Biochemical and Biophysical Research Communications **271,** 203–211 (2000)
doi:10.1006/bbrc.2000.2612, available online at http://www.idealibrary.com on IDEAL®

Mechanism of Regulation of HGF/SF Gene Expression in Fibroblasts by TGF-β1

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Received March 27, 2000

The effect of transforming growth factor β 1 (TGF-β1) on levels of hepatocyte growth factor/scatter factor (HGF/SF) gene transcripts was investigated in the human lung embryonic fibroblast cell line, MRC-5. TGF-β1 markedly reduced the expression of the 6.0-kb and 3.0-kb HGF/SF mRNA, which encode full-length HGF/SF, but it had little effect on the expression of the alternatively spliced 1.5-kb mRNA, which encodes NK2, a competitive HGF/SF antagonist. Using actinomycin D to block RNA synthesis, it was observed that TGF-β1 had little effect on the stability of the 1.5-kb NK2 mRNA but increased the rate of degradation of the 6.0- and 3.0-kb HGF/SF mRNA transcripts by a mechanism that was dependent on new protein synthesis. TGF-β1 minimally increased rather than reduced HGF/SF promoter activity in cells transiently transfected with chloramphenicol acetyltransferase (CAT) reporter genes driven by HGF/SF gene 5′-flanking sequences. In MRC-5 cells, TGF-β1 modulates HGF/SF gene transcripts at the posttranscriptional level in order to favour expression of the 1.5-kb mRNA that encodes the truncated protein NK2.

Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic factor produced by mesenchymal cells and is a humoral mediator of mesenchymal–epithelial interactions, which stimulates cell motility (1, 2), proliferation (3–7), and morphogenesis (8, 9), depending on the target cell and culture conditions. These main functions have implicated HGF/SF in many physiological processes such as organ regeneration (10–12), embryological development (13, 14) and angiogenesis (15–17). Although HGF/SF inhibits the growth of certain sarcoma and epidermoid carcinoma cell lines (18, 19), it is thought to have a pathophysiological role in tumour development because transgenic mice overexpressing the HGF/SF gene developed a number of histologically distinct tumours (20, 21) and HGF/SF has been shown to be associated with tumour cell growth (22–24) and invasion (25–28). Elevated circulating levels of HGF/SF have been associated with a poor outcome in some groups of cancer patients, suggesting that HGF/SF might also play an important role in tumour progression (29–33).

Mature HGF/SF protein consists of a 69 kD α-subunit and a 34 kD β-subunit that are encoded by a single open reading frame (ORF) in both the full length 6.0 kb HGF/SF mRNA and the alternatively spliced 3.0 kb mRNA (34, 35). It is thought that all biological signals of HGF/SF are transduced through the tyrosine kinase receptor encoded by the c-Met protooncogene (9). A functional domain in the N-terminal and first two kringle regions of the α-chain is sufficient for binding to the receptor but the complete heterodimer protein appears to be required for induction of full mitogenic activity (36, 37).

A variant 1.5 kb HGF/SF mRNA, generated by alternative exon splicing (38), is found in many cell lines and tissues that express the full length HGF/SF mRNA and in some, such as foreskin fibroblasts, it is the predominant transcript (39). It encodes a 290 amino acid polypeptide called NK2 that is identical to the N-terminal region and first 2 kringles of HGF/SF (38). A physiological function has not been ascribed to the NK2 protein but it has the properties of a naturally occurring HGF/SF antagonist because it binds to the HGF/SF receptor (36) and inhibits HGF/SF-induced DNA synthesis (39). Transgenic mice expressing NK2 do not exhibit the phenotype characteristic of HGF/SF transgenic mice. Rather, when coexpressed in NK2-HGF/SF bitransgenic mice, NK2 antagonises the pathological consequences of HGF/SF and discourages the development of tumours (40).

Whilst there has been much research into the functions of HGF/SF, less is known about the regulation of HGF/SF gene expression. HGF/SF production by fibroblasts (41, 42), mesangial cells and endothelial cells (43) is inhibited by transforming growth factor-β1 (TGF-β1), as a consequence of downregulation of

0006-291X/00 $35.00

HGF/SF mRNA expression (42). It has been assumed that TGF-β1 downregulates HGF/SF mRNA expression by inhibiting gene transcription as the HGF/SF promoter regions in both rat (44) and mouse (45) contain the sequence GAATTGGTGT, which is known as the TGF-β inhibitory element (TIE) and is conserved among other TGFβ-regulated genes. It has been demonstrated that the TIE binds a nuclear protein complex from TGFβ-treated fibroblasts and that this complex contains the c-*fos* proto-oncogene product (46). Using electrophoretic mobility shift assay, Odenthal *et al.*, found increased binding of nuclear protein to the putative TIE in the rat HGF/SF promoter in response to TGF-β1 and, when cloned into a CAT reporter plasmid, this TIE inhibited CAT activity in response to TGF-β1 (47). In contrast, Plaschke-Schlütter and colleagues reported that TGF-β1 had no effect on the promoter activity of the 5′ flanking region of the mouse HGF/SF gene (48). In the present study, we investigated the mechanism by which TGF-β1 reduces expression of HGF/SF mRNA in fibroblasts and examine its effect on levels of the alternatively spliced HGF/SF gene transcripts.

