once with 80% ice-cold ethanol. The precipitates were dissolved in SDS sample buffer and analyzed by 12% SDS-PAGE under reducing/nonreducing conditions.

Alternatively, the HYAL1/HYAL3 cDNAs or vector DNA were mixed with TNT® Quick master mix and 20 μM unlabeled L-methionine. The reaction was carried out at 30 °C for 90 min. The unlabeled *in vitro* translated products were analyzed for HAase activity by the ELISA-like assay.

**HAase ELISA-like Assay**—HAase ELISA-like assay has been described previously (17, 18, 38, 39). To assess HAase activity secreted by RT4 transfectants and bladder cancer cells, at ~60% confluence, various cultures were washed three times in phosphate-buffered saline and incubated in RPMI 1640 containing insulin, transferrin, and selenium solution (ITS supplement, Sigma-Aldrich). Following a 48-h incubation, the serum-free conditioned media were collected. For the HAase activity ELISA-like assay, various aliquots (0.5, 1.0, 2.5, 5.0, 7.5, and 10 μl)

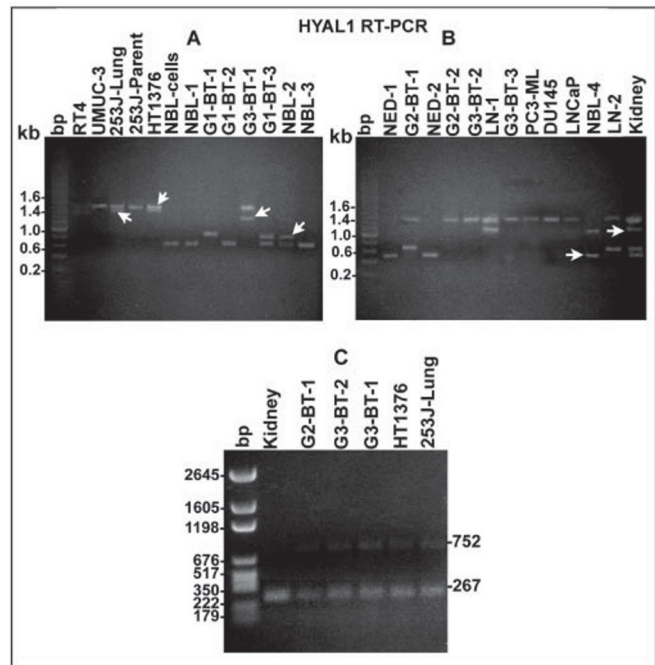
of either *in vitro* translated samples, serum-free culture-conditioned media of different cells/transfectants or urine from bladder cancer patients or normal individuals were incubated with 96-well HA-coated microtiter plates in the presence of HAase assay buffer. Following incubation, the degraded HA was washed off, and the HA remaining on the wells was detected using a biotinylated HA-binding protein and an avidin-biotin detection system. The HAase present in each sample was calculated using a standard graph, which was prepared by plotting  $A_{405\text{ nm}}$  versus HAase concentration (units  $\times 10^{-4}/\text{ml}$ ) (18, 39). In the *in vitro* translated samples, the  $A_{405\text{ nm}}$  of each HYAL1/HYAL3 sample was subtracted from the  $A_{405\text{ nm}}$  of the vector sample, and the difference was used to determine the HAase activity.

**Generation of Anti-HYAL1v1 Peptide Antibody**—A 13-aa HYAL1v1 peptide (NH<sub>2</sub>-TNHFPLESCQAI-COOH) was synthesized, conjugated to keyhole limpet hemocyanin, and injected in New Zealand rabbits. The anti-HYAL1v1 antibody was purified using the HYAL1v1 peptide-conjugated affinity chromatography. The synthesis of HYAL1v1 peptide, generation of rabbit polyclonal antibody, and affinity purification were carried out by ResGen™ Invitrogen Corp. (Huntsville, AL).

**Immunoblot Analysis**—Conditioned media of bladder cancer cells and stable transfectants and urine specimens were separated by 8.5% SDS-PAGE under nonreducing conditions. The gels were blotted on to polyvinylidene difluoride membranes. The blots were probed with either anti-HYAL1 peptide IgG (30) or anti-HYAL1v1peptide affinity-purified IgG at 4 °C for 16 h. The blots were developed using an alkaline phosphatase detection system, as described previously (30).

## RESULTS

**Detection of HYAL1 Splice Variants by RT-PCR, cDNA Cloning, and Sequence Analysis**—Based on the Human Genome Blast search, HYAL1 gene contains three exons separated by two introns (NT\_006014.7/HS3\_6171, *Homo sapiens* chromosome 3 working sequence). It has been previously shown that the untranslated region in exon 1 between nucleotides 110 and 596 is alternatively spliced (GenBank™ accession number AF173154). To detect the expression of HYAL1 in various cancer cells, tumor tissues, and normal kidney tissue, we performed RT-PCR analysis using a HYAL1-specific primer pair. This primer pair should amplify the entire HYAL1 coding region (bp 618–1926), an untranslated region in exon 1 that is alternatively spliced (nucleotide 110 joining 596), and the first 110 bp. Thus, the length of the expected PCR product is 1926 nucleotides. Fig. 1 (A and B) shows two gels in which PCR products from various samples were analyzed. The different tissue specimens are numbered (e.g. NBL-1 to NBL-4; G1-BT-1 to G1-BT-3; G2-BT-1 and G2-BT-2; G3-BT-1 to G3-BT-3; NED-1; NED-2; LN-1 and LN-2). An approximately 1.4-kb product is amplified from bladder cancer cells (i.e. HT1376, 253J-Lung, 253J-parent, and UMUC-3), prostate cancer cells (i.e. DU145, LNCaP, and PC3-ML), G2 and G3 bladder tumor tissues (G3-BT-1, G2-BT-1, G2-BT-2, G3-BT-2, and G3-BT-3), lymph node specimens invaded with bladder tumor (LN-1 and LN-2) and normal kidney tissue (Fig. 1, A and B). RT4 RNA sample showed negligible amplification of any HYAL1-related PCR products. A smaller ~1.3-kb PCR product is also amplified from 253J-Lung and HT1376 RNA samples (Fig. 1A). The same ~1.3-kb PCR product was also detected in the kidney tissue and some bladder tumor tissue specimens when electrophoresis was carried out for a longer period of time to allow separation between ~1.4- and ~1.3-kb PCR products (data not shown). As shown in Fig. 1 (A and B), an approximately 500-bp PCR product is amplified from RNA samples isolated from a primary normal bladder cell culture (NBL cells), normal bladder tissues (NBL-1, NBL-2, NBL-3, and NBL-4), kidney tissue, and G1 bladder tumor tissues (G1-BT-2 and G1-BT-3). An approximately 650-bp PCR product is also amplified from two G1 bladder tumor tissues (G1-BT-1 and G1-BT-3), NBL2 tissue, normal kidney tissue, and an LN-2 specimen (Fig. 1, A and B). An approximately 1.0-kb PCR product is amplified from a G3 bladder tumor tissue (G3-BT-1) and an LN-1 specimen (Fig. 1



