Characterization of the Murine Hyaluronidase Gene Region Reveals Complex Organization and Cotranscription of Hyal1 with Downstream Genes, Fus2 and Hyal3*

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Hyaluronidases are required for the breakdown of hyaluronic acid (HA), an abundant component of the extracellular matrix of vertebrate tissues. Multiple hyaluronidase genes have been identified, but the only clue to the function of their products has come from the identification of hyaluronidase 1 deficiency in a single patient with a mild clinical phenotype. As a first step in the generation of mice with hyaluronidase deficiency, we have used experimental and bioinformatic approaches to examine the organization of the mouse chromosome 9 region containing, in order, Hyal2, Hyal1, and Hyal3. This region was found to be complex, with Fus2 partially embedded in Hyal3, and Ifrd2 immediately downstream from Hyal3. The Hyal genes were all found to have four exons, and exons 2–4 exhibited the highest sequence conservation. Northern blot analysis demonstrated that the tissue expression profile for Hyal1 was similar in mice and humans, but a greater number of transcripts was detected in mouse tissues. Hyal3 was expressed more broadly in mice compared with humans and again exhibited additional transcripts. Reverse transcription-PCR demonstrated that some of the larger Hyal1 transcripts, seen on the Northern blot, were the result of cotranscription of Hyal1 with downstream genes, Fus2 or Hyal3. In vitro transcription/translation of one of the high abundance bicistronic transcripts produced Hyal 1, suggesting that Hyal 1 could be produced from all of the bicistronic transcripts. Characterization of the region including mouse Hyal1 and Hyal3 revealed complex organization and transcription that must be considered in the development and interpretation of mouse models involving genes in this region.

Hyaluronidases are endoglycosidases essential for the breakdown of hyaluronic acid (HA), a high molecular mass glycosaminoglycan abundant in the extracellular matrix of vertebrate tissues (1). High levels of HA confer important structural and functional properties to tissues, including viscosity to synovial fluid (2) and fluidity to the matrix surrounding proliferating and migrating cells involved in processes such as embryogenesis and wound healing (3, 4). HA degradation is an important component of these processes, and the recent identification of multiple related hyaluronidase genes (5, 6) suggests that this requirement is fulfilled through duplication and divergence.

Several mammalian hyaluronidases have now been recognized. The most extensively studied is PH-20, a neutrally active hyaluronidase that is specific to sperm and degrades the HA-rich cumulus enveloping the egg, allowing fertilization to occur (7). At least two acid-active hyaluronidases, HYAL 1 and HYAL 2, have been recognized (8, 9), whereas the identification of two other enzymes, HYAL 3 and HYAL 4, as hyaluronidases is based on homology to previously characterized hyaluronidases. They are believed to be lysosomal enzymes, but with differing substrate specificities (9) and expression profiles (5, 6). The existence of more than one functional lysosomal hyaluronidase is suggested by the mild phenotype and cell-specific HA accumulation observed in a patient with a deficiency of HYAL 1 (5, 10).

The existence of multiple hyaluronidases provides vast potential for both constitutive and regulated turnover of HA. At this time, little is known about the role of these individual hyaluronidases. It is interesting that three of the hyaluronidase-encoding genes, HYAL1, HYAL2, and HYAL3, were originally identified as candidates in a search for a tumor suppressor for small cell lung carcinoma, on human chromosome 3p21 (11). HYAL2 and HYAL1 remain in the minimum interval that has been defined for this disease (12), making it important to evaluate their potential role in lung carcinoma. Given that only a single hyaluronidase-deficient patient has been identified to date, the most direct way to examine the roles of these enzymes in health and disease is through the generation of mice deficient in each enzyme.

As a first step in the generation of mouse models with targeted disruptions of the Hyal1 or Hyal3 genes, we have isolated the mouse Hyal1, 2, and 3 genes and characterized the organization and expression profiles of Hyal1 and Hyal3. The mouse Hyal2 gene was characterized previously (13). During

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1 The abbreviations used are: HA, hyaluronan; EST, expressed sequence tag; PAC, P1 artificial chromosome; PIP, percent identity plot; RACE, rapid amplification of cDNA ends; RT, reverse transcription.
our analysis, unusual complexity in both the structure and expression of the hyaluronidases was detected. Hyal1 was found to overlap with another gene in the minimal interval for lung carcinoma, Fus2; the second exon of Fus2 was found imbedded in the first intron of Hyal3. This unusual organization was accompanied by complex transcription profiles that were accounted for by cotranscription of Hyal1 with the downstream genes, Fus2 or Hyal3. We used in vitro transcription/translation to show that one of the bicistronic mRNA species could be translated. Although cotranscription is considered to be rare in mammals, the abundance of the bicistronic transcripts encoding Hyal 1 and their ability to be translated indicate that they are biologically relevant. These factors must be considered in gene targeting strategies aimed at the generation of mouse models for hyaluronidase deficiency or lung carcinoma susceptibility, as the disruption of one gene in this region could impact on the expression of others.

