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Identification of a Cell-Type-Specific Transcriptional Repressor in the Promoter Region of the Mouse Hepatocyte Growth Factor Gene

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Hepatocyte growth factor (HGF), a cytokine with multiple functions, exhibits cell-type-specific as well as cytokine- and steroid hormone-regulated expression. The HGF gene is known to be expressed predominately in mesenchymal but not in epithelial cells. In this study, we report the identification of a cell-type-specific transcriptional repressor in the promoter region of the mouse HGF gene, which is evidently responsible for the suppression of HGF expression in epithelial cells. Gel mobility shift assays and DNase I footprinting studies revealed that a 27-bp element (−16 to +11) around the transcription initiation site is responsible for the binding of a nuclear protein which is present in epithelial but not in mesenchymally derived cells. Further analysis of the binding activity of the DNA region with nuclear protein revealed that an approximately 19-bp sequence containing a unique palindromic structure (5′-AACCGACCGGTT-3′) overlapped by a CAP box is essential for binding. Substitution of a single base (the contact site) within this region by site-directed mutagenesis resulted in total abrogation of the binding of the nuclear protein and a concomitant increase in the transcriptional activity of various lengths of HGF-chloramphenicol acetyltransferase fused genes when transfected into the epithelial cell line RL95-2 but not the mesenchymal cell line NIH 3T3. Southwestern (DNA-protein) analyses revealed that the nuclear protein which binds to this repressor element is a single polypeptide of ~70 kDa. Analysis of the nuclear extract prepared from regenerating mouse liver at various times after two-thirds partial hepatectomy by gel mobility shift assay revealed a substantial reduction (more than 75% within 3 h) in the binding of the repressor to its cognate binding site. Our results suggest that a *cis*-acting transcriptional repressor in the promoter region of the mouse HGF gene is involved in cell-type-specific regulation through binding to its cognate *trans*-acting protein which exists in epithelial cells but is absent in fibroblast cells.

Hepatocyte growth factor (HGF), also known as scatter factor, is a unique cytokine with multiple distinct functions. Extensive studies have shown that HGF has strong mitogenic effects on a wide variety of cells, including epithelial cells, endothelial cells, and melanocytes (6, 19, 33, 43, 60), although it was initially characterized as a potent mitogen for mature hepatocytes in primary culture (37, 62). In addition to mitogenic activity, HGF stimulates the motility of a variety of epithelial cells (motogenic activity) (51, 56) and includes the tubule formation of Madin-Darby canine kidney epithelial cells (morphogenic activity) in vitro (36). Furthermore, HGF has been shown to be cytostatic and cytotoxic to certain tumor cells (49, 52). Several lines of evidence suggest that these diverse biological actions of HGF are mediated via a single receptor, c-MET, the product of the c-*met* proto-oncogene encoding a 190-kDa transmembrane protein possessing an intracellular tyrosine kinase domain (4, 38). It is believed that HGF and its receptor c-MET play an essential role not only in cell growth and organ regeneration but also in embryogenesis, morphogenesis, and tumorigenesis.

Expression of the HGF gene is tissue and cell type specific (33, 43) and occurs primarily in mesenchymal cells such as cultured fibroblasts derived from many organs, Ito cells (fat-storing cells) of the liver, alveolar macrophages, peripheral blood leukocytes, and megakaryocytes (58, 63). Studies utilizing in situ hybridization and cell fractionation followed by Northern (RNA) blot analysis have indicated that the distribution of HGF transcripts is restricted to nonepithelial cells (28, 39). In addition, HGF gene expression has been found to be developmentally, hormonally, and cytokine regulated in a variety of tissues under normal and pathophysiological conditions (8, 23, 29, 50, 54). In response to tissue damage, such as partial hepatectomy, the level of HGF mRNA is dramatically increased in liver as well as in distal organs, including the lung and spleen (21, 40, 61). Similar results are also achieved in the remaining rat kidney and lung after unilateral nephrectomy and pneumonectomy, respectively (59). Recently, we have shown that steroid hormones such as estrogen play an important role in the regulation of HGF expression. A single injection of 17β-estradiol resulted in dramatic and transient induction of the HGF transcript in mouse ovaries (23). It has also been demonstrated that several cytokines, including interleukin 1 (IL-1) and tumor necrosis factor alpha, can upregulate HGF expression in primary cultured fibroblast cells and established cell lines (54), while transforming growth factor β and dexamethasone inhibited its expression (29). These results suggest that the regulation of HGF gene expression is a complex process involving cell-type-specific and inducible expression under various circumstances. However, little is known about the molecular mechanism underlying such complicated regulation of the HGF gene.

The cell-type-specific, developmental, hormonal, and cytokine regulation of HGF gene expression is controlled primarily at the level of transcription (23, 43, 54, 61). It is known that the

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transcription activation of eukaryotic genes via regulatory elements is achieved by the interplay between *cis*-acting DNA regulatory sequences and *trans*-acting proteins that recognize such DNA elements (17, 34, 53). In an attempt to understand the mechanism governing the regulation of HGF expression at the transcriptional level, we have previously isolated the entire mouse HGF gene (22) and delineated the structure and function of 2.8 kb of the 5′-flanking region (24). The data suggest that the presence of several cytokine- and hormone-responsive elements, such as IL-6-responsive elements (IL-6RE), estrogen-responsive elements (ERE), and transforming growth factor β inhibitory element, in the 5′-flanking region of the mouse HGF gene undoubtedly provide the molecular basis for the inducible expression of the HGF gene. Additionally, a cell-type-specific negative regulatory element has been identified between positions −964 and −699 by a combination of a series of 5′-end deletions and internal deletions, which may explain, in part, the specific suppression of HGF gene expression in epithelial cells. However, it appears that additional negative elements are likely required to totally repress HGF gene expression in epithelial cells, since the negative element located between −964 and −699 alone cannot completely silence the HGF gene in nonexpressing RL95-2 cells (24).

In this study, we have identified and characterized a novel *cis*-acting transcriptional repressor sequence located between positions −16 and +11 relative to the transcription initiation site in the promoter region of the mouse HGF gene. Using Southwestern (DNA-protein) analysis, we also identified a potential cell-type-specific *trans*-acting transcription factor, a 70-kDa polypeptide that is present in epithelial cells but not in fibroblast cells, which binds to this element. Our data therefore suggest the presence of complex DNA-protein interactions that are necessary for the compartmentalized cell-type-specific expression of the mouse HGF gene.

MATERIALS AND METHODS

Preparation of nuclear protein extract. Human endometrial carcinoma RL95-2 cells, human hepatoma HepG2 cells, mouse fibroblast NIH 3T3 cells, human embryonic lung fibroblast MRC-5 cells, human promyelocytic leukemia HL-60 cells, and mouse mastocytoma P815 cells were originally obtained from the American Type Culture Collection (Rockville, Md.). RL95-2 and NIH 3T3 cells were cultured in the conditions described previously (24). All of the other cell lines were maintained in the media recommended by the supplier. For preparation of nuclear protein extracts, cells in an exponential growth stage were washed twice with cold phosphate-buffered saline and scraped off the plate with a rubber policeman. Cells were collected and the nuclei were isolated by a method described elsewhere (25). Briefly, the pelleted cells were resuspended in 4 volumes of buffer A containing protease inhibitors (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 0.5 M sucrose, 1.5 mM NaCl, 60 mM KCl, 0.15 mM spermidine, 0.5 mM spermine, 0.5 mM EDTA, and 1 mM dithiothreitol, plus 1 μg each of leupeptin, soybean trypsin inhibitor, antipain, and chymostatin per ml). An equal volume of buffer A containing 0.6% Nonidet P-40 was added with gentle mixing to lyse the cells. Immediately after lysis, the solution was diluted with 8 volumes of buffer A, and the nuclei were collected by centrifugation at 5,000 × *g* for 30 min at 4°C. Nuclear protein was extracted with 0.4 M KCl-TGM buffer (10 mM Tris-HCl [pH 7.6], 10% glycerol, 3 mM $MgCl_2$, 3 mM EGTA) containing protease inhibitors as described above. The lysate was centrifuged for 45 min at 50,000 × *g* at 4°C, and the supernatant was then collected and dialyzed against 60 mM KCl-TGM buffer, using a mini-dialysis system (Gibco/BRL, Gaithersburg, Md.). The insoluble material was removed by centrifugation, and small aliquots of protein extract were quickly frozen and stored at −80°C after the protein concentration had been determined (protein assay; Bio-Rad, Richmond, Calif.). For heat treatment of the nuclear extracts, the nuclear extracts were treated at 65 or 75°C for either 10 or 30 min, and the insoluble fraction was removed by centrifugation at 15,000 × *g* for 6 min at 4°C. The heat-treated nuclear extracts were used directly or were divided into aliquots, frozen in liquid nitrogen, and stored at −80°C.