**FIG. 1. Examination of HYAL1 expression in tissues and cells.** Total RNA extracted from bladder tissues, bladder cells (tumor cells: RT4, UMUC-3, 253J-Lung, and 253J-parent and HT1376; NBL cells: normal bladder cells), prostate cancer cells (PC3-ML, DU145, and LNCaP) was subjected to RT-PCR analysis using HYAL1-specific primers as described under "Experimental Procedures." The PCR products were analyzed by agarose gel electrophoresis. Six PCR products of different lengths are marked. PCR products expressed in each of these tissues or cells were cloned and sequenced. The tissue specimens in each category are numbered (e.g. NBL-1, NBL-2, G1-BT-1, G3-BT-1, LN-1, etc.). A and B, RT-PCR analysis using primer pair HYAL1-L3 and HYAL1-R2. This primer pair should amplify an approximately 1.9-kb HYAL cDNA (i.e. 1–1926 bp) that contains the HYAL1 coding region (618–1925 bases). A 200-bp ladder (Promega) was used as a bp marker. C, RT-PCR analysis using the primer pair HYAL1-L3 and HYAL1-R3. This primer pair should amplify a 267- or 752-bp PCR product depending upon the splicing status of the HYAL1 transcript related to the untranslated region between nucleotides 110–596. pGEM markers (Promega) were used as a bp ladder.

A and B). A slightly larger PCR product (~1.1-kb) was detectable in NBL-4 and kidney tissue samples (Fig. 1B).

Because the RT-PCR analysis described above does not result in the amplification of an approximately 1.9-kb PCR product that is expected based on the primer pair design, there could be two possibilities. First, such a product may have been missed because of the large size of the PCR product, and second, bladder tissues/cells contain only the HYAL1 transcript in which the bp between 110 and 596 are spliced out. The second possibility is based on the sequences deposited in GenBank™, which show that the 5'-untranslated region between nucleotides 110 and 596 is alternatively spliced (e.g. accession numbers HSU03056 and AF173154). To test these possibilities we performed RT-PCR analysis using a HYAL1 primer pair (i.e. HYAL1-L3/HYAL1-R3) that lies outside the boundaries of the spliced region. This primer pair would generate a 267-bp PCR product if the region between nucleotides 110 and 596 is spliced out, and a 752-bp PCR product will be generated if the HYAL1 transcript is unspliced. We have previously used this primer pair to demonstrate the presence of HYAL1 splicing in this region in prostate cancer cells (17). As shown in Fig. 1C, a major 267-bp PCR-amplified product is present in all samples, which include kidney, G2-BT-1, G3-BT-2, G3-BT-1, HT1376, and 253J-Lung. However, a minor 752-bp product is visible in bladder cancer cells and bladder tumor tissues. The presence and absence of the region between nucleotides 110 and 596 was

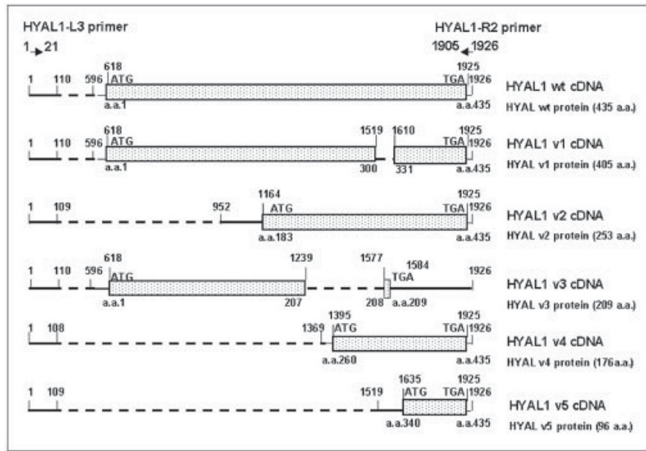


FIG. 2. A schematic representation of the HYAL1 splice variants. The figure shows HYAL1wt and different splice variant cDNAs. The numbering is based on the sequence of cDNA clone HSU03056 that is deposited in GenBank<sup>TM</sup>. However, the numbering is offset by 1. This is because HSU03056 clone lacks the nucleotide C that is present in the 5' terminus of all HYAL1 clones described in this study. The coding region is shown as a dotted block. The dashed line shows the region that is spliced out, and solid line represents untranslated regions. Each splice junction is marked by the nucleotides at the 5' and 3' boundaries that are joined because of splicing. The translation initiation and termination codons for HYAL1wt and HYAL1 variants are marked. The figure also shows the position of the forward (*i.e.* HYAL1-L3) and the reverse complementary (*i.e.* HYAL1-R2) primer pair used to amplify HYAL1 cDNA. The sequences of these primers are given under "Experimental Procedures."

confirmed by cDNA cloning and sequencing of the 267- and 752-bp PCR products as we described before (17). These results indicate that it is very likely that the HYAL1 transcripts in which the region between nucleotides 110 and 596 is unspliced are not the major HYAL1 transcripts expressed in bladder tumor tissues and cells. Furthermore, lack of amplification of an approximately 1.9-kb PCR product in tissue/cell specimens may be due to some technical difficulty associated with the amplification of large PCR products.

All of the different PCR products from each sample were gel-isolated and directly cloned into the eukaryotic expression vector pcDNA3.1/v5/His-TOPO. The sequence analyses revealed that various PCR products represent HYAL1 mRNA splice variants. The cDNA and amino acid sequences of each of the five HYAL1 splice variants described in this study have been deposited in GenBank<sup>TM</sup>. As shown in Fig. 2, the ~1.4-kb product is actually 1441 bases in length and is generated by splicing of the region from nucleotides 109 to 595 and, thus, joining nucleotides 110–596. This HYAL1 splice variant that arises from an internal splicing event in exon 1 has been identified previously (GenBank<sup>TM</sup> accession number AF173154 (gi number 5825510)). For the purpose of this study, we have designated the 1441-bp HYAL1 cDNA as HYAL1wt. The cloned HYAL1wt cDNA encodes the intact wild type HYAL1 protein consisting of 435 aa, the sequence of which is identical to that deposited in GenBank<sup>TM</sup> (accession number AAD09137.2)

The actual length of the ~1.3-kb product is 1351 bases. It lacks the region in Exon 1 between nucleotides 110 and 596 and, in addition, contains a deletion that joins nucleotides 1519 and 1610 (*i.e.* nucleotides 1520–1609 are deleted; Fig. 2). This cDNA, designated as HYAL1v1 (HYAL1 variant 1; GenBank<sup>TM</sup> accession number AF502904), can encode a protein that is 405 aa in length. When compared with the HYAL1wt protein sequence, the HYAL1v1 protein lacks 30 aa from aa 301–330. Thus, in the putative HYAL1v1 protein, aa 300 is joined to aa 331 (Fig. 2).