**EXPERIMENTAL PROCEDURES**

**Primer Extension**—To determine the transcription start site of Hyal1, a synthetic oligonucleotide (5′-ATCTTGTAGCATGCTGTA-AGAGATGCCA-3′) was used as a primer. A PCR product was amplified using H11032 forward (WPG199: 5′-CTGGATTATCAGTC-3′) and reverse (WPG288: 5′-AGTG-GAAGGTGTAGCGGCCCTC-3′) primers, an annealing temperature of 65°C. The PCR program consisted of an initial 3-min denaturation at 94°C followed by 35 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min. PCR products were cloned using Taq polymerase and a reverse adapter primer (5′-AGTG-GAAGGTGTAGCGGCCCTC-3′) to generate an approximately 636-bp fragment beginning at the 5′ end of Hyal1 and extending beyond the 5′ end of Hyal2. This fragment was amplified using Expand™ Reverse Transcriptase (Roche Molecular Biochemicals) at 94°C for 45 s, 55°C for 1 min, and a final extension at 72°C for 8 min. To determine the sequence of the gap between the unassembled sequence contigs containing Hyal2 and Hyal1 (GenBank AC025533), PCR amplification of PAC DNA was performed as above but using forward (WPG352: 5′-CTTGAGATCGACGATCGCTGCACTGCACTGCA-3′) and reverse (WPG348: 5′-ATAGTGTCACAGGTG-GAGAAAGAC-3′) primers, an annealing temperature of 65°C, and a 5-min extension at 72°C. All other PCR amplifications of PAC DNA were performed using Taq polymerase and a PCR program consisting of 35 cycles: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR fragments were cloned using the Original TA Cloning Kit as described above.

**Bioinformatic Analyses**—Comparative sequence alignments were generated using PipMaker (bio.cse.psu.edu) (17). DNA and protein sequences were compared with available public data bases using the various BLAST programs available through the network server at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). DNA and protein sequences were aligned with the DNAStar software. Multiple alignments of protein sequences were done with ClustalW (18). Repeat elements were characterized using RepeatMasker (ftp.genome.washington.edu/cgi-bin/RepeatMasker).2

Compiled, overlapping sets of mouse and human ESTs were downloaded as UniGene clusters (19), and the individual ESTs were assembled using DNAStar software. Additional overlapping EST sequences were then subcloned into the expression vector pCI/CMV (Promega) for DNA and protein analysis. Multiple alignments of protein sequences were done with ClustalW (18). Repeat elements were characterized using RepeatMasker (ftp.genome.washington.edu/cgi-bin/RepeatMasker).

The human genomic sequence from the HYAL2/SEMA3B locus at 3p21.3 was assembled from two separate GenBank sequences (U73167 and U90994). Mouse genomic sequence data (GenBank AC025533) came from a high throughput phase 1 draft of mouse clone RP23-93E19 and is found on three unordered contiguous stretches of DNA (contigs). Within GenBank AC025533, the sequence gap between the contigs containing Hyal2 and Hyal1 was determined to be 275 bp by PCR with primers WPG352 and WPG348, as described earlier. The second sequence gap within the Hyal3 gene was filled using the sequence that we generated from a full-length EST clone (GenBank AY048681).

**Northern Blot Analysis**—Adult male poly(A)+ RNA Northern blots (Schwarz Webester) and embryo multiple tissue Northern (MTN)21 blots (Schwarz Webester) (2 μg of poly(A)+ RNA per lane) were purchased from Origene and CLONTECH, respectively. Hybridizations were performed under stringent conditions in UltraHyb (Origene) for the adult blots or Express Hyb (CLONTECH) for the embryo blot, according to the manufacturers’ instructions. The embryo and the two

2 A. F. A. Smit and P. Green, unpublished data.
FIG. 1. Schematic diagram depicting the construction of the bicistronic Hyal1/Fus2 cDNA template and the constructs containing only Hyal1 or Fus2 cDNA, used in the in vitro transcription/translation studies. A, multiple cloning steps were performed to construct the Hyal1/Fus2 cDNA template, pSP64 (Hyal1/Fus2). B and C, cloning steps performed in the generation of the constructs containing only Hyal1 cDNA (pSP64(Hyal1)) or Fus2 cDNA (pSP64(Fus2)), respectively. Abbreviations: B, BamHI; E, EarI; GI, BsrGI; H, HindIII; Hd, HindIII; Nc, NcoI; RI, EcoRI; RV, EcoRV; S, SpeI; Sm, SmaI. Brackets surround sites that were lost during the cloning steps. Oligonucleotide primers used for PCR amplification of the fragments for cloning are shown as arrows.

adult blots were probed with a 353-bp BsaHI/XhoI fragment within exon 2 of mouse Hyal1. Subsequently, one of the adult blots was probed with a 211-bp HincII/BsrGI fragment located 2.5 kb downstream from Hyal1, within Fus2; the other adult blot was probed with a 636-bp HindIII RT-PCR fragment (WPG207/WPG245). The Northern blots were probed with human β-actin cDNA as a control. Probes were labeled with [α-32P]dATP by random priming (14). The hybridized blots were exposed to BioMax MS film (Kodak) between two intensifying screens at −80 °C. Stripping the blots consisted of heating the membrane in a 0.5% SDS solution, at 90–100 °C, for 10 min; the subsequent absence of a signal was confirmed by autoradiography.

In Vitro Transcription/Translation—A cotranscript with the potential to encode Hyal1 and Fus2 was in vitro translated using the TNT SP6 Coupled Reticulocyte Lysate System (Promega). DNA encoding luciferase, provided with the kit, was used as a positive control; negative controls contained pSP64(poly(A)) vector DNA (Promega) or no DNA. A construct containing a cDNA sequence for a Hyal1/Fus2 cotranscript, under the control of an SP6 promoter, was generated in three steps (Fig. 1A). First, the internal region of the transcript was obtained by restriction enzyme digestion of a clone (generated by RT-PCR with WPG199 and WPG288 as described earlier) with SpeI and EcoRV to generate a 1.6-kb fragment. The 5’-overhangs of the fragment were filled in with Klenow (New England Biolabs) and subcloned into the HincII site of pSP64(poly(A)) to generate pSP64(a). The 3’-end of the transcript was obtained by digestion of a clone (generated by PCR with WPG287 and WPG305 as described above) with SmaI and EarI to obtain a 0.8-kb fragment; the 5’-overhangs were filled in with Klenow and inserted into pSP64(a) digested with SmaI, to generate pSP64(b).

