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A Rapid Transient Increase in Hyaluronan Synthase-2 mRNA Initiates Secretion of Hyaluronan by Corneal Keratocytes in Response to Transforming Growth Factor β^*

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Abstract

Keratocytes of the corneal stroma produce transparent extracellular matrix devoid of hyaluronan (HA); however, in corneal pathologies and wounds, HA is abundant. We previously showed primary keratocytes cultured under serum-free conditions to secrete matrix similar to that of normal stroma, but serum and transforming growth factor β (TGF β) induced secretion of fibrotic matrix components, including HA. This study found HA secretion by primary bovine keratocytes to increase rapidly in response to TGF β , reaching a maximum in 12 h and then decreasing to <5% of the maximum by 48 h. Cell-free biosynthesis of HA by cell extracts also exhibited a transient peak at 12 h after TGF β treatment. mRNA for hyaluronan synthase enzymes *HAS1* and *HAS2* increased >10- and >50-fold, respectively, in 4–6 h, decreasing to near original levels after 24–48 h. Small interfering RNA against *HAS2* inhibited the transient increase of *HAS2* mRNA and completely blocked HA induction, but small interfering RNA to *HAS1* had no effect on HA secretion. *HAS2* mRNA was induced by a variety of mitogens, and TGF β acted synergistically to induce *HAS2* by as much as 150-fold. In addition to HA synthesis, treatment with TGF β induced degradation of fluorescein-HA added to culture medium. These results show HA secretion by keratocytes to be initiated by a rapid transient increase in the *HAS2* mRNA pool. The very rapid induction of HA expression in keratocytes suggests a functional role of this molecule in the fibrotic response of keratocytes to wound healing.

Hyaluronan (HA)² is a high molecular weight, nonsulfated glycosaminoglycan abundant in most tissues, where it acts as a hydrating agent and an organizer of extracellular matrix scaffolding via specific interactions with matrix proteins containing hyaluronectin domains (for review see Ref. 1). The corneal stroma, unlike most vertebrate tissues, is virtually devoid of HA. During active corneal wound healing and in corneas with various chronic pathologies, however, hyaluronan becomes abundant in the corneal stroma (2,3).

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²The abbreviations used are: HA, hyaluronan; TGF β , transforming growth factor beta; HAS, hyaluronan synthase; FBS, fetal bovine serum; FACE, fluorophore-assisted carbohydrate electrophoresis; HABP, hyaluronan-binding protein; qRT, quantitative reverse transcriptase; siRNA, small interfering RNA; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; DME, Dulbecco's modified Eagle's; PDGF, platelet-derived growth factor

The corneal stroma maintains transparency to light by virtue of the highly organized structure of its collagenous extracellular matrix. Collagen fibrils of the stroma exhibit highly regular parallel alignment and spacing. This spacing is controlled by collagen-associated small leucine-rich proteoglycans that form glycosaminoglycan cross-links between adjacent fibrils (4). Disruption of the fibril spacing is a major cause of loss of corneal transparency in scarring and stromal pathological conditions (5). In scars, interfibrillar glycosaminoglycan cross-links are altered or eliminated, and spaces without fibrils, known as “lakes,” have been identified (5). The almost ubiquitous presence of HA in nontransparent corneas suggests a relationship between the large hydrodynamic volume occupied by HA molecules and the disruption of the stromal ultrastructure. Additionally, the recent recognition of the diverse bioactivity of HA (6) raises the potential that matrix production by stromal keratocytes may be altered as a response to the HA present in pathological tissues. We have therefore undertaken a study to define the molecular mechanism by which HA is produced in the corneal stroma.

The stroma is populated by keratocytes, mesenchymal cells of neural crest origin. *In vitro*, keratocytes under quiescent serum-free conditions secrete matrix components similar or identical to those they produce *in vivo* (7–9). On exposure to serum and TGF β , keratocytes alter their morphology and matrix secretion in a manner similar to cells in healing stromal wounds (8,10). Expression of keratocan, a unique stromal keratan sulfate proteoglycan, is strongly down-regulated and that of biglycan, a dermatan sulfate proteoglycan not normally present in stroma, is increased markedly (10). The glycosaminoglycans also change in a manner similar to that seen in scar tissue (8). Secretion of sulfated keratan sulfate is reduced, and dermatan sulfate made by these cells is more highly sulfated and more abundant (10). HA is also up-regulated in this *in vitro* model of fibrosis (8). HA biosynthesis is not detected when keratocytes are cultured in serum-free medium, but after 6 days of exposure to FBS, HA rises to about 1% of the total glycosaminoglycan, increasing to about 5% in the presence of both TGF β and serum (8).

Recent studies have shown HA in mammalian cells to be the product of an enzyme known as hyaluronan synthase (HAS) of which there are three isoforms (HAS1, HAS2, and HAS3). Each isoform is the product of a separate gene (11,12). In several cellular systems, alteration in HA biosynthesis is correlated with increases in HAS mRNA pools, although the genes involved differ for different cell types (13–19). In this study we examined the temporal response of HA and HAS mRNA to activation of keratocytes with TGF β and mitogens. We found a rapid increase in HA biosynthesis resulting from increases in mRNA pools of the HAS2 gene. This mRNA undergoes a rapid and transient response, peaking at 4–6 h after stimulation and returning to near base line within 24 h. HA biosynthesis also increases rapidly, peaking at 12 h and decreasing thereafter.

EXPERIMENTAL PROCEDURES

Cell Culture

Corneal stromas from fresh bovine eyes (Pel-Freez Biologicals, Rogers, AR) were digested with collagenase as described previously (9). The cells were diluted in serum-free DME/F12 medium containing antibiotics and cultured on tissue culture-treated plastic at 4×10^4 cells/cm² in a humidified atmosphere containing 5% CO₂. Myofibroblastic transformation was induced by the addition of the same medium containing 2% FBS and 1 ng/ml recombinant human TGF β 1 (Sigma). Heparin-stripped horse serum was prepared as described previously (20).

Quantification of Glycosaminoglycans Using FACE

Glycosaminoglycans and proteoglycans were isolated from the culture medium of three identical 75-cm² flasks using ion exchange chromatography as described previously (10). Chondroitin/dermatan sulfate and hyaluronan were digested with chondroitinase ABC (catalog number C3667, Sigma), 0.2 units/ml for 16 h at 37 °C in 0.1 M NH₄ acetate, pH 7.5. Digested products were recovered by ultrafiltration through Microcon YM-3 microfiltration devices (Millipore). Mannose was added to aliquots of the digestion products as an internal standard, and the carbohydrates were fluorescently labeled with 5 μl of 0.1 M 2-aminoacridone in 3:17 (v/v) acetic acid/dimethyl sulfoxide for 15 min followed by addition of 5 μl of freshly dissolved 1 M sodium cyanoborohydride at 37 °C overnight (21). Borohydride was quenched with 30 μl of 25% glycerol containing 2 μl of 1 mg/ml bromophenol blue, and the derivatized disaccharides were separated on 8 × 10 × 0.05-cm gels of 27% acrylamide, 0.72% bisacrylamide containing 0.045 M Tris acetate, pH 7, and 0.25% glycerol. The running buffer was 0.089 M Tris borate, 2 mM EDTA, pH 8.3, chilled to 4 °C. Electrophoresis was carried out on ice at 8 watts of constant power per gel. Fluorescent bands were immediately photographed using a 12-bit Bio-Rad FluorS Max imaging system, and quantification was accomplished with Bio-Rad Quantity One software. FACE bands generated by chondroitinase were identified by co-electrophoresis with purified standards of fragments from chondroitin sulfate and hyaluronan (Sigma).

HA was also quantified directly in conditioned culture medium using a competitive ELISA based on HA binding to biotinylated HABP (Echelon Biosystems Inc., Salt Lake City, UT). 100 μl of each culture medium was assayed in triplicate using a standard curve of HA according to the manufacturer's directions.