For preparation of mouse liver nuclear protein extract, livers were removed from mice at 0, 3, 6, and 24 h after partial hepatectomy and homogenized in buffer A containing protease inhibitors. The nuclei were collected, and the nuclear protein was extracted with 0.4 M KCl-TGM buffer as described above.

Oligonucleotides. Oligonucleotides were chemically synthesized in the Department of Pathology, University of Pittsburgh Medical Center, and purified by gel electrophoresis. Complementary strands were annealed in 10 mM Tris-HCl (pH 8.0)–200 mM NaCl–1 mM EDTA by heating to 95°C and cooling to room temperature over a period of 3 h. The majority of the sequences of the oligonucleotides used in this study are described in the corresponding context. The oligonucleotides were PR2 (protected region 2 detected by DNase I footprinting; 5′-GATCTAGCTCATCGCAATAAAAG-3′ and 5′-GATCCTTTTATTGCGATGAGCTA-3′); PR3 (5′-GATCTTTCCAGTTAATCACACAACAAACTTAG-3′ and 5′-GATCCTAAGTTTGTTGTGTGATTAACTGGAAA-3′); ERE (5′-GATCAAGGTCAGAAAGACCAT-3′ and 5′-GATCATGGTCTTTCTGACCTT-3′); AP1 (5′-GATCTGCCTTGACTTAGCGAG-3′ and 5′-GATCCTCGCTAAGTCAAGGCA-3′); and IL-6RE (5′-GATCAGAGCTGGGATCTG-3′ and 5′-GATCCAGATCCCAGCTCT-3′). These oligonucleotides correspond to the specific elements of the mouse HGF gene and were used as competitor DNAs in gel mobility shift assays. In addition, oligonucleotides corresponding to Sp1 (5′-ATTCGATCGGGGCGGGGCGAGC-3′ and 5′-GCTCGCCCCGCCCCGATCGAAT-3′) and OCT-1 (5′-TGTCGAATGCAAATCACTAGAA-3′ and 5′-TTCTAGTGATTTGCATTCGACA-3′) were purchased from Promega (Madison, Wis.) and used in gel mobility shift assays.

Gel mobility shift assay. A DNA fragment (F1) corresponding to positions −70 to +29 of the 5′-flanking region of the mouse HGF gene was isolated from chimeric plasmid 0.3HGF-CAT, which contains 0.3 kb of the mouse HGF 5′-flanking region as described previously (24). Following restriction digestion of plasmid 0.3HGF-CAT with *Pst*I and *Bgl*II, the 99-bp fragment F1 was purified from an agarose gel and labeled with [α-^{32}P]dGTP (3,000 Ci/mmol; Amersham) by filling in and blunt ending with Klenow enzyme (Gibco/BRL) and nucleostide triphosphate (NTP). The double-stranded oligonucleotides corresponding to specific sequences as indicated in the text were also labeled with ^{32}P by end labeling with T4 kinase. The labeled probes were then gel purified and used in gel mobility shift assays as described previously (25). Four micrograms of poly(dI-dC) · poly(dI-dC) (Pharmacia, Piscataway, N.J.) was used as the nonspecific competitor in 10 μl of reaction mixture. The binding reactions were carried out at room temperature for 15 min before loading on 5% nondenaturing polyacrylamide (19:1, acrylamide/bisacrylamide) gels. The concentration of nuclear extract used in each reaction was 4 μg, and that of the labeled probe was between 0.3 and 0.4 ng. For competition experiments, a 100-fold molar excess of unlabeled DNA fragments or oligonucleotide was included in the reaction mixture. Gels were run in 0.5× TBE buffer (0.045

M Tris-borate, 0.001 M EDTA) at a constant voltage of 190 V, dried, and autoradiographed with intensifying screens.

DNase I footprinting. The binding reaction conditions and labeling of DNA fragment F1 were the same as described for gel mobility shift assays. Nuclear protein extract was incubated with ^{32}P-end-labeled DNA probe and poly(dI-dC) · poly(dI-dC) in a volume of 50 μl of the binding buffer for 15 min. DNase I (1 U) in 50 μl of buffer containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ was then added, and incubation continued for 2 min at room temperature. The DNase I reaction was then stopped by adding buffer containing 12.5 mM EDTA, 12.5 μg of proteinase K per ml, 125 μg of yeast tRNA per ml, and 0.1% sodium dodecyl sulfate (SDS). After incubation for 10 min at 37°C, the mixture was subjected to extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitation. The reaction products were separated in 12% polyacrylamide–8 M urea sequencing gels and subjected to autoradiography. The sequencing of the same ^{32}P-labeled probe was performed as described by Maxam and Gilbert (30), and the sequencing reaction mixture was loaded adjacent to the samples analyzed by DNase I footprinting.

Methylation interference assay. The methylation interference assay was performed as previously described (25). The 99-bp DNA fragment (F1) corresponding to positions −70 to +29 relative to the transcription initiation site was isolated and labeled with [^{32}P]dGTP by filling in with Klenow enzyme. The labeled F1 was purified on a nondenaturing polyacrylamide gel and methylated with dimethyl sulfate (45). The conditions for interactions between methylated labeled DNA and nuclear protein extract were identical to those used for gel mobility shift assays described above, with the exception of a threefold increase in the reaction mixtures. Following electrophoresis through a 5% polyacrylamide gel, the radioactive bands (both protein bound and free) were localized and excised according to the autoradiography of the wet gel. The DNA was then eluted with 400 μl of 0.3 M sodium acetate (pH 5.2) at room temperature, purified by a Sephadex G-25 column (5′-3′, Denver, Colo.), and precipitated with ethanol. The DNA was cleaved at guanine residues by 1 M piperidine, and the fragments were resolved on an 12% sequencing gel.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis was conducted with a Transformer site-directed mutagenesis kit as instructed by the manufacturer (Clontech, Palo Alto, Calif.). Briefly, a mutagenic primer containing a specific nucleotide mutation at DNA-protein contact site of the mouse HGF gene (5′-CAGAACCGAACGGTTTGCAA CAGGATTCT-3′) and a selection primer containing a specific mutation within either a unique *Bam*HI restriction site of plasmids 0.7HGF-CAT and 0.1HGF-CAT (5′-GTATCTTAT CATGTCTGAATCCCCCGGAA-3′) or a unique *Kpn*I restriction site of plasmid 2.8HGF-CAT (5′-CTTGCCCTTG GGTACCTCTGTATGGC-3′) were phosphorylated with T4 kinase and ATP and annealed with the denatured parental plasmids 2.8HGF-CAT, 0.7HGF-CAT, and 0.1HGF-CAT, which have been described elsewhere (24). Following addition of DNA polymerase and T4 DNA ligase, the reaction mixtures were subjected first to digestion with either *Bam*HI or *Kpn*I to linearize the parental plasmids. After transformation into repair-deficient bacterial cells, isolated plasmids were pooled and digested again with either *Bam*HI or *Kpn*I to further linearize the parental wild-type plasmids. Following a second round of transformation, plasmids from individual colonies were isolated and digested with either *Bam*HI or *Kpn*I. The plasmids resistant to digestion were further sequenced to confirm the specific nucleotide mutation.

Construction of plasmids. The wild-type chimeric plasmids 2.8HGF-CAT, 0.7HGF-CAT, and 0.1HGF-CAT, which contain 2.8, 0.7, and 0.1 kb, respectively, of the 5′-flanking region of the mouse HGF gene, have been described previously (24). The mutant constructs 2.8HGF-CAT, 0.7HGF-CAT, and 0.1HGF-CAT, with a point mutation at position −8, were generated by site-directed mutagenesis as described above. For construction of plasmid PR1-SV-CAT, an oligonucleotide corresponding to the PR1 sequence was synthesized and cloned into the *Bgl*II site in front of a simian virus 40 promoter vector (pCAT-promoter; Promega, Madison, Wis.). The sequence, copy number, and orientation of the insert had been confirmed by sequencing using a T7 DNA sequencing kit (U.S. Biochemical, Cleveland, Ohio) (45).