The HYAL1v2 cDNA consists of 1084 bases (Fig. 2). In this

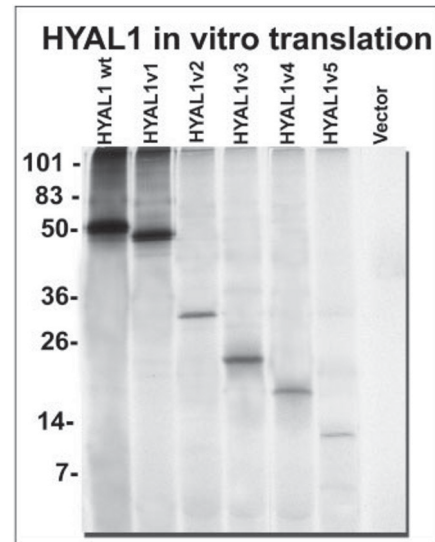


FIG. 3. *In vitro* transcription/translation of HYAL1wt and HYAL1 variant cDNAs. Various HYAL1 cDNAs and plasmid vector DNA were transcribed and translated *in vitro* using a coupled transcription/translation system and <sup>35</sup>S-labeled methionine, as described under "Experimental Procedures." The <sup>35</sup>S-labeled proteins were analyzed by SDS-PAGE along with molecular mass markers as described under "Experimental Procedures."

variant, nucleotide 109 is joined to nucleotide 952, *i.e.* the sequence from nucleotides 110 to 951 is deleted (GenBank<sup>TM</sup> accession number AF502905). If the HYAL1v2 variant is translated, using an ATG at position 1164 as the start codon, it will generate a protein consisting of 253 aa. The sequence of the putative HYAL1v2 protein is identical to the HYAL1wt protein sequence from aa 183 to 435.

The slightly larger ~1.1-kb HYAL1 variant is designated as HYAL1v3, and it is 1104 bases in length (Fig. 2). HYAL1v3 contains two spliced regions; in the first, nucleotide 110 joins nucleotide 596, and in the second, nucleotide 1239 joins nucleotide 1577 (*i.e.* bases from 1240 to 1576 are deleted; GenBank<sup>TM</sup> accession number AF502906). If HYAL1v3 is translated, it will utilize ATG<sup>618</sup> as the start codon and generate a protein consisting of 209 aa (Fig. 2). The first 207 aa of the putative HYAL1v3 protein are identical to the first 207 aa of the HYAL1wt protein.

The ~0.65-kb PCR product originates from a HYAL1v4 variant whose actual length is 666 bases (Fig. 2). The HYAL1v4 cDNA contains a deletion starting from nucleotide 109 and ending at nucleotide 1368. Thus, in the HYAL1v4 cDNA, nucleotide 108 is joined to nucleotide 1369 (GenBank<sup>TM</sup> accession number AF502907). The HYAL1v4 cDNA contains an open reading frame starting at ATG<sup>1395</sup> that allows translation of a polypeptide consisting of 176 aa. The aa sequence of the putative HYAL1v4 protein is identical to the aa sequence of HYAL1wt starting with aa 260–435 (Fig. 2).

The splice variant HYAL1v5 consists of 517 bases, and this variant is generated by splicing of nucleotides 109 and 1519 (GenBank<sup>TM</sup> accession number AF502908). If ATG<sup>1635</sup> is used as the start codon, the HYAL1v5 variant will encode a protein made up of 96 aa, the sequence of which is identical to the HYAL1wt protein sequence from aa 340 to 435 (Fig. 2).

*In Vitro Transcription and Analysis of HAase Activity of HYAL1 Variant Proteins*—To determine whether various HYAL1 variants encode HAase activity, we used a coupled *in vitro* transcription/translation system and <sup>35</sup>S-labeled L-methionine to generate HYAL1wt and HYAL1 variant proteins. As shown in Fig. 3, the *in vitro* translated HYAL1wt and HYAL1 variant (v1–v5) proteins are of molecular mass ~50, ~47, ~30,

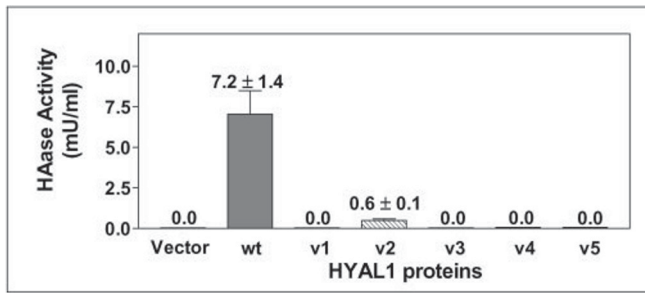


FIG. 4. Analysis of HAase activity of HYAL1wt and HYAL1 variant proteins. *In vitro* translated, unlabeled HYAL1wt and HYAL1 variant proteins, as well as vector-only control were analyzed for HAase activity using the HAase activity ELISA-like assay as described under "Experimental Procedures." Each sample was assayed at five different concentrations in duplicate. The results represent milliunits/ml  $\pm$  S.E. Each experiment was repeated twice and independently.

~20, and ~10 kDa. The molecular mass of these proteins is similar to the expected molecular mass of the HYAL1wt and various HYAL1 variant polypeptides, based on their deduced aa sequences. The reduced intensities of the  $^{35}$ S-labeled HYAL1v2, HYAL1v3, HYAL1v4, and HYAL1v5 proteins are most likely not due to reduced synthesis but to the reduced number of methionine residues present in these polypeptides. Based on the deduced aa sequence, HYAL1wt and HYAL1v1 proteins contain 9 methionines, whereas HYAL1v2, HYAL1v3, HYAL1v4, and HYAL1v5 proteins contain 6, 4, 4, and 3 methionines, respectively.

The HAase activity of *in vitro* translated HYAL1 variant proteins was measured using an ELISA-like assay (30, 39). Because we have previously shown that HYAL1 type HAase has a pH optimum at 4.2 (range 4.0–4.3; Ref. 30), the HAase ELISA-like assay was carried out at pH 4.2. As shown in Fig. 4, no HAase activity is detected in the vector only sample. However, the *in vitro* translated HYAL1wt protein has measurable HAase activity ( $7.2 \pm 1.4$  milliunits/ml). Interestingly, the HYAL1v1 protein that lacks only 30 aa (aa 301–330) has no HAase activity (Fig. 4). It is noteworthy that the HYAL1v1 protein contains both the putative HAase catalytic site (Asp<sup>131</sup> and Glu<sup>133</sup>) and Glu<sup>268</sup>, which are critical for HAase activity of HYAL1 (Figs. 2 and 4 and Ref. 31). The HYAL1v2 protein that lacks the putative catalytic site but retains Glu<sup>268</sup> and the 30-aa sequence shows less than 90% of HAase activity ( $0.6 \pm 0.1$  milliunits/ml) when compared with the HYAL1wt protein. No HAase activity is detected in HYAL1v3, HYAL1v4, and HYAL1v5 proteins. We also conducted the HAase ELISA-like assay at pH 3.7, 5.0, and 7.0, and none of the HYAL1 variants showed any activity at these pH levels (data not shown). These results demonstrate that although the catalytic site in HYAL1 may lie in the amino-terminal third of the protein, a 30-aa sequence from aa 301 to 330 is also critical for HAase activity.

