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CHARACTERIZATION OF THE 5'-FLANKING REGION OF THE HEPATOCYTE GROWTH FACTOR GENE

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The Hepatocyte growth factor is the most potent mitogen for hepatocytes in primary culture and is involved in liver regeneration. The expression of the gene appears to be tightly controlled by various humoral factors. To understand the molecular mechanism of the gene expression, we cloned and determined the nucleotide sequence of the 5'-flanking region of the gene. In this region, there are sequences homologous to responding elements of P53, Rb, IL-1, IL-6, glucocorticoids, TPA and TGF-β. We also identified three major transcriptional initiation sites by primer extension analysis of this region. Functional analyses of this region by constructing CAT reporter plasmids indicate that the sequence functions in a tissue specific manner and there is a negative regulatory region which suppresses the gene expression in rat transformed kidney cells. © 1993 Academic Press, Inc.

The Hepatocyte growth factor (HGF) was first identified in the sera of partially hepatectomized rats as the most potent mitogen for hepatocytes in a primary culture (1,2). Because of its strong hepatotrophic and renotrophic activities, HGF has been suggested to play a key role in the development and the regeneration of the liver and kidneys (3,4). Furthermore, HGF was recently found to be identical to the Scatter factor which enhances motility and invasiveness of cells of epithelial origin (5,6). Therefore, the pleiotropic activities of HGF are also implicated in the course of pathogenesis of various diseases such as hepatitis (7) and cirrhosis (8) and in tumorigenesis (9,10) and metastasis (11).

The expression of the HGF gene appears to be tightly controlled by a variety of factors in order to meet various physiological conditions. The level of HGF mRNA rapidly increases in the liver after hepatic injury and reaches a maximum at 24 h (12). This marked induction of mRNA appears to be triggered by a humoral factor called injurin which is released into the

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circulatory system immediately after organ injury (13). The transcription of the HGF gene is also known to be up-regulated by IL-1α (14), TPA (14), growth hormone (15) and IGF (15), and down-regulated by TGF-β and dexamethasone (16). However, little is known about the signal pathways and the molecular mechanisms of regulation of the HGF gene expression.

In order to understand the control mechanisms of the HGF gene expression in rat, we have analyzed the 5'-flanking region of the gene. Here, we report the primary structure of the 5'-upstream region of the gene and present evidence for the negative regulation of the gene expression.

MATERIALS AND METHODS

Cloning and sequencing of the 5'-flanking region of the HGF gene

A rat genomic library in EMBL3 vector was purchased from Clontech Co. The probe which was 214 bp [-139 - +75, from the cDNA sequence, (17)] was constructed by PCR using genomic DNA prepared from a female Sprague-Dawley rat. About a million plaques were screened as previously described (18). Phages of positive clones were prepared by the PEG/CsCl method (18) and the phage DNAs were prepared by the standard method (18). The cloned DNA was further analyzed by PCR and Southern blotting. A Pst I fragment, about 1360 bp long and located immediately upstream of the HGF gene, was subcloned into the M13 phage vector. The nucleotide sequence of this fragment was determined by constructing nested deletions (19) followed by sequencing by Sanger's method (20) using the USB sequencing kit.

RNA preparation and primer extension

A female Sprague-Dawley rat was injected intraperitoneally with 50% CCl_4 in mineral oil. It was sacrificed and the liver was taken for RNA preparation 16 h after the injection. Total RNA was isolated using the Guanidinium thiocyanate/CsCl method (21). Poly A^+ RNA was prepared from the total RNA using an oligo-dT cellulose column (22). For the primer extension analysis, an oligonucleotide of 23 base long (nucleotides -17 to +6) was end-labeled with γ-^{32}P ATP using T4 polynucleotide kinase. The radio-labeled probe (~10^6 cpm) was hybridized to 10 μg of poly A^+ RNA in the presence of 40 mM PIPES, 1 mM EDTA, 400 mM NaCl and 50% formamide. The resultant hybrids were reverse-transcribed using murine reverse transcriptase in the presence of 50 mM Tris-HCl (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM of each dNTP and RNase inhibitor (18). The primer-extended product was analyzed on an 8% polyacrylamide-urea gel along with a sequencing product of the HGF gene obtained using the same 23-mer as the primer.

Construction of CAT plasmids and deletion mutants

The PBLCAT3 plasmid which contains a multiple cloning site immediately upstream of the chloramphenicol acetyl transferase (CAT) gene was obtained from the American Type Culture Collection. The 1360 bp Pst I fragment containing the 5'-flanking region of the HGF gene was subcloned at the Pst I site of the pBLCAT3 in both ordered and reverse orientations. The resultant plasmids were designated pHGF(1400)CAT and pHGF(1400R)CAT, respectively.

