Natural Antisense mRNAs to Hyaluronan Synthase 2 Inhibit Hyaluronan Biosynthesis and Cell Proliferation*

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We report the identification of a natural antisense mRNA of hyaluronan synthase 2 that we have chosen to designate as HASNT (for HA synthase 2 antisense) in human and mouse. HASNT is transcribed from the opposite strand of the HAS2 gene locus and is represented by several independent expressed sequence tags in human. Portions of the mouse Hasnt gene were identified through an exon-trapping approach. Sequence conservation is extremely low between human and mouse HASNT, and it is not clear whether these mRNAs contain functional open reading frames. HASNT has an alternate splice site in both human and mouse. This splice site is located at an identical position within the gene in both species and results in mRNAs of two different lengths. In each species, the antisense portion of the HASNT gene is complementary to the first exon of HAS2, which represents the 5′-untranslated region. To study the biological activity of HASNT, two human expressed sequence tag clones, representing long and short HASNT splice variants, were cloned into a tetracycline-inducible vector and were stably transfected into human osteosarcoma U2-OS Tet-on cells. The long and short HASNT-expressing cells had a reduction in HAS2 mRNA levels up to 94 and 86%, respectively, whereas hyaluronan biosynthesis was inhibited by 40 and 37%, respectively. Cell proliferation was reduced throughout the time frame of the experiment.

Hyaluronan (HA) is a linear, unmodified glycosaminoglycan polymer composed of repeating (N-acetyl-d-glucosamine-β(1→4)-d-glucuronic acid-β(1→3)) disaccharide units. HA is both a common and necessary component of extracellular matrices within most vertebrate tissues (1–3). HA functions not only as a major structural component, but it can also regulate a variety of physiological and pathological functions, such as cell proliferation, cell adhesion, migration, differentiation, and metastatic spread of tumor cells (4, 5, 6). Mammalian HA is synthesized at the plasma membrane by one of three HA synthases (HASs), HAS1, HAS2, and HAS3 (7, 8). Three mammalian HASs are differentially expressed in response to external stimulation or physiopathological conditions (9–13).

Recent studies show that many cell types synthesize significant amounts of HA from an apparently minimal HAS2 mRNA pool (>50 copies) (4). Expression of HAS mRNAs is correlated with HA biosynthesis. In general, it can be stated that expression of HAS mRNAs correlates with HA biosynthesis; high levels of HAS mRNAs are mirrored by high levels of HA biosynthesis (14, 15). For instance, mouse cumulus cell oocyte complexes, isolated immediately after inducing an ovulatory cycle (at which time they do not synthesize HA), expressed no Has2 mRNA. When HA biosynthesis began ~3–4 h later, however, Has2 mRNA was expressed at high levels (15). HAS2 expression can be stimulated by many growth factors and cytokines. Platelet-derived growth factor-BB is able to dramatically stimulate HA biosynthesis by normal human mesothelial cells (14). This stimulation of HA biosynthesis corresponded closely with a rapid increase in HAS2 mRNA levels. Overall, studies suggest that HAS gene transcription represents the primary control mechanism that acts to regulate HA biosynthesis on a cellular level. It is not surprising, therefore, that reduction of the HAS2 copy number affects HA biosynthesis and HA-related cell functions, such as cell migration and proliferation (4). These studies highlight the importance of HAS gene transcript levels to both HA biosynthesis and basic cellular functions. Despite recent progress in studies focused upon the differential expression of three mammalian HAS isoforms and identification of the respective promoter sequences, the molecular mechanisms that regulate HAS mRNA levels remain unclear. Gene structures and promoter sequences have now been identified for all three HAS genes (16–18). The HAS2 and HAS3 genes are organized in a similar fashion, with the entire 5′-untranslated region encoded within the first exon. This gene organization has been maintained over at least 500 million years of evolution. The first exon of the Amphioxus (Branchiostoma floridana) has genes is composed of the entire 5′-untranslated region, whereas the remainder of the coding sequence is contained within one large exon. Glucocorticoids induce a rapid and sustained, near-total suppression of HAS2 mRNA levels in osteosarcoma cells and dermal fibroblasts, mediated through substantial decreases in both gene transcription and

Received for publication, October 12, 2004, and in revised form, March 31, 2005
Published, JBC Papers in Press, April 19, 2005, DOI 10.1074/jbc.M411544200
HAS2 mRNA stability (17). Interleukin-1β and transforming growth factor-β inhibited the HAS2 mRNA level in osteosarcoma and chondrocytes (12). Overall, several lines of evidence suggest that additional mechanisms may act in concert with changes in transcriptional activity to rapidly regulate HAS2 mRNA levels.

