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Regulation of HGF/SF Gene Expression in MRC-5 Cells by *N*-Acetylcysteine

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The effect of *N*-acetylcysteine (NAC) on levels of hepatocyte growth factor/scatter factor (HGF/SF) gene transcripts was investigated in the human lung embryonic fibroblast cell line, MRC-5. NAC increased expression of HGF/SF mRNA, in a dose- and time-dependent fashion, by a mechanism independent of glutathione synthesis but sensitive to oxidant stress induced by H_2O_2. Using actinomycin D to block RNA synthesis, it was observed that NAC had no effect on the stability of the HGF/SF mRNA transcripts. NAC increased HGF/SF promoter activity in cells transiently transfected with chloramphenicol acetyltransferase (CAT) reporter genes driven by HGF/SF gene 5′-flanking sequences. Primer extension analysis demonstrated that NAC enhanced the expression of HGF/SF mRNA transcribed from the main transcription initiation site. Although the 5′ flanking region of the HGF/SF gene contains a sequence at −1019 to −1011 with homology to the NF-κB response element, electrophoretic mobility shift assay demonstrated that this site did not bind nuclear factors in MRC-5 cells in the presence or absence of NAC. In contrast to the effect on HGF/SF mRNA, NAC did not increase HGF/SF protein production by MRC-5 cells.

Key Words: **buthionine-S,R-sulfoximine; chloramphenicol acetyltransferase reporter gene; glutathione; hydrogen peroxide; nuclear factor kappa B; Northern hybridisation; RNA stability; gene transcription.**

Hepatocyte growth factor/scatter factor (HGF/SF) is a potent mitogen for both rat and human hepatocytes in primary culture (1). Administration of HGF/SF triggered hepatocyte DNA synthesis in normal rats (2, 3) and mice (4) and the *in vivo* injection of a recombinant adenoviral vector encoding human HGF/SF in normal mice stimulated hepatocyte DNA synthesis and increased liver weight in a dose-dependent fashion without inducing significant liver injury (5). HGF/SF is thought to have a key role in liver regeneration, since plasma HGF/SF levels rose markedly following liver injury in the rat preceding by many hours the onset of hepatic DNA synthesis (6) and its administration enhanced liver growth in rats following both partial hepatectomy (2, 3, 7) and liver injury (7, 8). Following partial hepatectomy, livers from transgenic mice that over-expressed the HGF/SF gene under the control of albumin regulatory sequences recovered in half the time needed by normal mice and the labelling index of hepatocytes was doubled compared to that in wild-type mice (9). These findings suggest that increasing circulating HGF/SF by administration of an agent that increases HGF/SF production might be beneficial in patients with liver injury. Further work is required to identify non-toxic agents that can stimulate HGF/SF synthesis.

The antioxidant *N*-acetylcysteine (NAC) has been shown to regulate the expression of a number of genes by modulating DNA binding and transactivation by transcription factors including nuclear factor kappa B (NF-κB) (10, 11). Since the 5′ flanking region of the HGF/SF gene (12) contains a sequence motif 5′-GGGATTTCC-3′ (−1019 to −1011) with homology to the consensus NF-κB response element, we postulated that NAC might regulate expression of the HGF/SF gene. In this study, we investigated the effect of NAC on HGF/SF gene expression in MRC-5 cells, a human embryonic lung fibroblast cell line that produces high levels of HGF/SF (13).

MATERIALS AND METHODS

Culture of cells. MRC-5 cells (European collection of animal cell cultures), at passages 20 to 28, were cultured as monolayers on 90 mm diameter plastic dishes in 10 ml of Dulbecco's modified Eagle medium (DMEM), plus 10% foetal calf serum (FCS), and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin), at 37°C in a humidified atmosphere of 5% CO_2/95% air. When the cells had reached near confluence the experiments were started by changing to the same volume of fresh medium containing the test compounds.

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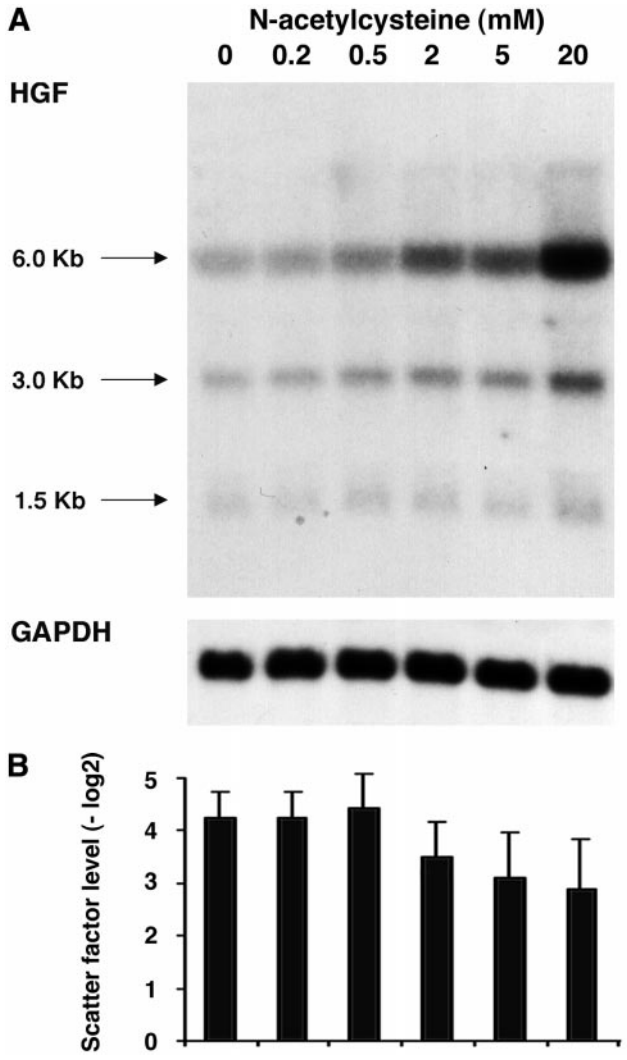