To introduce the 5’-end of the transcript, a RT-PCR product, generated with WPG333 and WPG183 as described earlier, was treated with Vent DNA polymerase (New England Biolabs) to remove the 3’-overhangs. Subsequently, the product was digested with NcoI to produce a 1.3-kb WPG333/NcoI fragment that was inserted into pSP64(b) digested with HindIII (filled in with Klenow) and NcoI, to generate pSP64(Hyal1/Fus2). A construct containing only Hyal1 cDNA was also generated (Fig. 1B). To obtain the Hyal1 cDNA, pSP64(Hyal1/Fus2) was digested with EcoRI, and the ends were filled in with Klenow. The product was subsequently digested with BsrGI and inserted into the BsrGI/HincII site of pSP64(poly(A)) to generate pSP64(Hyal1).

In vitro transcription/translation was performed using 1 μg of pSP64(Hyal1/Fus2), pSP64(Hyal1), pSP64(Fus2), or control plasmid DNA in a 50-μl total reaction containing 25 μCi of [35S]methionine (Amersham Biosciences) and 1 μl of TNT SP6 polymerase; the reaction was incubated at 30 °C for 90 min. 5-μl aliquots of the products were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane (20). The membrane was exposed to BioMax MS film with a TranScreen-LE intensifying screen (Kodak) and subsequently analyzed by Western blot (see “Western Blot Analysis”)

Production of HYAL 1 Monoclonal Antibody—To generate an antibody against human HYAL 1, the 1,100-bp NcoI fragment (filled with Klenow) of the HYAL1 EST clone (GenBank AA223264) was subcloned into the Klenow-filled HindIII site of pET-28a (+) (Novagen), and the construct was transformed into BL21 (DE3) cells. The expression of the HYAL 1 (amino acids 1–366)-His6 tag fusion was induced for 3 h with 1 mM isopropyl-β-D-thiogalactoside, and insoluble inclusion bodies were purified following the manufacturer’s (Novagen) instructions. The fusion protein was purified further by 10% SDS-PAGE. The protein bands were visualized by soaking the gel in 4 M sodium acetate for 1 h; the HYAL 1-His6 tag fusion was excised from the gel, reequilibrated with phosphate-buffered saline, and homogenized with Titer-Max Gold (Cedarlane Laboratories Inc.). Monoclonal antibodies against HYAL 1 were produced from BALB/c mice as described previously (21), in the Canadian Genetic Diseases Network Immunoprobes Facility (Winnipeg, MB). Clones that produced antibodies toward HYAL 1 were.
identified by enzyme-linked immunosorbent assay screening of the culture medium from individual clones for a reaction against amino acids 1–289 of HYAL 1 fused to maltose-binding protein. Positive clones were tested for their ability to recognize HYAL 1 but not HYAL 2 or HYAL 3 fusion proteins on a Western blot (data not shown). Clone 1D10 was found to work well for Western blot analysis and was specific for HYAL 1. Further characterization of the specificity of this antibody will be presented in a separate manuscript.

**Western Blot Analysis**—The nitrocellulose membrane was blocked for 1 h with 5% skim milk powder in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5) and 0.1% Tween 20 (TBST). The membrane was incubated with 0.5 μg/ml anti-HYAL 1 in TBST for 1 h and subsequently washed with TBST. This was followed by a 1-h incubation in horseradish peroxidase-conjugated sheep anti-mouse (1:15,000) in TBST and several washes with TBST, before visualization of the bound secondary antibody with the ECL Western blot detection system (Amersham Biosciences).

**RESULTS**

The development of strategies for the generation of mouse models deficient in Hyal1, Hyal2, or Hyal3 necessitated a careful evaluation of the organization and transcription profiles of their genes. Initial characterization of Hyal1 and Hyal3 gene organization and structure was performed (see “Experimental Analysis of Hyaluronidase Gene Structure and Organization”). As genomic sequence in the hyaluronidase region of mouse chromosome 9F became available, bioinformatic approaches were used to confirm and extend our knowledge of this region (see “Bioinformatic Comparisons and Analysis of the Hyal12/ Sema2b Region”). The complex mRNA expression profiles of mouse Hyal1 and Hyal3 were characterized and their potential biological significance assessed (see “Expression Profile of the Hyal1 to Hyal3 Region”). The murine Hyal2 gene structure and expression had already been examined in a previous study (13) and therefore was not included in the experimental analysis.

**Experimental Analysis of Hyaluronidase Gene Structure and Organization**

**Characterization of Murine Hyal1 and Hyal3 cDNA Sequences**—To determine the organization of the genes in the mouse chromosome 9 region and the intron/exon structures of murine Hyal1 and Hyal3, the full-length cDNA sequences were required. A 2,125-bp cDNA sequence containing the entire 1,386-bp open reading frame of murine Hyal1 had been reported previously (22), but it was not known whether this was the full-length cDNA. To determine this, the putative transcription start site of Hyal1 was obtained by primer extension as described under “Experimental Procedures.” A reproducible 105-nucleotide extension product, as seen in Fig. 2, corresponded to an initiation site 73 nucleotides upstream from the translation initiation codon and 30 nucleotides upstream from the published cDNA sequence (GenBank AF011567), extending the Hyal1 cDNA to a length of 2,155 bp. Although the 105-bp extension product is clearly the major species, other less prominent bands were detected and may be additional extension products. Because all of the ESTs corresponding to the 3′-end of Hyal1 terminated at position 2155, we concluded that a poly(A) cleavage site was present at this position, although no consensus poly(A) signal was identified upstream.