Natural antisense RNAs are endogenous transcripts, which are complementary to mRNA sequences of known function, i.e. their sense sequences (19). Natural antisense RNAs are capable of regulating prokaryotic and eukaryotic gene expression (19–21). Natural antisense RNAs exert their regulatory effects at multiple levels, including transcription, RNA editing, post-transcription, and translation (22–25). By altering the expression of a particular gene, natural antisense RNAs regulate biological functions, such as development, viral infection, or adaptive responses (26). Antisense transcription is predicted to be abundant in the human and mouse genomes (27). Through analyses of the mouse genome sequence, computer searches have predicted the expression of over 2000 putative sense-antisense RNA pairs (28). Many antisense RNAs are conserved among species, such as the c-myec antisense in human, bovine, and rodents (29–31). The wide distribution and conservation of endogenous antisense transcripts strongly suggest that antisense RNAs are not accidental and may play a general regulatory role in gene expression in many higher eukaryotes, including mammals. In our current investigation, we have discovered natural antisense mRNAs of human and mouse HAS2 that are transcribed from the opposite strand of the HAS2 gene locus. We have chosen to designate these natural HAS2 antisense genes as HASNT. We have demonstrated herein that HASNT mRNAs reduce HAS2 mRNA levels and HA biosynthesis in HASNT-transfected cells. Our data suggest that HASNT is a novel and important mechanism in the regulation of HAS2 mRNA levels, HAS2-associated HA biosynthesis and HA-related biological functions in vivo.

MATERIALS AND METHODS

Data Base Searches—Nucleotide-nucleotide BLAST searches were performed of the human and mouse expressed sequence tag (EST) data bases using the full-length human and mouse HAS2 cDNA sequences and partial genomic sequences. Candidate ESTs were used to screen the EST data base in an effort to extend sequences through creation of a contig of overlapping EST sequences. Putative polypeptide sequences were also searched against the protein data base using standard search parameters of the low complexity filter. Additional data bases that were used include the University of Santa Cruz Genome Site and the Ensembl site.

Reagents—All reagents were purchased from Sigma unless stated otherwise. Real time PCR reagents were purchased from Applied Biosystems (Foster City, CA). Tetracycline-inducible pTRE2hyg plasmid vector, doxycycline, genetin (G418), and hygromycin were purchased from BD Biosciences. pCIneo plasmid vector was purchased from Promega Corporation (Los Altos, CA). [3H]acetic acid (4.7 Ci/mmol) was purchased from BD Biociences. pCIneo plasmid vector was purchased from Promega. U2-OS cells were grown in McCoy's 5A medium with 100 μg/ml hygromycin. Stably transfected cells were maintained in McCoy's 5A medium with 1.5 mg/ml hygromycin. Transfection was performed using Superfect (Qiagen) according to the manufacturer's recommendation. Transfected cells were subjected to limiting dilu-
and were plated in 96-well plates. All transfectants were selected through 4–6 weeks of antibiotic selection in 400 μg/ml genetin and 300 μg/ml hygromycin until cell growth was stable. Stable transfectants were maintained in the parental medium supplemented with 100 μg/ml genetin and 100 μg/ml hygromycin.

**Cell Proliferation**—Cell proliferation assays were performed using the MTT cell proliferation kit (Roche Applied Science) (33). Standard curves were generated for each cell clone. Cells were synchronized by culture in fetuin bovine serum-free medium for 72 h. Cell numbers were determined using a hemocytometer. Two thousand cells in 100 μl of McCoy's 5A medium containing 5% fetal bovine serum and doxycycline (2 μg/ml), of individual L-, S-HASNT, and Vec-only clones, were seeded into 96-well plates and cultured for 1–5 days at 37 °C, 5% CO₂. Ten microliters of MTT reagent were added to the wells every 24 h during the experimental period and then incubated exactly for another 4 h before adding 100 μl of lysis buffer. After adding lysis buffer, the cells were incubated overnight at 37 °C, 5% CO₂, the absorbances at 590 nm were measured in a multwell spectrophotometer, and the cell numbers were calculated based on individual standard curves.