HA Biosynthesis by Keratocytes

Primary bovine keratocytes were seeded at 1×10^6 /well on FNC (Athena Environmental Service, Inc.) pre-coated 6-well plates in serum-free DME/F12 medium. Fibroblastic response was induced with 2% FBS and 1 ng/ml TGF-β1 in DME/F12 for 6, 12, 18, 24, and 48 h, respectively. The control was incubated in serum-free DME/F12. During the last 6 h of incubation, [³H]glucosamine (38.3 Ci/mmol; PerkinElmer Life Sciences) was added to the medium to a final concentration of 50 μCi/ml. After labeling, the medium was collected; the cells were rinsed twice with cold PBS. The wash and medium were combined, and glycosaminoglycans in the culture medium were purified by ion exchange chromatography, dialyzed against water, and lyophilized. The dried samples were dissolved in 60 μl of 0.02 M sodium acetate buffer, pH 6.0, containing 0.15 M NaCl. HA was captured on wells of a microtiter plate coated with hyaluronan-binding protein (HABP) (Corgenix Inc.). To confirm the captured radioactivity as radiolabeled HA, one-half of each sample was digested by 1 TRU/μl *Streptomyces* hyaluronidase (Sigma) at 50 °C overnight before adsorption by HABP. Triplicate 100-μl samples were added into a 96-well HABP-coated plate and incubated at room temperature overnight followed by four washes with PBS. The bound HA was released by digestion with 25 μg/ml proteinase K in 0.1% SDS and 0.1 M Tris-HCl, pH 7.4, at 37 °C for 2 h. The solution was transferred to scintillation vials, and the incorporation of [³H]GlcNAc into HA was determined as disintegrations/min (dpm) using a Beckman Coulter LS 3801 after the addition and complete emulsion of the samples in 4 ml of Scintisafe (Fisher) scintillant.

Membrane Preparation

Primary bovine keratocytes in DME/F12 were first seeded at $3-6 \times 10^6$ cells/150 cm² on collagen-coated culture dishes in serum-free DME/F12 for 48 h and then were induced with 2% FBS and 1 ng/ml TGFβ1 in DME/F12 for variable times. After induction, the cells were washed, harvested in cold PBS, and centrifuged at 2000 × g for 10 min. The cell pellets were

resuspended in cold hypotonic homogenization buffer, containing 10 mM HEPES, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 1 mM NaF, 1 mM sodium vanadate, 5 μl/ml protease inhibitor mixture (catalog number P8340, Sigma). After swelling on ice for 10 min, the cells were disrupted by sonication, and nuclei were pelleted by centrifugation at 4000 × *g* for 10 min. The supernatants containing crude membranes were centrifuged in a Beckman TLA100.2 rotor at 80,000 × *g* for 50 min. The membrane pellet was resuspended using homogenization buffer and centrifuged again at 80,000 × *g* for 30 min. The washed membrane pellets were resuspended in 100 μl of 15% glycerol, 50 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 1 mM EGTA and stored at -80 °C until use. The membrane protein concentration was measured by fluorescence using Nano-Orange reagent (Invitrogen).

In Vitro HA Synthase Activity

To measure HA polymerization, 0.1 mM unlabeled UDP-GlcNAc, 2.2 μM ³H-labeled UDP-GlcNAc (60 Ci/mmol; American Radiolabeled Chemicals Inc.), 0.5 mM UDP-GlcUA, and 1 mM dithiothreitol were added to 10 μg of the membrane protein in a total of 50 μl. One-half of each sample was incubated for 4 h at 37 °C, and the reactions were then terminated by boiling the mixture for 10 min. The second part was boiled for 10 min first and then subjected to incubation at 37 °C for 4 h. HA production was captured by using an HABP plate as described above. Captured HA was released by proteinase K. The solution was transferred to scintillation vials, and the incorporation of [³H]UDP-GlcNAc into HA was determined as disintegrations/min using a Beckman Coulter LS 3801.

Degradation of Fluorescein-labeled HA

Fluorescein-HA (Sigma) was isolated by initial chromatography on a Superose 6 gel size exclusion column, eluted in 0.02 M Tris, pH 7.4, 0.2 M NaCl. High molecular weight fractions were pooled, dialyzed against distilled water, dried, and dissolved in DME/F12, filter-sterilized, and stored at -20 °C until use.

Primary bovine keratocytes in DME/F12 were seeded at 1 × 10⁵ cells/well on FNC pre-coated 6-well plates and induced with 2% FBS and 1 ng/ml TGFβ1 in DME/F12 for 3 days. Control cells were plated at 1 × 10⁶ cells/well in serum-free DME/F12. Three days later, 7.5 μg/ml fluorescein HA was added to each well and incubated at 37 °C for 24 h. The medium was collected, and glycosaminoglycans in the culture medium were purified by ion exchange chromatography and subjected to size exclusion chromatography on Superose 6 gel using the conditions described above. The fluorescence of the fractions was measured by FLx800 Reader (Bio-Tek Instruments).

mRNA Quantification

Cells were collected by centrifugation after scraping into cold saline, and RNA was isolated using RNeasy Mini kit (Qiagen). RNA was treated with DNase I (Ambion) according to the supplier's protocol. RNA (400 ng) was transcribed to cDNA in a 100-μl reaction containing 1×PCR II buffer (Roche Applied Science), 1.5 mM MgCl₂, 800 μM dNTP mixture (Roche Applied Science), 2.5 μM random hexamers (Invitrogen), 0.4 units of RNase inhibitor, and 125 units of SuperScript II reverse transcriptase (Invitrogen). qRT-PCR was carried out for 45 cycles of 15 s at 95 °C, 60 s at 60 °C after an initial incubation at 95 °C for 10 min in an ABI 7700 thermocycler. Reaction volume was 25 μl containing 1× TaqMan reaction buffer (Applied Biosystems), primers, probe, and cDNA. Forward and reverse primers and fluorescent internal hybridization probes for each gene, as shown in Table 1, were used at optimized concentrations. Efficiency of the amplification was determined to be >90% in each case.

For each gene/cDNA combination, amplifications without reverse transcriptase were carried out as negative controls. Amplification of 18 S ribosomal RNA was carried out for each cDNA (in triplicate) for normalization of RNA content. Threshold cycle number (C_t) of amplification in each sample was determined by ABI software. Relative mRNA abundance was calculated as the C_t for amplification of a gene-specific cDNA minus average C_t for 18 S, expressed as a power of 2, *i.e.* $2^{-\Delta C_t}$. Three individual gene-specific values, thus calculated, were averaged to obtain standard errors.

Gene Knockdown Using siRNA

Uncultured primary cells at a concentration of 2.5×10^6 cells/ml in siPORT electroporation buffer (Ambion Inc.) were transfected with 21-bp double-stranded siRNA. For *HAS1* mRNA a mixture of four siRNAs was used (Smart Pool, Dharmacon), and for *HAS2* a single 21-bp chemically modified siRNA (Stealth RNA, Invitrogen) was transfected at $1 \mu\text{M}$ final concentration using electroporation with an ECM830 Square Wave electroporator (BTX Inc., San Diego) with three pulses of 3500 V/cm, each pulse having a duration of 300 μs with a delay of 125 ms between pulses. The cells were plated at 8×10^4 cells/cm² in serum-free medium on tissue culture plastic, precoated with FNC coating mixture. After 24 h, cells were treated with 2% FBS and 1 ng/ml TGF β for 6 h, and RNA was prepared for qRT-PCR analysis. For analysis of HA secretion, conditioned medium was collected from 24 to 72 h after electroporation as a negative control. At 72 h the medium was changed to 2% FBS, 1 ng/ml TGF β . After 48 h this medium was collected for analysis of HA by FACE gels.

RESULTS

We previously reported HA secretion by quiescent primary cultures of bovine keratocytes to increase from undetectable levels to 5% of the total glycosaminoglycan 6 days after induction of myofibroblastic transdifferentiation with FBS and TGF β . In this study we initially examined the kinetics of induction of the HA using FACE gel technology. As shown in Fig. 1A, the HA disaccharide (*diHA*) band could be detected within 6 h of exposure to FBS + TGF β 1, and its abundance increased after 12 h in the induction medium. Disaccharides containing 6-sulfate from chondroitin-dermatan sulfate (*diCS-6S*) also increased rapidly; however, total chondroitin/dermatan sulfate did not change for at least 24 h of treatment. Quantification of the HA from FACE analysis similar to that shown in Fig. 1A is presented in Fig. 1B. The HA recovered from culture medium increased at both 6 and 12 h of FBS + TGF β treatment, after which the HA level remained statistically unchanged for at least 72 h.

This plateau in HA accumulation suggests a decrease in the biosynthetic rate and/or increased degradation occurring after 12 h of TGF β exposure. Alterations in HA biosynthesis and degradation are explored in the experiments shown in Fig. 2. In Fig. 2A, secreted HA was recovered from culture medium after 6 h of metabolic labeling with [³H]glucosamine. Incorporation during the 6-h labeling period was minimal without TGF β treatment, but after exposure to TGF β , ³H-labeled HA increased markedly at 6 h, reaching a peak at 12 h of incorporation, ratio exhibiting a 75-fold increase compared with the untreated cultures. After 12 h, incorporation decreased rapidly. By 24 h metabolic labeling of the HA was about 3-fold that of untreated controls. By 48 h the rate had decreased to only 5% of the maximum rate but still remained about 2-fold that of the control.