Cell culture, DNA transfection, and CAT assay. Human endometrial carcinoma RL95-2 cells and mouse fibroblast NIH 3T3 cells were cultured as described previously (24). Twenty-four hours prior to transfection, the cells were seeded in six-well plates at 2×10^5 cells per well. The cells were then transiently transfected with various mouse HGF-chloramphenicol acetyltransferase (CAT) chimeric plasmids, using the DNA calcium phosphate method as instructed for the Cell-Phect transfection kit (Pharmacia). In each experiment, RL95-2 and NIH 3T3 cells were cotransfected with the same amount of plasmid DNA (5 μg per well) and 1 μg of the β-galactosidase reference plasmid pCH110 (Pharmacia) as an internal standard for transfection efficiency. After incubation with DNA-calcium phosphate coprecipitation buffer for 16 h, the NIH 3T3 and RL95-2 cells were washed twice with serum-free medium, complete medium containing 10% fetal calf serum was added, and then the cells were incubated for an additional 24 h before harvesting for CAT assays. After the cells were washed in phosphate-buffered saline, pelleted, resuspended in 150 μl of 0.25 M Tris HCl (pH 7.5), and disrupted by three freeze-thaw cycles, the protein suspension was clarified by centrifugation at 15,000 × *g* for 5 min at 4°C, and the supernatant was collected and assayed for CAT activity as described elsewhere (24). Reaction mixtures containing the cell extract, 0.2 μCi of [^{14}C]chloramphenicol, and 4.4 mM acetyl coenzyme A were incubated for 2 h at 37°C. The reaction products were separated by thin-layer chromatography (Kodak, Rochester, N.Y.) and visualized by autoradiography. Experimental results were quantitated by densitometric analysis using a Bio-Image analyzer (Millipore, Bedford, Mass.). All experiments were repeated at least three times (duplicated wells per experiment) to ensure reproducibility. Relative CAT activity was reported after normalization for transfection efficiency.

Southwestern blot analysis. Southwestern blot analysis was performed as described by Gao et al. (11). Twenty-microgram samples of nuclear protein from different cells were separated on a denaturing SDS-polyacrylamide gel. Following electrophoresis, the gel was electroblotted onto nitrocellulose filters (Schleicher & Schuell) in transfer buffer (50 mM Tris-HCl, 40 mM glycine, 0.04% SDS, 20% methanol), using a Bio-Rad Trans-Blot cell at 125 mA per slab for 15 h at room temperature. After the transfer was completed, the nitrocellulose filter was soaked in BLOTTO (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.6], 50 mM KCl, 1 mM EDTA, 5% Carnation nonfat milk powder, 5 μg of denatured and sonicated salmon sperm DNA per ml, 1 mM dithiothreitol), incubated at room temperature for 1 h with gentle shaking, and then rinsed twice with rinsing buffer (BLOTTO buffer without milk powder). The binding reaction was carried out by incubating the filter in rinsing buffer that contained 5×10^5 cpm of ^{32}P-labeled DNA probe per ml for 1 h at room temperature. The filter was then washed three times (10 min

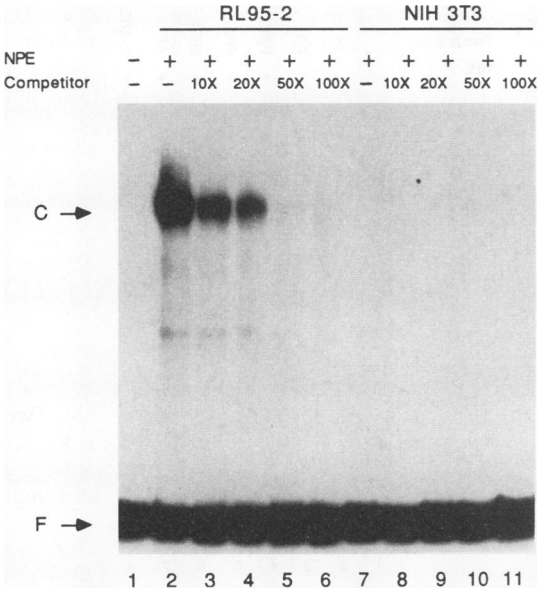

FIG. 1. Identification of specific binding of the mouse HGF promoter region to nuclear proteins from RL95-2 cells. DNA fragment F1 was labeled with ^{32}P and incubated with nuclear protein extracts (NPE) from human endometrial carcinoma RL95-2 cells and mouse fibroblast NIH 3T3 cells in the absence (−) or presence (+) of increasing amounts of unlabeled competitor DNA. The binding reactions were performed at room temperature for 15 min in the presence of poly(dI-dC) · poly(dI-dC) as a nonspecific competitor. C, binding complex; F, free ^{32}P-labeled DNA probe.

each) by gentle agitation at room temperature with rinsing buffer, air dried, and subjected to autoradiography to visualize the radiolabeled DNA-protein interaction.

RESULTS

Specific binding of the mouse HGF promoter region to nuclear proteins. We have recently isolated genomic clones containing the entire mouse HGF structural gene as well as 2.8 kb of the 5′-flanking sequence (22, 24). To identify in more detail the *cis*-acting elements and *trans*-acting factors which control HGF gene expression, a portion of the 5′-flanking region and first exon (from positions −956 to +29) of the mouse HGF gene were divided into six fragments, using a number of unique restriction enzymes. These fragments were end labeled by filling in with ^{32}P-labeled dNTP and analyzed for the ability to bind nuclear proteins extracted from various types of cells, using a gel mobility shift assay. Our initial results showed that five of six fragments were specifically bound to nuclear proteins derived from either established cell lines or mouse tissues (data not shown).

DNA fragment F1, corresponding to nucleotides −70 to +29 of the 5′-flanking region of mouse HGF gene, was found to bind nuclear proteins in a cell-type-specific manner. Examination of the DNA-protein complexes by polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions revealed a prominent band with a slow electrophoretic mobility when nuclear protein extracts from RL95-2 (human endometrial carcinoma) cells were used for the binding assay (Fig. 1, lane 2). The formation of this DNA-protein complex could be completely abolished by the addition of a 100-fold excess of the unlabeled DNA fragment, indicating specificity of the binding (Fig. 1, lane 6). Under the same conditions, addition of a 100-fold-excess molar ratio of the DNA fragment containing unrelated sequences did not affect binding (see Fig. 3). However, no binding with this fragment could be detected at the corresponding position with protein extracts from mouse fibroblast NIH 3T3 cells (Fig. 1, lane 7). The differences in the binding activity between RL95-2 and NIH 3T3 cell nuclear protein extracts are not due to the quality and the integrity of the nuclear extracts, since we have used these extracts in gel mobility shift assays using other DNA probes and have confirmed that the extracts are intact (see Fig. 7B). From these observations, we conclude that RL95-2 cells contain a binding protein that is not present in detectable levels in NIH 3T3 cells and that this protein binds to the DNA fragment corresponding to the nucleotide −70 to +29 region of the mouse HGF gene.

Identification of the binding region and DNA-protein contact sites. To identify the location of the binding site within DNA fragment F1, DNase I footprinting experiments were performed with a ^{32}P-labeled DNA probe corresponding to nucleotides −70 to +29 relative to the transcription initiation site. After the DNA fragment was incubated with RL95-2 cell nuclear extract, three protected regions, designated PR1, PR2, and PR3, were detected (Fig. 2). Increasing the protein concentration appeared to achieve more protection accordingly. As illustrated in Fig. 2, the PR1 region protected against DNase I digestion was located between nucleotides −16 and +11 relative to the transcription initiation site. Examination of the nucleotide sequence within this 27-bp region indicated that there is a palindromic sequence, 5′-AACCGACCGGTT-3′, at positions −14 to −3 and a so-called CAP box, 5′-GTTTGC-3′, at positions −5 to +1, which is assumed to serve as the binding site for RNA polymerase II complexes (27). The PR2 region located at positions −33 to −38 resides adjacent to the putative TATA box. The PR3 region is located at positions −59 to −44, and the nucleotide sequence within this region is extremely AC rich. In addition, hypersensitive sites can be seen between the PR2 and PR3 sequences (Fig. 2).