No sequences representing murine Hyal3 cDNA were initially available within the mouse EST data base; therefore, a fragment of the Hyal3 cDNA was isolated by RT-PCR amplification of cDNA from mouse testis using primers (WPG207/ WPG245) that were based on the human HYAL3 sequence. A BLAST search with the sequence of this RT-PCR fragment subsequently identified a single EST corresponding to Hyal3 (GenBank BE376250) which had become available in the data base. Complete sequencing of this EST clone and comparison with human HYAL3 (GenBank AF036035) suggested an ATG translation initiation codon at position 48 (numbering corresponding to GenBank AY048681) and a TGA stop codon at position 1,284, giving an open reading frame of 1,236 bp. Primer extension to determine the Hyal3 transcription initiation site was not performed because of the low level of Hyal3 transcripts.

Sequencing of the Hyal3 EST clone, GenBank BE376250, revealed a string of A nucleotides at the 3′-end which was initially believed to represent the poly(A) tail. However, this string was also found in the corresponding genomic DNA sequence (GenBank AC025353), suggesting that it may be an artifact resulting from priming upstream from the true poly(A) tail. To determine the true poly(A) cleavage site of Hyal3, we used 3′-RACE, and two products of ~0.53 and 0.3 kb were cloned and sequenced (data not shown). The 0.53-kb fragment identified a poly(A) cleavage site at position 1817–1819 (numbers corresponding to GenBank AY048681); the site could not be determined specifically because of the presence of A nucleotides at positions 1818 and 1819 in the cDNA as well as in the genomic sequence. A poly(A) signal (CATAAA) was located upstream from this cleavage site. An alternative poly(A) cleavage site, between nucleotides 1582 and 1604, was indicated by the 0.3-kb product and could not be specified because of the string of A nucleotides within the cDNA and genomic sequence. This site corresponded to the one predicted by the sequence of GenBank BE376250. The authenticity of this poly(A) site was confirmed by the presence of a lengthy poly(A) tail, at the 3′-end of the RACE product, which was much longer than the sequence of A nucleotides in the genomic DNA and by the absence of amplification in the negative control (i.e. no reverse transcriptase). A weak poly(A) signal (TATAAA) was identified upstream from this cleavage site. The full-length Hyal3 cDNA (GenBank AY048681) is determined to be 1.8 kb, and the upstream alternative poly(A) site generates a 1.6-kb cDNA.

**Isolation and Localization of Mouse Hyal1, Hyal2, and Hyal3**—As a first step in characterizing the structure and organization of the Hyal1 and Hyal3 genes, mouse genomic C129/Sv clones containing the Hyal1 and Hyal2 genes were identified by screening a mouse PAC library using human HYAL1 and HYAL2 cDNAs as hybridization probes. Three PAC clones, 452D10, 52408, and 562N23, were identified which hybridized to both probes. These clones exhibited identical hybridization patterns when the PAC DNA was digested with EcoRI, and multiple bands were found to be shared by all three clones when several restriction enzymes were used (data not shown).
shown). Taken together, these results suggested that the genomic region containing Hyal1 and Hyal2 was present in all of the clones. The PAC clone, 452D10, was chosen for further characterization because it had the smallest insert.

A restriction enzyme map of 452D10 was generated using a combination of single and double restriction enzyme digests. Summation of the fragment sizes generated by individual restriction enzymes gave a genomic DNA insert size of ∼145 kb (Fig. 3). To localize Hyal1, Hyal2, and Hyal3 genes within this PAC clone, restriction enzyme fragments from mouse Hyal1 and Hyal2 ESTs and a Hyal3 cDNA fragment (WPG207/WPG245) were used as hybridization probes against restriction enzyme digests of 452D10 (data not shown). As indicated in Fig. 3, the genes were found to span ∼23 kb, and the order of the genes was Hyal2, Hyal1, and Hyal3. Hyal1 and Hyal2 have been mapped previously to mouse chromosome 9F1-F2 (21, 13), suggesting that the PAC clone, 452D10, including the Hyal3 gene, can also be localized to chromosome 9F1-F2.

**Hyal1 and Hyal3 Gene Structures**—The Hyal1 and Hyal3 gene structures were determined by restriction enzyme mapping, PCR amplification, and DNA sequencing of the Hyal1 genomic subclone, pHLS1, and the PAC clone, 452D10, respectively. Genomic DNA containing human Hyal3 (GenBank U73167) was aligned with the available sequence of the Hyal3 cDNA (GenBank AF036035) to determine the gene structure of human Hyal3 (data not shown). Based on the available sequence of the mouse Hyal1 cDNA (GenBank AF011567), the human and mouse Hyal3 cDNAs (GenBank AF036035 and AF048681) and guided by the human Hyal1 (10) and the Hyal3 gene structures, PCR primers were specified to amplify the predicted Hyal1 and Hyal3 exons and introns, from pHLS1 and the PAC clone, 452D10, respectively. The data are shown as a traditional dot-plot (Fig. 5A) and percent identity plot (PIP; Fig. 5B), generated by submitting sequences and annotations to the PipMaker web site (see “Experimental Procedures”).