MATERIALS AND METHODS

Culture of cells. MRC-5 cells (passages 20 to 28) were grown as monolayers on plastic dishes of 90 mm diameter in 10 ml of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS), in the presence of antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air until the cells had reached near confluence, at which point, the experiments were started by changing to the same volume of fresh medium containing the test compounds. NIH3T3, mouse embryo fibroblasts, were grown as monolayers on plastic dishes of 90 mm diameter in 10 ml of DMEM plus 10% bovine serum (BS) and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin).

Northern blot analysis. After treatment of the MRC-5 cells for the specified times, the culture medium was removed and total RNA was isolated according to the acid guanidinium thiocyanate-phenol-chloroform method as described by Chomczynski (49). Each experiment was performed in triplicate and the RNA pooled. RNA samples (30 μg) were fractionated by gel electrophoresis and transferred to Genescreen (Du-Pont-NEN, Boston, USA). The membrane was prehybridised for 6 h at 42°C, in a solution containing 50% formamide, 10% dextran sulphate, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone (MW 40,000), 0.2% ficoll, 50 mM Tris-HCl (pH 7.5), 0.1% sodium pyrophosphate, 1% SDS and 150 μg/ml sheared salmon sperm DNA. It was then hybridised overnight at 42°C, after the addition to the prehybridisation solution of a ^{32}P-labelled DNA probe for HGF/SF, which had been labelled by the random primer labelling procedure using [^{32}P]αdATP (50). The filter was washed twice at room temperature for 5 minutes in 2× SSC plus 1% SDS, then twice at 65°C for 30 minutes in 0.2× SSC plus 0.1% SDS and then once at 65°C for 30 minutes in 0.2× SSC. After the last wash the membrane was left slightly damp, to facilitate subsequent removal of the probe, and sealed in thin polythene before being exposed to X-ray film with intensifying screens at −70°C. The labelled probe was then stripped from the membrane, which was rehybridised to a ^{32}P-labelled partial cDNA probe for GAPDH and exposed to X-ray film with intensifying screens at −70°C.

The HGF/SF probe was derived from a 1043 bp DNA fragment of the HGF/SF gene that had been amplified by PCR from human placental DNA: sense strand (nt −904 to −885) 5′-TCAGGGACAGGCTATGGACA-3′ and antisense strand (nt +120 to +139) 5′-AGGAGGAGATGCAGGAGGAC-3′ (51). The probe was a 408 bp Sac1-Pst1 fragment of this PCR product, spanning the 5′-flanking region and first exon of the HGF/SF gene. The 360 bp partial cDNA for GAPDH was amplified by PCR from first strand cDNA derived from normal liver RNA primed with random hexanucleotides and reverse transcribed using Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase (Gibco BRL, Gaithersburg, USA). The nested primers used were first round, sense strand (nt 61–80) 5′-ATGGGGAAGGTGAAGGTCGG-3′ and antisense strand (nt 921–940) 5′-TGGAGGAGTGGGTGTCGCTG-3′; second round, sense strand (nt 111–130) 5′-GGTCACCAGGGCTGCTTTTA-3′ and antisense strand (nt 451–470) 5′-TGGTTCACACCCATGACGAA-3′ (52). The PCR product was cloned into the BamH1-Xba1 sites of pGEM-3Z. The authenticity of the probes was confirmed by sequencing and no mutations were found in comparison with the reported sequences.

Ribonuclease protection assay. Nested primers were used in the synthesis of a 668 bp partial cDNA of HGF/SF: first round, sense strand (nucleotides (nt) 671–691) 5′-ATCATACAGAATCAGGCAAGA-3′ and antisense strand (nt 1385–1405) 5′-AACGAGAAATAGGGCAATAAT-3′; second round, sense strand (nt 700–720) 5′-CGCTGGGATCATCAGACACCA-3′ and antisense strand (nt 1347–1367) 5′-TTTCCCGTGTAGCACCAGGGT-3′ (35). The PCR product was cloned into the EcoR1-BamH1 sites of pGEM-3Z and the plasmid linearised using the unique BglII restriction site (nt 1219) within the HGF/SF cDNA (35). The vector containing the GAPDH cDNA, as described above, was linearised using the EcoR1 restriction site within the plasmid's cloning cassette. A specific NK2 cDNA was amplified by PCR from human placental DNA using primers specific for the unique 3′ UTR, sense strand (nt 939–958) 5′-TGCGAGACATAACATGGGCT-3′ and antisense strand (nt 1161–1142) 5′-GGGTAAGGGCCAGCATGTAG-3′ (38). The PCR product was cloned into the Xba1-Sal1 sites of pGEM-3Z and the vector was linearised using the EcoR1 restriction site within the plasmid's cloning cassette. The insert was sequenced to confirm the authenticity.