**Evaluation of HAase Expression in Stable Transfectants Expressing HYAL1wt and HYAL1v1 cDNA Constructs**—It is possible that when translated *in vitro*, the HYAL1v1 protein does not fold correctly and/or lacks some post-translational modifications that are necessary for HAase activity. To test this possibility, we stably transfected RT4 bladder cancer cells with plasmid vector DNA, HYAL1wt, or HYAL1v1 cDNA constructs. We have previously shown that HYAL1 is not expressed in RT4 cells and that these cells do not secrete any HAase activity in their culture-conditioned media. We analyzed 10 stable clones of RT4 cells/construct (*i.e.* vector, HYAL1wt, and HYAL1v1) for HAase activity and HYAL1-related protein expression. The data on two representative clones/construct are shown in Figs. 5 and 6. Fig. 5A shows immunoblot analysis of culture-con-

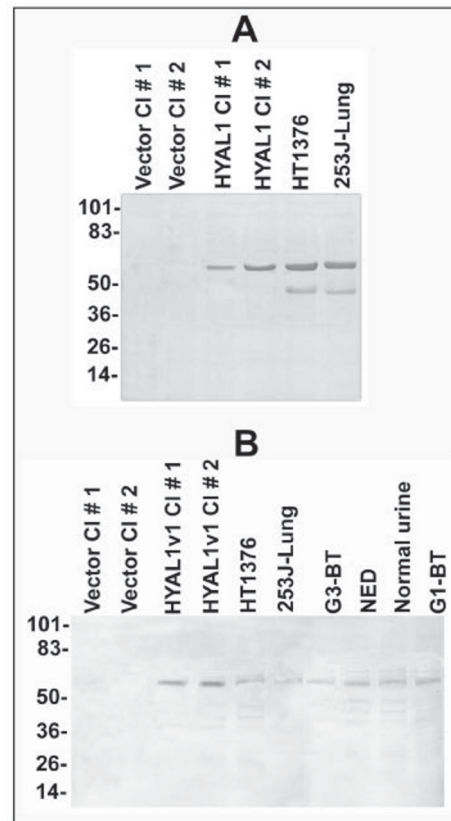


FIG. 5. Immunoblot analysis of HYAL1 and HYAL1v1 expression. Serum-free culture-conditioned media of RT4 stable transfectant (*i.e.* vector only, HYAL1wt, and HYAL1v1; two clones/construct), bladder cancer cells, and patient urine were subjected to either anti-HYAL1 or anti-HYAL1v1 peptide IgG immunoblot analysis as described under "Experimental Procedures." A, immunoblot analysis using anti-HYAL1 peptide IgG. HYAL1 indicates HYAL1wt. B, immunoblot analysis using an affinity-purified anti-HYAL1v1 IgG. G3-BT, urine specimen obtained from a patient with G3 bladder tumor; G1-BT, urine specimen obtained from a patient with G1 bladder tumor; NED, urine specimen obtained from a patient with a history of bladder cancer but no disease at the time of specimen collection.

ditioned media of RT4 clones and HT1376 and 253J-lung cells using an anti-HYAL1 peptide IgG (30). The anti-HYAL1 peptide IgG was generated against a peptide sequence in HYAL1 between aa 321 and 338 (30). Thus, this antibody will be able to recognize HYAL1wt and variant proteins HYAL1v2 and HYAL1v4. However, it will not detect other variant proteins (*i.e.* HYAL1v1, HYAL1v3, and HYAL1v5) that either partially or completely lack this sequence. Although no expression of HYAL1-related protein is observed in the vector only clones, an approximately 60-kDa HYAL1 protein is detected in the conditioned media of HYAL1wt clones and HT1376 and 253J-lung cells. The molecular mass of the HYAL1wt protein detected in the conditioned media is higher than that generated by *in vitro* translation (~50 kDa), indicating that HYAL1 protein is glycosylated or modified by other post-translational modifications. It is noteworthy that the anti-HYAL1 peptide IgG was able to immunoprecipitate [ $^{35}$ S]methionine-labeled HYAL1wt polypeptide generated by *in vitro* translation (data not shown). The smaller product observed in the conditioned media of HT1376 and 253J-Lung cells is most likely a degradation product. This is because these cells do not express HYAL1 v2 and HYAL1v4 transcripts (as seen in Fig. 1A), and furthermore, the molecular mass of this product (~45 kDa) is larger than those of the expected HYAL1 v2 and HYAL1 v4 products.

Fig. 5B shows immunoblot analysis of conditioned media of HYAL1 v1 transfectants of RT4, HT1376, and 253J-Lung cells

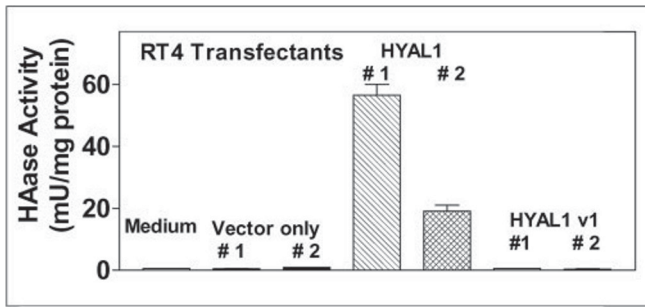


FIG. 6. Analysis of HAase activity secreted by RT4 stable transfectants. Serum-free culture-conditioned media of two clones each of vector only, HYAL1wt, and HYAL1v1 RT4 transfectants were assayed for HAase activity using the ELISA-like assay as described under "Experimental Procedures." Five concentrations of each sample were assayed in duplicate in each experiment, and each experiment was independently repeated three times. The HAase activity was normalized to total protein (mg/ml) and expressed as milliunits/mg protein  $\pm$  S.E.

and urine specimens using an anti-HYAL1v1 affinity-purified peptide IgG. The anti-HYAL1v1 antibody was generated against a 13-aa peptide in which the 300<sup>th</sup> aa present in the HYAL1 sequence is juxtaposed with 331<sup>st</sup> aa. Such a peptide can occur only in the HYAL1v1 protein, in which the aa 300 is joined to aa 331 because of mRNA splicing. Therefore, this antibody is specific for HYAL1v1 and does not detect other HYAL1 proteins. As expected, no HYAL1v1 protein is detected in the conditioned media of vector only clones. However, an approximately 57-kDa protein is detected in the conditioned media of HYAL1v1 clones. Interestingly, the protein detected by anti-HYAL1v1 peptide IgG is also present in the conditioned media of HT1376 and 253J-Lung cells and in the urine of bladder cancer patients (G3-BT and G1-BT), a patient with bladder cancer history but no disease at the time of urine collection, and normal individual (Fig. 5B). The faint smaller bands that are observed in some samples are possibly degradation products that may be generated during sample storage. The fact that the molecular mass of the HYAL1v1 protein is larger than the HYAL1v1 polypeptide generated by *in vitro* translation suggests that the HYAL1v1 protein is also post-translationally modified. The secretion of both the cloned and naturally occurring HYAL1v1 protein in the culture-conditioned media suggests that the HYAL1v1 protein is folded and processed correctly. It should be noted that the ~57-kDa protein detected by anti-HYAL1v1 IgG in the immunoblot analysis is HYAL1v1, because the affinity-purified anti-HYAL1v1 IgG was able to immunoprecipitate HYAL1v1 polypeptide generated by *in vitro* translation (data not shown).