FIG. 1. Effect of NAC on HGF/SF gene expression in MRC-5 cells. (A) Total RNA was isolated from subconfluent MRC-5 cells cultured in the presence of increasing concentrations of NAC for 24 h and 30 μg RNA subjected to sequential Northern blot analysis using: a 668 bp HGF/SF cDNA probe, 40 h autoradiographic exposure; and a GAPDH cDNA probe, as a loading control, 6 h autoradiographic exposure. (B) MRC-5 cells were cultured in the presence of increasing concentrations of NAC for 3 days and HGF/SF levels were measured in the conditioned media using the scatter factor assay.

Northern blot analysis. After treatment of the MRC-5 cells for the specified times, the culture medium was removed and total RNA was isolated from the cells according to the acid guanidinium thiocyanate-phenol-chloroform method as described by Chomczynski (14). Each condition was performed in triplicate and the RNA pooled. The RNA samples (30 μg) were fractionated by gel electrophoresis (1% agarose and 2.2% formaldehyde) and transferred to Genescreen (Du-Pont-NEN, Boston, MA) in 10 $\times$ SSC (1.5 M sodium chloride + 0.15 M trisodium citrate, pH 7.0). 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% polyvinyl-pyrrolidone (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 668 bp DNA probe for HGF/SF, corresponding to exons 6 to 11, which had been labelled by the random primer procedure using [^{32}P]αdATP (15). The filter was washed twice at room temperature for 5 min in 2 $\times$ SSC plus 1% SDS, twice at 65°C for 30 min in 0.2 $\times$ SSC plus 0.1% SDS and then once at 65°C for 30 min in 0.2 $\times$ SSC. It was exposed to X-ray film with intensifying screens at $-$70°C. After stripping the membrane was rehybridised to a ^{32}P-labelled 360 bp partial cDNA probe for GAPDH. The synthesis of the cDNA probes for HGF/SF and GAPDH has been described previously (16).

HGF/SF gene promoter activity. The 5′-flanking region of the human HGF/SF gene was amplified from human placental DNA by PCR using: sense strand nt $-$1033 to $-$1007 and antisense strand nt +56 to +74 (12). It was cloned into the chloramphenicol acetyltransferase (CAT) reporter plasmid pCAT-Basic (Promega, Madison, WI) and the new plasmid was called 1.0 HGF-CAT. A second HGF/SF promoter sequence with a 152 bp deletion of the region containing the putative NF-κB response element was also generated by PCR using: sense strand nt $-$886 to $-$840 (12) and the same antisense primer. It was cloned into pCAT-Basic and the plasmid was called 0.9 HGF-CAT. The authenticity of the sequences was confirmed by sequencing (ABI 373A automated DNA sequencer).

MRC-5 cells (5 $\times$ 10^5) were transiently transfected with 10 μg of the HGF/SF promoter-CAT chimeric plasmid and 2 μg of pSV-β-galactosidase control plasmid (Promega, Madison, WI) using the calcium phosphate method (17). CMV-CAT, was used as a positive control and the pCAT-basic plasmid not containing HGF/SF 5′-flanking sequence was used as a negative control. The cells were incubated with the DNA-calcium phosphate coprecipitate for 16 h followed by an additional 24 h in culture with or without NAC. The cells were harvested and disrupted by three cycles of freeze-thaw (freezing on dry ice and thawing at 37°C). 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 min to inactivate any endogenous acety-