**Cell Adhesion Assay**—Two-hundred thousand cells were seeded onto 10-cm cell culture plates. Cells were incubated for 30, 60, and 120 min at 37 °C, 5% CO₂. At the end of incubation, non-adherent cells were removed by gently washing three times with 10 ml of phosphate-buffered saline. Adherent cells were trypsinized and counted using a hemocytometer. The experiments were repeated six times.

**Quantitative Analysis of HAS2 Expression**—Total RNAs were isolated from individual clones using TRIzol reagent (Invitrogen) as recommended by the manufacturer. Relative expression levels of HAS2 were determined by quantitative real time PCR. Gene-specific primers and probes were designed using Primer Express software version 2.0 (Applied Biosystems) and were as follows: forward, 5′-TCAGCATTTTGCAAGATGGTTAG-3′, reverse, 5′-CAAGGACTATGTGATATTGTTG-3′, 5′-FAM-(CCAATAGCATG-3′, and probes were designed using Primer Express software version 2.0 (Applied Biosystems). Amplifications of CATAGAGCAACGTTCCA)-TAMRA-3′. SDS software version 1.7 (Applied Biosystems). Amplifications of β-actin, which was used as the internal control for normalization, were performed using the β-actin detection reagent (Applied Biosystems).

**RESULTS**

**Discovery of Natural Antisense mRNAs of HAS2 (HASNT)—** Data base searches using the complete published cDNA sequence for human and mouse HAS2 identified several EST clones (Fig. 1A) that shared sequence identity with the 5′-untranslated region (5′-UTR) of human HAS2 (Fig. 1B). Many of these clones were oriented in a direction consistent with their transcription from the opposite strand of the HAS2 locus, representing putative cis-encoded natural antisense mRNAs of HAS2. Through additional data base searches, a total sequence of 1.6 kb (Fig. 1B) was derived for the human transcript, which was split into 4 exons, all of which were flanked by a consenus splice acceptor and donor sequences (Figs. 1 and 2). The 1.6-kbp cDNA was initially assembled in silico from overlapping EST sequences and then confirmed as being part of a contiguous mRNA using RT-PCR (data not shown). A short ORF was predicted within the human sequence, although the functionality of this ORF remains unclear to date. The predicted polypeptide had no sequence identity to any other polypeptide sequence and was not conserved with any predicted polypeptide generated from the derived mouse sequence. Although we have been able to verify the 1.6-kbp cDNA sequence for human HASNT using RT-PCR, all efforts to determine the true transcription start site(s) of this transcript have failed to date. Based upon our RT-PCR, quantitative RT-PCR, and Northern analyses, HASNT represents an extremely rare transcript. We have screened two cDNA libraries without success and have made multiple attempts at 5′-RACE and primer extensions to determine the true transcription start site for HASNT. In the absence of complete sequence information and the confirmed presence or identity of a functional ORF, we have assigned this gene the name HASNT. Four exons were identified. The four HASNT exons were distributed as follows with respect to the previously described HAS2 gene structure. One HASNT exon was encoded by sequences located within intron 1 of HAS2, one exon was complementary to a portion of HAS2 exon 1, and two HASNT exons were encoded by sequences located within the proximal promoter region for HAS2 (Fig. 2). An alternate splice site was identified, which resulted in alternate mRNAs with long (L) and short (S) antisense regions. Long (L)-HASNT, represented by image clone 5171029 (GenBank accession number B1829151), has 257 nucleotides of perfect complementary sequence to a region starting ~70 bp from the presumed transcription start site of human HAS2 (accession number AJ604570), whereas Short (S)-HASNT, represented by image clone 2369939 (accession number AI761403) has 174 nucleotides of perfect complementary sequence (Figs. 1 and 2). In addition to alternate splicing, EST clones defined at least two polyadenylation sequences (Figs. 1 and 2).

Data base searches failed to identify similar EST clone sequences within the mouse or rat EST data bases. In an effort to identify putative exonic sequences for mouse Hasnt, we used a novel exon-trapping approach to scan genomic restriction fragments of the mouse Has2 gene that we had previously cloned (7). This utilized the pCIneo general purpose expression vector, as we discovered that genomic fragments could be easily cloned into the unique BsiI restriction site that is located within the chimeric intron of pCIneo (Fig. 3). Any exonic sequences that were present within genomic fragments cloned into this site would be spliced into mRNAs resulting from transfection of the