To further define the nucleotide sequences that are responsible for the binding of DNA fragment F1 with nuclear protein(s), gel mobility shift competition assays were performed with synthetic oligonucleotides corresponding to the individual protected regions shown in Fig. 2 and unrelated sequences as competitor DNAs. Three oligonucleotides corresponding to the individual protected regions PR1, PR2, and PR3 were incubated in the gel mobility shift reaction mixture as competitors, using DNA fragment F1 as a probe and nuclear protein(s) from RL95-2 cells. As shown in Fig. 3, only the PR1 oligonucleotide was capable of competing for binding to nuclear protein(s) with fragment F1. The binding was completely abolished when a 100-fold-excess molar ratio of the PR1 (−16 to +11) oligonucleotide was added to the reaction mixture (Fig. 3, lane 3). On the other hand, the PR2 (−38 to −33) and PR3 (−59 to −44) oligonucleotides at 100-fold-excess molar ratios did not compete (Fig. 3, lanes 4 and 5). As expected, other oligonucleotides corresponding to unrelated sequences, including ERE, AP1, and IL-6RE of the mouse HGF gene, also failed to compete with fragment F1 for binding. These results indicate the specificity of the binding of oligonucleotide PR1 with nuclear protein(s) from RL95-2 cells. In fact, when PR1 was ^{32}P labeled and incubated with RL95-2 cell nuclear protein(s), a specific binding complex of identical size was detected; however, when PR3 was labeled and incubated with the same nuclear protein(s), no such binding was observed (data not shown).

To examine the binding between DNA fragment F1 and nuclear protein from RL95-2 cells in further detail, methyl-

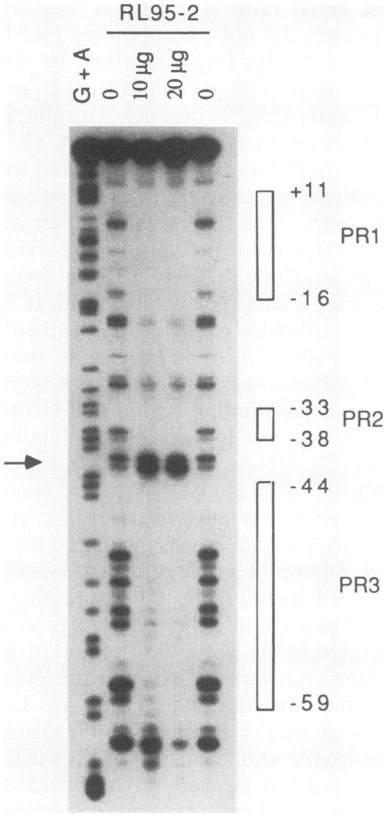

FIG. 2. DNase I footprinting analysis of the mouse HGF promoter region. DNA fragment F1 was end labeled and incubated with different amounts (10 and 20 μg) of crude nuclear protein extracted from RL95-2 cells in the presence of poly(dI-dC) · poly(dI-dC) as a nonspecific competitor. After 15 min of incubation at room temperature, the samples were treated with DNase I, and the DNA was analyzed on 12% polyacrylamide sequencing gels. The Maxam-Gilbert sequencing marker (G+A) (30), using the same ^{32}P-labeled DNA fragment, are displayed in the leftmost lane. The regions protected by RL95-2 cell nuclear proteins (PR1, PR2, and PR3) are indicated by boxes on the right. The arrow on the left side illustrates the hyposensitive site.

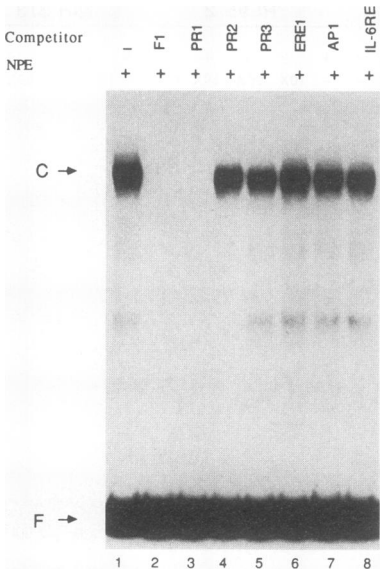

FIG. 3. Definition of PR1 in the promoter of HGF gene as the binding site for the nuclear protein(s). DNA fragment F1 was labeled with ^{32}P and incubated with nuclear protein extract (NPE) from RL95-2 cells. The binding reactions were performed at room temperature for 15 min in the presence of a nonspecific competitor. One hundred-fold-excess molar ratios of double-stranded oligonucleotides corresponding to the protected regions PR1, PR2, and PR3, respectively, as shown by DNase I footprinting (Fig. 2), were included in the reaction mixture as competitors. Some unrelated double-stranded oligonucleotide sequences, including ERE1, AP1, and IL-6RE, were also added to the reaction mixture to compete for binding as indicated. C, binding complex; F, free probe.

ation interference assays were used to define more precisely the nucleotide-protein contact sites of the complex. Fragment F1 was end labeled with ^{32}P on the bottom strand and partially methylated by dimethyl sulfate on guanine (G) residues. DNA was recovered from the binding complexes, and the free probe region of the gel mobility shift assay was cleaved at methylated residues by piperidine treatment. The results are presented in Fig. 4. The cleavage pattern revealed that methylation at the double G residues of the bottom strand at positions −7 and −8 strongly interfered with nuclear protein binding. A weak interference with nuclear protein binding at the G's at positions −12 and +4 was also observed (Fig. 4B). Thus, within the region consisting of 19 nucleotides, four G's acted as contact sites for nuclear protein binding.

Single-nucleotide mutation at the DNA-protein contact site abolishes binding. It is believed that the nucleotide-protein contact site is crucial for DNA-protein binding. To test whether the contact site is absolutely required for RL95-2 cell nuclear protein binding to the nucleotide sequence of PR1 within the HGF promoter, two mutant oligonucleotides in which either single- or double-nucleotide mutations were introduced at the contact sites were used in gel mobility shift assays as competitor DNAs. As shown in Fig. 5, the wild-type PR1 oligonucleotide could form a binding complex which is identical to that formed by fragment F1 and nuclear protein(s) from RL95-2 cells (Fig. 5A), and such binding could be abrogated by addition of a 20-fold excess of unlabeled PR1 (Fig. 5A). However, when a 20-fold excess of the double-stranded mutant oligonucleotide (Mut1) in which the C at position of −8 was substituted by A (Fig. 5C) was included in the reaction mixture, no competition for binding was found, as the intensity of the shifted band was similar to that obtained without competitor DNA (Fig. 5). Similar results were obtained with the Mut2 oligonucleotide, in which two contact sites at positions −12 and −8 had been mutated (Fig. 5). Likewise, when the Mut1 PR1 oligonucleotide was labeled and used in gel mobility shift assays, no binding activity was detected (Fig. 5B), indicating that a single-nucleotide mutation at the DNA-protein contact site is sufficient to abolish binding.