Riboprobes were synthesised *in vitro* from 0.5 μg of linearised vector containing the specific cDNA template and 100 μCi [α-^{32}P] UTP (400 Ci/mmol) (Amersham, UK), using either SP6 or T7 RNA polymerases as appropriate. Cold UTP was included in the transcription reaction for the synthesis of antisense GAPDH riboprobe to reduce the probe's specific activity. The antisense riboprobe for HGF/SF was 157 nucleotides long, of which 148 nucleotides were complementary to a portion of the 6.0 kb HGF/SF mRNA encoding part of the fourth kringle and derived from exons 10 and 11. The antisense riboprobe for NK2 was 287 nucleotides long of which 223 nucleotides were complementary to a portion of the 1.5 kb NK2 mRNA derived from exon 7b. The antisense riboprobe for GAPDH was 424 nucleotides long of which 360 nucleotides were complementary to GAPDH mRNA.

RNA samples (10 μg) were hybridised at 42°C in 80% formamide, 400 mM sodium chloride, 40 mM PIPES and 1 mM EDTA for 16 h with riboprobes for HGF/SF (5×10^5 cpm) and NK2 (5×10^5 cpm). Antisense GAPDH riboprobe (1×10^5 cpm) was included in each reaction to control for the amount of input RNA. The hybridisation products were digested with 40 μg/ml RNAse A and 2 μg/ml RNAse T1 for 1 h at 30°C, treated with 50 μg/ml proteinase K and extracted with phenol/chloroform. The riboprobe/mRNA hybrids were denatured and fractionated by electrophoresis on a denaturing 7 M urea/6% polyacrylamide gel. Dried gels were exposed to X-ray film with an intensifying screen at −70°C.

Ribonuclease protection mapping analysis. The 408 bp Sac1-Pst1 fragment of the HGF/SF gene, spanning the 5′-flanking region and first exon as described above, was cloned into pGEM-3Z vector and the plasmid was linearised using the EcoR1 restriction site within the cloning cassette. A 435 nucleotide antisense riboprobe was synthesised *in vitro* from 0.5 μg of linearised vector and 100 μCi [α-^{32}P]

UTP (400 Ci/mmol) (Amersham, UK), using SP6 RNA polymerase. The riboprobe (5×10^5 cpm) was hybridised with 10 μg of total RNA from MRC-5 cells and ribonuclease protection assay performed, as described above.

HGF/SF gene promoter activity. The 5′-flanking region of the human HGF/SF gene was cloned by PCR amplification from human placental DNA to produce fragments identical at the 3′ end at +42 bp downstream of the transcription initiation site and progressively deleted from the 5′ end. The positions given are in relation to the main transcription initiation site described by Miyazawa (51). The PCR primers were designed to incorporate restriction sites to facilitate subsequent cloning.

Downstream primer:

+42/+22 5′-GCTTCTAGAGATGCCTGGGTGAAAGA-3′
*Xba*I

Upstream primers:

−1025/−1005 5′-TTGGTCGACTTGAGGGATTTCCGGTGAA-3′
−906/−886 5′-TTGGTCGACTTGAGGGATTTCCGGTGAA-3′
−715/−695 5′-TTGGTCGACATCTGACAATCTGCGTGCT-3′
−491/−471 5′-TTGGTCGACGCACATTTGGCTGAAAGACA-3′
−371/−353 5′-TTCGTCGACCTGCCTGTGCCTTGATTTA-3′
Sal1

The *Sal*I and *Xba*I digest fragments of the PCR products were cloned into the CAT reporter plasmid pCAT-Basic (Promega, Madison, WI), which lacks eukaryotic promoter and enhancer sequences, to form chimeric HGF/SF-CAT plasmids. Each pCAT-HGF/SF construct was confirmed by DNA sequencing.

MRC-5 cells were found to transfect with the HGF/SF-CAT plasmids only at very low efficiency (data not shown) and, therefore, NIH3T3 mouse embryo fibroblasts were used instead. NIH3T3 cells at 50–60% confluence were transiently transfected with the HGF/SF promoter-CAT chimeric plasmid DNA using the calcium phosphate method (53). The cells were incubated with the DNA-calcium phosphate co-precipitate for 6 h, then washed twice with serum-free medium, followed by an additional 24 h in culture in DMEM containing 10% BS with or without TGF-β1 before the cell protein was harvested.

