A Novel Transcriptional Regulatory Region within the Core Promoter of the Hepatocyte Growth Factor Gene Is Responsible for Its Inducibility by Cytokines via the C/EBP Family of Transcription Factors

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Hepatocyte growth factor (HGF) is an inducible cytokine that is essential for the normal growth and development of various tissues, such as the liver. To decipher the molecular mechanisms that regulate HGF gene induction at the transcriptional level, we carried out *in vitro* and *in vivo* studies on the mouse HGF gene promoter. We have identified a novel regulatory element, located between -6 and $+7$ bp (from the transcription start site) in the HGF basal promoter region, which binds to inducible transcription factors and dictates responsiveness to extracellular stimuli that activate this gene. The core binding sequence for the inducible *cis*-acting factors was determined to be TTTGCAA (-4 to $+3$ bp) within the HGF promoter. Competition and gel mobility supershift assays showed that these binding complexes are composed of C/EBP β (CCAAT/enhancer-binding protein β) and C/EBP δ . DNA binding analysis also revealed that the binding site for the C/EBP family of transcription factors in the HGF promoter region overlaps that of another binding protein (complex C1), which binds specifically to a novel sequence with a core binding site of ACCGGT located adjacent to the C/EBP site (-9 to -4 bp). C1 binds to this region of the promoter and represses the inducible upregulation by C/EBP through direct competition for their individual binding sites. Partial hepatectomy, which is known to activate HGF gene expression in the liver, increased C/EBP (especially C/EBP β) binding activity to this region of the HGF promoter. Thus, our present results provide a mechanistic explanation for the transcriptional induction of the HGF gene by extracellular signals (i.e., cytokines) that induce tissue growth and regeneration.

HGF is a secreted glycoprotein that is produced by stromal cells in a variety of tissues and affects the neighboring epithelial cells in a paracrine fashion. HGF has multiple distinct functions. It stimulates cell growth, cell motility, and morphogenesis on its target cells through binding and activating its specific tyrosine kinase cell surface receptor, known as c-Met (for reviews on HGF, see references 24, 39, 40 and 57). HGF plays an essential role in embryonic development (41, 47) as well as in adulthood by supporting the maintenance and renewal of cells in various organs, including liver, lung, and kidney. Experimental and clinical studies have also shown that abnormal expression of HGF results in the development and/or progression of various human neoplasms (12, 39, 53). Recent studies have shown that HGF acts as a limb mesenchyme-derived chemoattractant and as a muscle-derived survival factor for motor neurons, facts which indicate that HGF can function as a guidance and survival factor in the developing nervous system (11).

HGF gene expression is restricted to nonepithelial cells, such as fibroblasts of various tissues, Ito cells of the liver, macrophages, peripheral blood leukocytes, endothelial cells, and megakaryocytes. In these cells, HGF gene expression has been found to be regulated by hormones, cytokines, and growth factors. *In vitro* studies have shown that dexamethasone and transforming growth factor β 1 downregulate HGF expression in fibroblasts (27). In contrast, IL-1, TNF- α , EGF,

fibroblast growth factor, platelet-derived growth factor, prostaglandins, serum, and TPA increase HGF expression in stromal cells, such as fibroblasts (15, 25, 26, 42) and monocytes (8). *In vivo*, the level of HGF mRNA increases dramatically in the liver as well as in distal organs, like lung and spleen, after partial hepatectomy or hepatotoxin injury. Similarly, after injury to a lung or kidney (by toxins or by surgical operation such as unilateral nephrectomy or pneumonectomy), HGF gene expression is induced in these organs to facilitate tissue regeneration (18, 56). We reported previously that 2.7 kb of the mouse HGF promoter region (-2674 to $+29$ bp) fused to the CAT reporter gene confers responsiveness to IL-6 in NIH 3T3 cells (23). In other *in vivo* models, a single injection of estradiol was shown to result in a marked elevation of HGF mRNA levels in the ovaries of immature mice (22). This treatment also induces HGF promoter activity in the ovaries, as we demonstrated in transgenic mice harboring 2.7 kb of the HGF promoter region linked to the CAT reporter gene (16). We found that the 2.7- and 0.7-kb HGF-CAT promoter constructs (2.7 HGF-CAT and 0.7 HGF-CAT, respectively) are active only in stromal cells and not in epithelial cells of various tissues, including the livers of transgenic mice, akin to the endogenous HGF gene expression pattern. Moreover, in this transgenic mouse model, partial (two-thirds) hepatectomy also dramatically induces HGF promoter activity in the liver, lung, and spleen (4). Thus, the 2.7-kb region of the HGF promoter contains the necessary response elements to dictate cell-type-specific and inducible HGF gene expression.

Our laboratory, as well as others, has shown that there are several potential transcriptional regulatory elements in the 5'-

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flanking region of the human, mouse, and rat HGF gene promoters (3, 23, 30, 33, 34). Subsequent work in our laboratory found that there are two major negative elements in the HGF promoter: one at -16 to +11 bp (20) and one at -872 to -860 bp (16). This latter element turned out to be an imperfect estrogen-responsive element to which the nuclear orphan receptor COUP-TF binds avidly. Estrogen receptor can compete with COUP-TF and release the repressive function of COUP-TF on the HGF promoter through binding to the same imperfect estrogen-responsive element (16). We also further characterized an enhancer element in the mouse HGF promoter region. The enhancer element was mapped to an Sp1 binding region (-328 to -297 bp) which has a CTCCC motif to which Sp1 and Sp3 bind and activate the HGF promoter (17). Although these findings partially explain why some cells express HGF, we still do not know the mechanism through which extracellular signals such as IL-6, TNF- α , EGF, serum, or TPA or tissue injury upregulate HGF gene transcription.