Since the PR1 region contains a palindromic structure consisting of 12 bp and since many transcription factors bind to DNA elements that have palindromic sequence (2, 34), we suspect that this 12-bp palindromic sequence (5′-AACCGAC CGGTT-3′) may be sufficient to bind nuclear protein from RL95-2 cells. However, when a double-stranded oligonucleotide corresponding to this 12-bp palindromic sequence was used in gel mobility shift assays to compete for binding with the PR1 probe, no competition for binding was detected (data not shown). Comparison of the nucleotide sequences of PR1 and the 12-bp palindrome revealed that one nucleotide-protein contact site at position +4 was not included in the palindromic structure. These results further suggest that the nucleotide-protein contact site is critical for DNA element binding to

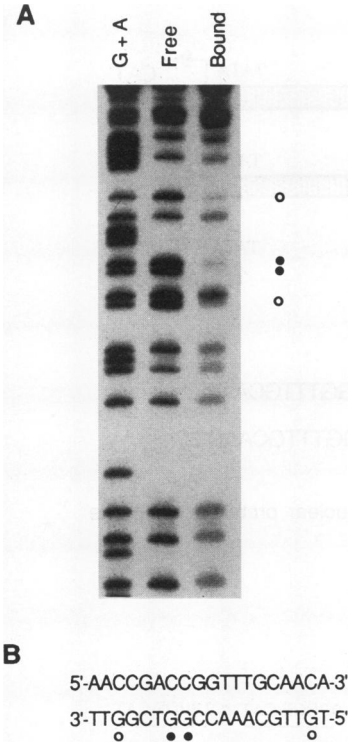

FIG. 4. Determination of the nucleotide-protein contact sites within the PR1 region of the HGF promoter. DNA fragment F1 was labeled on the bottom strand and partially methylated with dimethyl sulfate. The specific complexes formed between the methylated DNA and nuclear protein from RL95-2 cells were separated from free probe, and the recovered complex and free probe were cleaved by piperidine. The products were then resolved on a 12% polyacrylamide sequencing gel as described in Materials and Methods. (A) Autoradiographic photograph of the methylation interference analysis. Bound, DNA recovered from the DNA-nuclear protein complexes; Free, unbound DNA recovered from the DNA probe-nuclear protein reactions; G+A, Maxam-Gilbert sequencing reactions (30) of the same DNA fragment as a marker. (B) Nucleotide sequence of the binding region. The nucleotide contact sites are indicated. Solid circles depict strong contacts; open circles indicate those that are weak.

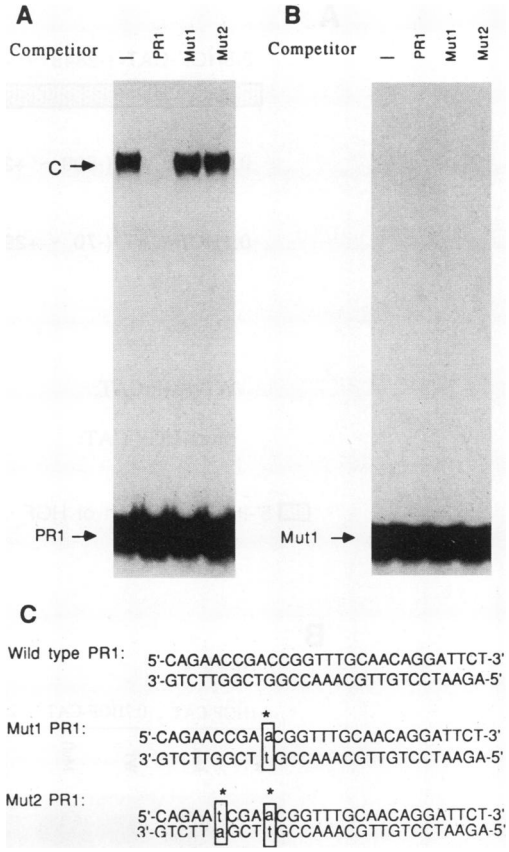

FIG. 5. Single-nucleotide mutation abolishes binding of the PR1 oligonucleotide to nuclear protein(s). (A) An oligonucleotide corresponding to wild-type PR1 sequence (−17 to +12) was labeled with ^{32}P and incubated with nuclear protein extract from RL95-2 cells. Twentyfold molar excesses of unlabeled wild-type PR1 and PR1 Mut1 and Mut2 oligonucleotides were included in the reaction mixture as competitors. The binding complex (C) and free probe (PR1) are indicated by arrows. (B) Similar gel mobility shift experiments except that the PR1 Mut1 oligonucleotide was labeled and used as a probe. (C) Nucleotide sequences of wild-type and mutated PR1 oligonucleotides. The nucleotides mutated are indicated by boxes and asterisks.

nuclear protein. In fact, these four nucleotides (Fig. 4B) are conserved in the human, mouse, and rat HGF promoters.

The DNA element that binds to RL95-2 cell nuclear protein functions as a cell-type-specific transcriptional repressor. To test the function of the binding region, site-directed mutagenesis was performed to mutate the DNA-protein contact site to prevent binding of the protein factor(s). On the basis of the results of the methylation interference (Fig. 4) and gel mobility shift (Fig. 5) assays, a single-nucleotide site-directed mutation (substitution of C at position −8 by A, which prevents the protein factor binding to the cognate DNA region) was introduced to the promoters of constructs 0.1HGF-CAT, 0.7HGF-CAT, and 2.7HGF-CAT (Fig. 6A). These mutated HGF reporter constructs and their wild-type counterparts were transfected into the RL95-2 carcinoma cell line, and the relative CAT activity of each construct was then determined. As shown in Fig. 6B, the transcriptional activity of the mutated promoter containing various lengths of the 5′-flanking regions of the HGF gene was increased dramatically (Fig. 6B and C). These results indicate that the DNA binding region acts as a transcriptional repressor for HGF gene activity in epithelial cells which express the binding factor(s). These results also show that the repression of the promoter activity is functional not only in the basal HGF promoter (0.1HGF-CAT) but also in the context of the HGF promoter containing up to 2.6 kb of the 5′-flanking region of the HGF gene. However, when the same wild-type and mutant 2.8HGF-CAT, 0.7HGF-CAT, and 0.1HGF-CAT constructs were transfected into NIH 3T3 cells which contain undetectable levels of the protein factor that binds the DNA region, the CAT activities of wild-type and mutant constructs were virtually identical (Fig. 6B and C). The results presented here clearly indicate that the DNA-binding element of HGF gene acts as a cell-type-specific transcriptional repressor which functions via the nuclear protein factor that is present in epithelial cells and absent in fibroblast cells.

To test whether the negative transcriptional activity of the DNA region is also functional in a heterologous promoter, the oligonucleotide corresponding to this *cis*-acting regulatory element (−16 to +11) was placed in front of a heterologous simian virus 40 promoter-CAT construct. This construct, PR1-SV-CAT, was then transiently transfected into RL95-2 cells and NIH 3T3 cells in parallel with the parental pSV-CAT