In each experiment, the cells were co-transfected with 10 μg of a HGF/SF promoter-CAT chimeric plasmid and 1 μg of pCMVβ, a β-galactosidase reporter plasmid under the control of the strong CMV promoter (Promega, Madison, WI). The latter was used as an internal control for monitoring transfection efficiency. Each set of experiments also included cells transfected with control DNA. A CAT chimeric plasmid containing the CMV promoter sequence (CMV-CAT) was used as a positive CAT control, since it produces strong CAT activity. The pCAT-basic plasmid not containing HGF/SF 5′-flanking sequence and salmon sperm DNA (ssDNA) were used as a negative controls. Each transfection assay was performed 5 times.

At 30 h after the start of the transfection, the cells were washed three times in phosphate buffered saline (PBS) then harvested into 1 ml of PBS on ice using a rubber policeman. The cells were spun at 12,000 g for 10 minutes at 4°C and the pellet resuspended in 150 μl of 0.25 M Tris pH 8.0 before being disrupted by three cycles of freeze-thaw (freezing on dry ice and thawing at 37°C) with vortexing after each thaw cycle. The cell debris was pelleted by centrifugation at 12,000 g for 5 minutes at room temperature and aliquots of the supernatant were taken for both β-galactosidase and CAT activity. The portion of the cell extract to be used in the subsequent CAT assay was heated to 60°C for 10 minutes to inactivate any endogenous acetylase or deacetylase activity. All extracts were stored at −70°C until assay.

The β-galactosidase assay was performed on 50 μl of the cell extract according to the manufacturer's instructions (Promega, Madison, WI). The β-galactosidase hydrolyses the o-nitrophenyl-β-D-galactopyranosde (ONPG) in the reaction buffer to o-nitrophenyl, which is yellow. The β-galactosidase activity was quantified by measuring the spectrophotometer absorbance at 420 nm and used as an indicator of transfection efficiency. The volume of cell extract assayed for CAT activity was adjusted for transfection efficiency according to β-galactosidase activity.

Chloramphenicol acetyl transferase is encoded by a bacterial drug-resistance gene which is not found in eukaryotes and is widely used as a reporter gene to study mammalian gene expression. CAT inactivates chloramphenicol by acetylating one or both of the hydroxyl groups of the drug. In order to measure CAT activity in the cell extracts, each aliquot, which had been adjusted for transfection efficiency, was mixed with 3 μl ^{14}C-chloramphenicol and 5 μl n-butyryl Coenzyme A (at 5 mg/ml), made up to a final volume of 125 μl with 0.25 M Tris-Cl pH 8.0 and incubated at 37°C overnight. CAT transfers the n-butyryl moiety from the cofactor to the chloramphenicol at one or two of its hydroxyl groups. The butyrylated chloramphenicol is more hydrophobic than free chloramphenicol and is therefore easily separated by a simple phase extraction (54). Reaction products were separated by 2:1 tetramethylpentadecane (TMPD)/xylene (v/v) extraction in which 300 μl is added to the reaction and vigorously vortexed for 45 s then centrifuged for 5 minutes to give a good phase separation. The upper, organic phase contains the converted chloramphenicol whilst the aqueous phase contains the unmodified chloramphenicol. The organic phase was then transferred to a scintillation vial and 4 ml scintillation fluid added and counted in a scintillation counter for 5 minutes.

RESULTS

The Effect of TGF-β1 on HGF/SF Gene Expression

Subconfluent MRC-5 cells were cultured in the presence of increasing concentrations of recombinant human TGF-β1 for 24 h. The RNA was extracted and analysed by Northern hybridisation using the HGF/SF exon 1 cDNA probe. The 6.0 kb and 3.0 kb HGF/SF transcripts and the 1.5 kb NK2 transcript were detected. In addition, a previously unreported 0.4 kb transcript was also detected. TGF-β1, at concentrations greater than 100 pg/ml, reduced the expression of both the 6.0 kb and 3.0 kb HGF/SF mRNA transcripts in a dose dependent fashion but it had little effect on the expression of the 1.5 kb NK2 mRNA and 0.4 kb species, even at high doses (Fig. 1A). RNAse protection assay using specific riboprobes confirmed that TGF-β1 down-regulated HGF/SF mRNA but not NK2 mRNA (Fig. 1B).