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resultant plasmids into a given cell line, such as COS-1 cells. Primers were designed for RT-PCR. The reverse primer was designed to anneal to the immediate 5'-end of any transcript originating from the pCIneo plasmid. Using this approach, a 237-bp fragment would be amplified by RT-PCR of transfected COS-1 cell total RNA when no exonic sequences were present within the genomic fragment being scanned. If exonic sequences were present, however, a longer DNA fragment would be amplified, corresponding to an mRNA in which exons were spliced into the pCIneo-derived transcript (Fig. 3). Exonic sequences were trapped from within a large 9-kbp HindIII restriction fragment that included exon 1 of mouse Has2 plus ~6 kbp of the first intron. Exonic sequences were not trapped from within intron 2 nor from the region immediately upstream of Has2 exon 1 (Fig. 3 and data not shown). Sequence analyses indicated that 4 exons had been trapped and that two RNA species had been trapped, corresponding to the short and long splice variants similar to those seen for human HASNT. Significantly, the alternate splice site was located in an identical position in human and mouse. Two of the four trapped exons shared complementary sequence to exon 1 of mouse Has2 (Fig. 3). The total antisense sequence amounted to 266 nucleotides for L-Hasnt and 181 for S-Hasnt. Sequence analyses revealed that all of the putative mouse Hasnt exons were flanked by conserved splice acceptor and donor sequences. Scanning of sequences located within the proximal promoter region for mouse Has2 resulted in the identification of consensus poly(A) signals encoded on the opposite (antisense) strand of the mouse Has2 locus in a location equivalent to those poly(A) signals that were identified for the human HASNT gene. EST data base searches identified one putative transcript (GenBank™ accession number BY724531) that included one of the antisense exons for mouse Hasnt, in addition to what appears to be a sequence split into two additional exons that are derived from a region ~92–110 kbp further downstream with respect to our Hasnt exons, i.e., located 5' of the mouse Has2 gene. Thus, it is formally possible that Hasnt exons in the mouse are part of a transcript in which exons are distributed over a much greater distance than that observed for human HASNT. Overall, our data suggest that the mouse Hasnt gene is made up of at least 6 exons; the first exon would not have been trapped using our exon-trapping approach, which relies upon the presence of flanking splice donor and acceptor sequences. A short ORF was predicted in the mouse Hasnt cDNA sequence, but the predicted polypeptide shared no significant sequence identity to any known gene product nor to the short polypeptide predicted from within the human HASNT sequence. The possible functionality of this predicted polypeptide is considered questionable.

Expression of HASNT in U2-OS Cells and Human Tissues—The expression of HASNT in U2-OS cells and in different tissues was examined by RT-PCR. The HASNT RT-PCR amplified a 264-bp fragment, which represented the expected size of the HASNT RT-PCR amplicon (Fig. 4). It should be stressed that only HASNT cDNAs (and not HAS2 cDNAs) would be amplified using this approach. First, we used a gene-specific reverse primer corresponding to the HASNT sequence during first strand synthesis. Second, the primers were designed to flank an intron of HASNT, such that a fragment of the expected size would only be amplified from first strand cDNA generated off a correctly spliced HASNT mRNA. The identity of this RT-PCR fragment was confirmed as HASNT by automated DNA sequencing. Northern analyses of 30 μg of U2-OS cell total RNA using the HASNT probe showed a HASNT-positive fragment of ~6 kbp (data not shown). In addition, Northern analyses identified a transcript of ~6.6 kbp in skeletal muscle and ~2.0 kbp in liver. This would suggest that the 1.6-kbp sequence that we have derived may be part of a larger transcript in most human cells and tissues. We examined the tissue expression pattern by RT-PCR and automated DNA sequenc-
ing of amplified products. Our preliminary semiquantitative data showed that HASNT was expressed at higher levels in skin, prostate gland, uterus, and salivary gland tissues (data not shown). Furthermore, both splice variants of many cell lines could be detected by RT-PCR, including HuT12 (human tumor cell line), WI38 (human embryonic lung fibroblast), and primary human dermal fibroblasts, along with mouse 3T6 (embryonic fibroblast) cells (data not shown). This demonstrated that HASNT mRNAs are normally expressed in human and mouse.