Incorporation of label under the conditions in Fig. 2A represents a balance between biosynthesis and degradation. To explore the extent to which HA degradation contributed to the results of Fig. 2A, degradation of fluorescent HA added to culture medium was examined using size exclusion chromatography. As shown in Fig. 2B, HA exposed to quiescent keratocytes for 24 h was recovered quantitatively and showed no change in

molecular size or release of small fragments. In cultures treated with FBS + TGF β 1, all of the added HA was recovered, but ~25% was degraded to smaller fragments. These results suggest that HA secreted during a 6-hour labeling period (as in Fig. 2A) would be expected to be recovered quantitatively and largely intact (94–100%). The changes in accumulation in HA observed in Fig. 2A, therefore, may be viewed as largely the result of alteration in HA biosynthesis with relatively minor contribution by catabolic action.

In Fig. 2C, membrane preparations from cells treated with FBS + TGF β were used in a cell-free assay of HA biosynthesis. Cells treated for 12 h showed >40-fold stimulation over controls. At 24, 48, and 72 h of treatment the synthetic activity was maintained at ~3-fold of the control ($p < 0.05$). These results are consistent with those of Fig. 2A in support of the conclusion that TGF β produces a transient induction of HA biosynthesis reaching a maximum within 12 h and decreasing thereafter to a level 2–3 times that of untreated cells.

Three HAS genes have been described, and their expression varies with different tissues. To determine which might be expressed in keratocytes, cDNA from keratocyte cultures untreated and treated for 6 h with FBS + TGF β 1 was amplified using primers specific for each of three bovine HAS genes. As seen in Fig. 3, each of the primer sets amplified a single product of the expected length. Keratocytes therefore appear to express mRNA for all three of the HAS mammalian genes. In the TGF β -treated cells, the bands for *HAS1* and *HAS2* were considerably stronger, suggesting an increase in mRNA pool size for these genes in response to TGF β .

Quantitative RT-PCR assays were designed with the primers used in Fig. 3 to determine the changes of the HAS mRNA pools in response to FBS + TGF β 1. As shown in Fig. 4, *HAS1* and *HAS2* each exhibited a rapid increase after stimulation of the cells, reaching a maximum in 4–6 h. *HAS3*, however, showed no change in pool size over 72 h of treatment. *HAS2* increased 30–50-fold under these conditions, whereas *HAS1* increased about 10-fold. The pools of mRNA for both genes decreased rapidly after reaching maximum. *HAS1*, in fact, decreased to almost the same level as untreated cells within 24 h, whereas *HAS2* was about 2-fold the original level after 48 h.

The very rapid increase in both mRNA pools and HA biosynthesis suggests a causal relationship. To test this relationship siRNA probes were designed for *HAS1* and *HAS2*. As shown in Fig. 5, treatment of the keratocytes with *HAS1* siRNA for 24 h before growth factor treatment completely blocked increases in *HAS1* mRNA pools but had no effect on the increased pool of *HAS2* transcripts. Conversely, siRNA with *HAS2*-specific sequence was able to block about 95% of the up-regulation of *HAS2* mRNA but had no effect on the pool size of *HAS1*. Electroporation of a mixture of the two siRNAs completely blocked both *HAS1* and *HAS2* up-regulation.

Secretion of HA by the keratocytes in the presence of the siRNA showed HA biosynthesis to be correlated only with *HAS2* mRNA levels. In the experiment shown in Fig. 6A, HA was measured using an ELISA based on binding of HABP. HA in medium was determined both before and after induction of HA biosynthesis by FBS + TGF β 1. In each case the pre-induction levels of secreted HA were near the lower limits of detection (Fig. 6A, *open bars on left*.) After induction (Fig. 6A, *gray bars*), *HAS1* siRNA transfection resulted in no statistically significant alteration in the HA amount compared with a mock transfection control. *HAS2* siRNA, however, blocked the stimulation of HA secretion by about 93% ($p < 0.01$). A combination of siRNA to *HAS1* and *HAS2* in the same cultures reduced the HA secretion by 92% ($p < 0.03$).

In a similar experiment shown in Fig. 6B, HA and chondroitin sulfate secretion were measured by FACE. The *diCS-0S* band of chondroitin/dermatan sulfate (Fig. 6B, *gray bars*),

measured on the same gel as the HA, showed no change as a result of HAS siRNA transfection, whereas *HAS2* siRNA reduced the *DiHA* bands by >90%. These results show the specificity of the *HAS2* siRNA in blocking only HA secretion and support the hypothesis that the up-regulation of HA biosynthesis by keratocytes in response to FBS + TGF β 1 stems directly from the rapid increase in *HAS2* mRNA pool. *HAS1* mRNA appears to play no role in this response.

We previously found synthesis of HA to be up-regulated after 6 days of exposure to FBS + TGF β 1; however, the data in this study show a marked decrease in HAS mRNA and HA synthesis after 24 h. To understand the temporal expression patterns, *HAS2* mRNA was examined for longer times. As seen in Fig. 7A, *HAS2* mRNA pools after the initial transient peak remained slightly elevated (about 2-fold) compared with levels of the untreated controls for at least 7 days. To test if *HAS2* was involved in continued HA biosynthesis, we transfected *HAS2* siRNA into keratocytes 24 h after growth factor treatment, a time at which mRNA pools had stabilized at a lower level. As shown in Fig. 7B, HA secretion over the 48–96-h period was suppressed by about 65% by this treatment, suggesting continued secretion of HA to be the result of the slight (*i.e.* 2-fold) long term increase in *HAS2*.

Earlier we reported HA biosynthesis to be stimulated by FBS alone as well as with TGF β . We pursued these observations in the experiment shown in Table 2, in which *HAS2* transcript increase was determined in response to addition of a number of agents to the keratocytes in serum-free medium. As shown in Table 2, FBS, as well as purified mitogens such as insulin-like growth factor 1 (IGF1), platelet-derived growth factor BB (PDGF), fibroblast growth factor 2 (FGF2), and TGF β alone, all stimulated *HAS2* significantly. To our surprise, agents that have no mitotic activity in these cells such as lipid-rich bovine serum albumin and heparin-stripped horse serum also activated *HAS2* by about the same degree as active mitogens. These stimulatory effects were not as strong as the combination of TGF β and FBS together, suggesting a synergistic effect of TGF β on increases in *HAS2* transcripts.

This synergy was further documented in Fig. 8. As seen in Fig. 8A, the increase in *HAS2* levels in the presence of TGF β was markedly stimulated (*dark bars*) beyond the level achieved using each of the agents tested alone (*light bars*). In Fig. 8B the degree of stimulation provided by TGF β is displayed. In each case TGF β stimulated *HAS2* mRNA by 5–10-fold regardless of the original level. TGF β stimulation of *HAS2*, therefore, is not an additive effect but multiplicative. Highest absolute levels of the *HAS2* (about 150-fold over quiescent cells) were observed in the presence of TGF β and PDGF.

DISCUSSION

In this study we found HA biosynthesis by keratocytes to respond rapidly to TGF β reaching a maximum rate of synthesis within 12 h. This increase in HA was preceded by a rapid and transient increase in *HAS1* and *HAS2* mRNA pools, peaking at 4–6 h and declining within 24–48 h to levels close to that of the quiescent cells. The increase in *HAS2* mRNA alone was found to be responsible for the induction of HA secretion by use of siRNAs, which completely and selectively blocked the increases in *HAS1* and *HAS2* mRNA.

HAS mRNAs have been found to be responsive to TGF β in a wide variety of cultured cells (13,15,16,23–32). The particular HAS gene involved, however, appears to be highly cell- and tissue-dependent. In synovial fibroblasts TGF β activates mostly *HAS1* (15,23,31), whereas *HAS3* is up-regulated in chondrocytes and down-regulated in keratinocytes by TGF β (15,27,32,33). It is interesting to note that two other cell types reported to respond to

TGF β with up-regulation of *HAS2* are both derived from the anterior segment of the eye: trabecular meshwork cells and corneal endothelial cells (13,25).