We next measured the HAase activity secreted in the conditioned media of various RT4 transfectants, and the activity was normalized to total protein in the conditioned media. As shown in Fig. 6, there is no detectable HAase activity in the medium control and in the conditioned media of the vector only clones. However, the two HYAL1wt clones secrete high levels of HAase activity (*i.e.* clone number 1:  $56.5 \pm 3.5$  milliunits/mg protein and clone number 2:  $27 \pm 4$  milliunits/mg protein). However, no HAase activity is detected in the conditioned media of RT4 clones that express and secrete HYAL1v1 protein. These results demonstrate that the HYAL1v1 protein is enzymatically inactive and that the sequence from aa 301 to 330 is critical for the activity of HYAL1 protein.

**Detection of HYAL3 Splice Variants, cDNA Cloning, and Sequencing**—Because the chromosomal locations are similar in HYAL1, HYAL2, and HYAL3 genes (7, 32, 40), we examined whether HYAL3 might also be similarly spliced and generate splice variants. The Human Genome Blast search reveals that

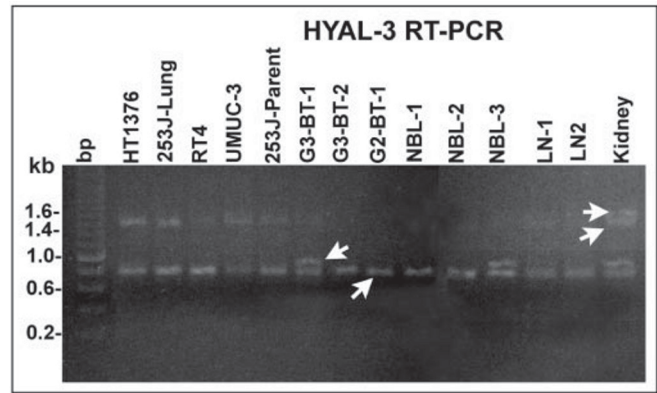
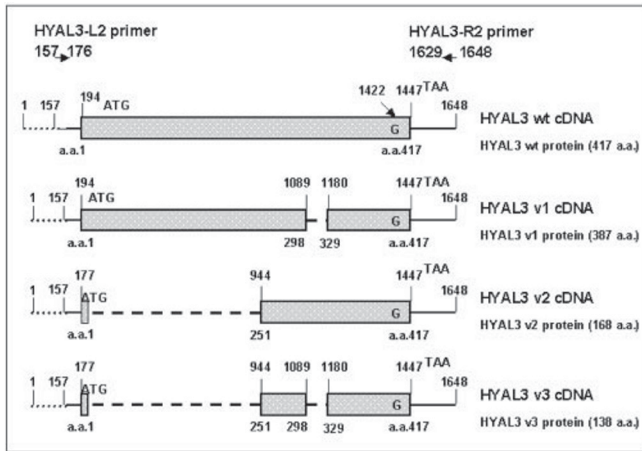


FIG. 7. Examination of HYAL3 expression in tissues and cells. Total RNA extracted from bladder tissues, bladder cells (RT4, UMUC-3, 253J-Lung, 253J-parent, and HT1376), and lymph node specimens invaded with bladder tumor was subjected to RT-PCR analysis using HYAL3 specific primers as described under "Experimental Procedures." The PCR products were analyzed by agarose gel electrophoresis. Four PCR products of different lengths are marked. PCR products expressed in each of these tissues or cells were cloned and sequenced. A 200-bp ladder (Promega) was used as a bp marker.

HYAL3 gene contains four exons separated by three introns (NT\_006014.7/HS3\_6171, *H. sapiens* chromosome 3 working sequence). To examine HYAL3 expression, we performed RT-PCR analysis on RNA extracted from bladder cancer lines (*i.e.* HT1376, 253J-Lung, RT4, UMUC-3, and 253J-parent), bladder tissues (*i.e.* normal and tumor), lymph node positive for tumor, and normal kidney tissue. The primer pair that we used for PCR analysis was designed to amplify a full-length HYAL3 cDNA, 1.5-kb in length. As shown in Fig. 7, an approximately 1.5-kb product is amplified from normal kidney tissue RNA. A smaller ~1.4-kb product is also visible in the kidney tissue sample that is detectable in many bladder cancer cell lines. The major PCR amplification product that is present in all bladder cancer cells, tissues, and lymph node specimens is ~600 bp in length (Fig. 7). This product is also present in the kidney tissue sample, along with an approximately 700-bp product. In addition, the 700-bp product is amplified from RNA extracted from G3 bladder tumor tissue (*i.e.* G3-BT-1), normal bladder tissue (*i.e.* NBL-3), and normal kidney tissue.

The ~1.5, ~1.4, ~0.7, and 0.6-kb PCR products were gel-isolated and directly cloned into the eukaryotic expression vector pcDNA3.1/v5/His-TOPO. The sequence analyses revealed that different PCR products represent HYAL3 splice variants. As shown in Fig. 8, the ~1.5-kb splice variant is actually 1492 bases in length. The sequence of this cDNA is 100% homologous to the HYAL3 clone AF040710 from nucleotides 157 to 1647, except that our cDNA has an extra G at position 1422 (GenBank<sup>TM</sup> accession number AF502912). This G is present in other HYAL3 cDNA clones (*e.g.* BC012892) and in the cosmid clone LUCA14 from 3p21.3, sequences of which are deposited in GenBank<sup>TM</sup> (accession numbers gi16164953, gi15208650, gi13543476, and AF036035). The G at position 1422 is also present in all of the HYAL3 splice variants identified in this study. Because of the presence of this G, translation of the HYAL3 polypeptide terminates at position 1447 (TAA termination codon position 1445–1447) and generates a 417-aa protein (Fig. 8).