HASNT Effects on HAS2 mRNA Levels and HA Biosynthesis—To investigate the biological activities of HASNT and their significance with respect to the regulation of HAS2 mRNA levels and HA biosynthesis, both L- and S-HASNT EST-derived cDNA sequences were inserted into a tetracycline-inducible plasmid vector, pTRE2hyg, which was transfected into U2-OS cells to generate stable transfectants. Several independent clones were randomly selected to determine the relative expression level of HAS2 mRNA. Expression of L- or S-HASNTs strongly reduced HAS2 mRNA levels. L-HASNT-expressing clones had an inhibition of the HAS2 mRNA level ranging from 56 to 94%, and S-HASNT-expressing clones had an inhibition of the HAS2 mRNA level from 46 to 86%, compared with the clones transfected with vector only (Table I). The TaqMan® RT-PCR amplification of HAS2 had an amplification efficiency between 80 and 85%, which generated a standard curve slope between −3.7 and −3.8. The amplification efficiency of the internal control β-actin was >99%. Rarer transcripts tend to have lower amplification efficiencies.

Expression of HASNTs also inhibited HA biosynthesis. Tritium-labeled HA, synthesized and released into the culture medium and associated with the cell surface and inside the cells, were collected, and the relative amounts of labeled hyaluronate lyase-sensitive material were quantitated. The L-HASNT-expressing clones had a reduction in HA biosynthesis ranging from 36 to 39%, and the S-HASNT-expressing clones had an inhibition from 8 to 37%, respectively (Fig. 5 and Table II).

HASNT Effect on Cell Proliferation—During routine expansion of cell cultures corresponding to vector control clones and HASNT-expressing clones, we observed that the cell growth was noticeably slower for the L- and S-HASNT-expressing clones. When 1 × 10⁶ cells of the L, S, and control clones were seeded on 10-cm plates and cultured at 37 °C, 5% CO₂, the control clones reached confluence within 72-hours, at which time the L- and S-HASNT clones were still obviously subconfluent (data not shown). We further investigated the apparent cell growth effect of HASNT expression on cell proliferation and cell adhesion using the MTT cell proliferation assay and direct cell counting after allowing attachment to proceed for defined amounts of time. Our cell proliferation data demonstrated that cell proliferation was inhibited in both L- and S-HASNT-expressing cells through the entire experimental period from day 1 to day 5 compared with vector control cells.
maximal inhibition of 32% was observed on day 2 and 23% on day 3 for L- and S-HASNT clones, respectively (Table III and Fig. 6A). This data were reproduced 5 times. Although L- and S-HASNT-expressing clones had reproducible defects in cell proliferation, neither class of clone showed any obvious defect related to cell attachment (Fig. 6B). When 4 x 10^5 cells were seeded into 10-cm culture plates and cultured in complete growth medium containing fetal bovine serum, followed by 30-, 60-, and 90-min incubations at 37 °C, 5% CO2, there was no difference in the absolute cell number that had adhered to each plate at the respective time points (Fig. 6B). This demonstrated that HASNT expression did not grossly affect the attachment of cells to the culture dish and suggested that the observed inhibitory effect of HASNT expression on cell proliferation was not because of defects in initial cell attachment but rather was more likely to result from defects in the cell proliferation machinery.
HASNT transcripts of compared in a panel of human adult tissues using Northern analyses. markers; weight DNA ladder showing the 250- and 300-bp molecular weight confirmed by automated DNA sequencing. cells and human adult tissues. most strongly in the heart in this tissue panel. sifier screens. In comparison, the human HAS2 blot was exposed for 1
nous high molecular mass HA failed to rescue the hindered cell
mination reduced both HAS2 mRNA levels and HA biosynthesis. To investigate whether the observed reduction in cell proliferation was due simply to a loss or reduction of extracellular or pericellular HA, we examined the ability of exogenous high molecular mass HA to rescue the cell proliferation defects in cell culture. Exogenous high molecular mass HA failed to rescue the hindered cell
Treatment of HAS2 mRNA levels and hyaluronan biosynthesis

**TABLE I**

| Clone no. | Relative to pTRE2hyg | % Inhibition |
|-----------|----------------------|-------------|
| pTRE2hyg  | 1.00 ± 0.18          | 0           |
| L-HASNT   | 0.97 ± 0.230         | 3.3 ± 0.78  |
| 2"        | 0.25 ± 0.015         | 76.6 ± 5.0  |
| 7"        | 0.12 ± 0.009         | 87.6 ± 6.6  |
| 8"        | 0.34 ± 0.024         | 65.9 ± 4.7  |
| 16"       | 0.44 ± 0.070         | 55.6 ± 8.8  |
| 65"       | 0.06 ± 0.008         | 94.0 ± 12   |
| S-HASNT   |                      |             |
| 10"       | 0.14 ± 0.020         | 86.2 ± 12.3 |
| 40"       | 0.24 ± 0.022         | 76.3 ± 7.0  |
| 42"       | 0.35 ± 0.052         | 65.3 ± 9.7  |
| 46"       | 0.39 ± 0.042         | 61.5 ± 6.6  |
| 102"      | 0.54 ± 0.056         | 46.0 ± 4.8  |

*p < 0.05.