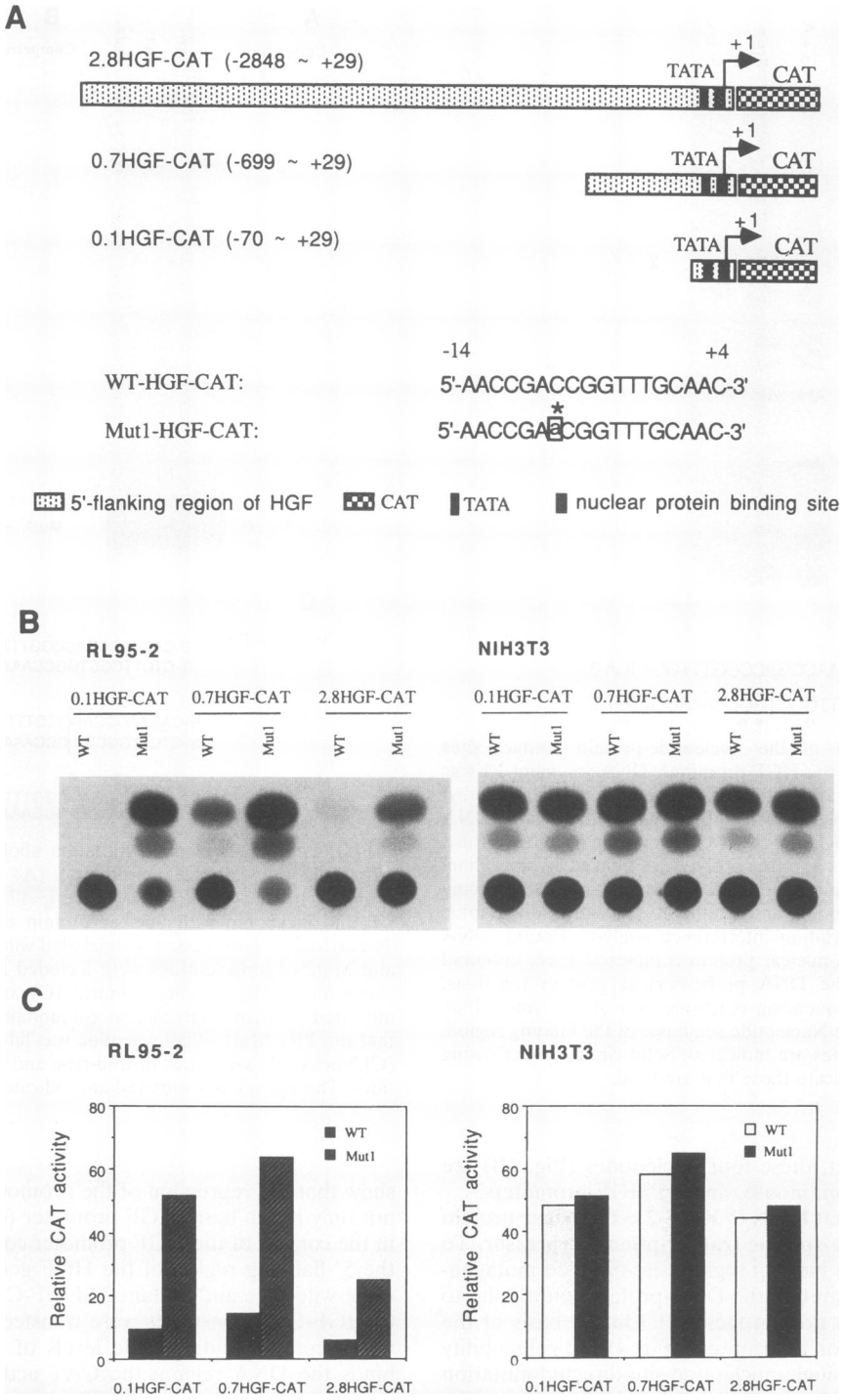

FIG. 6. Functional analysis of the effects of wild-type and mutated PR1 regions of the HGF promoter on expression of HGF-CAT chimeric genes in epithelial and fibroblast cell lines. (A) Diagrammatic representation of construction of the wild-type HGF-CAT (WT-HGF-CAT) and mutated HGF-CAT plasmids generated by site-directed mutagenesis. A partial nucleotide sequence corresponding to positions −14 to +4 of the 5′-flanking region of the mouse HGF gene is shown, and the mutated nucleotide is indicated by a lowercase letter. (B) Representative CAT assay following transfection of wild-type (WT) and mutated HGF-CAT chimeric plasmids into either RL95-2 or NIH 3T3 cells. (C) Relative CAT activity in cell lysates after transient transfection of either the wild-type (WT) or mutated HGF-CAT chimeric plasmid into RL95-2 and NIH 3T3 cells. Data represent averages of four independent experiments performed in duplicate.

plasmid. The results of these transfections indicated that the construct containing the PR1 sequence 5′ of pSV-CAT showed little if any suppression in RL95-2 cells (data not shown). As expected, no difference in NIH 3T3 cells was noted compared with the CAT activity between PR1-SV-CAT and its parental pSV-CAT construct. These results suggest that the negative role of the nuclear protein binding sequence might be unique to the HGF promoter (see Discussion).

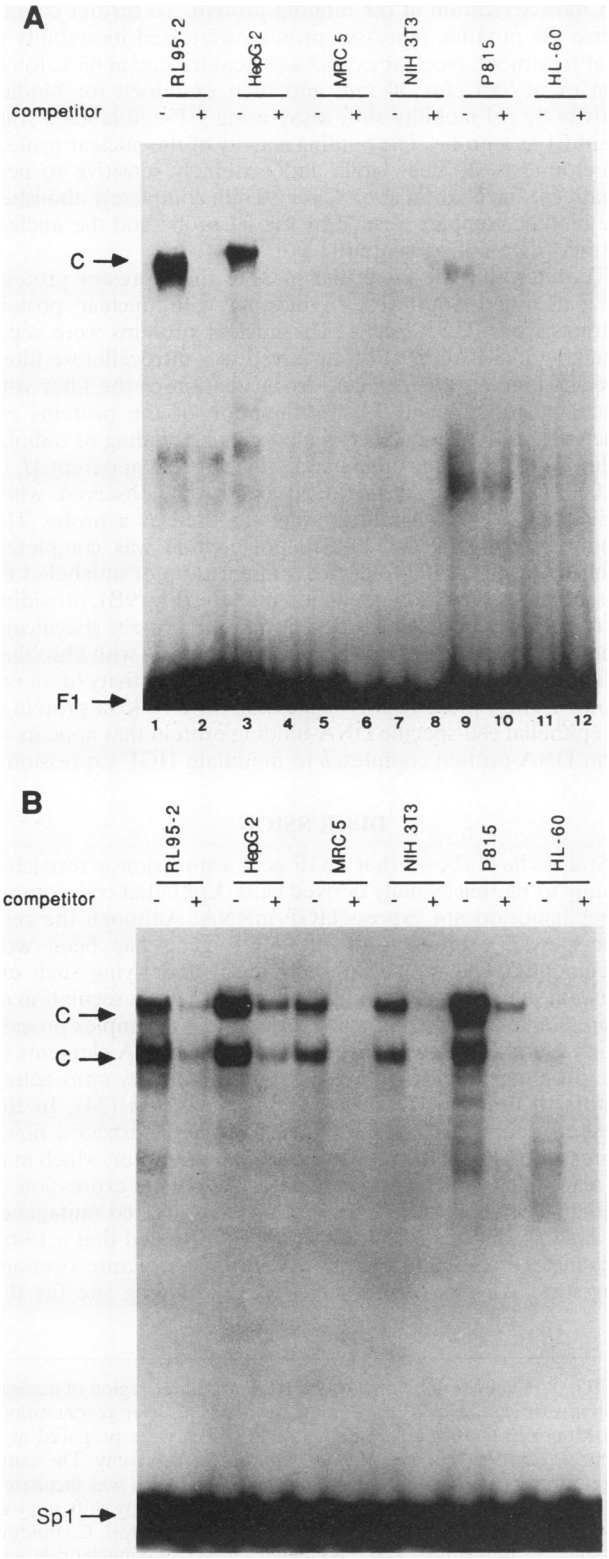

FIG. 7. Binding activities of nuclear proteins from different cell types with the PR1 oligonucleotide. (A) Oligonucleotide PR1 was labeled with ^{32}P and incubated with nuclear proteins extracted from different types of cells as indicated. The binding reactions were performed at room temperature for 15 min in the presence of a nonspecific competitor. A 100-fold-excess molar ratio of a double-stranded oligonucleotide corresponding to PR1 was included in the reaction mixture as a specific competitor. (B) Binding reactions performed as for panel A except that ^{32}P-labeled Sp1 oligonucleotide was used as a probe to serve as an internal control for monitoring the quality of the nuclear protein extracts. A 100-fold excess of Sp1 oligonucleotide was included in the reaction mixture as a competitor. C, binding complex.

Correlation between the lack of HGF gene expression and binding activity of the repressor protein. To examine the possible correlation between HGF gene expression and binding of nuclear protein to the repressor element, nuclear proteins prepared from different types of cells, including two epithelial cell lines (RL95-2 and HepG2), two fibroblast cell lines (MRC-5 and NIH 3T3), one mastocytoma cell line (P815), and one leukemia cell line (HL-60), were incubated with radiolabeled PR1 probe, and binding activity was determined by gel mobility shift assays. Figure 7A shows that a strong specific shifted band was observed in nuclear extracts from the two epithelial cells which do not express HGF mRNA. No binding activity was found in nuclear proteins extracted from two fibroblast cells cell lines which are known to express HGF mRNA (Fig. 7A, lanes 5 and 7). Incubation of the PR1 probe with protein extract from P815 cells resulted in one faint band with a slightly faster mobility (Fig. 7A, lane 9), while no detectable binding activity was found when the PR1 probe was incubated with nuclear protein from HL-60 cells. The difference in binding activity of the nuclear extracts prepared from various cells appears not to be due to the quality and integrity of the nuclear extracts, as the oligonucleotides corresponding to Sp1 (Fig. 7B) and other ubiquitous transcription factor binding sites, including OCT-1 and TATA (data not shown), could form specific binding complexes when they were incubated with the same nuclear extracts in gel mobility shift assays. It has been well documented that HGF is primarily expressed in fibroblast cells but not in epithelial cells (43). It is also known that HL-60 cells produce a considerable amount of HGF only after stimulation with tetradecanyl phorbol acetate (16), while in P815 cells, no HGF mRNA can be detected by Northern blot analysis (data not shown). These findings demonstrate that a correlation exists between the suppression of HGF gene expression and nuclear protein binding to the repressor element, providing further evidence that the DNA element located at −16 to +11 functions as *cis*-acting transcriptional repressor through binding to *trans*-acting cell-type-specific nuclear protein.