The dot-plot display (Fig. 5A) was generated using parameters that allowed one block of DNA to align to more than one region on the second sequence. This not only reveals the similarity between the orthologous Hyal2 and HYAL2 genes, but also the similarity between the coding regions of exons 2–4 of Hyal1 and the corresponding exons from the paralogous human genes HYAL1 and HYAL3 (see highlighted region in Fig. 5A). Similarity between exons 2–4 of Hyal1 and Hyal3 and the human paralogs is also evident, although this was not highlighted in Fig. 5A.

In Fig. 5B, the mouse sequence is drawn along the x axis with exons and repetitive elements being labeled. The percent identity between mouse and human genomic DNA is indicated as a series of horizontal lines ranging from 50 to 100% similarity (y axis). The parameter of “single coverage” was used so as to allow only one alignment at each sequence position to be displayed on the PIP. Because of gaps in the alignment, the horizontal lines on the x axis are discontinuous and are most visible in regions corresponding to exons. Outside of the exon regions, the horizontal lines appear as a scattering of dots that correspond to smaller gap-free DNA alignments. Dark pink (translated) and light pink (untranslated) regions in Fig. 5B represent alignments of exons found on the top DNA strand, including Hyal2, Hyal1, Fus2, Hyal3, and Ifrd2. Dark (translated) and light (untranslated) yellow coloring identifies alignments for Sema3b found on the bottom strand. The red stripe marks the 275-bp gap between Hyal2 and Hyal1 whose size was defined by PCR amplification; this fragment was highly repetitive and could not be fully sequenced. The blue shaded box represents an assembly of 14 ESTs that are unspliced and appear to be noncoding. Through the above comparisons and analyses, the sizes of Hyal1 and Hyal3, as well as the order of the mouse genes, determined experimentally, were confirmed.

The clustering of the Hyal1, Fus2, Hyal3, and Ifrd2 genes, together with evidence that Hyal1, Fus2, and Hyal3 are sometimes coordinately regulated, led us to search for a putative promoter for this region. A 488-bp sequence (GenBank AF290430), upstream from the published cDNA sequence of Hyal1 (GenBank AF011567), was examined by visual scanning...
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TABLE I
Nucleotide sequences of the exon/intron boundaries of the mouse Hyal1 gene

| Exon | Exon size | 5’ Splice donor | Intron | 3’ Splice acceptor |
|------|-----------|----------------|--------|-------------------|
| 1    | 134       | CTGAG/gtaag     | 0.48   | gccag/GTTTC       |
| 2    | 924       | CCGTGG/tgtggag  | 0.45   | ccccg/GAGGA       |
| 3    | 90        | CCAAG/gtaag     | 0.13   | ctcat/GAATC       |
| 4    | 998       |                |        |                   |

TABLE II
Nucleotide sequences of the exon/intron boundaries of the mouse Hyal3 gene

| Exon | Exon size | 5’ Splice donor | Intron | 3’ Splice acceptor |
|------|-----------|----------------|--------|-------------------|
| 1    | 30        | GCCAG/gtaag     | 3.0    | tgcag/GTCTTC      |
| 2    | 914       | CTCTG/gtaag     | 0.71   | tgcag/GACGA       |
| 3    | 90        | CCGTGG/tgtggag  | 0.10   | tgcag/GAAAA       |
| 4    | 783       |                |        |                   |

and using TFSEARCH (23). No classical TATA box was identified, although a potential CAAT box was detected, with reference to GenBank AF290430, at position 411 and two GC boxes at positions 397 and 402. A number of small alignments upstream from Hyal1 were evident in the PIP (Fig. 5B) which may be significant, but only two of these conserved regions are predicted to bind known transcription factors (Sp1 sites at positions 368 and 386) (GenBank AF290430). Functional studies are needed to identify DNA segments in this region which may be involved in the coordinated regulation of Hyal1, Fus2, and Hyal3.

Analysis of Expressed Sequences—The analysis of ESTs using BLAST was used to confirm the intron/exon junctions as well as the organization of the Fus2 and Hyal3 genes. These comparisons also suggested a slightly different organization in humans, with Fus2 and Hyal3 sometimes sharing the same first exon. There is no evidence for these findings in mouse, possibly because of the limited number of Hyal3 ESTs. It was also noted that the 5’-coding region of IFRD2 was within the 3’-untranslated region of Hyal3, but no evidence of this has been seen in the mouse.

Alignment of Protein Sequences—To demonstrate the homology between the predicted protein sequence of mouse Hyal3 and the previously identified hyaluronidases, the amino acid sequences of human hyaluronidases, Hyal 1, 2, and 3 and mouse Hyal 1, 2, and 3 were aligned using ClustalW (Fig. 6).

Expression Profile of the Hyal1 to Hyal3 Region

Tissue Expression Profiles of Hyal1 and Hyal3—To determine whether the tissue expression profiles of the mouse Hyal1 and Hyal3 genes were similar to their human counterparts, we used Northern blot analysis. The steady-state expression of Hyal1 and Hyal3 mRNA in adult mouse tissues was examined by hybridization using mouse cDNA fragments as probes under stringent conditions. The highest Hyal1 expression levels in adult tissues were in liver, followed by kidney and skin (Fig. 7A), consistent with similar studies by Coska et al. (22) and revealing an expression profile similar to the human gene (5). For Hyal3, the expression profile in the mouse tissues (Fig. 7D) was broader than in human tissues (5), with an expression profile similar to Hyal1, except also present in brain, stomach, and testis. Hyal1 mRNA was detected in mouse embryos of all developmental stages, with the strongest and the greatest number of bands at the 7 day stage (Fig. 7E).