**TABLE II**

| Clone no. | [3H] hyaluronan % Inhibition |
|-----------|-----------------------------|
| pTRE2hyg  | 0.053 ± 4.24 e-3 0 ± 0.59  |
| L-HASNT   | 0.032 ± 6.50 e-3 39.1 ± 0.79 |
| 8"        | 0.034 ± 4.76 e-3 35.9 ± 0.64 |
| 65"       | 0.033 ± 8.00 e-3 37.4 ± 0.93 |
| S-HASNT   | 0.049 ± 2.00 e-3 7.8 ± 2.0  |
| 40"       | 0.033 ± 6.80 e-3 37.0 ± 0.80 |
| 102"      | 0.037 ± 5.40 e-3 30.9 ± 0.68 |

*p < 0.05.

**TABLE III**

| Clone no. | % pTRE2hyg | HAS2 mRNA | [3H] hyaluronan |
|-----------|------------|-----------|----------------|
| U2-OS Cell| 100 ± 18   | 102 ± 0.56 |                 |
| pTRE2hyg  | 100 ± 18   | 100 ± 0.60 |                 |
| L-HASNT   | 23.4 ± 1.5 | 60.9 ± 0.12 |                 |
| 8"        | 34.1 ± 2.4 | 64.1 ± 0.09 |                 |
| 65"       | 6.0 ± 0.8  | 62.6 ± 0.16 |                 |
| S-HASNT   | 13.8 ± 2.0 | 92.2 ± 0.38 |                 |
| 40"       | 23.7 ± 2.2 | 63.0 ± 0.13 |                 |
| 102"      | 54.0 ± 5.6 | 69.1 ± 0.10 |                 |

*p < 0.05.

*Data represent the mean of six repeats.

**FIG. 5. Effect of HASNT expression on HA biosynthesis.** HASNT effect on HA biosynthesis was examined by in vitro HA metabolic assay. Data reveal that the expression of L- and S-HASNT inhibited HA biosynthesis up to 39 and 37%, respectively, compared with parental U2-OS cells and the control pTRE2hyg vector-only transfected cell line.

**DISCUSSION**

As a component of the vertebrate extracellular matrix, HA is quite unique (35–37). HA is composed entirely of a simple, linear carbohydrate polymer and is not synthesized in a form that is covalently attached to any polypeptide. HA is involved in the body’s ability to maintain extracellular matrix integrity and to regulate cell–matrix interactions. It is involved in a variety of biological processes, including cell adhesion, proliferation, migration, differentiation, and survival. [Figure 5](#).

**Effect of Exogenous High Molecular Mass HA and Endogenously Synthesized HA on Cell Proliferation—HASNT expression reduced both HAS2 mRNA levels and HA biosynthesis.** Our cell proliferation data suggested that cell proliferation defects resulted from expression of HASNT and presumably from the resultant reduction in HA biosynthesis. To investigate whether the observed reduction in cell proliferation was due simply to a loss or reduction of extracellular or pericellular HA, we examined the ability of exogenous high molecular mass HA to rescue the cell proliferation defects in cell culture. Exogenous high molecular mass HA failed to rescue the hindered cell proliferation (Fig. 7). We asked whether or not the reduced cell proliferation could be rescued by restoration of endogenous HA biosynthesis through adenoviral-mediated expression of human HAS3. Expression of the human HAS3 cDNA effectively rescued the reduced cell proliferation rate (Figs. 8, top and center). Indeed, the HAS3-expressing HASNT clones synthesized HA at a rate that was elevated above that seen in control cells (Fig. 8, bottom, and Table IV). This increased HA biosynthesis was reflected by an elevated cell proliferation rate, above and beyond the control cells. Thus, in this cell type, higher levels of endogenously synthesized HA were reflected in a higher cell proliferation rate. These results highlight the importance of endogenous biosynthesis of HA in cell proliferation and the differing biological activities of exogenous HA versus endogenously synthesized HA.
in many physiopathological processes, including multiple aspects of embryonic development, angiogenesis, tumor transformation, and metastasis (38, 39). HA is synthesized by any one of three HA synthase isoforms, HAS1, HAS2, and HAS3. The three HAS isoforms have distinct expression patterns, enzymatic properties, and roles in the formation of HA-dependent matrix (7, 8). In many cases, HAS2 is the dominant isoform (4, 5, 10, 13, 40, 41). For example, mice deficient in Has2 function fail to develop to term and die at mid-gestation because of multiple developmental abnormalities (40).