In order to examine the time course of these changes, RNA was isolated at time points after the addition of 1 ng/ml TGF-β1 to sub-confluent MRC-5 cells and analysed by Northern hybridisation. The reduction in levels of the 6.0 kb HGF/SF mRNA species was apparent at 3 h and was maximal by 9 h after exposure to TGF-β1 (Fig. 2). Changes in expression of the 3.0 kb HGF/SF mRNA species mirrored those of the 6.0 kb mRNA transcript and again TGF-β1 had little effect at any time point on the expression of the 1.5 kb NK2 mRNA transcript.

In order to determine whether TGF-β1 decreased the levels of the 6.0 kb and 3.0 kb HGF/SF mRNA transcripts by increasing their degradation, the half-lives

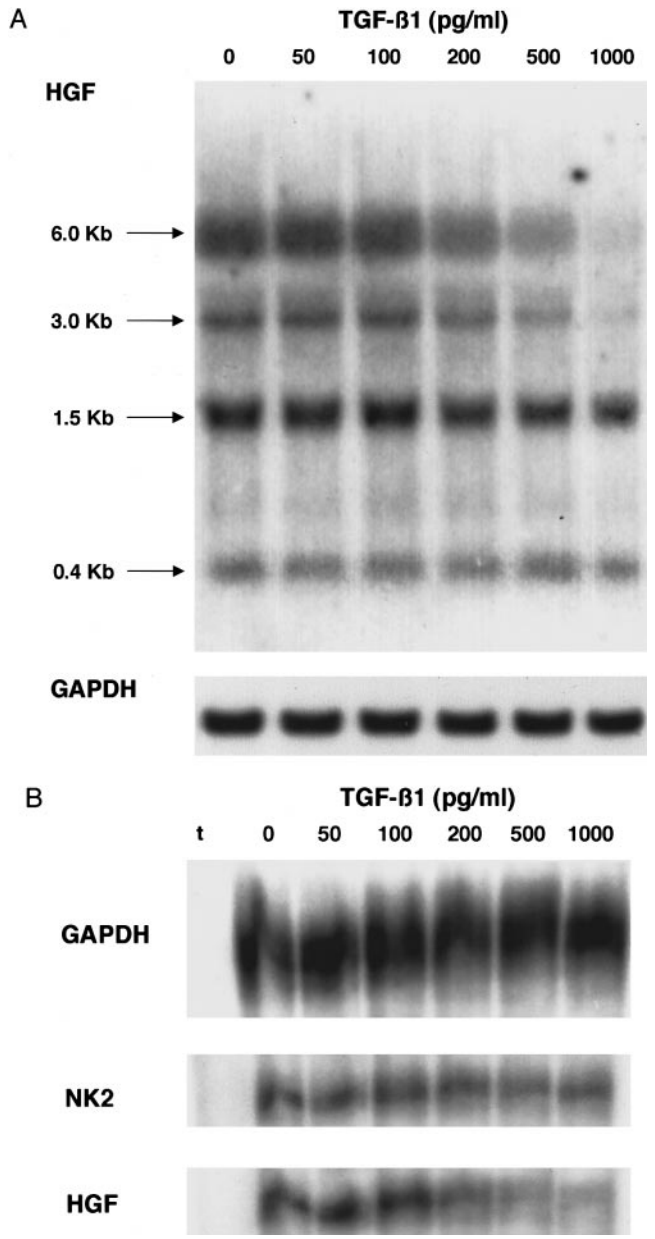

FIG. 1. Effect of TGF-β1 on HGF/SF gene expression in MRC-5 cells. Total RNA was isolated from subconfluent MRC-5 cells cultured in the presence of increasing concentrations of recombinant human TGF-β1 for 24 h. In (A) 30 μg RNA was subjected to sequential Northern blot analysis using: a HGF/SF exon 1 cDNA probe, 40 h autoradiographic exposure; and a GAPDH cDNA probe, as a loading control, 6 h autoradiographic exposure. The 6.0 kb and 3.0 kb HGF/SF mRNA, the 1.5 kb NK2 mRNA and the 400 base transcripts are indicated. In (B) RNase protection assay was performed by hybridising specific riboprobes for HGF/SF, NK2 and GAPDH with 10 μg of RNA. HGF/SF mRNA was detected a 148 nucleotide protected fragment, NK2 mRNA was detected as a 223 nucleotide protected species and GAPDH mRNA was detected as a 360 nucleotide protected species. tRNA was used as a negative control.