In the present work, we carried out experiments to address this question. By using transient transfection of various HGF-CAT promoter constructs into NIH 3T3 fibroblasts and DNA binding studies, we have identified a novel regulatory element, located between -6 and +7 bp (from the transcription start site) in the mouse HGF basal promoter region, that confers inducibility to serum, TPA, TNF- α , IL-6, and EGF. DNA binding studies using nuclear protein extracts from NIH 3T3 fibroblasts that have been treated with the agents mentioned above revealed that the binding activity of two specific complexes, C2 and C3, for the HGF promoter was dramatically induced. The core binding sequence for these two complexes was narrowed down to the sequence TTTGCAA (-4 to +3 bp) within the HGF promoter. Mutation of the core binding site abolished the binding activities and produced a concomitant loss of the promoter response to stimulation by cytokines. Competition and gel mobility supershift assays showed that these binding complexes are mainly composed of C/EBP β and C/EBP δ . DNA binding analysis also revealed that the binding site for the C/EBP family of transcription factors in the HGF promoter region overlaps that of another binding protein (C1), which binds specifically to a novel sequence with a core binding site of ACCGGT located immediately upstream of the C/EBP site (-9 to -4 bp). C1 binds to this region of the promoter and represses the inducible upregulation by C2 and C3 (C/EBP) of the basal HGF promoter activity through competition for their individual binding sites. A 2-bp mutation in the C1 core binding sequence abolished C1 binding while enhancing C2 and C3 (C/EBP) binding. On the other hand, mutation in the C/EBP core binding site diminished promoter activation in response to stimuli but had no effect on C1 binding. Cotransfection studies using expression vectors encoding one of the three isoforms of the C/EBP transcription factor (α , β , or δ) resulted in a marked induction of HGF promoter activity. Partial hepatectomy, which is known to activate HGF gene expression in the liver, increased C/EBP β binding activity for this region of the HGF promoter. This study thus reveals that the mouse HGF gene is upregulated via induction of C/EBP β and - δ , which are activated by some cytokines, such as IL-1, IL-6, TNF- α , or EGF-transforming growth factor α (TGF- α) in response to signals that induce tissue growth and regeneration.

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this article are as follows: HGF, hepatocyte growth factor; C/EBP, CCAAT/enhancer-binding protein; CAT, chloramphenicol acetyltransferase; IL-1, interleukin-1; IL-6, interleukin-6; TNF- α , tumor necrosis factor alpha; EGF, epithelial growth factor; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; IL-6RE, IL-6 re-

sponsive element; NPC, nonparenchymal cells; C1, complex 1; C2, complex 2; C3, complex 3; FBS, fetal bovine serum; TPA, tetradecanoyl phorbol acetate; SRE, serum response element; SRF, serum response factor.

Plasmids. Construction of the 2.7 mouse HGF-CAT (-2674 to +29 bp), 1.0 mouse HGF-CAT (-964 to +29 bp), 0.3 mouse HGF-CAT (-274 to +29 bp), and 0.1 mouse HGF-CAT (-70 to +29 bp) was described in an earlier work from our laboratory (23). The 0.05 mouse HGF-CAT (-40 to +12 bp) was created by inserting the DNA fragment from -40 to +12 bp of the mouse HGF basal promoter into the *HindIII/XbaI* site of the pCAT-Basic vector (Promega, Madison, Wis.). To generate various mutants of the 0.05 mouse HGF-CAT construct, different mutated oligonucleotides corresponding to the sequence from -40 to +12 bp in the mouse HGF gene promoter were synthesized and then cloned into the *HindIII/XbaI* site of pCAT-Basic vector. All the constructs were confirmed by DNA sequencing. pMEX-C/EBP α , pMEX-C/EBP β , and MSV-C/EBP δ expression plasmids were kindly provided by S. L. McKnight (Tularik, San Francisco, Calif.).

Cell culture, DNA transfection, and CAT assay. Mouse NIH 3T3 fibroblasts were cultured in 6-well plates for 24 h and then transfected with various mouse HGF promoter-CAT chimeric plasmids by using the DNA calcium phosphate method according to the instructions of the CellPfect transfection kit (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) as described previously (16). The β -galactosidase reference plasmid pCH110 (Pharmacia) was used as an internal standard for monitoring the transfection efficiency and normalizing the data accordingly. The amounts of plasmid used in transfection per well were 5 μ g of one chimerical CAT construct and 1 μ g of pCH110. After incubation of the cells with DNA-calcium phosphate coprecipitation for 16 h in serum-containing medium, the cells were washed twice with serum-free medium and incubated overnight in serum-free medium. The cells were kept in serum-free medium and treated with different reagents as indicated for an additional 24 h before harvesting for the determination of CAT activity. The concentrations of the reagents were 20% FBS and 100, 50, 60, and 50 ng/ml, respectively, for TPA, TNF- α , IL-6, or EGF. CAT activity was determined as described previously (16). Transfections were performed at least four separate times with two independent preparations of purified plasmid DNA.

For cotransfection with pMEX-C/EBP α , pMEX-C/EBP β , and MSV-C/EBP δ in NIH 3T3 cells, the amounts of plasmid used in transfection per well were 5 μ g of one chimerical CAT construct, 1 μ g of pCH110, and different amounts of expression plasmids. The total amount of DNA per well was equilibrated by addition of empty expression plasmids.

Preparation of nuclear extracts. Mouse NIH 3T3 fibroblasts were originally obtained from the American Type Culture Collection (Rockville, Md.) and cultured under the conditions described previously (16). Cells were grown to approximately 80% confluence, washed with serum-free medium, and maintained in serum-free medium overnight. Then the cells were cultured in serum-free medium with different stimuli as indicated. Cells were washed twice with cold phosphate-buffered saline and scraped with a rubber policeman in the same buffer. Nuclear protein extracts were prepared as we described previously (16, 17).

For preparation of mouse liver nuclear protein extracts, livers were removed from mice at 0, 3, 12, and 24 h after partial hepatectomy and homogenized in buffer A containing protease inhibitors (20). The nuclei were collected, and the nuclear protein was extracted by the method as described above. Liver NPC were isolated by differential centrifugation of the collagenase-digested liver perfusate. Cells were washed twice with cold phosphate-buffered saline and then used to make nuclear extracts by the method described above.

Oligonucleotides and antibodies. The following oligonucleotides and antibodies were purchased from Santa Cruz Biotechnology, Inc., and were used in gel mobility shift and supershift assays: C/EBP, 5'-TGCAGATTGCGCAATCTGCA-3'; C/EBP mutant, 5'-TGCAGAGACTAGTCTCTGCA-3'; SRE, 5'-GGATGTCCATATTAGGACATCT-3'; SRE mutant, 5'-GGATGTCCATATTATTA CATCT-3'; Oct-1, 5'-TGTCGAATGCAATCACTAGAA-3'; Oct-1 mutant, 5'-TGTCGAATGCAAGCCACTAGAA-3'; AP-1, 5'-CGCTTGATGACTCAG CCGGAA-3'; AP-2, 5'-GATCGAAGTACCGCCCGCGGCCGT-3'; NF- κ B, 5'-AGTTGAGGGGACTTCCCAAGGC-3'; Stat3, 5'-GATCCTTCTGGAATT CCTAGATC-3'; YY1, 5'-CGTCCCGCGCCATCTTGGCGGCTGGT-3'; and antibodies against C/EBP α , C/EBP β , C/EBP δ , SRF, Elk-1, Oct-1, and Stat3. In addition, oligonucleotides corresponding to Sp1 (5'-GGGCTGAGCCTTGCTC CTCCTTCCCTACT-3') derived from the mouse HGF promoter region (17) were used in gel mobility shift assays.