For both Hyal1 and Hyal3, the number of transcripts in the mouse tissues was greater than in the human tissues, and the higher expression of Hyal1 compared with Hyal3 made it easier to characterize the Hyal1 transcripts. Major Hyal1 transcripts of 6.2, 4.5, 3.3, and 1.3 kb in size, as well as a weak signal at 2.1 kb, were detected in both embryo and adult mouse tissues (Fig. 7, A and B). A 2.1-kb Hyal1 mRNA corresponds to the size of the published Hyal1 cDNA sequence, GenBank AF011567, and a mouse Hyal1 EST, GenBank AA716890, may be represented by the 1.3-kb transcript (data not shown). No ESTs corresponding to the larger Hyal1 transcripts were identified. The consistent 1.8-kb Hyal3 transcript observed on the Northern blot (Fig. 7D) putatively corresponds to the Hyal3 full-length cDNA (GenBank AY048681). Additional Hyal3 transcripts (3.8, 3.3, and 2.4 kB) were also detected in tissues with higher expression levels (Fig. 7D).

Alternative Polyadenylation of Hyal1—The high abundance of the large Hyal1 transcripts (6.2, 4.5, and 3.3 kb) suggested that they might have biological relevance. We were unable to explain the larger-sized transcripts by alternate splicing within the gene or alternative transcription initiation (data not shown). To determine whether alternative polyadenylation could explain the larger transcripts observed on the Hyal1 Northern blot, we used several fragments downstream from Hyal1 to probe the adult Northern blot. One probe, a genomic HincII/BsrGI fragment located 3.0 kb downstream from Hyal1 and containing sequence from the coding region of mouse Fus2, detected five transcripts (Fig. 7C). The 1.5-kb transcript that is highly expressed in testis is likely to correspond to the Fus2 published cDNA, GenBank AF172275. Three larger bands on the Fus2 Northern blot (6.2, 4.5, and 2.4 kb) (Fig. 7C) appeared to be of the same size and tissue distribution as three from the Hyal1 Northern (Fig. 7A), suggesting that some of the larger transcripts resulted from cotranscription of Hyal1 and Fus2. Similar comparisons of Hyal1 and Hyal3 Northern blots revealed a transcript of 3.3 kb in both blots (Fig. 7, B and D), implying that the two genes could be within a single transcript as well and use the poly(A) signal of Hyal3.

Cotranscription of Hyal1/Fus2 and Hyal1/Hyal3—To determine whether Hyal1 and downstream genes, Fus2 or Hyal3, are sometimes cotranscribed, RT-PCR was performed on mouse liver cDNA. The cDNA was amplified using a forward primer (WPG199) within exon 3 of Hyal1 and a reverse primer (WPG288) within exon 2 of Fus2 (Fig. 8A). A number of PCR amplification products were observed (data not shown). These products were cloned and sequenced, and the sequences of the
RT-PCR products have all been submitted to GenBank. The products demonstrated that Hyal1 and Fus2 were both found within seven differently spliced transcripts (Fig. 8B, i–vii). Using a forward Hyal1 primer (WPG199) and a reverse primer (WPG283) within exon 3 of Hyal3 (Fig. 8C), a single RT-PCR product was amplified from mouse liver cDNA (data not shown); sequencing revealed that Hyal1 and Hyal3 were found within one transcript as well (Fig. 8D).

At least seven bicistronic mRNA species containing Hyal1 and Fus2 were identified in mouse liver using RT-PCR. The level of these transcripts was not quantitated, but the intensity of the ethidium bromide staining of these species allowed a qualitative assessment of their abundance (Fig. 8). These various mRNA species have the potential to encode several novel protein products (Fig. 8).

In Vitro Transcription/Translation of Hyal1 and Fus2—The bicistronic transcripts containing both Hyal1 and Fus2 are expressed at relatively high levels and thus may contribute significantly to biological function if they are translated. To determine whether the bicistronic transcripts containing both Hyal1 and Fus2 could be translated, we generated a construct expressing the most abundant mRNA species (Fig. 8B), as well as constructs containing only Hyal1 or Fus2 cDNA, and tested their ability to be translated in vitro. pSP64(Hyal1/Fus2) DNA (expressing the abundant Hyal1/Fus2 transcript), pSP64(Hyal1), pSP64(Fus2), positive control DNA expressing luciferase and negative controls with the pSP64(poly(A)) vector or no added DNA, were introduced into the TNT Coupled Reticulocyte Lysate System, and the DNA templates were transcribed and translated. Aliquots of the resulting samples were separated on a SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (20), and analyzed by both autoradiography and Western blot. Autoradiography detected three identical bands in the lanes containing in vitro transcribed/translated pSP64(Hyal1/Fus2) and pSP64(Hyal1) (Fig. 9A). Bands of different molecular mass were also detected in the lane containing in vitro transcribed/translated pSP64(Fus2) (Fig. 9A), suggesting that Hyal 1, but not Fus 2, could be expressed from the bicistronic mRNA. The predicted molecular mass of Fus 2 expressed from pSP64(Fus2) is 34.6...
kDa, correlating to the top band detected in the lane containing in vitro transcribed/translated pSP64(Fus2) (Fig. 9). The smaller bands observed in the positive control and experimental samples are assumed to result from leaky scanning, resulting in translation initiation at downstream AUG codons. Products smaller than the full-length protein are commonly observed with these systems (Reticulocyte Lysate System technical notes). No bands were detected in the negative control lanes.

**Western Blot Analysis**—To determine which protein bands corresponded to Hyal 1, Western blot analysis of the in vitro translated samples was performed, using anti-HYAL 1. In the lanes containing the in vitro translated proteins from pSP64(Hyal1/Fus2) and pSP64(Hyal1), the top three bands were detected with anti-HYAL 1 (Fig. 9).