of the HGF/SF mRNA species were measured in the presence of actinomycin-D, which blocks gene transcription. RNA was isolated at time points after the addition of 5 μg/ml actinomycin-D to sub-confluent MRC-5 cells and analysed by Northern hybridisation. In the absence of TGF-β1, the 6.0 kb and 3.0 kb HGF/SF mRNA transcripts had a half-life of around 4 h but in the presence of TGF-β1, added 3 h before the addition of actinomycin D, the half-life of the 6.0 and 3.0 kb HGF/SF mRNA transcripts was reduced by 50%, whereas the 1.5 kb NK2 mRNA transcript remained stable (Fig. 3). In order to determine whether the down regulation of the 6.0 kb and 3.0 kb HGF/SF mRNA transcripts by TGF-β1 was dependent on new protein synthesis, subconfluent MRC-5 cells were treated with the protein synthesis inhibitor cyclohexamide before exposure to TGF-β1. RNA was again isolated at time points after the addition of actinomycin-D and analysed by Northern hybridisation. Cyclohexamide completely inhibited the effect of TGF-β1 (Fig. 4), providing evidence that TGF-β1 stimulates the synthesis of a protein which causes the degradation of the 6.0 and 3.0 kb HGF/SF mRNA transcripts.

Ribonuclease Protection Mapping Analysis

The ribonuclease protection mapping of the transcription initiation sites of the HGF/SF gene revealed 1 major cluster and 3 minor clusters of protected riboprobe fragments in MRC-5 cells. The strongest protected fragment in the major cluster was that of 102 nucleotides and since the Pst1 restriction site is 39 nucleotides downstream of the translation start site, the major transcription initiation site of the HGF/SF gene in MRC-5 cells mapped to 63 nucleotides up-

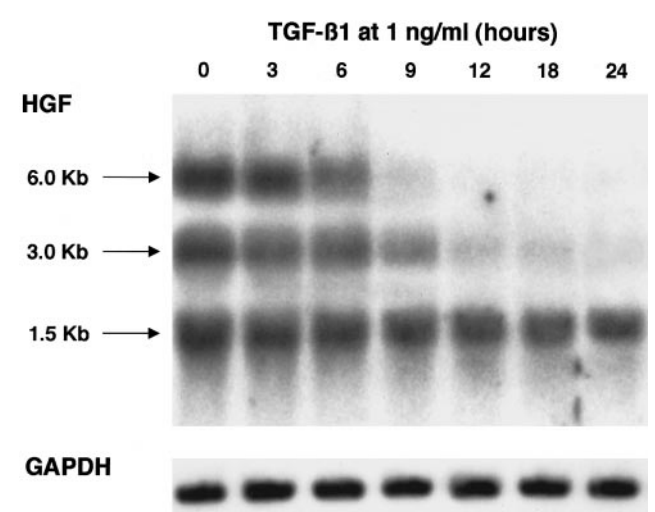

FIG. 2. Northern blot analysis of RNA from MRC-5 cells at time points after treatment with TGF-β1. Total RNA was isolated from subconfluent MRC-5 cells at time points up to 48 h after the addition of 1 ng/ml TGF-β1. Sequential Northern blot analysis was performed using: a HGF/SF exon 1 cDNA probe, 40 h autoradiographic exposure; and a GAPDH cDNA probe, 6 h autoradiographic exposure. The 6.0 kb and 3.0 kb HGF/SF mRNA and 1.5 kb NK2 mRNA transcripts are indicated.

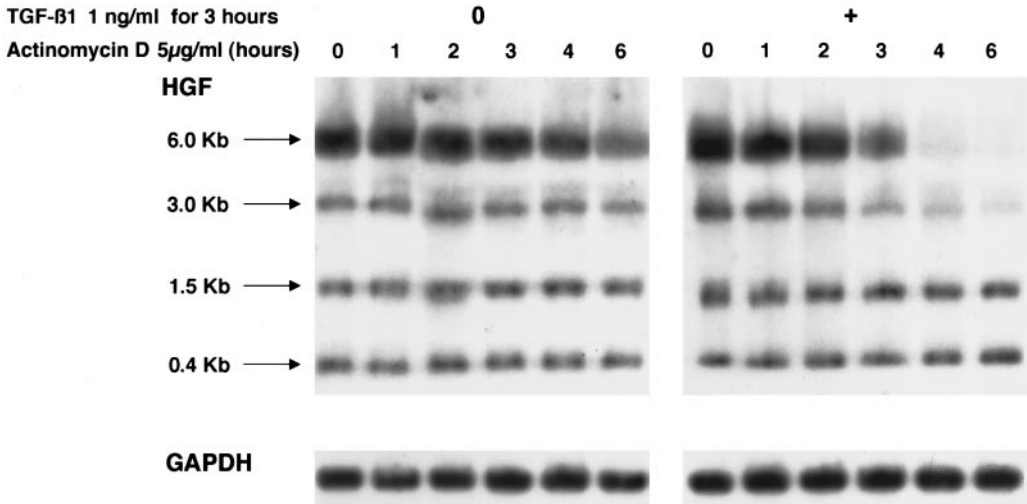

FIG. 3. The effect of TGF-β1 on the half-lives of HGF/SF mRNA transcripts. Subconfluent MRC-5 cells were cultured in the presence or absence of 1 ng/ml TGF-β1 for 3 h and then total RNA was isolated at time points after the addition of 5 μg/ml actinomycin-D. Sequential Northern blot analysis was performed using: the HGF/SF exon 1 cDNA probe, 48 h autoradiographic exposure; and a GAPDH cDNA probe, 6 h autoradiographic exposure. The 6.0 kb and 3.0 kb HGF/SF mRNA and the 1.5 kb NK2 mRNA transcripts are indicated.