Gel retardation assays. The double-stranded oligonucleotides used in gel mobility shift assays were labeled with [α - 32 P]dCTP by end labeling with Klenow fragment of DNA polymerase. The labeled oligonucleotide probes were then gel purified and used in gel mobility shift assays as described previously (16). One microgram of poly(dI-dC) (Pharmacia) was used as the nonspecific competitor in 10 μ l of reaction mixture. When antibodies for supershift were used (1 or 2 μ l), they were incubated with nuclear extracts for 20 min at room temperature before performance of the DNA binding shift assay. The binding reactions were carried out at room temperature for 20 min before loading on 5% nondenaturing polyacrylamide (19:1, acrylamide to bisacrylamide) gels. The amount of the nuclear protein extracts used in each reaction was 4 μ g, and that of the labeled probe was between 0.2 and 0.4 ng. For competition experiments, a 100-fold molar excess of unlabeled oligonucleotides, unless indicated otherwise, was included in

FIG. 1. Localization of an inducible enhancer element in the mouse HGF basal promoter region. Five different HGF-CAT constructs (2.7, 1.0, 0.3, 0.1, and 0.05 HGF-CAT) were transiently transfected into mouse NIH 3T3 fibroblasts. After transfection, cells were incubated in serum-free medium overnight and then treated with one of the following agents: control (untreated serum-free medium), serum (20% FBS), TPA (100 ng/ml), TNF- α (50 ng/ml), IL-6 (60 ng/ml), or EGF (50 ng/ml) for 24 h. Relative CAT activities were determined after normalization against β -galactosidase. The results presented are from four independent experiments performed in duplicate, and each error bar represents the standard deviation. pCAT-Basic (Promega Corporation), which contains no promoter region, was used as a negative control.

reaction mixtures. Gels were run in 0.5 $\times$ TBE buffer (0.045 M Tris-borate–0.001 M EDTA) at a constant voltage of 190 V, dried, and autoradiographed with intensifying screens.

Western blot (immunoblot) analysis. Twenty micrograms of nuclear extract from each sample were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12% polyacrylamide gel) and Western immunoblotting using antibodies against C/EBP β and C/EBP δ (purchased from Santa Cruz Biotechnology, Inc.). Horseradish peroxidase-linked secondary antibody was then added at a dilution of 1:2,000 for 1 h. The signals were then detected by chemiluminescence (ECL; Amersham, Arlington Heights, Ill.) according to the instructions of the manufacturer. Primary antibodies were used at a final dilution of 1:1,000 and incubated with the membrane for 2 h at room temperature.

RESULTS

Identification of a *cis*-acting enhancer element within the HGF core promoter that confers responsiveness to stimulation by serum, TPA, TNF- α , IL-6, and EGF. Previous work from our laboratory has partially characterized the mouse HGF gene and its promoter region through transient transfection studies *in vitro* and *in vivo* using transgenic mice (16, 17, 20, 21, 23). In those studies, we reported that IL-6 stimulates HGF promoter activity in NIH 3T3 fibroblasts that were stably transfected with a HGF-CAT promoter construct harboring 2.7 kb of the HGF promoter and its 5'-flanking region (23). Other studies have shown that HGF gene expression may be induced by serum, TPA, TNF- α , IL-1, and EGF in fibroblast cell lines in culture (14, 15, 32, 45). To further identify the *cis*-acting elements and *trans*-acting factors which mediate induction of the HGF gene in response to cytokines, a series of 5' deletion constructs of the mouse HGF gene fused to the CAT gene was used to analyze HGF promoter regulation. NIH 3T3 fibroblasts were transiently transfected with these HGF-CAT constructs and treated with serum, TPA, TNF- α , IL-6, or EGF. The results show that all of the HGF-CAT constructs, including 2.7 HGF-CAT, 1.0 HGF-CAT, 0.3 HGF-CAT, 0.1 HGF-CAT, and 0.05 HGF-CAT, respond to treatment with serum,

TPA, TNF- α , IL-6, or EGF in NIH 3T3 cells (Fig. 1). These experiments indicate that the basal HGF promoter (0.05 HGF-CAT, from –40 to +12 bp) and the element(s) in this region are responsible for induction by serum, TPA, TNF- α , IL-6, or EGF. To test whether any transcription factor(s) binds to the mouse HGF basal promoter region in response to extracellular stimuli, the 52-bp oligonucleotide from –40 to +12 bp was labeled and used in gel mobility shift assays in the presence of nuclear protein extracts from NIH 3T3 cells that had been treated with serum, TPA, TNF- α , IL-6, or EGF. Figure 2 is representative of results from gel mobility shift assays. As depicted, the oligonucleotide probe corresponding to the HGF basal promoter region formed three different specific complexes (named C1, C2, and C3) in the presence of nuclear extracts from NIH 3T3 cells that had been treated with serum, TNF- α , IL-6, or EGF for 5 h or with TPA for 24 h (Fig. 2, lanes 4, 6, 8, 10, and 12). Figure 2 also reveals that only one complex (C1) was detected in the nuclear extract from control cells (untreated NIH 3T3 cells) and that no obvious differences in C1 binding activity were noted in the nuclear extracts of treated cells as compared to the control nuclear extract (Fig. 2, lane 2). However, the binding activities of the lower two complexes, C2 and C3, were dramatically induced by these stimuli (Fig. 2; compare lanes 2 and 4). All of the three complexes were specific, because a 100-fold molar excess of cold oligonucleotide abolished these binding complexes (Fig. 2, lanes 3, 5, 7, 9, 11, and 13). According to these results, we concluded that stimulation of fibroblasts with cytokines results in the induction of binding activities of the two major complexes (C2 and C3) formed through interaction with the HGF basal promoter region (from –40 to +12 bp). Taken together, the results of Fig. 1 and 2 reveal that the inducibility of the mouse HGF promoter by serum, TPA, TNF- α , IL-6, and EGF may result from the formation and interaction of complexes C2 and C3 with the HGF basal promoter region.