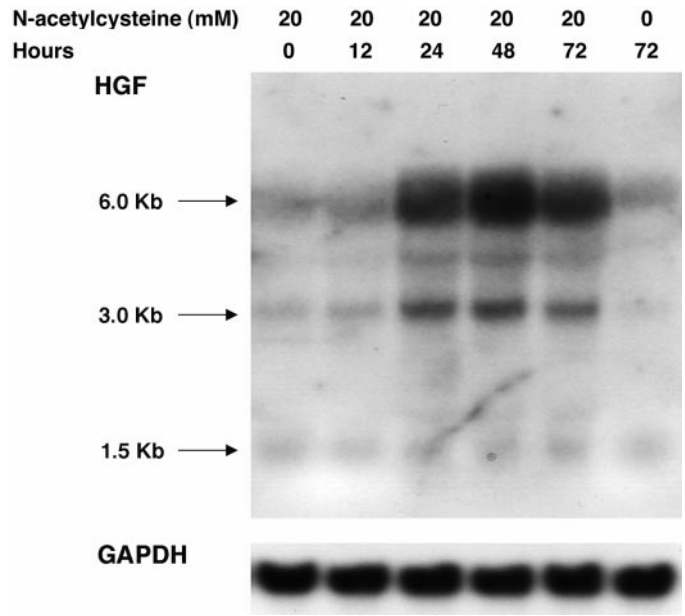

FIG. 2. Time course of the increase in HGF/SF mRNA in MRC-5 cells after treatment with NAC. Total RNA was isolated from subconfluent MRC-5 cells at time points up to 72 h after the addition of 20 mM NAC. Sequential Northern blot analysis was performed using: a 668 bp HGF/SF cDNA probe, 40 h autoradiographic exposure; and a GAPDH cDNA probe, as a loading control, 6 h autoradiographic exposure.

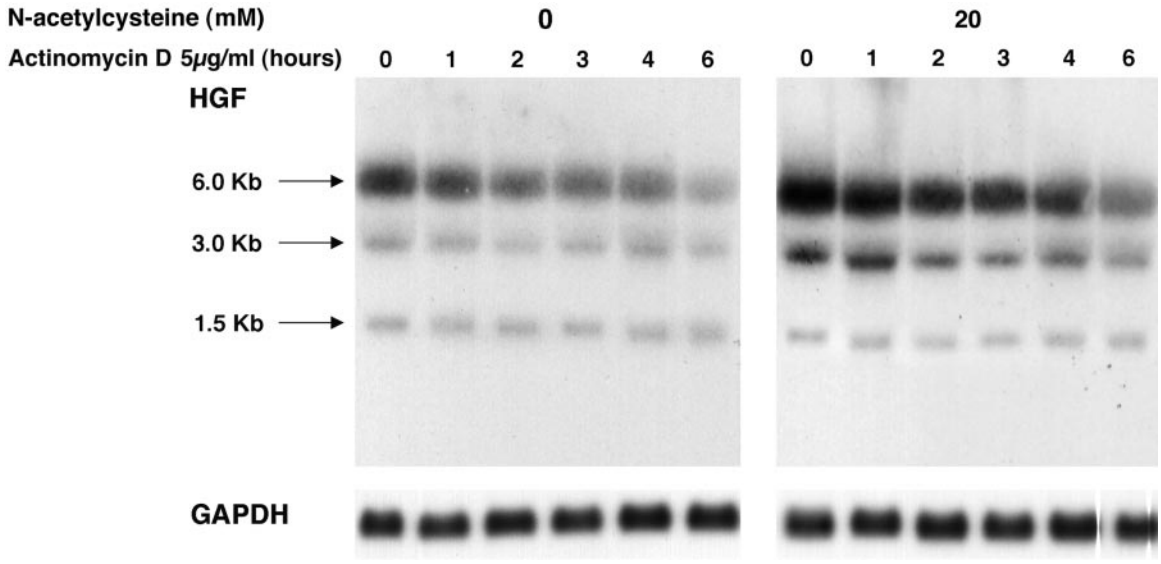

FIG. 3. The effect of NAC on the half-lives of HGF/SF mRNA transcripts in MRC-5 cells. Subconfluent MRC-5 cells were cultured in the presence or absence of 20 mM NAC for 24 h and then total RNA was isolated at time points after the addition of 5 μg/ml actinomycin-D. Sequential Northern blot analysis was performed using: a 668 bp HGF/SF cDNA probe, 40 h autoradiographic exposure; and a GAPDH cDNA probe, as a loading control, 6 h autoradiographic exposure.

lase or deacetylase activity. All extracts were stored at −70°C until assay. The β-galactosidase activity was quantified according to the manufacturer's instructions (Promega, Madison, WI) and used as a measure of transfection efficiency.

CAT activity in the cell extract was measured using the thin layer chromatography (TLC) assay, according to the manufacturer's instructions (Promega, Madison, WI). Briefly, a volume of cell extract adjusted for β-galactosidase activity was mixed with 4 μl of [^{14}C]chloramphenicol (55 mCi/mmol at 0.1 μCi/μl), 20 μl of acetyl coenzyme A (at 3.5 mg/ml) and made up to a final volume of 130 μl with 1 M Tris–HCl buffer pH 7.8. The reaction was incubated at 37°C for 2 h and then terminated by adding 1 ml ethyl acetate. The organic phase was evaporated under vacuum (Savant SpeedVac), resuspended in 25 μl of ethyl acetate and run on a silica gel thin layer chromatography (TLC) plate in a chloroform/methanol:95/5 chromatography chamber. The dried plate was exposed to X-ray film at room temperature for 3 days.