The dramatic and transient stimulation of HAS mRNA in response to TGF β appears to be a novel observation of this study, not previously reported in ocular or any other cultured cells. The study also reports several other novel findings. Two HAS mRNAs are up-regulated by TGF β and one is not, but only one of the two up-regulated HAS mRNAs appears to be involved in induction of HA secreted by these cells. This study provides the first demonstration of knockdown of HAS enzymes *in vitro* using siRNA, showing that HA but not chondroitin sulfate is altered by this knockdown. Finally, we observed that a wide variety of mitogenic agents up-regulate *HAS2* in addition to TGF β and these act synergistically with TGF β .

Transient changes in mRNA for signaling molecules in response to TGF β have been documented (34), but mRNA pools for extracellular matrix molecules typically change much more slowly (10). The *HAS2* burst initiates HA secretion by these cells, but after the peak of *HAS2* mRNA at 4–6 h the amount drops to only about 2-fold of that in quiescent cells within 48 h (Fig. 4) and remains at the level for at least 7 days (Fig. 7). This is consistent with the rate of HA biosynthesis determined by metabolic labeling (Fig. 2A) and the cell-free biosynthesis rate (Fig. 2C), which do not return to quiescent levels. Thus the conclusion of this study is that HA secretion by keratocytes is closely tied to pool levels for *HAS2* mRNA.

The untreated keratocytes do not appear to degrade extracellular HA, but treatment with TGF β + FBS induced some degradation of exogenously added HA (Fig. 2B) to smaller sized fragments. The increased degradation may contribute to the reduced HA recovered from culture medium after TGF β treatment, but the data showing that the biosynthetic rate markedly decreases after 12 h (Fig. 2C) suggest that degradation is not the major cause of decreased recovery of HA. Jenkins *et al.* (22) have shown that in cultured lung fibroblasts, increased HA in the culture medium in response to TGF β resulted from decreased degradation of extracellular HA via action of secreted hyaluronidases 1 and 2. HA accumulation by primary keratocytes, however, cannot be attributed to a similar mechanism, because the quiescent cells do not appear to degrade exogenous HA. Differences in the results of the two studies may be due to differing culture conditions, *i.e.* passaged fibroblasts in serum *versus* primary keratocytes in serum-free media, or from intrinsic differences in the cell types.

Despite up-regulation of two HAS mRNAs, our results show that only *HAS2* appears to be involved in the secretion of HA by these cells. Quantification of these two mRNAs indicated that at their maximum concentrations *HAS2* was about 40-fold more abundant than *HAS1* on a molecules/cell basis (data not shown). *HAS1* mRNA pools fully returned to the level seen in quiescent cells, whereas *HAS2* did not. It should be noted that the data in this study link *HAS2* only with HA recovered from the culture medium. Intracellular HA has been observed in some cells, and cell-associated HA is common in cultured cells. Our results do not rule out the possibility that HA polymerized by *HAS1* could be present as a cell-associated or intracellular form, whereas *HAS2* is responsible for secreted HA.

We found *HAS2* to be up-regulated by a variety of mitogens inducing cell division in keratocytes, including PDGF, fibroblast growth factor 2, insulin-like growth factor 1, and FBS. It was surprising to find that heparin-stripped horse serum and lipid-rich bovine serum albumin, agents with little or no mitogenic activity in keratocytes, also stimulated *HAS2* to the same level. Previous studies have shown that extracellular matrix components such as collagen and fibronectin can be up-regulated by albumin and by fatty acids (such as are

present in horse serum) (35–37). The response of the *HAS2* gene to low mitogen serum and to serum albumin in keratocytes may represent a similar mode of response.

We also observed TGF β to be strongly stimulatory and synergistic with other mitogens in inducing *HAS2*. The *HAS2* gene contains a number of promoter elements, presenting the possibility of multiple modes of transcriptional activation of *HAS2* gene expression (38). The presence of such multiple activation sites is consistent with a molecular mechanism involving separate and synergistic stimulatory pathways by TGF β combined with other growth factors.

The increases in HAS mRNA and HA secretion found by this study take place much more rapidly than previously documented keratocyte responses to serum or TGF β . Our previous studies found changes in secretion of dermatan sulfate and keratan sulfate by these cells to occur over a period of 2 or more days after exposure to TGF β . Maximum alterations in the mRNA pools for collagen III, EDA-fibronectin, and biglycan also required several days of stimulation by TGF β . The magnitude of the changes in *HAS2* mRNA in response to TGF β was large (up to 150-fold) compared with the changes we documented for mRNAs of other matrix components. The rapidity, magnitude, and transient nature of the response of *HAS2* suggest a functional role for HA in later events of the transdifferentiation of keratocytes to myofibroblasts. HA and HA fragments elicit motility and cell cycle entry from a number of cell types (6,39,40). It therefore seems likely that a burst of HA synthesis coupled with increased HA degradation could elicit a similar response from keratocytes as well. Initial responses of keratocytes to corneal wounding involve migration toward the site of the injury followed by mitosis. Thus, the HA secreted by keratocytes early in the healing process may play a functional role in initiating the complex program of responses exhibited by cell wound healing *in vivo*.

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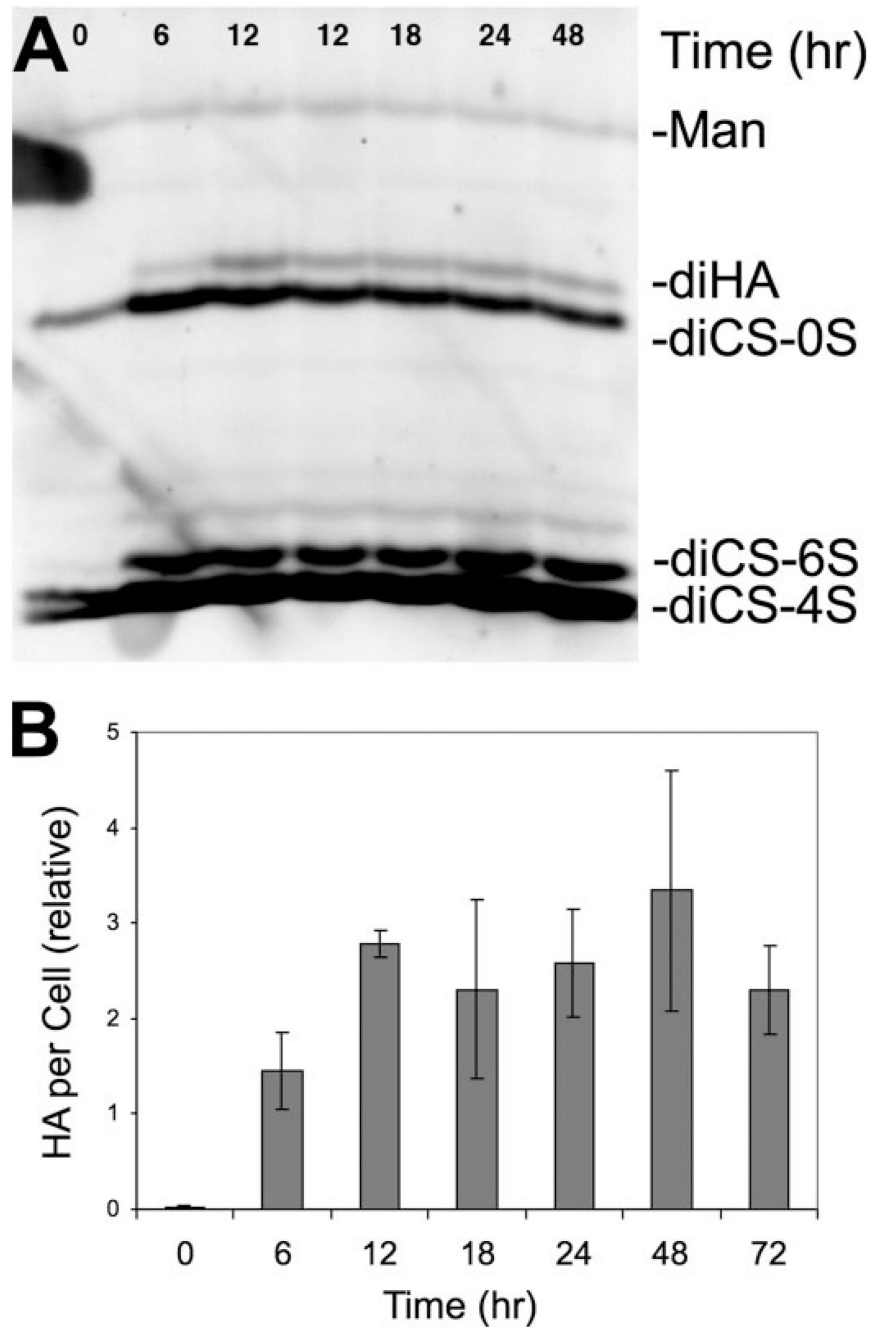