FIG. 2. Specific interaction of the mouse HGF basal promoter region with inducible factors present in the nuclear protein extracts from cytokine-treated NIH 3T3 fibroblasts. The 52-bp DNA fragment (from -40 to +12 bp) of the HGF promoter which confers responsiveness to TPA and cytokines was labeled with 32 P and incubated with nuclear protein extracts from NIH 3T3 cells that had been treated with serum, TNF- α , IL-6, or EGF for 5 h or with TPA for 24 h. The binding reactions were performed at room temperature for 20 min in the presence of the nonspecific competitor poly(dI-dC). Self competitor (unlabeled probe at a 100-fold molar excess) was used to show specific binding. C₁, complex 1; C₂, complex 2; C₃, complex 3; F, free 32 P-labeled DNA probe.

Localization of the binding region of the inducible complexes within the HGF basal promoter. To define the binding sites of complexes C1, C2, and C3 within the HGF promoter region, gel mobility shift assays were performed by using the DNA fragment from -40 to +12 bp as a probe, two short oligonucleotides which cover different regions from -40 to +12 bp as competitors, and nuclear protein extracts from TPA-treated NIH 3T3 cells. The oligonucleotide corresponding to the region from bp -17 to +12 bp totally competed with the labeled probe for binding to all three complexes, C1, C2, and C3 (Fig. 3A, lane 5). However, the oligonucleotide corresponding to the region from bp -40 to -15 showed no competition (Fig. 3A, lane 4). These results revealed that the binding site for all three complexes is located within the region from -17 to +12 bp in the HGF basal promoter. To confirm this conclusion, the synthetic oligonucleotide from -17 to +12 bp was labeled and used as a probe to perform gel mobility shift assays. The results demonstrated that this region is sufficient for binding to the three complexes (Fig. 3B).

It is possible that the three different complexes bind to individual sites or to the same site within the region from -17 to +12 bp. To answer this question, six different oligonucleotides were synthesized and used as competitors in gel mobility shift assays. As depicted in Fig. 4A, oligonucleotides S1 and S2 (-17 to +10 bp and -17 to +7 bp, respectively) showed the same ability to compete for the formation of all three complexes (Fig. 4A, lanes 4 and 5). On the other hand, the S3 oligonucleotide, which corresponds to the region from -17 to +5 bp, did not show any competition (Fig. 4A, lane 6). The oligonucleotides S4 and S5, which encompass the nucleotides

FIG. 3. Identification of the binding region within the HGF basal promoter. (A) The 52-bp DNA fragment (from -40 to +12 bp) was labeled with 32 P and incubated with the nuclear protein extract from TPA-treated NIH 3T3 cells. Two sets of double-stranded oligonucleotides which cover different regions of the 52-bp DNA fragment were synthesized (the sequences of these oligonucleotides are shown beneath the gel) and added to the reaction mixtures to compete for binding, as indicated above the gel. Lane 1, migration position of the free probe (F). Lane 3, self competition (100-fold molar excess of unlabeled probe). As shown, only the fragment from -17 to +12 bp competed for binding. (B) The DNA fragment from -17 to +12 bp was labeled and used directly in electrophoretic mobility gel shift assays as a probe. Nuclear extracts from NIH 3T3 cells that were treated with various agents, as indicated, were used.

FIG. 4. Complex C1 and complexes C2 and C3 bind distinct yet overlapping regions within the mouse HGF basal promoter. (A) Six different shorter versions (S1 through S6) of the binding region (−17 to +12 bp, as shown beneath the gel) within the HGF basal promoter were used as competitors in gel mobility band shift assays. Lane 1, migration of the free labeled probe (−17 to +12 bp). Lane 3, self competition (S). (B) Nine different mutant oligonucleotides, all of the same length (−17 to +12 bp) but with various nucleotides mutated (the mutated

from −11 to +12 bp and from −6 to +12 bp, respectively, abolished the formation of complexes C2 and C3 only (Fig. 4A, lanes 7 and 8). Based on these data, the binding region of C1 was localized to the region from −17 to +7 bp and that of C2 and C3 was localized to the region from −6 to +7 bp. The results indicate that C2 and C3 may bind to the same site, while the binding site of C1 overlaps with that of C2 and C3. To define the precise essential binding nucleotides of these complexes, a series of mutant oligonucleotides (M1 through M9) was synthesized and used as competitors in gel mobility shift assays (Fig. 4B). M4 to M8 interfered with the binding ability of C1 (Fig. 4B, lanes 5 to 9); the binding motif for C1 was therefore defined as GACCGGTTTG (−10 to −1 bp). On the other hand, mutants M7 to M9, which cover the region TTTGCAA at −4 to +3 bp, abrogated formation of C2 and C3, indicating that the essential binding site for C2 and C3 is from −4 to +3 bp (Fig. 4B, lanes 8 to 10). These data clearly demonstrated that the C1 complex and the C2 and C3 complexes bind to two different regions and that these two regions overlap. Fig. 4C summarizes the results of these experiments.

Competition between complex C1 and complexes C2 and C3 for their binding sites and the time course of TPA-mediated induction of C2 and C3. Considering the fact that the binding site for C1 overlaps that of C2 and C3, it is plausible that C1 competes with C2 and C3 for their cognate binding site. C1 may compete and block the binding of C2 and C3, and therefore suppress HGF promoter activity. On the other hand, C2 and C3 may relieve the repression of C1 by competing for the binding of C1 and subsequently induce HGF promoter activity. To test this hypothesis, a mutant C1 binding site in which nucleotides CC at −8 and −7 bp were changed to AA (M5) was labeled and used as a probe to carry out electrophoretic band shift assays. As shown in Fig. 5A, this mutation dramatically diminished the binding of C1 and concomitantly enhanced C2 and C3 binding (in Fig. 5A, compare lanes 2 through 5, in which the probe is wild type, to lanes 8 through 11, in which the probe harbors a mutation in the C1 binding site). The interesting finding was that the formation of C2 and C3 was readily detectable in the nuclear protein extracts from the control untreated cells when the C1 binding site was mutated to prevent C1 binding (Fig. 5A; compare lanes 2 and 8). This result may imply that small amounts of C2 and C3 are present in the untreated nuclear cell extract but cannot bind to the HGF promoter region because C1 is occupying the binding site and blocking the binding of C2 and C3. After treatment with various stimuli which increase C2 and C3 levels (see below), high levels of C2 and C3 in NIH 3T3 cells were sufficient to compete with C1 for binding to the HGF promoter.