Electrophoretic mobility shift assay (EMSA). The 5′-flanking region of the HGF/SF gene containing the putative NF-κB response element was subjected to EMSA. Nuclear protein was extracted from MRC-5 cells as described (18). Briefly, the cells were washed in 1 ml Tris-buffered saline and resuspended in 0.4 ml 10 mM Hepes, pH 7.8, 1 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, and 0.1 mM PMSF on ice for 15 min. The cell membranes were lysed by the addition of 25 μl 10% Nonidet-P40 for 15 s and the nuclei pelleted at 4°C. The nuclei were resuspended in 50 μl 50 mM Hepes pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol for 20 min. Following high speed centrifugation for 5 min at 4°C, the supernatant containing the nuclear protein was aliquoted and stored at −70°C. The concentration of nuclear protein was measured by the BCA Protein Assay.

The plasmid 1.0 HGF-CAT was cut with *Sal*I at −1025 bp and the phosphate group removed with calf intestinal phosphatase (CIP). The linear plasmid was end-labelled with γ[^{32}P]dATP using T4 polynucleotide kinase for 1 h at 37°C and cut with *Pst*I at −875 bp. The 150 bp DNA probe was isolated from a 4% polyacrylamide gel and then hybridised to 2 μg of nuclear protein in binding buffer (20 mM Tris, 2 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA, 10% glycerol (v/v), 0.1% Nonidet P-40 (v/v), 1 mM DTT, 300 μg/ml BSA) plus 1 μg poly(dI-dC).poly(dI-dC) with or without 100 ng of unlabelled competitor DNA for 30 min at 4°C. To determine whether a shifted band was to due specific binding of a protein to the DNA sequence, excess unlabelled double stranded HGF/SF DNA sequences, designated 1 and 2, were added to compete for binding to the protein. A non-specific DNA sequence, designated E3, which has no relation to the HGF/SF sequence, was used to control for non-specific binding. The competitor DNA sequences used are given below, only the upper strand sequence is shown and the putative NF-κB response element is underlined: (1), −1026/−1002 AGCTTGA<u>GGGATTTCC</u>GGTGAAAGT; (2), −1001/−977 CAGTCCTAACCAGTGTATACCTACA; (E3) GATCGGTCATGTGGCAAGGCTATTTGGG.

The reactions were fractionated by electrophoresis on a 6% polyacrylamide gel in a high ionic strength Tris-glycine buffer for 2–3 h at 4°C and visualised by autoradiography.

Primer extension analysis of the transcription initiation sites. A DNA oligonucleotide, 5′-TCCCTCTGGAGGAGATGCCTGGGTGAAAG, complementary to HGF/SF mRNA, was labelled with ^{32}P at the 5′ end using T4 polynucleotide kinase and 1×10^5 cpm was hybridised to 20 μg of total RNA from MRC-5 cells in S1 hybridisation solution (80% deionised formamide, 40 mM PIPES pH 6.4, 400 mM sodium chloride, 1 mM EDTA pH 8.0) at 30°C for 16 h. The DNA–RNA hybrids were reverse transcribed at 37°C for 90 min using M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) and the primer extended cDNA, after treatment with RNAse A, was fractionated by electrophoresis on a 6% polyacrylamide gel.

Biological assay of HGF/SF activity in MRC-5 cell conditioned media. HGF/SF also stimulates cell motility (19, 20), depending on the target cell and culture conditions, and is also termed "scatter factor." HGF/SF levels can be measured in conditioned media by the scatter factor assay (20), which utilises the dose-dependent effect of HGF/SF on scattering of Madin-Darby canine kidney cells (MDCK cells).

MRC-5 cells were grown in 24-well plates to near confluence, under the same conditions as for the Northern blot analysis, and then exposed to NAC for 3 days. HGF/SF levels in MRC-5 cell conditioned media were measured by the scatter factor assay (19). Briefly, using 96-well plates, twofold serial dilutions of the test