FIGURE 1. Stimulation of HA secretion by TGF β and FBS

Primary keratocyte cultures were exposed to 2% FBS and 1 ng/ml TGF β for different lengths of time, and proteoglycans were isolated from conditioned medium and digested with chondroitinase as described under "Experimental Procedures." Disaccharides from HA and chondroitin/dermatan sulfate were analyzed by FACE. *A*, gel image illustrating the marked increase in HA during the first 12 h of exposure to FBS and TGF β . *B*, quantitative analysis of the HA bands in this experiment carried out on triplicate samples normalized to the mannose internal standards (*Man*). The disaccharides used are as follows: *diHA*, hyaluronan disaccharide; *diCS-0S*, nonsulfated chondroitin sulfate disaccharide; *diCS-6S*,

chondroitin sulfate disaccharide GlcNAC-6 sulfate; *diCS-4S*, chondroitin sulfate disaccharide GlcNAC-4 sulfate.

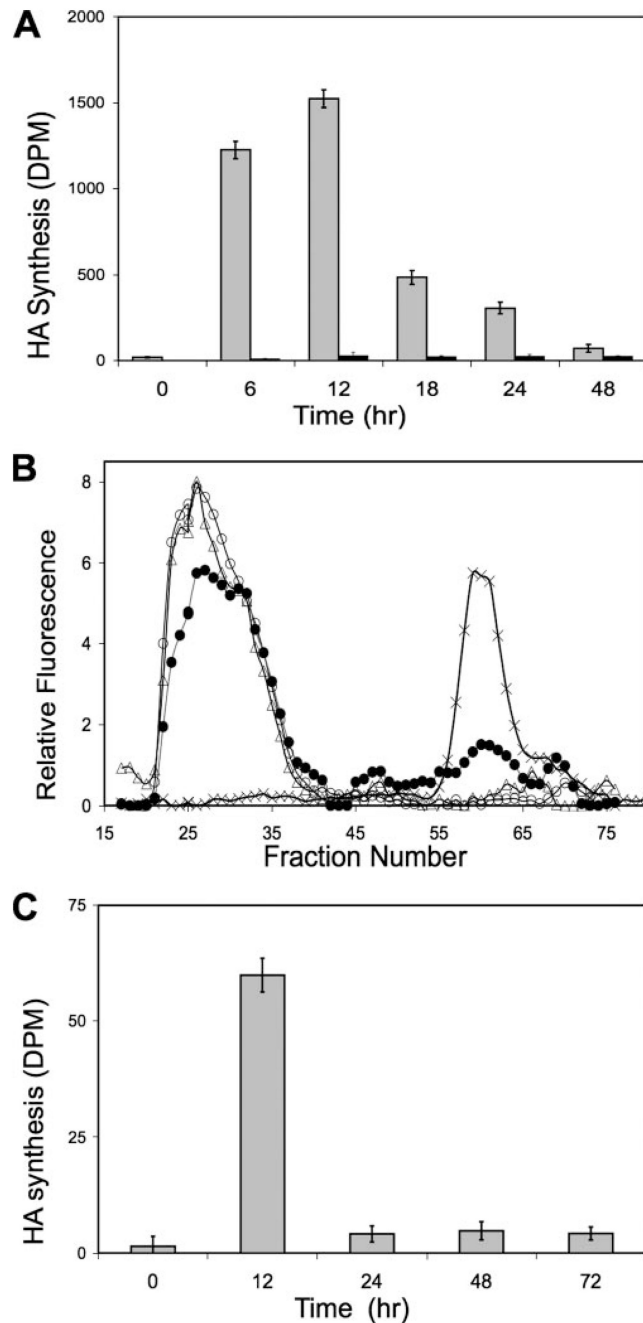


FIGURE 2. Rate of HA synthesis in response to TGF β and FBS

A, primary keratocytes were stimulated with TGF β and FBS for variable lengths of time as in Fig. 1. [^3H]Glucosamine was added during the final 6 h of culture, and proteoglycans were recovered from the culture medium by ion exchange as described under “Experimental Procedures.” HA was captured by immobilized HABP before (*gray bars*) or after treatment with hyaluronidase (*white bars*) as described under “Experimental Procedures.” Data are expressed in terms of radioactivity (*DPM*) of HA per 10^6 cells. *B*, degradation of exogenous HA by keratocytes. High molecular weight fluorescein-labeled HA was added to keratocyte cultures at a concentration of $7.5 \mu\text{g/ml}$ for 24 h and then recovered from the culture medium by ion exchange chromatography. Samples of the starting material (*triangles*) were

compared with HA incubated with keratocytes in serum-free medium (*open circles*) or with keratocytes after treatment with FBS and TGF β for 48 h (*filled circles*), or hyaluronidase-digested HA (*crosses*) on Superose-6 size exclusion chromatography. Fluorescence values are normalized to those of the control. C, cell-free HA biosynthesis. Membrane preparations from keratocyte cultures treated for various lengths of time with FBS and TGF β were used for cell-free biosynthesis of HA as described under "Experimental Procedures." Bars show labeled HA synthesized in terms of disintegrations/min per μ g of protein. Error bars show standard deviation of triplicate measurements.