The ~1.4-kb PCR product contains 1402 bases. As shown in Fig. 8, this sequence contains a 90-base deletion that joins nucleotides 1089 and 1180 (*i.e.* nucleotides 1090–1179 are deleted). This cDNA is designated as HYAL3v1 and can encode a protein made up of 387 aa (GenBank<sup>TM</sup> accession number AF502909). The putative HYAL3v1 protein contains a 30-aa deletion that involves aa 299–328. Thus, in the HYAL3v1

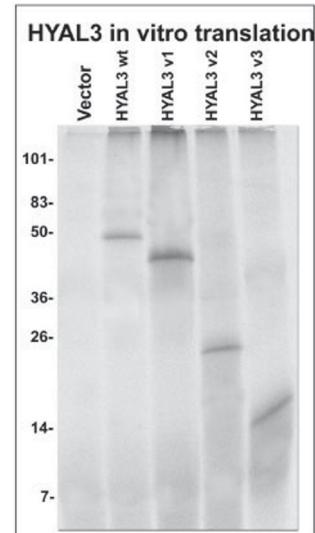


**FIG. 8. A schematic representation of the HYAL3 splice variants.** The figure shows HYAL3wt and different splice variant cDNAs. The numbering is based on the sequence of cDNA clone AF040710 that is deposited in GenBank™. However, the numbering is offset by 1 after nucleotide 1422. This is because the AF040710 is missing a G at position 1422. The coding region is shown as a *dotted block*. The *dashed line* shows the region that is spliced out, and the *solid line* represents untranslated regions. The *dotted line* between nucleotides 1 and 157 indicates the portion missing in our cDNAs. Each splice junction is marked by the nucleotides at the 5' and 3' boundaries that are joined because of splicing. The translation initiation and termination codons for HYAL1wt and HYAL1 variants are marked. The figure also shows the position of the forward (*i.e.* HYAL3-L2) and the reverse complementary (*i.e.* HYAL3-R2) primer pair used to amplify HYAL3 cDNA. The sequences of these primers are given under "Experimental Procedures," and the numbering is according to HYAL3 clone deposited in GenBank™ (accession number AF502912).

protein, aa 298 is joined to aa 329. The ~0.7-kb HYAL3 PCR product is actually 726 bases in length. The sequence analysis reveals that this variant (designated as HYAL3v2) is generated by a 766-base deletion that joins nucleotides 177 and 944 (Fig. 8; GenBank™ accession number AF502910). The HYAL3v2 variant would encode a 168-aa polypeptide in which aa 2–168 are identical to aa 251–417 in HYAL3wt protein. The smallest HYAL3 variant, HYAL3v3, is 636 bases in length. It is generated by two splicing events; in the first event, nucleotides 177 and 944 join, and in the second, the 90 bases between nucleotides 1089 and 1180 are deleted (as observed in HYAL3v1, Fig. 8; GenBank™ accession number AF502911). As shown in Fig. 8, the HYAL3v3 cDNA would encode a 138-aa protein, which like the HYAL3v2 protein is 100% homologous to aa 251–417 in the HYAL3wt sequence, except that it lacks the 30 aa sequence from aa 299 to 328.

**In Vitro Translation and Examination of HAase Activity of HYAL3wt and Variant Proteins**—Various HYAL3 cDNAs were *in vitro* translated using the coupled transcription/translation system and <sup>35</sup>S-labeled methionine. As shown in Fig. 9, HYAL3wt, HYAL3v1, HYAL3v2, and HYAL3v3 cDNAs upon *in vitro* transcription and translation generate polypeptide chains with molecular masses ~48, ~45, ~20, and ~16 kDa, respectively. The molecular masses of these proteins are comparable with the expected molecular masses based on the number of aa present in HYAL3wt and variant polypeptide chains. No protein is detected with vector only (Fig. 9, *Vector*).

We next measured the HAase activity of *in vitro* translated HYAL3 proteins. Because the HYAL3 gene is located in the same chromosomal location as the HYAL1 and HYAL2 genes, we assumed that like the HYAL1 and HYAL2 proteins, the HYAL3 protein may also have an acidic pH optimum (32). Therefore, we assayed the HAase activity of *in vitro* translated HYAL3 proteins at pH 4.2, using the HAase activity ELSA-like assay (17). As shown in Fig. 10, no HAase activity is detected



**FIG. 9. In vitro transcription/translation of HYAL3wt and HYAL3 variant cDNAs.** Various HYAL3 cDNAs and plasmid vector DNA were transcribed and translated *in vitro* using a coupled transcription/translation system and <sup>35</sup>S-labeled methionine, as described under "Experimental Procedures." The <sup>35</sup>S-labeled proteins were analyzed by SDS-PAGE along with molecular mass markers as described in "Experimental Procedures."

with vector only. However, HYAL3wt protein has measurable HAase activity ( $7.4 \pm 1.4$  milliunits/ml). The HYAL3v1 variant that lacks the 30-aa sequence from aa 299 to 328 but retains the putative enzyme catalytic site (Asp<sup>127</sup> and Glu<sup>129</sup>; Ref. 32) and Glu<sup>266</sup>, which may also be important for HAase activity (31), has no HAase activity (Fig. 10). The HYAL3v2 variant that retains the 30-aa sequence and Glu<sup>266</sup> but lacks the putative enzyme catalytic site is also enzymatically inactive (Fig. 10). The HYAL3v3 variant that contains only Glu<sup>266</sup> and lacks both the putative enzyme catalytic site and the 30-aa sequence has no HAase activity. These results demonstrate that in addition to the putative catalytic site and possibly Glu<sup>266</sup>, the 30-aa sequence from aa 299 to 328 is critical for HAase activity of the HYAL3 protein.

**Comparison of the 30-aa Sequence between HYAL1, HYAL3, and Other HAases**—Because in both HYAL1 and HYAL3 proteins a 30-aa sequence appears to be critical for HAase activity, we compared this sequence present in both proteins. As shown in Fig. 11, the 30-aa sequence present in HYAL1 (aa 301–330) and HYAL3 (aa 299–328) appears to be similarly situated in both proteins. In this 30-aa sequence, 17 aa among the first 22 are either identical or conserved substitutions. When a comparable 30-aa sequence among other human HAases (*i.e.* HYAL2, HYAL4, and PH20) is aligned, 17 of the first 22 aa are highly conserved (*i.e.* identical or conserved substitutions). Furthermore, the conserved aa occur at positions 2, 3, 6–8, 10–12, and 14–22. In addition, at position 29, either a Ser or a Thr residue is present in all HAases. In HYALP1, which is possibly a pseudogene (7), the 30-aa region also appears to be conserved, and of the first 22 aa in this sequence, 16 are conserved. In HYALP1, a Ser residue is present at position 29. We also compared a similarly positioned 30-aa sequence in bee HAase. As shown in Fig. 11, of the first 22, 12 are conserved (either identical or conserved substitutions) that occur at positions 2, 3, 6, 7, 14, 15, and 17–22. In addition, Thr at position 29 is also conserved. The high sequence homology among mammalian and invertebrate HAases with respect to the 30-aa sequence suggests that this sequence is critical for HAase structure and activity.