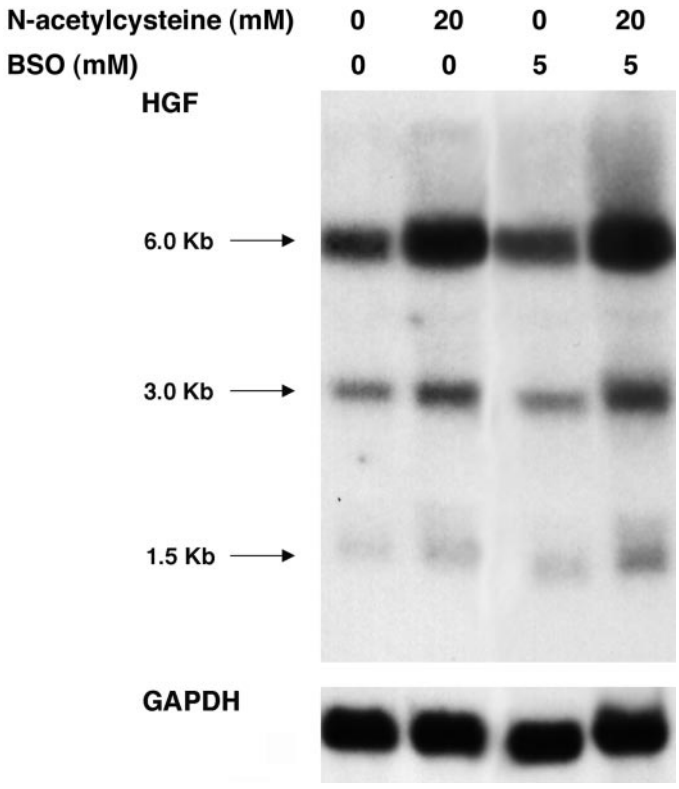

FIG. 4. Effect of BSO on the up-regulation of HGF/SF mRNA expression by NAC in MRC-5 cells. Total RNA was isolated from MRC-5 cells, cultured in the presence or absence of 5 mM BSO and 20 mM NAC for 24 h, and 30 μg RNA subjected to sequential Northern blot analysis using: a 668 bp HGF/SF cDNA probe, 40 h autoradiographic exposure; and a GAPDH cDNA probe, as a loading control, 6 h autoradiographic exposure.

samples were made from 1/2 to 1/256 in 0.15 ml of DMEM with 5% FCS before 3000 MDCK cells in 0.15 ml DMEM with 5% FCS were added and incubated for 16 h. The cells were fixed with 4% formaldehyde for 10 min and stained with 0.2% Coomassie blue. The plates were viewed under microscopy and the degree of scattering was recorded. The assay end point was defined as the highest dilution at which scattering could still be clearly observed and was recorded as the $-\log_2$. Each experiment was performed in quadruplicate.

RESULTS

The Effect of NAC on HGF/SF Gene Expression

In the Northern hybridisation, the 6.0 kb and 3.0 kb HGF/SF mRNA plus the alternatively spliced 1.5 kb mRNA transcripts were detected. *N*-acetylcysteine (Sigma Chemical Company, Poole, UK), at concentrations greater than 0.5 mM for 24 h, enhanced the expression of all HGF/SF gene transcripts in a dose dependent fashion (Fig. 1A). In contrast, there were no significant changes in HGF/SF protein production, as measured by the scatter factor assay, in media conditioned by MRC-5 cells exposed to NAC for 3 days (Fig. 1B). Culture media containing NAC that had not been conditioned by MRC-5 cells had no scatter factor activity (data not shown). In order to examine the time course of the mRNA changes, RNA was isolated at time points after the addition of 20 mM NAC to subconfluent MRC-5 cells and analysed by Northern hybridisation. A rise in HGF/SF mRNA expression was detected at 24 h, reached a peak at 48 h, and was still evident at 72 h (Fig. 2).

To investigate the mechanism of action of NAC on expression of HGF/SF mRNA, we determined its effect on HGF/SF mRNA stability. RNA was isolated from subconfluent MRC-5 cells at time points after the addition of 5 μg/ml actinomycin-D, which blocks gene transcription, and 30 μg analysed by Northern blot. Pretreatment with 20 mM NAC for 24 h before the addition of actinomycin D increased expression of the 6.0 kb HGF/SF mRNA but had no effect on its half-life, which was around 3 h (Fig. 3).

Further experiments were performed to determine whether glutathione and the cellular redox state are important in the regulation of HGF/SF mRNA expression. The enzyme γ-glutamylcysteine synthetase catalyses the first rate-limiting step in glutathione synthesis and is specifically and irreversibly inhibited by buthionine-S,R-sulfoximine (BSO) (37). Treatment of cultured cells with 5 mM BSO reduces glutathione levels to 40% of control values after 24 h with minimal cellular toxicity (37). BSO at 5 mM for 24 h had no effect on levels of any of the HGF/SF mRNA transcripts

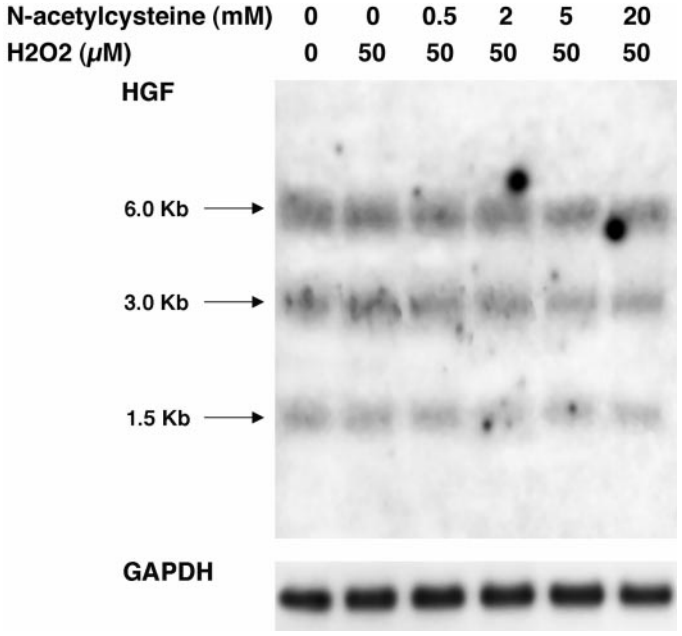

FIG. 5. Effect of H_2O_2 on the up-regulation of HGF/SF mRNA expression by NAC in MRC-5 cells. Total RNA was isolated from MRC-5 cells, cultured in the presence or absence of 50 μM H_2O_2 and increasing concentrations of NAC for 24 h, and 30 μg RNA subjected to sequential Northern blot analysis using: a 668 bp HGF/SF cDNA probe, 40 h autoradiographic exposure; and a GAPDH cDNA probe, as a loading control, 6 h autoradiographic exposure.