To investigate binding activity of the repressor protein during liver regeneration, nuclear proteins were extracted from mouse liver at 0, 3, 6, and 24 h after two-thirds hepatectomy and incubated with ^{32}P-labeled F1 probe. The binding activity of mouse liver nuclear protein extracts to the F1 probe was dramatically decreased at 3 h postoperation (Fig. 8A, lane 2; Fig. 8C). The decrease of the relative binding activity was extended to at least 24 h posthepatectomy, although a slight recovery of binding was noted at later time points (Fig. 8C). However, when the same nuclear protein extracts were incubated with OCT-1 oligonucleotide, a ubiquitous transcription factor (OCT-1) binding site, no detectable change was observed (Fig. 8B). The binding activities of the F1 and OCT-1 probes to mouse liver nuclear proteins are indeed specific, since addition of unlabeled probes completely abolished the binding (Fig. 8A, lanes 5 to 7; Fig. 8B, lanes 5 to 7). It has been previously reported that the expression of HGF is remarkably increased in liver after partial hepatectomy (61). These results clearly indicate that the induction of HGF expression coincides well with the decrease of binding activity of repressor protein in mouse liver during liver regeneration.

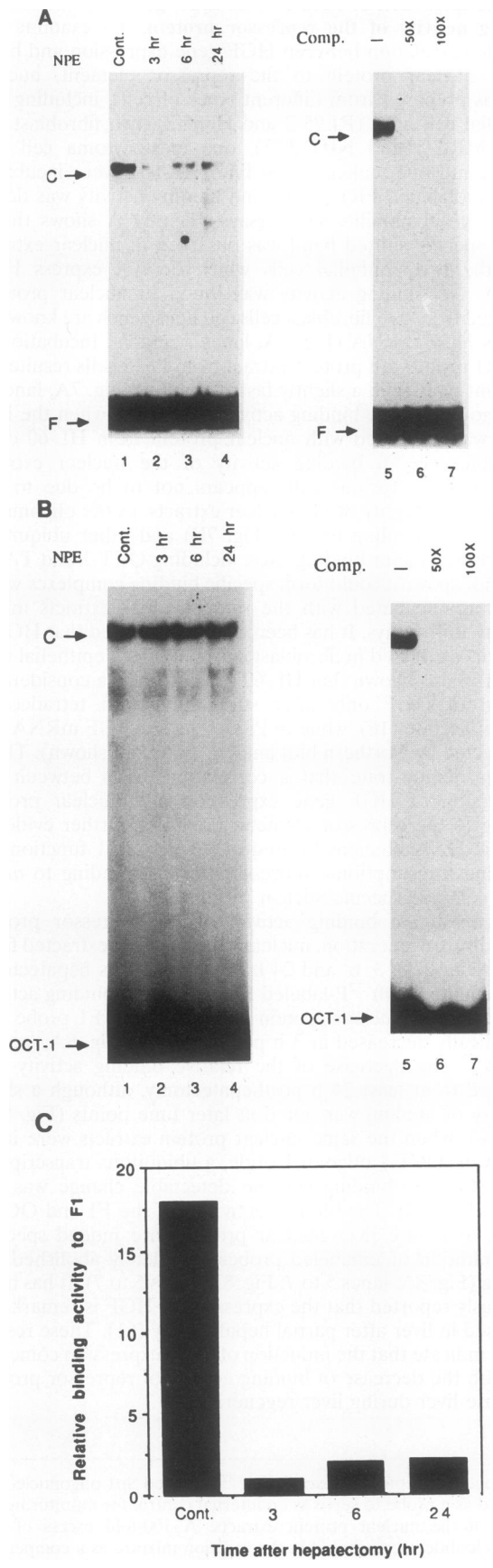

FIG. 8. Changes in binding to the HGF promoter region of nuclear protein extracts derived from mouse liver during liver regeneration. (A) Mouse liver nuclear protein extracts (NPE) were prepared at 0 (control [Cont.]), 3, 6, and 24 h after partial hepatectomy. The same amount of nuclear protein extract for each time point was incubated with ^{32}P-labeled F1 probe and subjected to a gel mobility shift assay as described in the legend to Fig. 1. Comp., competitor; C, binding complex; F, free probe. (B) ^{32}P-labeled OCT-1 oligonucleotide was incubated with mouse liver nuclear protein extract described for panel A as an internal control for monitoring the quality of the nuclear protein extracts. (C) Graphic representation of changes in relative binding to the F1 probe of nuclear protein extracts from mouse liver after partial hepatectomy. The autoradiographs shown in panels A and B (lanes 1 to 4) were scanned with a Bio-Image computing laser densitometer, and the changes in binding to F1 are plotted after normalization to the binding to OCT-1.

Characterization of the binding protein. To further characterize the putative repressor protein, we tested its stability to heat treatment. Nuclear extract was heat treated at 65°C for 10 min or at 75°C for 30 min and then examined for binding activity by gel mobility shift assay using ^{32}P-end-labeled fragment F1 as a probe. The binding activity of the nuclear protein was found to be heat labile and extremely sensitive to heat treatment; incubation at 65°C for 10 min completely abolished the binding complex formed by the F1 probe and the nuclear extract (data not presented).

To determine the molecular mass of the repressor protein, we performed Southwestern blotting with nuclear protein extracts from RL95-2 cells. The nuclear proteins were separated by SDS-PAGE and transferred to a nitrocellulose filter. Specific binding was detected by incubation of the filter with radiolabeled fragment F1. Examination of the proteins extracted from RL95-2 cells revealed specific binding of radiolabeled F1 to a single protein band, which had an apparent M_r of 70,000 (Fig. 9A). An identical band was observed when radiolabeled PR1 oligonucleotide was used as a probe. The binding activity of the 70-kDa polypeptide was completely abolished when a 100-fold-excess molar ratio of unlabeled F1 was added to the binding reaction mixture (Fig. 9B), providing further evidence that the 70-kDa nuclear protein specifically binds to F1 DNA fragment. Similar experiments with fibroblast cell nuclear extracts did not show any binding activity (data not shown). Thus, these results suggest that the 70-kDa protein is an epithelial cell-specific DNA-binding protein that appears to form DNA-protein complexes to modulate HGF expression.

DISCUSSION

Studies have shown that HGF gene expression is restricted mainly to mesenchymally derived cells. Epithelial cells, on the other hand, do not express HGF mRNA. Although the cell-type-specific expression of the HGF gene has been well documented, the molecular mechanism underlying such expression remains unknown. It is believed that the regulation of expression of a particular eukaryotic gene is a complex process which usually involves a cluster of *cis*-acting DNA elements in the promoter region interacting with multiple *trans*-acting factors to produce variable expression patterns (34). In the present report, we have identified and characterized a novel repressor region in the mouse HGF gene promoter, which may be responsible for the suppression of HGF gene expression in epithelial cells. DNase I footprinting, site-directed mutagenesis analysis, and gel mobility shift assays revealed that a 19-bp sequence containing a unique palindromic structure overlapping the CAP box region serves as the binding site for the

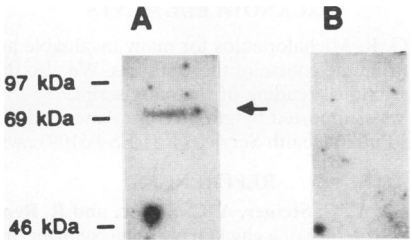

FIG. 9. Characterization of the HGF promoter-binding protein by Southwestern blotting. Nuclear protein from RL95-2 cells was separated by denaturing SDS-PAGE, transferred to a nitrocellulose membrane, and probed with ^{32}P-labeled 99-bp DNA fragment F1 probe in the absence (A) or presence (B) of a 100-fold excess of unlabeled F1 as a specific competitor DNA. Protein-DNA interactions were analyzed by autoradiography. The relative positions of molecular weight markers are illustrated on the left. The arrow indicates the molecular mass of the specific binding protein.

repressor, a 70-kDa polypeptide. This repressor is present in HGF nonexpressing epithelial cells but absent in HGF-expressing fibroblast cells. Therefore, our current results on the identification of an epithelial cell-specific transcriptional repressor in the promoter region of the mouse HGF gene undoubtedly shed new light on the understanding of the molecular mechanism involved in the cell-type-specific expression of the HGF gene.