To characterize the time course of the induction of C2 and C3 binding, we prepared nuclear extracts from NIH 3T3 cells that had been incubated in the presence of TPA for various lengths of time (Fig. 5B). C2 and C3 binding increased gradually after 60 min, reaching maximum levels by 24 h (Fig. 5B, lane 7, and 5C). But C2 and C3 binding activities increased dramatically in the nuclear extracts from NIH 3T3 cells that were treated with 20% FBS and EGF, reaching the maximum level in 3 to 5 h (data not shown). The differences in the binding activities of the nuclear extracts prepared from TPA-

nucleotides are underlined in the sequences shown below the gel), were synthesized and used as competitors in band shift assays to define the core binding sites for C1, C2, and C3. The experiment was performed as described for panel A. In both panel A and panel B, the competitors were used at a 100-fold molar excess. (C) Summary of the data obtained from experiments for which results are shown in panels A and B. Solid rectangles, core binding motifs for C1, C2, and C3.

FIG. 5. (A) Competition of complex C1 with complexes C2 and C3 for their overlapping binding sites within the HGF basal promoter. Wild-type (WT) and mutant M5 (see Fig. 4B) oligonucleotides were labeled to the same specific activities and used as probes to perform electrophoretic mobility shift assays using nuclear protein extract from NIH 3T3 cells that had been treated without (control) or with various reagents. In lanes 1 through 6, the probe used was wild type, and in lanes 7 through 12, the probe was M5, to which the binding of C1 is diminished. In lanes 1 and 7, no protein extract was added to the reaction mixture, in order to show the migration of the free probes (F). (B and C) Time course of TPA-mediated induction of C2 and C3. (B) M5 was labeled and used as a probe in electrophoretic mobility shift assays using nuclear extracts prepared from NIH 3T3 cells that had been treated with TPA for different times, as indicated. (C) Graphic representation of data shown in panel B. The levels of the binding activities presented here are summations of those for C2 and C3. The complex level in control nuclear protein extract was arbitrarily set at 1.0. (D) Binding reactions were performed as for panel B except that the Sp1 oligonucleotide was labeled and 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 last reaction mixture as a competitor. C, specific binding complex.

treated cells at various time points were not due to the quality and integrity of the nuclear extracts, since the oligonucleotide corresponding to the Sp1 binding site formed specific Sp1 and Sp3 binding complexes (17) when it was incubated with these same nuclear extracts in gel mobility shift assays (Fig. 5D).

Identification of the inducible transcription factors as C/EBP family members. To identify the inducible factors that bound to the HGF promoter region (-17 to +12 bp), a computer search of known transcription factor binding sequences was performed (36). Five potential binding sites for transcrip-

tion factors C/EBP, SRE, Elk-1, Oct-1, and YY1 were found in the HGF basal promoter region from bp -7 to +7 (Fig. 6A). We used oligonucleotides corresponding to these consensus sequences as competitors and the labeled M5 as a probe (since M5 mutation diminished C1 binding ability without affecting C2 and C3 binding) to perform gel mobility band shift assays. Additional competitors (i.e., binding sites for various factors known to be induced by cytokines), including AP-1, AP-2, IL-6RE, NF- κ B, and Stat3, were also tested. It was surprising to find that wild-type C/EBP oligonucleotide at a 20-fold molar

FIG. 6. Identification of the inducible HGF promoter binding proteins as members of the C/EBP family of transcription factors. (A) Analysis of potential transcription binding sites within the HGF basal promoter region (from -17 to +12 bp, which binds C1, C2, and C3). Computer searches, including those available via the Internet, were performed to identify potential binding sites in the HGF basal promoter region (36). Significant homology to some transcription factor binding sites was found, as illustrated. The binding region of C2 and C3 is underlined. (B and C) Competition experiments were performed with oligonucleotides corresponding to the potential binding sites for several transcription factors that are present in the HGF basal promoter. AP-1, AP-2, IL-6RE, NF- κ B, and Stat3 sites were also used, since these factors are known to be induced by cytokines. Competitors were added at a 20-fold (B) or a 100-fold (C) molar excess. (D) C2 and C3 bound to the HGF basal promoter (M5 probe) were supershifted by anti-C/EBP β and/or anti-C/EBP δ antibodies (1 $\times$, 1 μ l; 2 $\times$, 2 μ l). Anti-SRF, anti-Elk-1, and anti-Oct-1, and anti-Stat3 antibodies were also used for comparison. The binding assays were performed as described in Materials and Methods. In these experiments, M5 oligonucleotide was labeled and used as a probe and the nuclear protein extracts were from NIH 3T3 cells treated with TPA for 24 h. S, supershifted complex or complexes.

excess totally competed with these complexes but a mutant variant did not (Fig. 6B, lanes 4 and 5, respectively). Interestingly, a 100-fold molar excess of wild-type SRE, Oct-1, and YY1 (but not their mutant forms) also competed for C2 and C3 (Fig. 6C, lanes 6 through 9 and 13). This is perhaps due to the fact that these sites also contain an imperfect C/EBP binding site. On the other hand, AP-1, AP-2, IL-6RE, NF- κ B, and Stat3 did not have any competitive ability even at a 100-fold molar excess (Fig. 6C, lanes 10 through 12, 14, and 15). The results from these experiments strongly suggested that the inducible complexes (C2 and C3) may be composed of members of the C/EBP family of transcription factors. To determine the identities of these inducible transcription factors that bound the HGF promoter region, supershift assays were performed with antibodies against these potential factors. As shown in Fig. 6D, antibody against C/EBP β reacted with C2 and C3 (Fig. 6D, lanes 4 and 5). Antibody against C/EBP δ also partially shifted the complexes C2 and C3 (Fig. 6D, lanes 6 and 7). The mixture of anti-C/EBP β and anti-C/EBP δ , however, totally supershifted complexes C2 and C3 (Fig. 6D, lane 10). Other antibodies, including anti-C/EBP α , anti-SRF, anti-

Elk-1, anti-Oct-1, and anti-Stat3, did not recognize C2 and C3 (Fig. 6D, lanes 2, 3, and 12 through 15). These results show that C2 and C3 contain transcription factors C/EBP β and C/EBP δ of the C/EBP family.