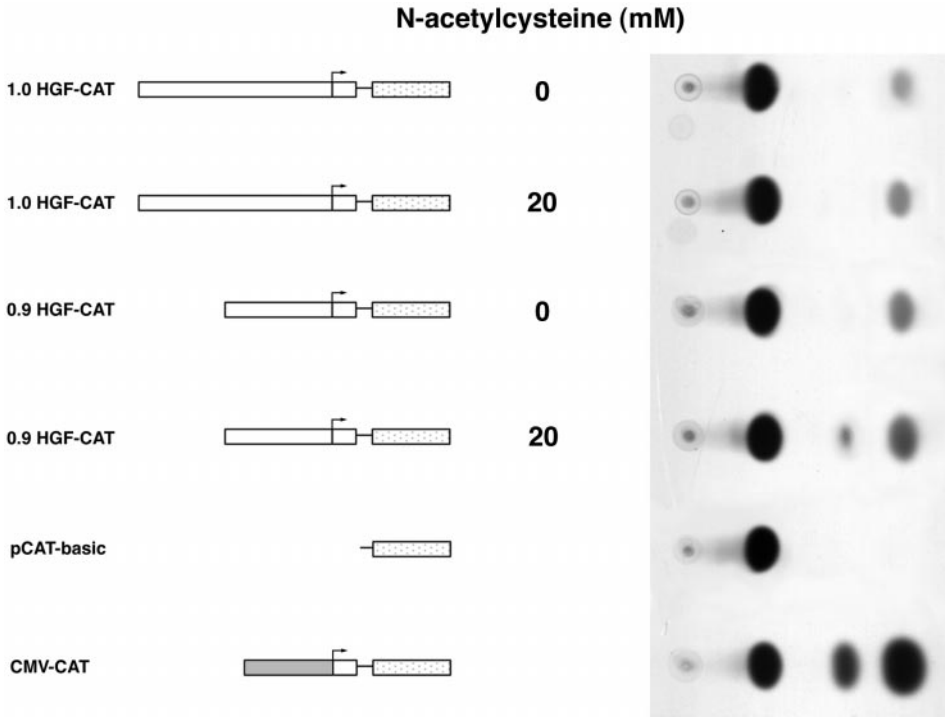

FIG. 6. Activity of human HGF/SF promoter-CAT chimeric genes. The 5′-flanking region of the human HGF/SF gene was amplified by PCR to produce two fragments that were identical at the 3′ end but one had a 152 bp deletion of the region containing the sequence motif with homology to the NF-κB response element. They were cloned into the reporter vector pCAT-basic to create the chimeric HGF/SF-CAT constructs, 1.0 and 0.9 HGF-CAT, respectively. The plasmids were transiently transfected into MRC-5 cells by the calcium phosphate method and cotransfection with pCMV-β-galactosidase plasmid was used to control for transfection efficiency. After 16 h NAC at 20 mM was added for 24 h. The cells were then harvested and disrupted by three cycles of freeze-thaw. The supernatants, adjusted for galactosidase activity, were taken for standard TLC CAT assay. CMV-CAT was used as a positive control and pCAT-basic not containing HGF/SF 5′-flanking sequence was used as a negative control.

and pretreatment of the cells for 24 h with 5 mM BSO did not block the up-regulation of HGF/SF mRNA expression by 20 mM NAC at 24 h (Fig. 4).

Hydrogen peroxide (H_2O_2) is a membrane permeable oxidant that at micromolar concentrations has been shown to modulate the expression of many genes by the activation of transcription factors (21, 22). H_2O_2 at a concentration of 50 μM for 24 h had no effect on HGF/SF mRNA expression on its own but it completely suppressed the induction of HGF/SF mRNA expression by NAC (Fig. 5).

HGF/SF Gene Promoter Activity

Following transfection into MRC-5 cells, 0.9 HGF-CAT produced higher CAT activity than 1.0 HGF-CAT, indicating there is an element present in the region between nucleotides −1026 and −875 that reduces transcriptional activity (Fig. 6). The nature of this inhibitory element was not investigated further in this study. Treatment of transfected cells with 20 mM NAC for 24 h increased by around twofold the CAT activity produced by both constructs but did not alter their relative CAT activities (Fig. 6). This indicates that the NAC response element is located between −875 and +74 and that the region containing the putative NF-κB response element is not required for the stimulation of transcriptional activity by NAC.

Primer Extension Analysis

Primer extension of MRC-5 cell mRNA produced two DNA fragments, 11 and 24 nucleotides longer than the oligonucleotide (Fig. 7). Hence, the transcription initiation sites of the HGF/SF gene in MRC-5 cells mapped to 64 and 77 nucleotides upstream of the translation initiation site. NAC only increased the expression of HGF/SF mRNA transcribed from the most 5′ of the two sites.