The binding site for the cell-type-specific repressor is located 3′ of the putative TATA box, as shown by gel mobility shift assays, DNase I footprinting, and methylation interference assays. On the basis of the sequences, it is likely that the repressor element is a novel binding site, since no homology to other characterized repressors (1, 7, 12, 26, 27, 47) was found. It is also interesting that although the sequence of the repressor element is not identical among HGF promoters from mouse, rat, and human species (24, 35, 41, 48), the nucleotide-protein contact sites which are crucial for protein factor binding are well conserved among the three species, indicating that the same regulation pathway may be shared. In fact, in DNA-protein binding experiments, DNA sequences from the mouse HGF promoter were actually used for interacting with nuclear protein extract derived from human cell lines.

Functional analysis of the repressor within the native context of the HGF gene indicated that the repressor acts as a potent suppressor (80% suppression) of HGF gene expression in epithelial cells (Fig. 6). The reduction in transcription was partial, however, as the wild-type 0.1HGF-CAT and 0.7HGF-CAT chimeric constructs still drove a significant level of CAT expression in epithelial RL95-2 cells (Fig. 6) (24, 25). These results suggest that the repressor is required but not sufficient to completely repress HGF gene expression. The relative activity of CAT expression in the 2.8HGF-CAT chimeric construct, even after the introduction of a mutation in the repressor element at position −8, was approximately 50% of those of the 0.7HGF-CAT and 0.1HGF-CAT mutant constructs (Fig. 6C). These findings support the conclusion that the HGF gene contains multiple cell-type-specific negative elements which are required for total silencing of transcription in HGF-nonexpressing cells. In fact, in addition to the repressor described in this study, we have identified another cell-type-specific negative regulatory element at −964 to −699, using a series of 5′-end deletions and internal deletions (24). The presence of multiple negative elements is not unique to the mouse HGF gene. It has been documented that multiple negative elements are also found in chicken (12), hamster (57), and human (44) vimentin, chicken lysozyme (1), rat glutathione *P*-transferase (15), human ε-globin (7), IL-4 (26), and collagen (47) genes. It is becoming clear that gene regulation is an integration of a number of effects resulting from interactions of factors from multiple sites on the promoter. It is suggested that some kind of stabilizing interaction occurs across multiple repressor elements to attain higher levels of transcriptional repression (12). A requirement for the presence of multiple repressors may prove to be a general rule in genes utilizing this type of negative transcriptional control. Further experiments will be needed to determine whether and/or how these multiple elements cooperate in repressing HGF gene transcription.

How the *cis*-acting repressor element of the mouse HGF gene actually exerts its repressing activity by binding to the *trans*-acting transcription factor remains unclear at this stage. It has been suggested that the repressor protein(s) might interfere with the assembly of a stable and functional transcriptional preinitiation complex, which is a prerequisite to accurate transcription initiation by mammalian RNA polymerase II (5, 31, 55). Repressor proteins are thought to act directly to block transcription initiation by interfering with the binding or association of one or more of the general transcription factors (TFIIA to TFIIF). In this case, the repressor protein(s) might be involved in the formation of a tertiary structure which is inhibitory to transcription directly through protein-protein interactions or might influence one or more of the steps involved in the preinitiation complex assembly process. However, it is unlikely that the repressor acts in a similar manner in the mouse HGF gene, since in the cases described above, the repressor acts indirectly and does not bind to a specific DNA sequence. Additionally, the type of repressor activity exhibited by the mouse HGF gene is also different from the negative regulation seen, for instance, by protein Id (inhibitor of DNA binding) (3) in genes activated by myoD, E12, and E47, or by the protein IκBβ (20), or in genes activated by Rel and NF-κB. In these cases, repressor proteins bind to positive transcription factors and form transcriptionally inactive heterodimers, inhibiting their capacity to activate cell-type-specific gene expression by preventing DNA binding.

The possible mechanism of the repressor action in the mouse HGF gene is more likely similar to the type of negative regulation in which positive DNA elements are partially overlapped by negative regulatory domains (13, 25) or a single DNA element binds two different factors with opposite and mutually exclusive effects (9, 18). The fact that within the 19-bp sequence the binding site for the repressor protein is overlapped by a CAP box, which is assumed to be the binding site for the RNA polymerase II complex (27), makes it likely that the binding of a repressor protein to its cognate DNA element will physically block and prevent the RNA polymerase II complex binding, simply because of a lack of space to house both the repressor protein and the RNA polymerase II complex. This type of repression by direct competition of two different proteins for binding in the overlapping region in a mutually exclusive manner has been found in many other genes (9, 13, 18, 25). For example, COUP transcription factor functionally represses estrogen receptor-mediated activation of mouse lactoferrin gene expression, and such repression has been demonstrated to be due to direct competition between the COUP transcription factor and the estrogen receptor for binding at identical nucleotide-protein contact sites in the overlapping region (25). The potential competition for binding makes the repressor protein a unique candidate for determination of cell-type-specific suppression of HGF expression. It is likely that the relative amount and concentration of the

repressor protein within a particular type of cell will dictate the HGF expression pattern. Indeed, a large amount of the repressor protein was seen in epithelial cells (Fig. 7), which may predominantly block the transcriptional initiation of the HGF gene.

It is noteworthy that the repressor sequence in the mouse HGF gene acts in a promoter-dependent manner, as the *cis*-acting repressor element was unable to function in a heterologous TATA-containing simian virus 40 promoter. This dependence of the repressor element's action within its own promoter context probably is unique to the HGF gene, since most characterized repressors in other genes act in a promoter-, distance-, and orientiation-independent manner (12, 14, 25). These results further imply that the mechanism of repression of HGF expression by the repressor protein is implicated in direct competition for binding between the repressor protein and RNA polymerase II complex. It is reasonable to anticipate that within the native context of the HGF promoter, repressor protein binding will prevent transcription initiation by blocking binding of the transcription machinery to the same region. This view is further supported by the fact that when the oligonucleotide containing the repressor sequence was placed in front of a heterologous promoter, it did not block transcription of the CAT reporter gene.

The repressor protein likely binds DNA as a monomer or homodimer, as suggested by in vitro binding studies (Fig. 9). If DNA binding is due to a heterodimer, the subunits would have to be of the same molecular weight, since only a single band is detected under the denaturing gel conditions of the Southwestern blot. The possibility remains that the factor is multimeric in vivo, but this could occur only if the 70-kDa monomer is capable of DNA binding or the heterodimer subunits are a single band in the Southwestern blot. A resolution of this question will have to await purification of the binding protein and/or production of an antibody which can be used to purify the binding complexes from cellular extracts; then a detailed examination of all associated components can be performed. Experiments to purify the repressor protein with a DNA affinity column and cloning of its cDNA are currently in progress.

The suppression of HGF expression in epithelial cells seems to be critical for normal tissue growth and development because of its diverse biological effects on a broad spectrum of cellular targets. It is believed that HGF exerts its mitogenic, motogenic, and morphogenic effects as well as cytostatic and cytotoxic activities on a variety of target cells in a paracrine fashion, although the presence of circulating HGF and induction of its transcription in distant organs support the possibility of an endocrine mechanism as well (21). The pattern of mesenchymally derived sources adjacent to epithelial targets renders HGF a prime candidate for mediating mesenchymal-epithelial cell interactions; such interactions have been proven to be absolutely required for embryogenesis, tissue repair, organ regeneration, and maintenance of a multicellular organism (43). It is likely that suppression of HGF expression in epithelial cells is a crucial step to maintain the paracrine mechanism of HGF action for normal development and function. Failure of the suppression of HGF expression in epithelial cells will result in an autocrine loop, which will expectedly contribute to abnormal growth and aberrant differentiation. In fact, in an experimental model, coexpression of HGF and its receptor c-MET was associated with tumorigenicity (42). Thus, the cell-type-specific transcriptional repressors likely play crucial roles in ensuring accurate and prompt repression of HGF expression in normal epithelial cells to avoid autocrine loop formation.

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