stream of the translation initiation codon. The 3 minor transcription initiation sites detected upstream of the major site were centred around 75, 84 and 96 nucleotides upstream of the translation initiation codon. The addition of TGF-β1 at 1 ng/ml for 24 h reduced the levels of all protected riboprobe fragments but had no effect on the relative use of the transcription initiation sites (data not shown).

HGF/SF Gene Promoter Activity

The sequence of the 5′-flanking region amplified by PCR from human placental DNA was identical to that cloned from human placental genomic DNA libraries (51, 55). NIH3T3 cells showed a high level of transfection efficiency. The transcriptional activity of each construct is shown (Fig. 5). The HGF/SF promoter region up to approximately 1 kb is highly regulated and there are inhibitory regions between −1025 to −906 and −715 to −491 bp and upregulatory regions at −491 to −371 and −906 to −715 bp.

The level of CAT activity and hence transcriptional activity was upregulated by TGF-β1 at 1 ng/ml for 24 h in the constructs HGF/SF-CAT1025, 906, 491 and 371 by 2.7-, 1.7-, 1.2- and 1.8-fold, respectively. The activity of pCAT-HGF/SF715 was unaffected by TGF-β1 (Fig. 5). These data show that TGF-β1 has a small stimulatory effect on HGF/SF gene promoter activity.

DISCUSSION

The present study demonstrated that although TGF-β1 markedly reduced expression of the 6.0 kb

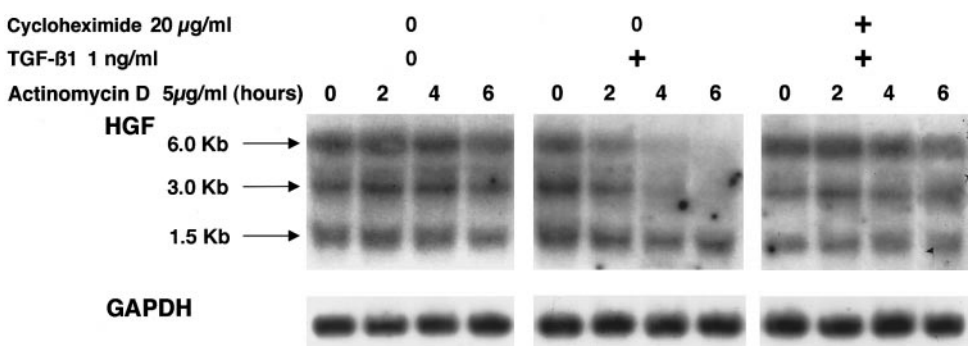

FIG. 4. The effect of cyclohexamide on the half-lives of HGF/SF mRNA transcripts. Subconfluent MRC-5 cells were treated with cyclohexamide at 20 μg/ml for 1 h before exposing the cells to TGF-β1 at 1 ng/ml for 3 h. Total RNA was then isolated at time points after the addition of 5 μg/ml actinomycin-D. Sequential Northern blot analysis was performed using: the HGF/SF exon 1 cDNA probe, 48 h autoradiographic exposure; and a GAPDH cDNA probe, 6 h autoradiographic exposure. The 6.0 kb and 3.0 kb HGF/SF mRNA and the 1.5 kb NK2 mRNA transcripts are indicated.