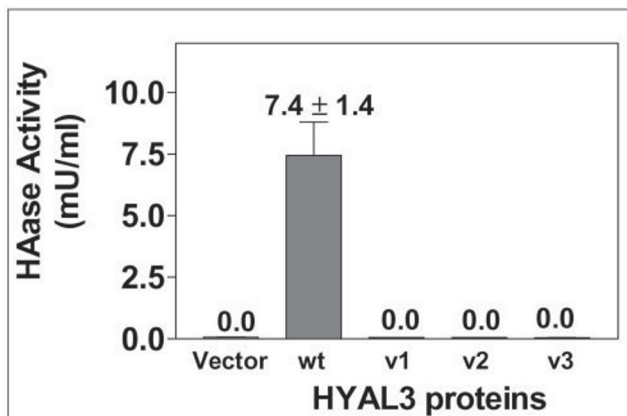


FIG. 10. Analysis of HAase activity of HYAL3wt and HYAL3 variant proteins. *In vitro* translated, unlabeled HYAL3wt and HYAL3 variant proteins, as well as vector-only control were analyzed for HAase activity using the HAase activity ELISA-like assay as described under "Experimental Procedures." Each sample was assayed at five different concentrations in duplicate. The results represent milli-units/ml  $\pm$  S.E. Each experiment was repeated twice and independently.

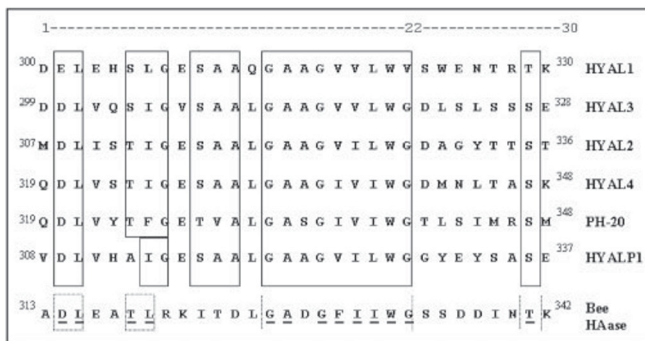


FIG. 11. Comparison of a 30-aa sequence in various human and bee HAases. The 30-aa sequences identified in HYAL1 and HYAL3 type HAases were compared with each other and with the corresponding 30-aa sequences in other human and bee HAases. The numbers indicate the positions of this sequence in various HAases. The boxes highlight the conserved (*i.e.* identical or conserved substitutions) aa between various HAases.

## DISCUSSION

In this study we have identified a 30-aa sequence that is well conserved in several HAases and is required for enzyme activity of at least two human HAases (*i.e.* HYAL1 and HYAL3). The nucleotide sequence of HYAL1 gene reveals this gene contains three exons and two introns. Exons 1 (~1.5 kb) and 3 (0.9 kb) are relatively large compared with exon 2 (90 bp). The HYAL1 genomic sequence shows that the 90-bp sequence (nucleotides 1520–1609) that is missing in HYAL1v1 is the entire exon 2. Other HYAL1 variants appear to be generated by internal exon splicing events that usually involve exon 1. For example, the HYAL1wt described in this study is generated by an internal exon 1 splicing event, involving nucleotides 110 and 596. This splice variant has been described previously (GenBank™ accession number AF173154). HYAL1v2 appears to be generated by internal exon splicing involving nucleotides 109 and 952 as donor/acceptor sites, respectively, both of which are present in exon 1. Generation of HYAL1v3 variant is interesting in that it involves 2 splicing events. The first is the same internal exon 1 splicing involving nucleotides 110 and 596. The second splicing involves splicing of nucleotide 1239 present in exon 1 to nucleotide 1577 that is present in exon 2. Generation of HYAL1v4 again involves an internal exon splicing event involving exon 1; both the donor (nucleotide 108) and acceptor (nucleotide 1369)

sites are present in exon 1. Generation of HYAL1v5 involves internal exon 1 splicing that starts at nucleotide 109 and ends nearly at the end of exon 1 (nucleotide 1519). Thus, the analysis of HYAL1 variants demonstrates that exon 1 in HYAL1 gene has several internal donor and acceptor sites suitable for alternative mRNA splicing resulting in the generation of various HYAL1 splice variants. In addition, exon 2 can be alternatively spliced. It is interesting to note that each of these splicing events (either internal exon splicing or splicing of two exons) maintain the same open reading frame as the HYAL1 protein, resulting in different HYAL1 variant proteins.

Our study on HYAL3 splice variants confirms that the 30-aa sequence that is important for enzyme activity is encoded by an independent exon. The nucleotide sequence of HYAL3 gene reveals that this gene contains four exons separated by three introns. Exon 3 is 90 bp in length and corresponds to the 90-bp v1 region (nucleotides 1090–1179). Thus, splicing out of the independent exon 3 generates HYAL3v1. As observed for HYAL1 variants, HYAL3v2 and HYAL3v3 splice variants are generated by an internal exon splicing events that involve exons 2 and 3. For example, HYAL3v2 variant is generated by an internal splicing event involving exon 2 (nucleotides 178–943 are deleted), and HYAL3v3 is generated by the same internal splicing event involving exon 2 and also splicing of the independent exon 3.

It is interesting to note that different bladder and lymph node specimens and cells show differences in the pattern and types of HYAL1 and HYAL3 splice variant that are expressed. The reason for this heterogeneity is unknown at present. It is noteworthy that bladder tumors show heterogeneity in their ability to progress and recur. This heterogeneity in turn relates to the differences in the biological behavior of different bladder tumors (41). It is possible that the heterogeneous expression of the HYAL1 or HYAL3 variant may be related to the different biological behavior of different bladder tumors. Because in both HYAL1 and HYAL3 proteins the loss of the 30-aa sequence results in the loss of HAase activity and because the 30-aa sequence is coded by one single independent exon, the 90-bp-long independent exon in both genes seems to encode an aa sequence that is critical for HAase activity.

Other studies involving site-directed mutagenesis of PH20, identification of a naturally occurring mutation in HYAL1, and the crystal structure of bee HAase have identified several aa present in different parts of HAase that are conserved and are important for activity (31, 35, 42). The crystal structure of bee HAase reveals that the insect and possibly mammalian HAases have a classical ( $\beta/\alpha$ )<sub>8</sub> TIM barrel structure (35). The dominant feature of the HAase structure is a large groove that extends perpendicular to the barrel axis. In bee HAase, loops following the  $\beta$  strands 2, 3, and 4 form one wall of the groove, and those of 1, 5, 6, and 7 form the other wall. This groove is large enough to accommodate a hexasaccharide (35). Co-crystallization of bee HAase with a hexasaccharide shows that the catalytic site that cleaves the glycosidic bond between *N*-acetyl-D-glucosamine and D-glucuronic acid lies in aa residues Asp<sup>143</sup> and Glu<sup>145</sup> (numbering according to GenBank™ accession number AAA27730.1). In a substrate-assisted acid-base catalytic mechanism, Glu<sup>145</sup> acts as the proton donor, and the *N*-acetyl group of the substrate acts as the nucleophile. In all human HAases, this Glu residue is conserved along with Asp and is believed to be responsible for substrate cleavage (32). In the HYAL1 sequence this Glu is aa 131 (numbering according to GenBank™ accession number AAD09137.2), and in HYAL3 it is aa 129 (numbering according to GenBank™ accession number AAH12892.1). Thus, the 30-aa sequence that we have identi-

fied in HYAL1 (aa 301–300) and in HYAL3 (aa 299–328) most likely is not a part of the catalytic site.