Expression of the HAS2 gene can be influenced by different physiopathological conditions and external stimuli, such as ovulation, tumor transformation, and many exogenous factors, including growth factors, cytokines, and glucocorticoids (14, 15, 17). Dexamethasone induced a near-total suppression of HAS2 mRNA in dermal fibroblast cells and osteosarcoma cells within a 2-h incubation (17), whereas hydrocortisone dramatically inhibited HAS2 mRNA (but not HAS1 or HAS3) after a 6-h treatment (shortest treatment in the experiment) (14). This suggested a mechanism through which HAS2 mRNA expression could be rapidly shut down or repressed. Significantly, dexamethasone treatment reduced both the transcriptional rate at the HAS2 locus
mRNAs of human and mouse HAS2 (HASNT), which are transcribed rapidly. In this manuscript, we have presented data on the logical importance of natural antisense RNAs in mammals (42). Our knowledge and acceptance of the biological importance of natural antisense RNAs in mammals, including human and mouse (19). Indeed, computer predictions have estimated that natural antisense gene evolution (42). Their functions are proposed to include gene regulation and stability to their sense transcripts, at least partially, and be the most susceptible targets for antisense RNA. It is significant that the complementary sequences that are shared between HASNT and HAS2 are localized to the first exon of HAS2, corresponding to the 5′-untranslated region and start codon and other regions involved in translation initiation appear to be the most susceptible targets for antisense RNA. It is significant, therefore, that the complementary sequences that are shared between HASNT and HAS2 are localized to the first exon of HAS2, corresponding to the 5′-UTR. This sequence includes the transcription initiation site and a portion of the translation initiation site (all except the AUG codon). This led to our hypothesis that HASNT plays an important and novel role in the regulation of HAS2 expression and HA biosynthesis. Although natural antisense transcripts were identified for both human and mouse HAS2, no positive evidence was found to support the existence of equivalent natural antisense transcripts within the HAS1 or HAS3 gene loci (data not shown).

Human HASNT transcripts were identified in the U2-OS cells used in this experiment and in several other human and mouse cell lines and tissues. Furthermore, 12 human HASNT EST clones are now present within the genetic data base (Fig. 1A), several of which contain consensus poly(A) signals at the position immediately upstream of the site at which the oligo(dT) primer annealed. This indicated that HASNT is a bona fide mRNA that is expressed in vivo. We have obtained data that suggests that HASNT transcripts may range from a high of ∼6 kbp to a low of ∼2 kbp, depending on the tissue or cell type (Fig. 4). HASNT transcripts could only be detected using RT-PCR or through Northern analyses, with a large relative amount of total RNA or poly(A)+ RNA. This and the fact that only 12 EST clones have been identified for human HASNT and only one putative clone to date for mouse Hasnt strongly suggest that HASNT transcripts are rare and that HASNT transcription and/or stability may be under tight control. That being said, HAS2 is also a rare transcript in many cell types and tissues. Indeed, HAS2 expression was difficult to detect by Northern analyses of U2-OS total RNA, whereas it could be detected by RT-PCR.

**Expression of HASNT inhibits cell proliferation**

| Day | L-HASNT no. 8 | S-HASNT no. 102 |
|-----|---------------|-----------------|
| 1   | 8.6 ± 0.49a   | 15.5 ± 1.24a    |
| 2   | 27.1 ± 1.43a  | 22.5 ± 1.45a    |
| 3   | 32.2 ± 0.83a  | 13.9 ± 0.59a    |
| 4   | 25.3 ± 1.67a  | 10.3 ± 0.12a    |
| 5   | 19.4 ± 1.00a  | 3.6 ± 0.09a     |

*p < 0.05.

As an approach to elucidate the biological activity of HASNT in vitro, L- and S-HASNT EST-derived cDNAs were cloned into a “Tet-on” pTRE2hyg vector, which was designed to drive HASNT expression under a minimal cytomegalovirus promoter in the presence of doxycycline. We chose to use a previously derived Tet-on U2-OS cell line, as this cell line expressed HAS2 as the dominant HAS gene (data not shown). Expression of either L- or S-HASNT resulted in a dramatic inhibition of HAS2 mRNA levels and HA biosynthesis. This demonstrated that L- and S-HASNT were functionally active in the regulation of HAS2 mRNA levels and HA biosynthesis in this model cell culture system. It is highly likely that HASNT is a biologically active natural antisense RNA in vivo.