Deletion mutants of pHGF(1400)CAT were constructed by the complete digestion of the plasmid with Hind III followed by a partial digestion with Bam HI, Sma I or Bgl II. The partially digested fragments with expected sizes were isolated by agarose gel electrophoresis, filled-in, re-ligated and transformed into *E.coli* DH10. Resultant mutant clones were designated pHGF(800)CAT, pHGF(600)CAT and pHGF(130)CAT, respectively.

CAT assay

The CAT assay was performed essentially as described by Gorman et al. (23). Briefly, various plasmid constructs were transfected into different cell lines using Lipofectin (BRL) following the methods recommended by the manufacturer. Cells were incubated for two days and cell extracts were assayed for CAT activity.

RESULTS

Primary structure of 5'-flanking region of the HGF gene

In order to clone the 5'-flanking region of the HGF gene, a rat genomic library constructed in EMBL3 was screened by a DNA probe that covered the 5' end of the c-DNA sequence. Screening of approximately a million plaques gave eight positive clones of which one was chosen for further analysis. Based on the physical mapping and the Southern blot analyses, we located a 1360 bp long Pst I fragment at the immediate upstream of the HGF gene. This Pst I fragment was subcloned into the M13 phage vector and its nucleotide sequence was determined. As shown in Fig. 1, this region contains binding sites for several protein factors that have been reported to be involved in the regulation of transcription of many genes. There are two regions homologous to the IL-6 responding element at positions -240 and -271 upstream of the initiation codon. At positions -817 and -1011, sequences with almost complete homology to the IL-1 responding element were found. A nuclear factor I (NF-1) binding site was found at position -520 and a binding site for the NF-IL-6 complex at -1149. A sequence with complete homology to the essential region of the glucocorticoid response element (GRE) was located at -1184. There are two TRE (TPA response element)-like sequences at -383 and at -1173. In addition to these sequences, there are at least three regions homologous to negative regulatory elements. A TGF-β-inhibitory element (TIE) was found to be located at -1116 and at -1341. Regions homologous to the retinoblastoma protein (Rb) control element (RCE) and two potential half-sites necessary for the binding of P53 were identified at positions -254 and -752, respectively.