To determine whether the observed changes in the DNA binding activities of C/EBP isoforms (β and δ) after treatment with serum, TPA, TNF- α , IL-6, or EGF correlate with the actual protein content of the individual C/EBP members in the nuclear protein extracts from NIH 3T3 cells, we performed Western immunoblot analyses (Fig. 7). Consistent with the DNA binding activities, no detectable C/EBP δ and a very small amount of C/EBP β were seen in the nuclear extracts from uninduced NIH 3T3 cells (Fig. 7, lane 1). After treatment with serum, TNF- α , or IL-6, the amounts of C/EBP β and C/EBP δ in the nuclear extracts of NIH 3T3 cells increased dramatically (Fig. 7, lanes 2 through 6).

It is well known that HGF gene expression is induced in the remnant liver after partial (two-thirds) hepatectomy, reaching a maximum by 12 to 24 h (56). It is also documented that C/EBP β increases after partial hepatectomy in rat and mouse livers (46). It is possible that partial hepatectomy induces the

FIG. 7. Protein levels of C/EBP β and C/EBP δ in the nuclei of NIH 3T3 fibroblasts treated with different stimuli. Nuclear protein extracts (20 μ g/lane) from NIH 3T3 cells treated with serum, TNF- α , IL-6, or EGF for 5 h, or with TPA for 24 h, were analyzed by Western immunoblot using anti-C/EBP β (upper panel) and anti-C/EBP δ (lower panel) antibodies. Both isoforms of C/EBP are detected readily after induction, as indicated by arrows. LIP is a truncated form of C/EBP β .

binding of C/EBP β to the basal HGF promoter region, leading to the subsequent activation of HGF gene expression. To test this notion, oligonucleotide DNA corresponding to the HGF basal promoter from -17 to +12 bp (M5) was labeled and used to perform gel mobility shift assays with the nuclear protein extracts from mouse liver at different time points following two-thirds hepatectomy. M5 was used because its ability to bind to C1 was dramatically reduced, thus allowing easier detection of C2 and C3. As shown in Fig. 8A, four complexes were detected in the mouse liver nuclear extracts. These were C1, C2, C3, and a faint band above C2, which is C/EBP α (see below). The C2 and C3 binding activities increased by 24 h, and a concomitant decrease in C1 binding was noted (Fig. 8A, lanes 2 through 4). However, when the same nuclear protein extracts were incubated with the labeled Sp1 oligonucleotide, no detectable changes were detected, indicating that the observed changes in C1, C2, and C3 are not due to quality of the liver nuclear extracts (Fig. 8A, lanes 6 through 9). We did gel mobility supershift assays with the nuclear protein extracts from the mouse liver at 24 h after partial hepatectomy, using the HGF basal promoter region as a probe. Antibodies against C/EBP α and C/EBP δ showed a very weak reaction with C2 and C3, and only anti-C/EBP α shifted the complex above C2 (Fig. 8B, lane 2). Anti-C/EBP β , however, reacted strongly with these complexes (Fig. 8B, lane 3). Thus, the results indicated that the complexes that bound to the HGF promoter during liver regeneration consist mainly of C/EBP β and very small amounts of C/EBP α and - δ . Because the NPC of the liver (Ito cells, Kupffer cells, and endothelial cells) are the HGF-expressing cells (hepatocytes do not express HGF) in this organ, NPC were isolated from liver tissue and used to make nuclear protein extract. Band shift and supershift assays revealed that one major C/EBP complex was detected and only antibody against C/EBP β reacted with it (Fig. 8C, lane 3). These data imply that the complex in the liver NPC that bound to the HGF basal promoter contained C/EBP β .

Activation by individual C/EBP family members and suppression by C1 of the mouse basal HGF promoter. To demonstrate the functionality of the C/EBP sites and the role of the C/EBP family of transcription factors in the activation of the

HGF promoter, and also to test the suppressive function of C1 on the HGF promoter, we created two different mutant HGF-CAT constructs: 0.05 M1HGF-CAT and 0.05 M2HGF-CAT. The binding site of C1 was mutated in 0.05 M1HGF-CAT, whereas the binding site of the C/EBP complexes C2 and C3 was changed in 0.05 M2HGF-CAT (Fig. 9A). Wild-type 0.05 HGF-CAT, 0.05 M1HGF-CAT, and 0.05 M2HGF-CAT were used to perform transient transfection and CAT assays. In serum-free medium, no obvious differences between the basal promoter activity of the 0.05 HGF-CAT and the 0.05 M2HGF-CAT constructs were observed but the promoter activity of the 0.05 M1HGF-CAT was slightly greater than that of the wild-type 0.05 HGF-CAT (Fig. 9B). With the 0.05 M1HGF-CAT construct, in which the binding site of C1 was mutated, an increased response to treatments with serum, TPA, TNF- α , IL-6, or EGF was obtained (Fig. 9B). In the 0.05 M2HGF-CAT, in which the C/EBP site was mutated, no stimulation by these stimuli was noted (Fig. 9B). It should be noted that the M2 mutation did not abrogate the basal activity of the HGF promoter (Fig. 9). This may suggest that the mutation has not interfered with the activity of the transcriptional machinery. These results suggest that the C/EBP site is essential for the activation of HGF transcription and that C1 suppresses the induction of the HGF promoter, which is achieved by the overlap of the C1 binding site with that of C/EBP.

Gel mobility supershift assays showed that the HGF basal promoter region from -6 to +7 bp could form complexes with C/EBP α , C/EBP β , and C/EBP δ (Fig. 6 and 8). Although the major isoforms of the C/EBP family that were found to bind the HGF promoter appeared to be C/EBP β and C/EBP δ (based on supershift assays), it is possible that C/EBP α also is stimulatory for the HGF promoter in some HGF-expressing cells which also have high levels of C/EBP α , such as adipocytes and myeloblasts. To test whether the three different isoforms of C/EBP can stimulate HGF promoter activity, the expression vectors encoding these three isoforms were used to perform cotransfection experiments into NIH 3T3 cells with different HGF-CAT constructs. Not only C/EBP β and C/EBP δ , but also C/EBP α , strongly stimulated the HGF-CAT promoter constructs, and they did not stimulate the negative-control construct pCAT-Basic, which does not contain a promoter (Fig. 10A). Fig. 10B shows that the stimulation by C/EBP β on the HGF promoter was dose dependent and that an intact C/EBP site is essential for responsiveness to C/EBP β cotransfection. C/EBP α and C/EBP δ had the same behavior as C/EBP β (data not shown). These experiments revealed that all three of the C/EBP isoforms have a stimulatory function for the HGF gene promoter.