Down-regulation of HAS2 gene expression using a synthetic HAS2 antisense construct reduced HAS2 mRNA levels, HA biosynthesis, and cell proliferation in a rat keratinocyte cell line (4). In our experiments, expression of natural antisense HAS2 RNA in U2-OS cells resulted in similar effects on HA biosynthesis and cell proliferation. Although previous studies have reported that the initial stage of cell matrix adhesion could be mediated and modulated by cell surface HA (43), the cell attachment abilities of HASNT-expressing U2-OS cells were not affected when assayed on standard cell culture plastic in serum-containing growth medium. We have not, however, investigated the ability of HASNT-expressing cells to attach to defined extracellular matrix substrates. The addition of high molecular mass HA to the culture medium did not rescue the hindered cell proliferation resulting from HASNT expression. In contrast, overexpression of human Has3 in HASNT-expressing cells stimulated HA biosynthesis and effectively rescued the cell proliferation defect. Indeed, Has3 overexpression resulted in HA biosynthesis levels that were over and above those seen by control cells and that correlated with higher cell proliferation rates than those observed for control cells. We did not test the ability of low molecular mass HA to rescue the cell proliferation effect observed in these experiments. Our previous data has reported that the various HAS isoforms are responsible for the biosynthesis of HA polymers of distinct molecular masses in vitro (8). These experiments were performed on isolated cell membrane fragments and we have found that, in intact cell cultures, the expression of all three HAS polypeptides resulted in the biosynthesis and liberation of high molecular mass HA into the culture medium. In at least one in vivo condition, however, the expression and function of mouse Has3 has been associated with the appearance of low molecular mass HA (44).

Exogenously added high molecular mass HA and endogenously synthesized Has3-dependent HA profoundly different effects on the cell proliferation of HASNT-expressing U2-OS cells. In contrast, in another in vitro culture system, we have demonstrated previously that cellular transformation of Has2-deficient mouse endocardial cushion cells could be equally rescued by exogenously added high molecular mass HA or by expression of Has2 or Has3 (40). In this context, HA appears to function as a signaling molecule, interacting with members of the erbB receptor tyrosine kinase family to ultimately activate a ras-dependent signaling cascade, which is required for the cellular transformation event. Significantly, we have observed that the overall cellularity of many tissues within Has2-deficient mouse embryos is reduced. Is it possible that HA biosynthesis is a general factor that plays a role in the regulation of cell proliferation rates? Our expression data has demonstrated that Has2 expression is associated with rapidly

**TABLE IV**

| RNA | Inhibition (%) |
|-----|---------------|
| L-HASNT no. 8 | 15.5 ± 1.24a |
| S-HASNT no. 102 | 22.5 ± 1.45a |

**Natural HAS2 Antisense RNA Regulates HA Biosynthesis**

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A. Spicer, unpublished data.

A. Spicer, J. Y. Tien, and A. Joo, unpublished data.
proliferating mesenchymal cell populations within the developing mouse embryo (45). We hypothesize that de novo HA biosynthesis plays a novel, as yet undefined, role in the positive regulation of the proliferation rate of certain cell types. Furthermore, we predict that exogenous HA cannot act in a similar capacity.

In summary, natural antisense mRNAs of HAS2 were identified in human and mouse and will presumably be found in other mammals and possibly all vertebrates. These natural antisense mRNAs were functionally active to regulate HAS2 mRNA, de novo HA biosynthesis, and HA-related cell functions in a cell culture model system. We hypothesize that HASNT represents a novel post-transcriptional regulation mechanism to control HAS2 mRNA and/or polypeptide levels and HA-related functions in vivo. We predict that HAS2 and HASNT transcriptional activity are independent and mutually exclusive. HASNT transcription may represent a mechanism through which HAS2 mRNAs can be rapidly targeted for degradation. This may be critically important as small increases in HAS2 mRNA copy number are able to elicit dramatic responses in cell behavior (4). Further investigations will focus on the complete elucidation of human and mouse HASNT gene structures and promoter/enhancer sequences, along with investigation of the significance of HASNT transcription and function, both in a developmental context and in physiopathological conditions.

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