**DISCUSSION**

We have analyzed the organization of the Hyal1, Hyal2, and Hyal3 genes on mouse chromosome 9F1-F2 using both experimental and bioinformatic approaches. The region encompassing these genes was found to be complex and gene dense (one gene/6 kb). Six mouse genes were identified on 40 kb of mouse chromosome 9, and their order was determined to be Hyal2, Hyal1, Fus2, Hyal3, Ifrd2, and Sema3b (Figs. 3 and 5). This is identical to the order of the orthologous human genes located on chromosome 3p21.3, but the interval in humans was 17 kb larger, in part because of the insertion of 10 kb of repeat elements into the intergenic region between HyAL2 and HyAL1 (Fig. 5A). Our analysis revealed the second exon of Fus2, a gene encoding an N-acetyltransferase, within the first intron of Hyal3. Further, Ifrd2, a gene encoding an interferon-related developmental regulator, was found immediately 5’ to Hyal3 (Fig. 5B). Although the general gene organization was conserved in humans and mice, the analysis of ESTs from this region suggested some differences. Human HyAL3 and Fus2 sometimes share the same first exon, but no ESTs were identified to support this usage in the mouse. Further, in humans, the 3’-untranslated region of HyAL3 overlaps with the putative 5’-coding region of Ifrd2 (GenBank Y12395), again a finding not observed in the mouse. Northern blot analysis revealed further complexity in the transcription of this region, which is discussed later.

The order of a second group of three related HyAL genes on human chromosome 7q31 has been determined to be SPAM1, HyAL4, and HyALP1 (6). Mouse Spam1 has been localized to chromosome 6 (24), suggesting that there is a second group of hyaluronidase genes, as in humans, on mouse chromosome 6. An analysis has not been done to determine whether genes related to Fus2, Ifrd2, and Sema3b are also duplicated with the hyaluronidase genes in these regions. If the six linked genes (Hyal2, Hyal1, Fus2, Hyal3, Ifrd2, and Sema3b) are completely duplicated, it would be interesting to determine whether there is increased transcriptional complexity in any parts of this
region. A full characterization of this region may also provide insight into the evolutionary history of these two groupings of hyaluronidase genes.

The mouse Hyal1, Hyal2, and Hyal3 genes were found, using bioinformatic approaches, to consist of four exons and three introns (Fig. 5B). This general structure was confirmed for Hyal1 and Hyal3 using experimental approaches and published information for Hyal2 (13). This structure is also conserved in human HYAL2 and SPAM1 (5, 25), mouse Spam1 (26), and human HYAL3 (data not shown). The human HYAL1 structure is similar, except that the first exon junction in the 5′-untranslated region is not always recognized, leading to the retention of the first intron a significant proportion of the time (5, 27). There is no evidence in the EST data base that this intron is retained in the mouse Hyal1 gene, although RT-PCR does detect a low level of alternative splicing in this region.

Fig. 7. Tissue expression profiles of adult Hyal1, Fus2, and Hyal3, and embryo Hyal1 mRNA. A and C, adult mouse Northern blot probed with Hyal1 and Fus2, respectively; 17-h exposures for both. Arrows denote bands shared between Hyal1 and Fus2. B and D, adult Northern blot (different from A and C) probed with Hyal1 (17.5-h exposure) and Hyal3 (22-h exposure), respectively. * denotes a band shared between Hyal1 and Hyal3. E, mouse embryo Northern blot probed with Hyal1. F, adult Northern blots probed with actin. Developmental stages are shown above the embryo blot. Molecular weight markers in kb are shown on the right.

Fig. 8. Cotranscription of Hyal1/Fus2 and Hyal1/Hyal3. RT-PCR products of mouse liver cDNA were generated using a forward primer in Hyal1 (→) and reverse primers in Fus2 and Hyal3 (→). Hyal1 and Fus2 (A) and Hyal1 and Hyal3 (C) genomic arrangements are shown. B, i–vii, RT-PCR products consisting of Hyal1 and Fus2 within the same transcripts. Sequences were deposited as GenBank AF417494, AF417493, AF417495, AF417492, AF417498, AF417497, and AF417496. D, RT-PCR product containing Hyal1 and Hyal3 within a single transcript. The abundance of the RT-PCR products for Hyal1/Fus2, determined qualitatively (with 1 being the most abundant and 4, the least) and the predicted putative protein products for both are stated within the tables. Hyal1 exons are depicted by unshaded boxes, Fus2 exons are shown by light gray boxes, and dark gray boxes represent Hyal3 exons. Black boxes depict the sequence between Hyal1 and Fus2 which is present in some of the bicistronic transcripts and may contain an internal ribosome entry site. Start (ATG) and stop (TGA) codons are indicated. Hyal 1 followed by the caret or prime or asterisk correspond to putative Hyal 1 proteins with different C termini. Fus 2** and Fus 2′ represent putative Fus 2 proteins with different N termini.
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In vitro transcription/translation and Western blot analysis of Hyal1. A bicistronic mRNA species encoding both Hyal1 and Fus2 was translated in vitro using [3H]leucine, SP6 polymerase, and the TNT-coupled reticulocyte lysate system. Constructs containing Hyal1 or Fus2 cDNA were also transcribed and translated. Luciferase cDNA was used as a positive control, and negative controls consisted of pSP64(polyA) vector alone or no added DNA. The samples were separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and analyzed by autoradiography and Western blot. A, autoradiograph of H-labeled cotranscript products; 2.5-h exposure. B, Western blot detection of in vitro translated products using anti-HYAL 1 (α-HYAL 1). In vitro transcribed/translated products from pSP64(Hyal1/Fus2) (lane 1), pSP64(Hyal1) (lane 2), pSP64(Fus2) (lane 3), expressed luciferase protein (lane 4), pSP64(polyA) negative control (lane 5), and no DNA (lane 6) are shown. Molecular mass markers in kDa are shown on the left.