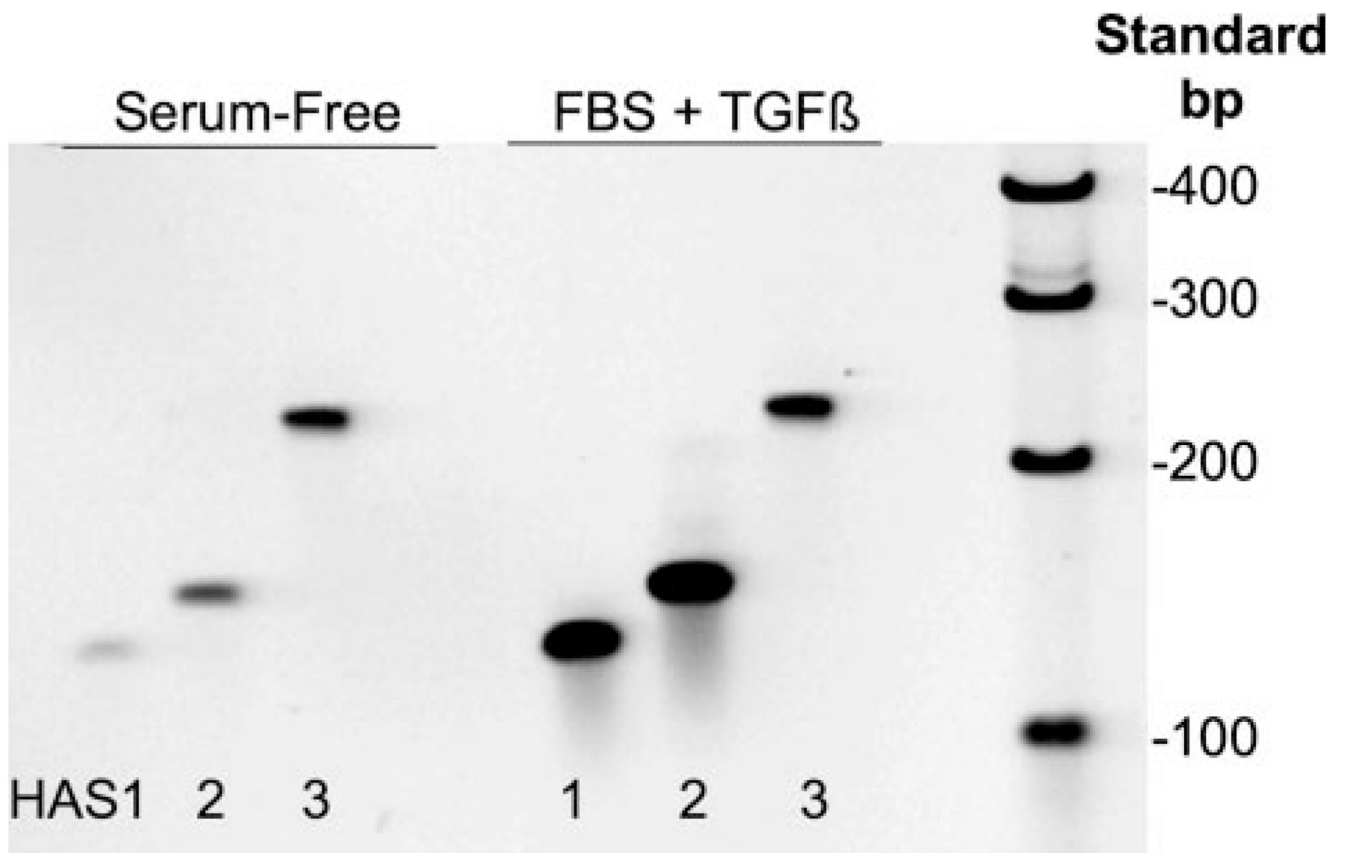


FIGURE 3. Detecting mRNA for hyaluronan synthase genes in keratocytes

Total RNA was extracted from primary bovine keratocytes cultured in serum-free medium or treated for 6 h with FBS and TGF β as in Fig. 1. RNA was reverse-transcribed using random primers, and DNA was amplified for 35 cycles using primers representing unique regions from bovine *HAS1*, 128 bp; *HAS2*, 134 bp; and *HAS3*, 234 bp (Table 1). The products were separated on 6% acrylamide gels and stained using SYBR Gold. Lane 7 shows DNA molecular size standards.

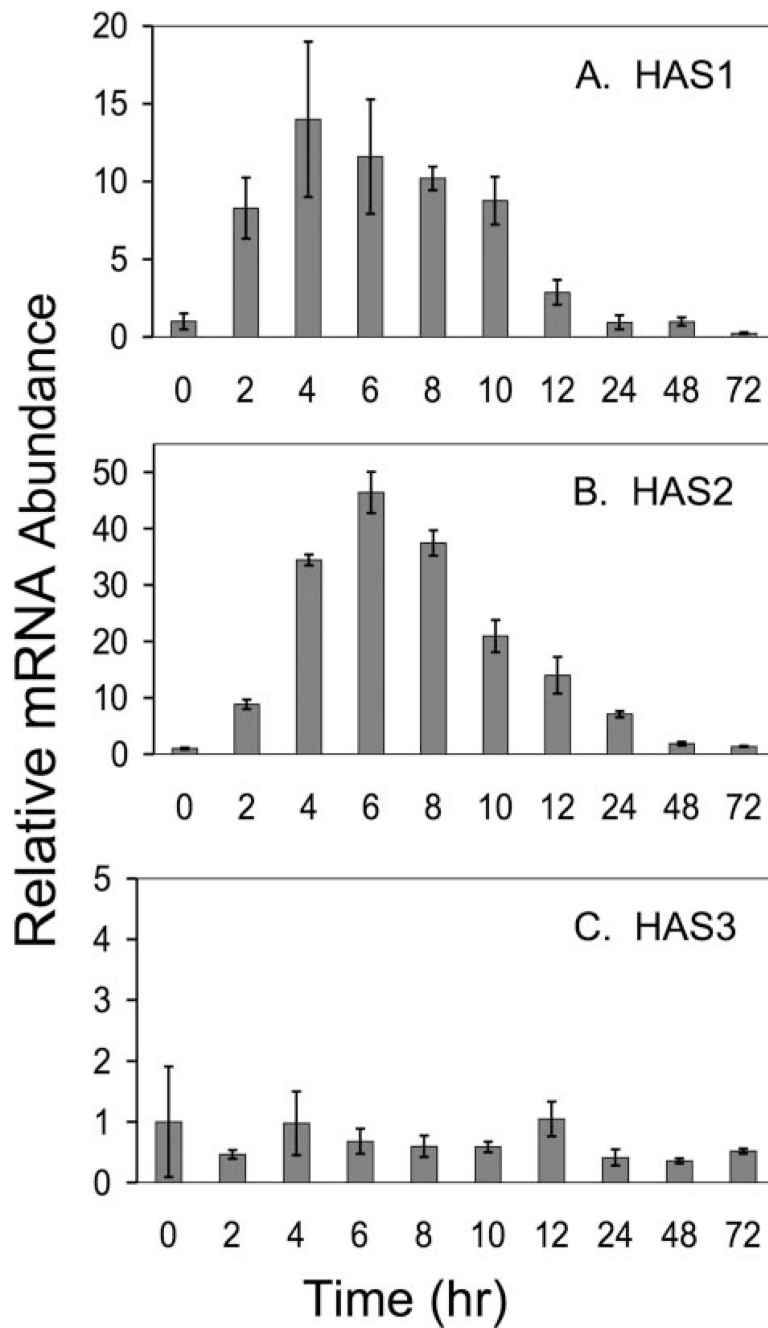


FIGURE 4. Temporal response of HAS mRNA to TGF β

Primary bovine keratocytes were exposed to FBS and TGF β as in Fig. 1, for the intervals shown, and the total RNA was analyzed by qRT-PCR for each gene product compared with 18 S RNA in the same sample as described under "Experimental Procedures." Bars show the standard error of triplicate analyses carried on duplicate cultures. For each gene, mRNA abundance at time 0 was set to 1.

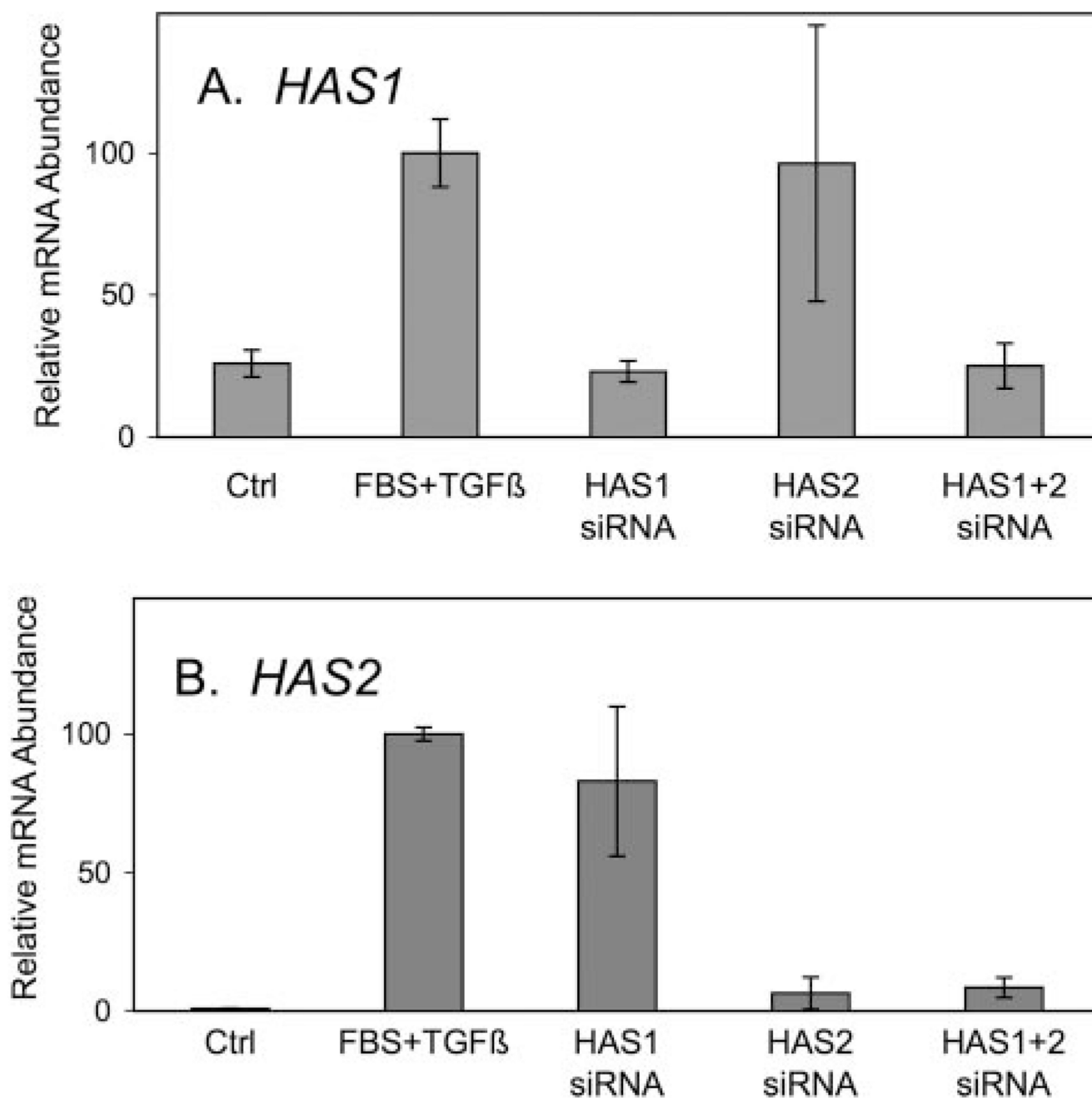


FIGURE 5. Inhibition of HAS up-regulation by siRNA

Primary uncultured keratocytes were transfected with siRNA for *HAS1*, *HAS2*, or a combination, and cultured for 20 h in serum-free conditions. *HAS* gene expression was induced with TGFβ and FBS for 6 h as in Fig. 1, and total cellular RNA was assayed for mRNA abundance of *HAS1* (A) and *HAS2* (B). mRNA levels are normalized so that the induced samples = 100. Error bars show triplicate analyses of RNA from three pooled cultures. *Ctrl*, control.