DISCUSSION

It is well documented in the literature through *in vivo* and *in vitro* studies that the HGF gene is induced in response to signals that cue cell growth and tissue regeneration. These investigations have revealed that HGF gene expression is tissue and cell type specific (i.e., expression in stromal but not epithelial cells) and that the induction of this gene is controlled at the transcriptional level. However, the molecular bases for these phenomena have remained poorly defined. In our previous reports, we described the cloning and functional characterization of the mouse HGF promoter and partially explained the issue of the cell-type-specific pattern of HGF gene transcription (16, 17, 20, 22, 23). In the present studies, we carried out experiments to address how the HGF gene is induced in response to extracellular signals like IL-1, IL-6, TNF- α , EGF, serum, TPA, and tissue regeneration. The data presented in

FIG. 8. Induction of binding activities for members of the C/EBP family to the HGF basal promoter in liver after two-thirds hepatectomy. (A) Electrophoretic mobility band shift assays were performed in the presence of mouse liver nuclear extracts prepared at different time points, including 0 (control), 3, 12, and 24 h after partial hepatectomy (PHX). In the left panel (lanes 1 through 4), M5 was labeled and used as a probe. In the right panel (lanes 5 through 9), Sp1 was labeled and used as a probe to control for the quality of the liver nuclear protein extracts. (B) Supershift assays were performed on the regenerating mouse liver nuclear protein extract (24 h PHX) by using anti-C/EBPβ, anti-C/EBPδ antibodies, as indicated. As shown, the majority of the complexes were supershifted by anti-C/EBPβ. (C) The experiment was performed as described for panel B, but the nuclear extract from liver NPC was used to perform the binding. S, supershift complex; C, complex. M5 oligonucleotide was labeled and used as a probe in these experiments.

that the promoter is not selective for any one C/EBP family member per se and that it may potentially be activated by any of these factors alone or in combination, depending on their availability. For example, the CYP2D5 P-45 gene in the liver was recently shown to be activated by C/EBPβ but not by C/EBPα, and the mechanism was found to be related to the ability of Sp1 or Sp1-like protein to cooperate with C/EBPβ but not with C/EBPα (19).

In NIH 3T3 fibroblasts, which are known to express C/EBPβ and C/EBPδ but not the α isoform, we showed that not only were the binding activities of these two C/EBPs (β and δ) induced in response to treatments with serum, TPA, TNF-α, IL-6, or EGF (Fig. 2 and 3), but their protein levels were also increased (Fig. 7). In the normal liver, in which HGF expression is low yet detectable, we detected the isoforms of the C/EBP family (α and β) binding to the HGF promoter. The binding of C/EBPβ to the HGF promoter was dramatically increased in the regenerating liver after partial hepatectomy (Fig. 8). This result is consistent with previous reports which have shown an increase in the level of C/EBPβ protein and its binding activity during the early phase of liver regeneration (46). As mentioned earlier, our laboratory, as well as those of others, has reported that HGF gene expression is induced in the remnant liver after partial hepatectomy, reaching a maximum by 24 h (24, 56). Moreover, we have recently analyzed HGF promoter activity in transgenic mice. We demonstrated that the 2.7 and 0.7 HGF-CAT promoter constructs are active only in stromal cells, not in epithelial cells, of various tissues,

Fig. 1 through 5 mapped the region of the HGF promoter that confers inducibility in response to these agents and identified a unique response element within the HGF basal promoter that harbors a C/EBP binding site which binds an inducible factor(s). The results presented in Fig. 6 through 8 demonstrated that these inducible factors are members of the C/EBP family of transcription factors (C/EBPα, C/EBPβ, and C/EBPδ). The experiments for which results are shown in Fig. 9 and 10 tested the functionality of the C/EBP binding site within the HGF basal promoter and clearly demonstrated that an intact C/EBP site is indeed required for the induction of HGF promoter activity by cytokines. The significance of the interactions of different C/EBP isoforms (α, β, and δ) with the HGF basal promoter region was evaluated by *in vivo* transactivation assays (Fig. 9). These results also showed that forced overexpression of any of the three C/EBP isoforms (α, β, or δ) effectively transactivated the HGF promoter (Fig. 10A). This indicates

A **B**

FIG. 9. Functional analysis of the binding sites for C1 and for C/EBP within the HGF basal promoter. (A) Diagrammatic representation of the wild-type HGF-CAT (0.05 HGF-CAT) and its mutated HGF-CAT reporter plasmids (0.05 M1HGF-CAT and 0.05 M2HGF-CAT) which harbor mutations in the C1 and C/EBP binding sites, respectively. The nucleotide sequence corresponding to the positions from -17 to +12 bp in the mouse HGF basal promoter region is shown, with mutated nucleotides indicated by lowercase letters. (B) NIH 3T3 cells were transiently transfected with the HGF-CAT constructs described for panel A and then treated with serum, TPA, TNF- α , IL-6, or EGF as described in Materials and Methods and the legend to Fig. 1. Data are presented as relative CAT activity (% conversion) and are from four independent experiments performed in duplicate. Error bars represent standard deviations.

including the liver, and that in this transgenic mouse model, partial (two-thirds) hepatectomy also induces HGF promoter activity in the liver (4). Taken together with our present findings, these results suggest that the C/EBP family may contribute to the transcriptional activation of the mouse HGF gene. In this regard, it should be noted that we have previously shown by DNase I hypersensitivity assays using nuclei from NIH 3T3 cells that there are five hypersensitive sites within the 2.7-kb region of the HGF promoter, one of which is within 0.1 kb from the transcriptional start site (17). We have also used nuclei isolated from regenerating mouse liver (12 h after two-thirds hepatectomy), performed DNase I hypersensitivity assays, and found three hypersensitive sites. Interestingly, one of these sites is also located within the basal promoter region, indicating that this site is bound and/or accessible to transcription factors (4).