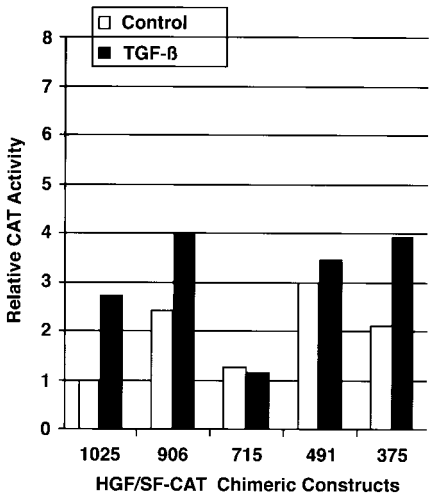

FIG. 5. Activity of human HGF/SF promoter-CAT chimeric gene. The 5′-flanking region of the human HGF/SF gene was cloned by PCR amplification from human placental DNA, to produce fragments identical at the 3′ end and progressively deleted from the 5′ end, and then cloned into the CAT reporter vector pCAT-basic (−1025 to +42, 1025 HGF/SF-CAT; −906 to +42, 906 HGF/SF-CAT; −715 to +42, 715 HGF/SF-CAT; −491 to +42, 491 HGF/SF-CAT; −371 to +42, 371 HGF/SF-CAT). The chimeric constructs were transiently transfected into NIH3T3 fibroblasts by the calcium phosphate method. Cotransfection with pCMV-β-galactosidase plasmid was used to control for transfection efficiency. After 6 h recombinant human TGF-β1 at 1 ng/ml was added for 24 h. The cells were then harvested into 1 ml of PBS on ice, pelleted, and disrupted by three cycles of freeze–thaw. After centrifugation, aliquots of the supernatant, adjusted for galactosidase activity, were taken for standard CAT assay. The CAT activity of each construct is expressed in relation to that of 1025 HGF/SF-CAT without TGF-β1 stimulation and each result is the median of five transfection assays.

HGF/SF mRNA in fibroblasts, in a dose and time dependent fashion, it had little effect on the expression of the 1.5 kb NK2 mRNA. This differential effect of TGF-β1 on the expression of the alternatively spliced HGF/SF transcripts led to the 1.5 kb NK2 mRNA becoming the predominant mRNA species in MRC-5 cells after 6 h.

It has been assumed that TGF-β1 downregulates HGF/SF mRNA expression by inhibiting gene transcription as there is a non-consensus potential TIE site in the human HGF/SF promoter (GAATTGGTCC) at −382 to −373 bp (51). However, in the present study, we demonstrated that TGF-β1 increased the transcriptional activity of the 5′ flanking region of the HGF/SF gene, at a dose that markedly inhibited expression of the 6.0 kb HGF/SF mRNA. This suggests that either additional elements outside of the studied region of the human HGF/SF promoter are required for an inhibitory response to TGF-β1 or the down regulation of HGF/SF mRNA by TGF-β1 is mediated at the post transcriptional level. The mechanism for the observed stimulatory effect of TGF-β1 on human HGF/SF promoter activity is unknown but it is not mediated through the putative TIE because we found that the activity of HGF/SF-CAT371, which does not contain the TIE sequence, was also increased.

The main transcription initiation site observed in MRC-5 cells in the present study coincides with that found in both rat (44) and mouse liver (48, 56). The major transcription initiation site of the HGF/SF gene detected in both placenta and MRC-5 cells by Miyazawa *et al.* using S1-nuclease analysis (51) accords with a minor rather than the main transcription initiation site determined in the present study. This minor transcription initiation site corresponds with the 5′ end of the cDNA for the 1.5 kb NK2 mRNA (38, 39) but the finding that TGF-β1 had no effect on the relative use of the transcription initiation sites suggests that the 1.5 kb NK2 mRNA is not transcribed from a unique site. Interestingly, no transcription initiation site was observed corresponding to the 5′ end of the HGF/SF cDNA cloned from a liver cDNA library by Nakamura *et al.* (35) suggesting that either the Nakamura clone may have been derived from a transcript of very low abundance or there is differential utilisation of transcription initiation sites in liver compared with fibroblasts in culture.

The finding that TGF-β1 increased the rate of degradation of the 6.0 kb and 3.0 kb mRNA transcripts demonstrates that TGF-β1 reduced the steady state expression of HGF/SF mRNA species encoding the full length protein in MRC-5 cells by a post-transcriptional mechanism. TGF-β1 has recently been shown to inhibit the expression of a number of other genes in cultured cells as a consequence of reducing mRNA stability, including c-kit (57), inducible nitric oxide synthase (iNOS) (58), somatostatin (59), Pax-2 (60), collagenase (61), IL-12p40 subunit (62) and indoleamine 2,3-dioxygenase (63). However, since TGF-β1 had little effect on levels of the 1.5 kb NK2 mRNA transcript, this is the first report to our knowledge of differential regulation of alternatively spliced gene transcripts by TGF-β1 at the post-transcriptional level. We found that cyclohexamide blocked the effect of TGF-β1 indicating that TGF-β1 stimulates the synthesis of one or more proteins that then cause the degradation of the 6.0 kb and 3.0 kb HGF/SF mRNA transcripts but not the 1.5 kb mRNA species. TGF-β1 has been shown to increase the stability of mRNA transcripts for The Receptor for Hyaluronan-Mediated Motility (64) and ribonucleotide reductase R2 (65, 66), and these transcripts have cis-acting elements within the 3′ UTR, which interact specifically with cytoplasmic trans-factors following treatment with TGF-β1. However to date, no such elements have been identified within the 3′ UTRs of mRNA transcripts down-regulated by TGF-β1.

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