Based on the bee HAase crystal structure, the 30-aa sequence from aa 313 to 342 (Fig. 11), that is homologous to the 30-aa sequences in HYAL1 and HYAL3, forms  $\beta$  sheets 6 and 7,  $\alpha$ -helix 8, and the loops in between (35). Because the loops following  $\beta$  strands 6 and 7 are involved in forming one of the walls of the substrate binding groove, loss of the 30-aa sequence in HYAL1 and HYAL3 proteins may result in the loss of substrate binding. The substrate-associated catalytic cleavage of the glycosidic bond between *N*-acetyl-D-glucosamine and D-glucuronic acid requires accurate positioning of the *N*-acetyl side chain of the substrate with respect to the catalytic site (35). This is achieved by two hydrogen bonding interactions and a hydrophobic interaction. The crystal structure shows that in bee HAase, Trp<sup>333</sup> is involved in hydrophobic interaction with the *N*-acetyl side chain (35). This Trp<sup>333</sup> corresponds to Trp<sup>321</sup> in HYAL1 and Trp<sup>319</sup> in HYAL3, both of which are present in the respective 30-aa sequences (Fig. 11). This Trp residue is also conserved in all HAases and other chitinolytic enzymes (43, 44). Thus, the absence of this Trp residue in HYAL1v1 and HYAL3v1 mutant proteins may lead to improper positioning of the substrate resulting in no catalysis. It remains to be determined why this 30-aa sequence is so well conserved in various HAases and what role other conserved residues play in terms of substrate binding and catalysis. It should be noted that the loss of  $\beta$  sheets 6 and 7,  $\alpha$ -helix 8, and the loops in between, because of the 30-aa deletion in HYAL1v1 and HYAL3v1, may also result in complete loss of the TIM barrel structure, making these proteins enzymatically inactive.

Other HYAL1 and HYAL3 variant proteins also illustrate the importance of various structural domains for HAase activity. For example, HYAL1v3 variant, which contains the putative catalytic site (*i.e.* Asp<sup>129</sup> and Glu<sup>131</sup>) but lacks aa 208–435, has no HAase activity. It is likely that the HYAL1v3 protein does not form a proper TIM barrel structure, and at the very least, it lacks the substrate-binding groove. HYAL1v2 (aa 183–435) and HYAL1v4 (aa 260–435) proteins retain the 30-aa sequence and also Glu<sup>268</sup>, which has been shown to be critical for HAase activity. However, these variants are enzymatically inactive, because they lack the putative catalytic site as well as parts of the substrate-binding groove. A similar situation may hold true for HYAL3v2 variant (aa 251–435), which also does not have any HAase activity. The variants HYAL1v5 (aa 340–435) and HYAL3v3 (aa 251–435 but lacking aa 299–328) lack more than two-thirds of the respective molecules and hence will not fold properly, lack the HAase catalytic site, and have no substrate binding groove. Thus, this study illustrates the involvement of several structural domains that are conserved in various HAases and are important for enzyme activity.

Detection of various HYAL1/HYAL3 variants in bladder and prostate cancer lines, bladder tumor tissues, as well as normal kidney tissue suggest that the expression of functionally active HAase in various cells and tissues may be regulated by alternative mRNA splicing. We have previously shown that HAase levels are elevated (3–7-fold) in the urine of bladder cancer patients who have high grade (*i.e.* G2/G3) disease (39). Furthermore, in a study of 504 patients, we demonstrated that the HAase level serves as a highly sensitive (82%) and specific (83%) marker for detecting G2/G3 bladder cancer (18). Urinary HAase levels together with HA levels (*i.e.* HA-HAase test) are sensitive and specific in detecting bladder cancer and monitoring its recurrence (18, 45). We have also shown that both invasive bladder and prostate cancer cells secrete high levels of HAase activity, and HYAL1 is the major HAase expressed in these carcinomas (17, 30, 46). Consistent with these observa-

tions, in this study we observed that the full-length HYAL1wt transcript that encodes a functional HAase is expressed only in bladder and prostate cancer cells, G2/G3 bladder tumor tissues, and lymph node specimens showing tumor invasion. In normal bladder and G1 bladder tumor tissues, in a normal bladder primary culture, and in bladder tissues showing no evidence of tumor, the major HYAL1 transcript that is expressed is the HYAL1v5 variant. Because this transcript or other HYAL1 variant transcripts (*i.e.* HYAL1v2-v4) that are also expressed in some of these specimens do not encode a functional HAase, it may explain why in these tissues or cells no HAase activity and wild type HYAL1 protein are detected (30, 46, 47).

HAase protein has been shown to be associated with tumor angiogenesis and/or invasion (48). However, because HYAL1 (and also HYAL2 and HYAL3) is present on chromosome 3p 21.3 locus, and this region is a critical tumor homozygous deletion region in lung and breast cancers, it has been suggested that HYAL1 may be a tumor suppressor (7, 49). A recent study clearly demonstrates that the RASSF1 gene and not HYAL1 is the tumor suppressor gene present in that locus (50). Thus, several observations, including elevation of HAase levels in bladder and prostate tumors (18, 30), suggest that HYAL1 may have a role in promoting tumor progression. Normal cells may suppress the HAase activity of HYAL1 by generating HYAL1 splice variants that encode an inactive enzyme. It remains to be determined why the normal bladder cells/tissues, G1 bladder tumor tissues, and normal kidney tissues express different splice variants that encode inactive HYAL1 proteins.

The expression of HYAL3 protein has been previously detected in prostate cancer cells by RT-PCR analysis (51). Our results show that in all bladder tumor cells and prostate cancer cells,<sup>2</sup> and in all of the bladder tumor tissues that we have tested, the major HYAL3 transcript appears to be HYAL3v3 variant. This variant is enzymatically inactive. In fact we were unable to clone HYAL3wt from various cancer cells and tissues. The minor ~1.4-kb transcript expressed in cancer cells/tissues is HYAL3v1. Once again it remains unclear what is the function of these enzymatically inactive HYAL3 variant proteins.

This study identifies a 30-aa sequence that is highly conserved among all human and insect HAases and is critical for HAase activity. This study also identifies alternative mRNA splicing as the likely mechanism of regulating the expression of enzymatically active HAase in various tissues and cells. The expression of HYAL1wt mRNA that encodes the functional HYAL1 protein in high grade bladder tumor tissues and in invasive bladder/prostate cancer cells provides an insight into why HAase levels are elevated and serve as a marker for tumor progression.

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