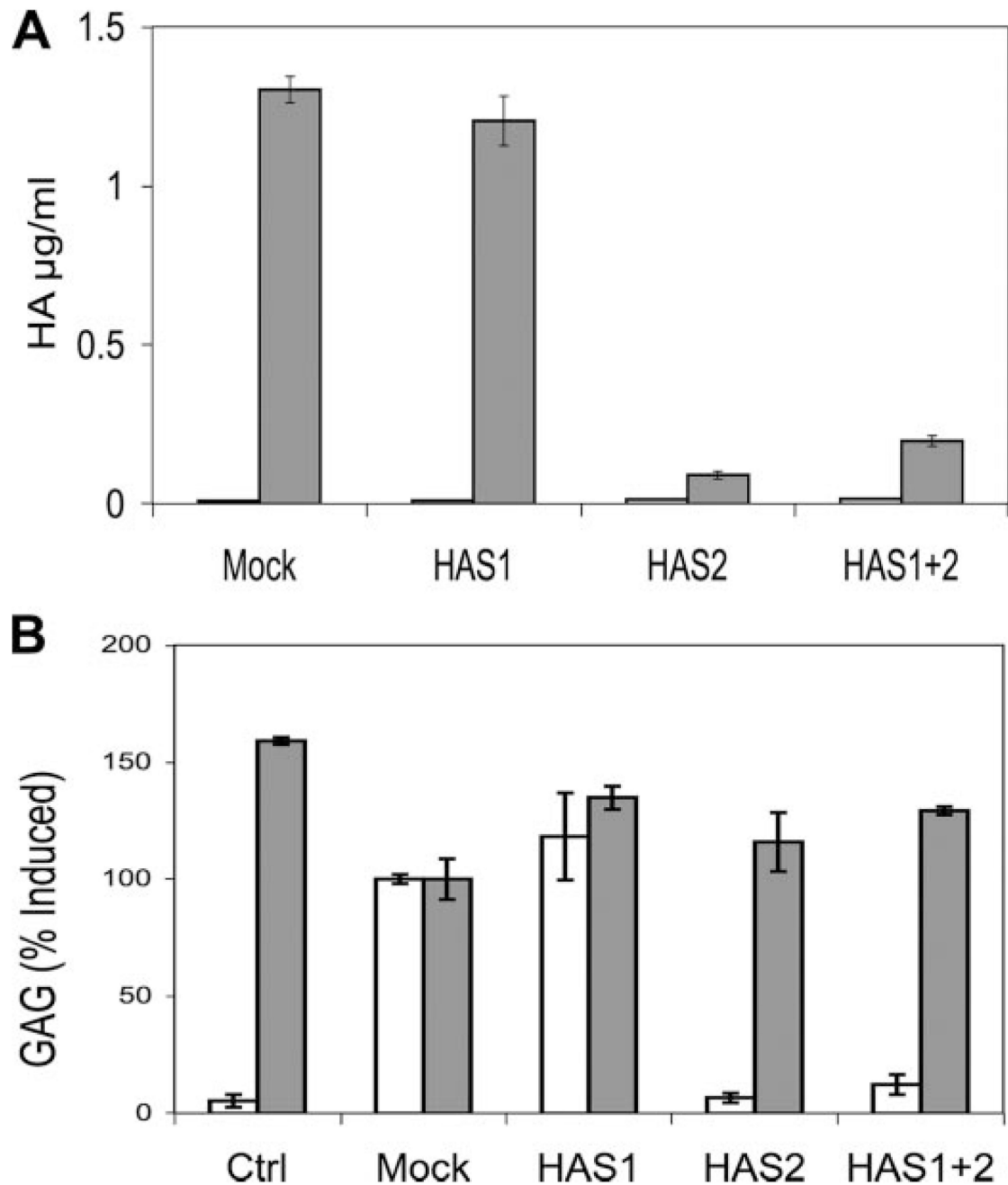


FIGURE 6. Knockdown of HA biosynthesis by *HAS2* siRNA

Primary bovine keratocytes were transfected with siRNA against *HAS1*, *HAS2*, or a combination as described under “Experimental Procedures.” The cells were cultured in serum-free medium for 48 h and then exposed to FBS and TGFβ for 48 h. *A*, HA in serum-free medium (left, open bars) or TGFβ-FBS (right, gray bars) was analyzed by HABP-ELISA as described “Experimental Procedures.” *B*, chondroitin/dermatan sulfate (CS) (gray bars) and HA (open bars) were analyzed by FACE gels similar to those in Fig. 1. *Ctrl* (control) shows HA and CS expression in serum-free medium for 48 h. All other samples show expression during 48 h after induction with TGFβ-FBS. *Mock* indicates no siRNA. HA

and chondroitin/dermatan sulfate values are normalized to the Mock samples. *Bars* show standard deviation of triplicate analyses.