The C/EBP proteins form a family of transcription factors with at least seven members (50), which include C/EBP α , C/EBP β (also known as NF-IL6), C/EBP δ , GADD153 (also known as CHOP), and a recently characterized isoform, C/EBP ϵ (9). These proteins consist of three structural components, which include a C-terminal leucine zipper, a basic DNA binding region, and an N-terminal transactivating region (49). Dimerization through the leucine zipper domain leads to formation of homo- and heterodimers, which then bind with their

two basic regions to often asymmetric DNA sequences in the promoter/enhancer regions of a variety of genes (7, 52) and control diverse cellular functions, such as growth and differentiation (6, 10, 38, 48, 50, 55). Not only do they interact with themselves, but members of this family of transcription factors also interact with a number of other transcription factors. For example, C/EBP β has been shown to interact or cooperate with c-Jun, cyclic AMP response element binding protein, glucocorticoid receptor, Myb, NF- κ B, and Sp1 (19, 50, 55). The C/EBP family of transcription factors is known to mediate signal transduction induced by cytokines and growth factors. IL-1 and IL-6 have been shown to increase the respective DNA binding activities of C/EBP β and - δ through a posttranslational mechanism (1, 2, 5, 35, 37). TNF- α also induces a transient increase in the nuclear concentrations of C/EBP β and C/EBP δ without altering the expression of their mRNAs or increasing total cellular concentrations of these C/EBP isoforms. These TNF- α -initiated changes in the subcellular distribution of C/EBP β and C/EBP δ are involved in posttranscriptional regulation of C/EBP activity (54). Phosphorylation of C/EBP β has been documented, and various protein kinases, including protein kinase A (28), Ca²⁺-calmodulin-dependent protein kinase II (51), and mitogen-activated protein kinase (31), have been indirectly or directly implicated in its phosphorylation. Thus,

A **B**

FIG. 10. Activation of HGF promoter activity by C/EBPα, C/EBPβ, and C/EBPδ in NIH 3T3 cells. (A) Five micrograms of each of the HGF-CAT reporter plasmids (2.7, 1.0, 0.3, 0.1, and 0.05 HGF-CAT) and negative control pCAT-Basic vectors (promoterless) were cotransfected along with 2 μg of C/EBPα, C/EBPβ, or C/EBPδ expression vectors into NIH 3T3 cells, and the activity of each promoter construct was determined and expressed as relative CAT activity as described in Materials and Methods. Controls received no C/EBP expression vectors. (B) Five micrograms of 0.05 HGF-CAT or 0.05 M2HGF-CAT reporter constructs, along with increasing amounts of the C/EBPβ expression vector, was cotransfected into NIH 3T3 fibroblasts. The activity of each promoter construct (relative CAT activity) was normalized by β-galactosidase, and the results are from four independent experiments performed in duplicate. Error bars represent standard deviations.

C/EBPβ and C/EBPδ are good candidates to mediate signal transduction induced by various extracellular cues.

The members of the C/EBP family recognize similar DNA motifs; however, GCAA has been reported by some investigators to be the core sequence (36). An optimal palindromic C/EBP binding site, GATTGC GCAATC, has been artificially designed (44). The C/EBP binding site in the HGF basal promoter (GGTTTGCAACAGGA) is similar to this optimal palindrome. The interesting finding from our work is that the C/EBP core binding site is within the +1 site of the HGF basal promoter. It is important that several independent groups, including ours, by using S1 nuclease mapping, RNase protection assays, and primer extension, have confirmed the designated +1 site and shown that this site is the major transcriptional start site for the rodent (mouse and rat) HGF gene. These studies have utilized RNA transcribed from the endogenous HGF gene as well as RNA transcribed from HGF-CAT promoter constructs (3, 30, 33, 34). Moreover, Plaschke-Schlutter et al., who have also independently cloned and partially characterized the mouse HGF promoter, showed that a

short region of the HGF promoter (containing only the region from -7 to +14 bp) is sufficient to drive CAT expression in NIH 3T3 fibroblasts, indicating that that region is the basal HGF promoter (34). We show that this region in the HGF basal promoter can be recognized by the C/EBP family. Upstream (18 bp) of the major +1 site in the mouse and rat HGF promoter, a minor transcriptional initiation site also exists (33, 34). However, Okajima et al., by using S1 nuclease mapping and primer extension analysis, reported that this major site is the preferential site in the rat HGF gene promoter utilized in the liver and spleen in response to acute liver injury (33). These authors showed that the levels of major-site-initiated HGF mRNA increased about 10-fold in the liver and about 20-fold in the spleen over levels in the normal unoperated controls. In contrast, the levels of minor-site-initiated HGF mRNA increased only twofold in the liver and about fourfold in the spleen. Because the C/EBP binding region of the rat HGF gene is the same as that of the mouse HGF gene and the promoter regions of both are essentially identical, and on the basis of our current findings, it is quite possible that HGF promoter activation is indeed achieved by the binding of the C/EBP family members to the HGF basal promoter. It is tempting to speculate that the C/EBP family members bound to the +1 site in the HGF promoter not only elicit enhancer activity but also function as initiators of transcription. The TATA-like element in the HGF promoter (at -30), which we have found to bind to the TATA binding protein TBP, may be necessary for the basal activity of the HGF gene promoter. TBP could also cooperate with C/EBP bound to the +1 site of

the HGF gene promoter and induce the expression of the HGF gene. The HGF gene promoter may be similar to the adeno-associated virus P5 promoter in which YY1 functions as an initiator, and its activity is enhanced in the presence of a TATA element (43).

Another interesting finding from our studies is the identification of the C1 complex, which seems to be composed of a novel transcription factor(s). Computer analysis of the C1 binding sequence, as well as gel mobility competition and supershift assays, did not identify any known transcription factor candidates. Although our data presented here did not show that C1 may directly suppress the HGF promoter, the results shown in Fig. 5A and 8A suggest that C1 can block C/EBP binding, thereby indirectly repressing the HGF promoter. In experiments in which the binding site for C1 was mutated, the binding of C/EBP to the HGF promoter was increased, indicating that competition between C1 and C/EBP may occur for binding to the HGF basal promoter. Moreover, this mutation also increased HGF promoter activity, thus supporting the notion that C1 represses the HGF promoter (see Fig. 9B).

In summary, we show here that the HGF gene promoter binds to and is transcriptionally activated by members of the C/EBP family of transcription factors through a novel site within the HGF basal promoter. Partial hepatectomy and tissue injury initiate a signaling network which includes increases in the cytokines, which in turn activate transcription factors that upregulate the expression of genes which control tissue regeneration (for reviews see references 13, 29, and 46). In the case of HGF gene induction by cytokines, one mechanism appears to involve the induction of C/EBP β and C/EBP δ , which in turn bind to the HGF gene promoter to activate its transcription. Future studies on the contribution of the C/EBP family of transcription factors to the induction of the HGF gene should involve experiments carried out on *c/ebp*-null mice (single and double knockout mice) so as to better understand the contribution of these factors to the activation of the HGF gene.

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