(data not shown). Surprisingly, in contrast to mouse Hyal2 and Hyal3 and human HYAL2, HYAL3, and SPAM1 where the translation start codon (ATG) is found near the 5′-end of exon 2, the addition of a new upstream sequence in the mouse gene places the ATG start codon within exon 1. The strongest sequence conservation among the members of the HYAL1 family is found in exons 2–4 (Fig. 5A), which, except for Hyal1, contains the entirety of the coding sequence.

Northern blot analysis of mouse Hyal1 demonstrated a tissue expression profile similar to that of the human gene, but more transcripts were detected. In humans, only two major HYAL1 transcripts (1.9 and 2.6 kb) have been identified (5) compared with four major transcripts in mice (6.2, 4.5, 3.3, and 1.3 kb) (Fig. 7, A and B), indicating that transcriptional regulation differs between mice and humans. During a search for the basis of the larger Hyal1 transcripts, shared bands between Hyal1 and Fus2 (Fig. 7, A and C), as well as between Hyal1 and Hyal3 (Fig. 7, B and D), were recognized. RT-PCR confirmed the cotranscription of Hyal1 and Fus2 as well as Hyal1 and Hyal3 (Fig. 8). In humans, no evidence for significant levels of cotranscription of HYAL1 with other genes is indicated from Northern blot analysis, suggesting that this region has evolved since the divergence of humans and mice.

Hyal3 expression was found to be lower than that of Hyal1 (Fig. 7, B and D), as had been observed previously for the human gene (5). However, the mouse Hyal3 tissue expression profile differed from that of human HYAL3 in that in addition to expression in the same tissues as the human gene, Hyal3 also had acquired expression in the same tissues as Hyal1. Interestingly, a similar phenomenon was observed for Fus2, suggesting some type of coordinate regulation for this region in mouse.

The steady-state levels of bicistronic mRNA species containing both Hyal1 and Fus2 are relatively high (Fig. 7), suggesting that they may have biological relevance. Several of the bicistronic species, characterized by RT-PCR (Fig. 8), encode unique protein products. “Wild-type” Hyal 1 protein is encoded by the cDNAs depicted in Fig. 8B, v–vii, but because of alternative splicing to form different bicistronic mRNA species, Hyal 1 proteins with different C termini would be generated by species i–iv of Fig. 8. Differing forms of Fus 2 are encoded by the bicistronic transcripts (Fig. 8B, ii, vi, and vii), but our in vitro transcription/translation studies suggest that they are not produced. The bicistronic transcript comprised of both Hyal1 and Hyal3 encodes a Hyal 1:Hyal 3 fusion protein (Fig. 8D). It is interesting that the most abundant bicistronic transcript, detected by RT-PCR (Fig. 8), encodes Hyal 1 that has the last eight amino acids removed and replaced by four novel amino acids. Given that the C termini of other hyaluronidases (PH-20 and HYAL 2) were found to be important for its localization (28, 29), this could have implications for the targeting and localization of Hyal 1.

In vitro transcription/translation studies of the most abundant bicistronic transcript, identified by RT-PCR (Fig. 8), showed that only Hyal 1 could be expressed from this transcript. A protein product, translated in the in vitro system from pSP64(Fus2), corresponded to the predicted size of Fus 2 but was not detected in the lane containing protein products expressed from the bicistronic transcript (Fig. 9A). Based on the analysis of this transcript, it is likely that Hyal 1, but not Fus 2 protein products, can be generated from all of the cotranscripts.

Reports of polycistronic transcription in mammals are rare (30–33), but more will probably arise as complete analysis of mRNA species becomes possible with the availability of the human genome sequence. Polycistronic transcripts may result from the close proximity of unrelated genes. In prokaryotes, the proteins encoded on operons are functionally related, but whether this is the case in eukaryotes is yet to be determined. We currently have no evidence that Hyal 1 and Fus 2 have a related biological function.

In contrast to Hyal2, the Hyal1, Fus2, Hyal3, and Ifrd2 genes on mouse chromosome 9 form a cluster that exhibits complex organization and transcription. The cotranscription of Hyal1 with downstream genes, Fus2 and Hyal3, indicates that the same promoter can be used to express multiple genes in this region. It is interesting that the tissue distribution of Fus2 and Hyal3 in mouse differs from that in humans and now appears similar to the profile of Hyal1. It is possible that this entire cluster of genes has evolved some coordinate regulation in mouse or lost this regulation in humans.

The characterization of the region encompassing Hyal2, Hyal1, and Hyal3 revealed unexpected complexity in structure and expression which has significant implications for the targeting of all of the genes in this region. We found the genes in this region to be densely packed, in both humans and mice, and in some cases, overlapping. In mice, the dense organization was accompanied by significant levels of cotranscription as well as coordinated tissue expression of some of the genes. Experimental studies showed that Hyal 1 is likely to be translated from the bicistronic transcripts encoding Hyal 1 and either Fus 2 or Hyal 3. The high abundance of the cotranscripts, together with their ability to be translated, suggests that they contribute significantly to the production of Hyal 1. The complexity in this region of mouse chromosome 9 may make it difficult to target genes without impacting on neighboring genes. Strategies for targeting genes in this region and analyzing the mouse knockouts of genes in this region should take this information into account.

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