Electrophoretic Mobility Shift Assay

Nuclear protein extracted from MRC-5 cells produced a binding complex in EMSA with the −1025/−876 HGF/SF DNA probe (Fig. 8). The 25 bp HGF/SF DNA sequence 1 competitor (−1026 to −1002), containing the putative NF-κB response element, did not compete in EMSA for binding protein, taken from cells

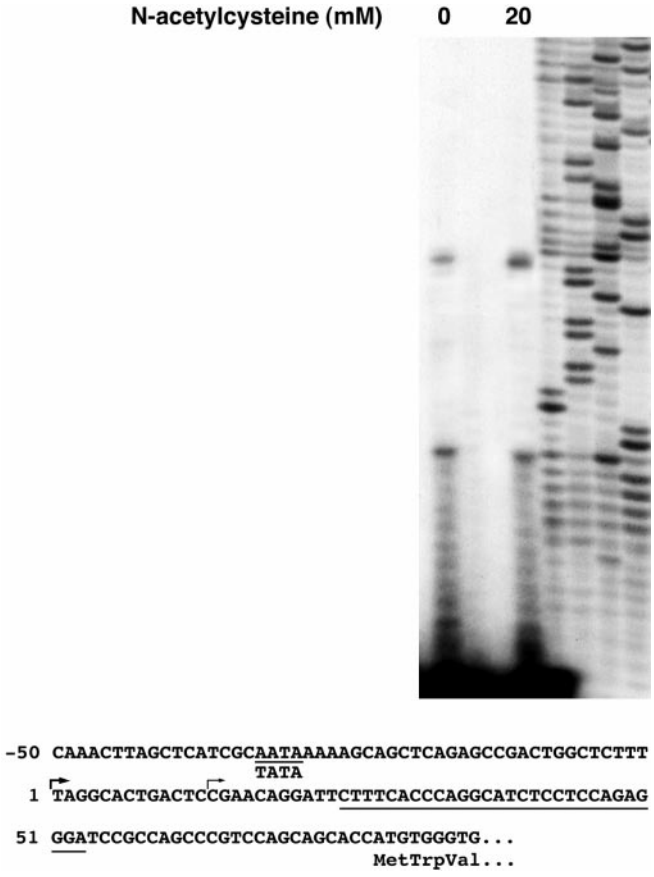

FIG. 7. Primer extension analysis of HGF/SF mRNA from MRC-5 cells treated with NAC. An oligonucleotide complementary to HGF/SF mRNA (underlined sequence), was labelled with ^{32}P at the 5′ end using T4 polynucleotide kinase and hybridised (1×10^5 cpm) in S1 hybridisation solution to 20 μg of total RNA from MRC-5 cells cultured in the presence or absence of 20 mM NAC for 24 h. The DNA–RNA hybrids were reverse transcribed at 37°C for 90 min. using M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) and the primer extended cDNA, after treatment with RNAse A, was fractionated by electrophoresis on a denaturing 6% polyacrylamide/7 M urea gel. The dried gel was exposed to X-ray film with an intensifying screen at −70°C for 48 h. The size marker is an unrelated sequence ladder. The HGF/SF gene sequence demonstrates the relationship of the transcription initiation sites to the putative TATA box and the translation initiation codon.

cultured in the presence or absence of 20 mM *N*-acetylcysteine for 24 h, indicating that the HGF/SF gene is not regulated in MRC-5 cells by factors binding to this element. The addition of cold HGF/SF DNA sequence 2 (−1001 to −977) to the EMSA completely abolished the binding complex demonstrating that a nuclear factor reacted with the HGF/SF promoter between −1001 and −977 (Fig. 8). This factor was not further characterised in the present study.

DISCUSSION

This study demonstrated that NAC increased the expression of HGF/SF mRNA transcripts in MRC-5 cells, in a dose- and time-dependent fashion. This effect was not mediated by enhancing HGF/SF mRNA stability, rather further experiments using HGF-CAT chimeric genes found that NAC stimulated HGF/SF gene transcription. Using primer extension analysis, we found that the transcription initiation sites of the HGF/SF gene in MRC-5 cells mapped to 64 and 77 nucleotides upstream of the translation initiation codon. The addition of NAC increased the expression of HGF/SF mRNA transcribed from the site 77 nucleotides upstream of the translation initiation site, which coincides with the major transcription initiation site detected in both placenta and MRC-5 cells by Miyazawa *et al.* using S1-nuclease analysis (12). The second transcription initiation site observed in the present study coincides with the major site found in both rat (23) and mouse liver (24, 25) but HGF/SF mRNA transcription from this site in MRC-5 cells was not effected by NAC.