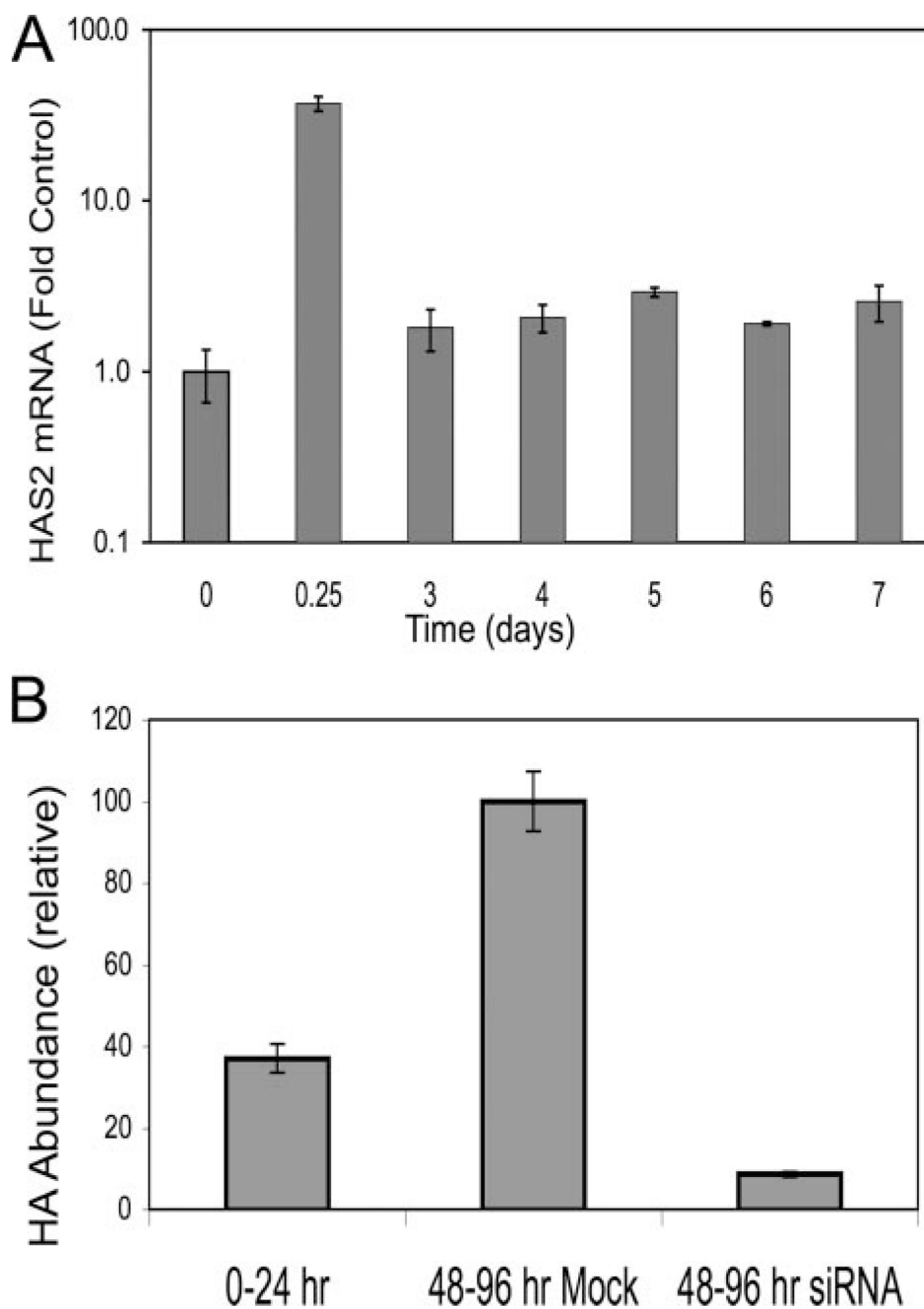


FIGURE 7. Long term HA biosynthesis is HAS2-related

A, primary bovine keratocytes were exposed to FBS and TGF β similarly to Fig. 1 for the intervals shown, and the *HAS2* mRNA was determined by qRT-PCR as described in Fig. 4. *Bars* show the standard error of triplicate analyses of duplicate cultures. RNA abundance is compared with untreated cells = 1. Note the log scale in the y axis. *B*, HA biosynthesis was induced in keratocytes for 24 h followed by transfection with siRNA against *HAS2*. Conditioned medium was collected for the period 24–72 h after siRNA transfection (48–96 h after TGF β treatment). HA was determined in the collected media using FACE. Mock cells were subject to electroporation without siRNA.

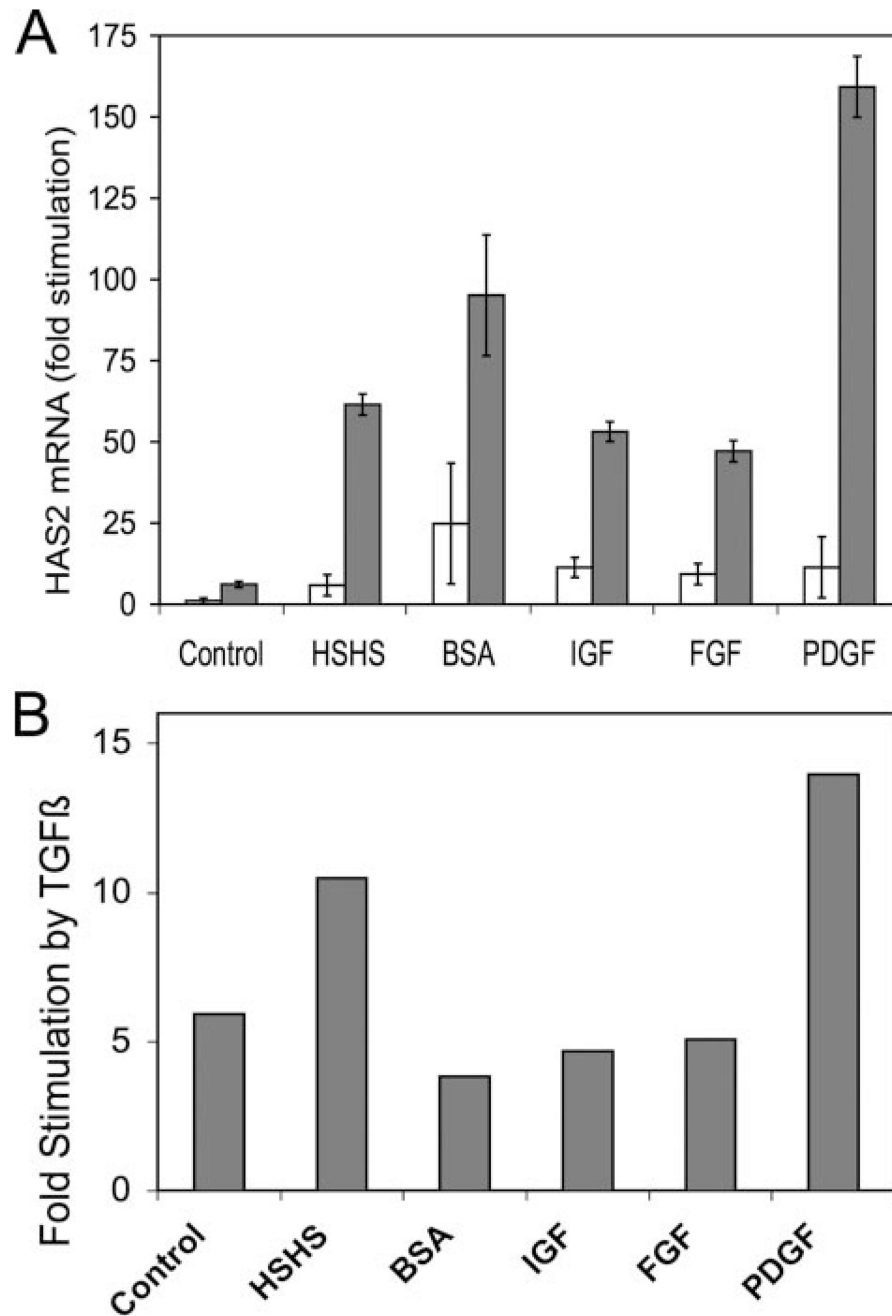


FIGURE 8. Synergistic stimulation of *HAS2* levels by TGF β and mitogens

A, primary bovine keratocytes were exposed to various agents as in Table 1 in the presence (dark bars) or absence (open bars) of 1 ng/ml TGF β . *HAS2* mRNA was determined by qRT-PCR after 6 h of exposure. Error bars show standard deviation of triplicate analyses. mRNA levels in untreated cells were set = 1. *B*, shows the ratio of mRNA abundance for samples in *A* (TGF β treated/no TGF β). HSHS, 2% heparin-stripped horse serum; BSA, 1 mg/ml Albumax, lipid-rich bovine serum albumin; IGF, 50 ng/ml insulin-like growth factor 1; FGF, 10 ng/ml fibroblast growth factor-2; PDGF, 10 ng/ml platelet-derived growth factor BB.

TABLE 1

Primers and siRNA

Gene	Primer	Sequence
<i>HAS1</i>	Forward	AGGCTGGTACAACCAGAAG
	Reverse	TCTCCGAGTAGCAGCGAGAC
	Taqman probe	FAM-AGCATGGGCTATGCCACCAAGTACAC-TAMRA
	siRNA 1	GUGCUGCTCTCACUCUAUA
	siRNA 2	GGAACAACCUCCUGCAGCA
	siRNA 3	GCGAGUGGUGUACAAUGC
	siRNA 4	ACGCGUGGAUGACCUACGA
<i>HAS2</i>	Forward	CCGTCATCACTGGGTTCTTC
	Reverse	TAAGGCAGCTGGCAAAGAT
	Taqman probe	FAM-TCATTGCCACGGTAATCCAGCTCTTC-TAMRA
	siRNA	CCUUGGAAUCACAGCUGCUUAUAUU
<i>HAS3</i>	Forward	CCAACCGAGTCCTGAGTCTT
	Reverse	TGTATGACCGTGGCAATGAG
	Taqman probe	FAM-CTCCCGGAAGTAAGACTTGCTCCAGC-TAMRA

TABLE 2

Stimulation of *HAS2* mRNA

Culture condition	<i>HAS2</i>	S.D.
Serum-free (control)	1.0	0.2
2% FBS	11.3	2.5
0.2 ng/ml TGF β + 2% FBS	38.4	11.8
1 ng/ml TGF β + 2% FBS	43.3	2.5
2 ng/ml TGF β + 2% FBS	25.1	4.4
10 ng/ml TGF β + 2% FBS	28.4	9.9
1 ng/ml TGF β serum-free	8.5	1.2
IGF1 50 ng/ml serum-free	9.0	0.6
FGF2 10 ng/ml serum-free	7.9	0.7
PDGF BB 10 ng/ml serum-free	23.8	1.5
1% Heparin-stripped horse serum	10.8	2.7
3 mg/ml Bovine serum albumin	16.7	4.8