Following exposure to NAC, the time scale of the induction of HGF/SF mRNA in MRC-5 cells was slow suggesting it did not act directly but rather via activa-

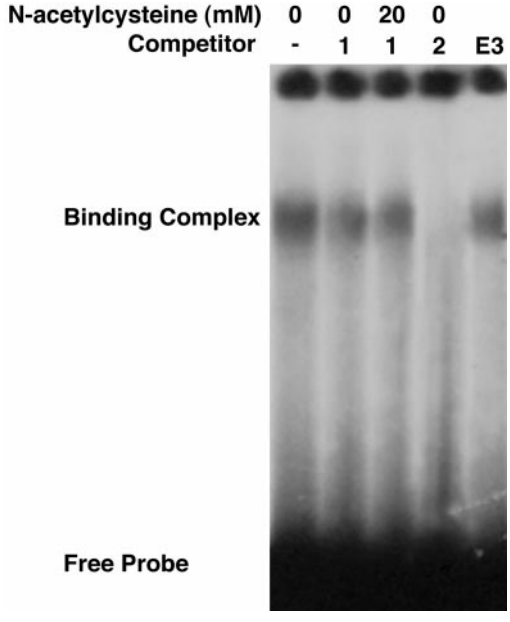

FIG. 8. EMSA with the −1025 to −876 HGF/SF promoter DNA probe. The 150 bp end-labelled [^{32}P]DNA probe was hybridised to 2 μg of nuclear protein from MRC-5 cells in binding buffer (20 mM Tris, 2 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA, 10% glycerol (v/v), 0.1% Nonidet P-40 (v/v), 1 mM DTT, 300 μg/ml BSA) plus 1 μg poly(dI-dC).poly(dI-dC) with or without 100 ng of unlabelled competitor DNA for 30 min at 4°C. The 25 bp competitor HGF/SF DNA sequence (−1026 to −1002), containing the putative NF-κB response element, was designated 1 and the other HGF/SF DNA sequence (−1001 to −977) designated 2. A nonspecific 28 bp DNA sequence, which has no relation to the HGF/SF promoter sequence, was designated E3 and used to control for nonspecific binding. The reactions were fractionated by electrophoresis on a 6% polyacrylamide gel in a high ionic strength Tris-glycine buffer for 2–3 h at 4°C and visualised by autoradiography.

tion of an intermediate signalling pathway. NAC has previously been reported to regulate DNA binding and transactivation by several transcription factors. The 5′ flanking region of the HGF/SF gene contains a sequence at −1019 to −1011 with homology to the NF-κB response element but, although NAC has previously been reported to modulate transactivation by NF-κB in other cell lines (10, 11), the EMSA data demonstrate that this site does not bind nuclear factors in MRC-5 cells in the presence or absence of NAC. In addition, the finding that NAC increased the CAT activity of the HGF-CAT chimeric construct with a deletion of the region containing the putative NF-κB response element supports the EMSA data showing that the stimulation of HGF/SF gene transcription by NAC is not mediated by modulating DNA binding at this site and indicates that the NAC response element is located between −875 and +74. No other potential NF-κB binding sites were identified in the 5′-flanking region of the HGF/SF gene between −875 and +74.

DNA binding and transactivation by the activating protein-1 (AP-1) family of transcription factors is also modulated by the cellular redox state (26–28). In contrast to NF-kB, which is usually activated by oxidants, AP-1 is activated by antioxidants, showing reduced activity in the presence of oxidants (22). NAC primarily modulates the cellular redox state by stimulating synthesis of intracellular reduced glutathione, an abundant low molecular weight nonprotein thiol (29). However, we found that inhibition of glutathione synthetase by BSO had no effect on HGF/SF mRNA expression and did not block the up-regulation of HGF/SF mRNA expression due to NAC, indicating that the effect of NAC on HGF/SF gene transcription is independent of stimulation of glutathione synthesis. However, our observation that H_2O_2 inhibits the increase in HGF/SF mRNA expression by NAC is consistent with the HGF/SF gene being regulated by AP-1. Sequence analysis of the 5′ flanking region of the human HGF/SF gene revealed one possible AP-1 binding site located at −56 to −62 bp on the negative strand. Further work is required to investigate regulation of HGF/SF gene expression via the AP-1 binding site.

NAC has also been reported to inhibit binding and transactivation by nuclear factor-IL6 (NF-IL6) (30), which binds a response element in the IL-6 gene and several other acute phase genes (31). There is a potential binding site for NF-IL6 located at −151 to −143 bp in the HGF/SF gene promoter but it is uncertain whether inhibition of NF-IL6 binding at this site by NAC would increase HGF/SF transcription. NAC has also been found to activate the Ras-extracellular signal-regulated kinase pathway in PC12 cells (30) by a mechanism that is dependent on ongoing transcription (30) but unrelated to any change intracellular glutathione levels. The effect of NAC on the Ras-extracellular signal-regulated kinase pathway in MRC-5 cells has not been studied.

Given the marked rise in HGF/SF mRNA expression in MRC-5 cells following NAC treatment, we anticipated a concomitant increase in HGF/SF protein production but conversely NAC had no significant effect on HGF/SF protein production. This observation suggests that, in addition to its effect on stimulating HGF/SF gene transcription, NAC also impaired translation or induced degradation of newly translated protein, as has previously been observed in LPS-treated cells of monocyte/macrophage lineage (32).

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