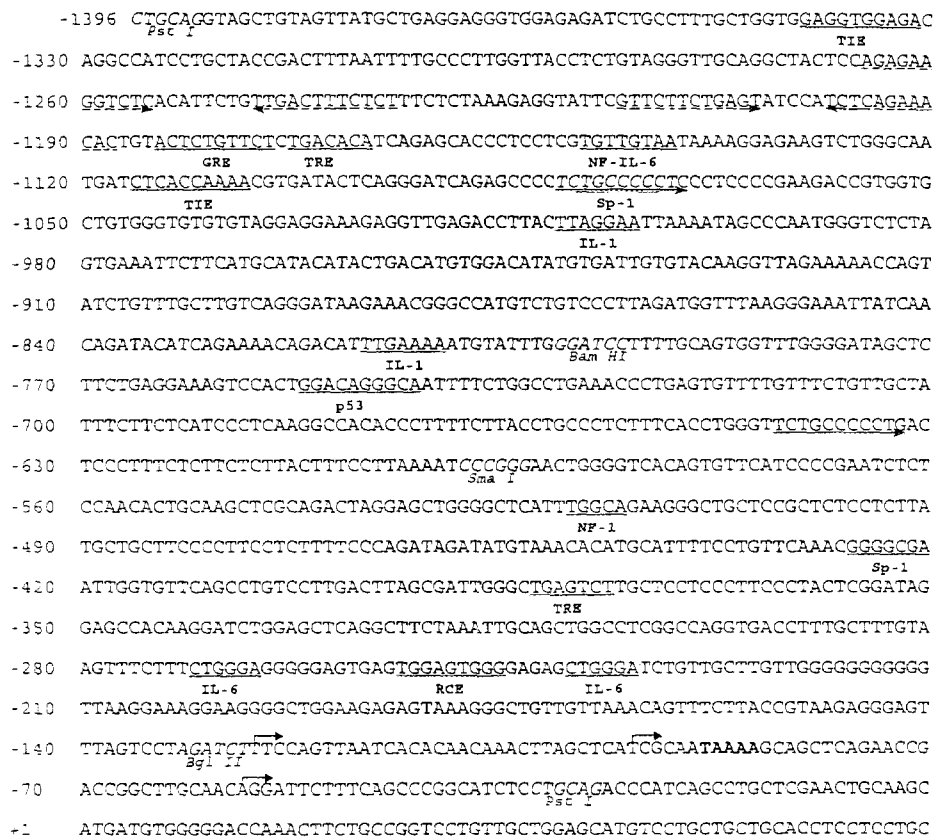

Fig.1. Primary structure of the 5'-flanking region of the HGF gene.
1360bp Pst fragment located immediately upstream of the HGF gene was subcloned to the M13mp19 vector. After constructing nested deletions, nucleotide sequence was determined by Sanger's method. Nucleotide sequences homologous to TIE, GRE, TRE, RCE, IL-1 RE and IL-6RE and the binding sites for P53, NF-1 and NF-IL-6 are underlined. Transcriptional initiation sites identified by primer extension analysis (see Fig.2) are indicated by arrows. Restriction sites are printed in italics. The TATA-like sequence is indicated in bold letters. A single underline with an arrow marks direct repeats and a broken underline with an arrow marks possible hair-pin-loop structure.

In order to determine the transcriptional initiation site of the gene, a primer extension analysis was conducted using poly A^+ RNA prepared from livers of CCl_4 treated or non-treated rats. As shown in Fig. 2, we identified three major bands corresponding to positions -57, -95 and -126. Since none of these bands were detected when poly A^+ RNA from normal rat liver was used, all three initiation sites are considered to be activated due to liver injury caused by CCl_4.

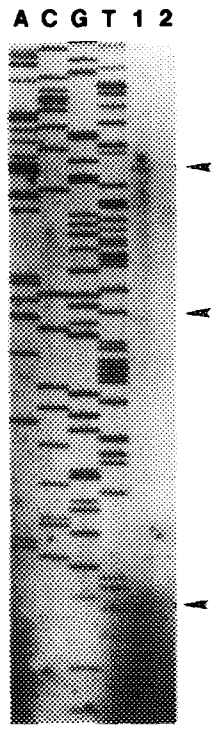

Fig.2. Primer extension analysis of the 5'-flanking region. Primer extension analysis was performed as described in the text using poly A$^+$ RNA prepared from CCl_4-treated (lane 1) or untreated (lane 2) rat liver. Lanes A,C,G and T are the sequencing reactions for the same region using the same primer used for the primer extension experiment. Major bands are indicated by arrowheads.

Tissue specificity and negative regulation of the HGF gene expression

The expression of HGF is normally tissue specific and restricted to fibroblastic cells (12). To test functional activities of the 5'-flanking sequences in different tissues, the 1360 bp Pst I fragment was placed immediately upstream to the CAT gene in the pBLCAT3 plasmid. The resultant plasmid pHGF(1400)CAT was transfected to five different tissue cells including KNRK (rat kidney cells transformed by MSV), Rat2 (rat embryonic fibroblast cells), 49F (rat normal kidney, fibroblast cells), MM55(mouse liver epithelial cells) and IMR90 (human lung fibroblastic cells). After 48 h of incubation, cell extracts were prepared and assayed for CAT activity. As shown in Fig. 3, only KNRK cells showed significant CAT activity, while expression in Rat2 cells was low but detectable. None of the other cell lines showed measurable CAT activity. These results suggest that the 5'-flanking sequence functions in a tissue-specific manner.

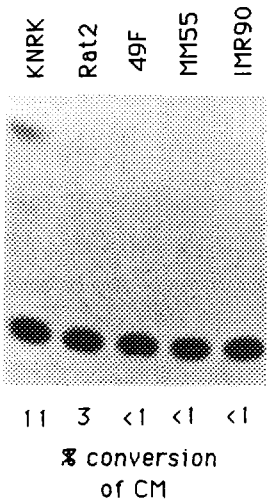

Fig.3. Tissue specificity of function of 5'-flanking sequence. pHGF(1400)CAT and β-galactosidase expression plasmid pRSVZ were transfected to tissue cells including KNRK, Rat2, 49F, MM55 and IMR90 using Lipofectin(BRL) according to the method recommended by the manufacturer. Forty-eight hours after the transfection, cells were collected and cell extracts were prepared as described in the text and CAT and β-gal activities of the extracts were measured by the standard methods. After autoradiography of the TLC plate, each spot was cut and counted for radioactivities to calculate percentage conversion of ^{14}C-CM to its acetylated form.

In order to analyze regulatory domains in the 5'-flanking region, we constructed a series of deletion mutants in the region by restriction enzyme digestions. The KNRK cells were transfected with mutants pHGF(800)CAT, pHGF(600)CAT and pHGF(130)CAT and then the expression of CAT was measured. As shown in Fig. 4, transfection with pHGF(130)CAT showed a significantly higher level

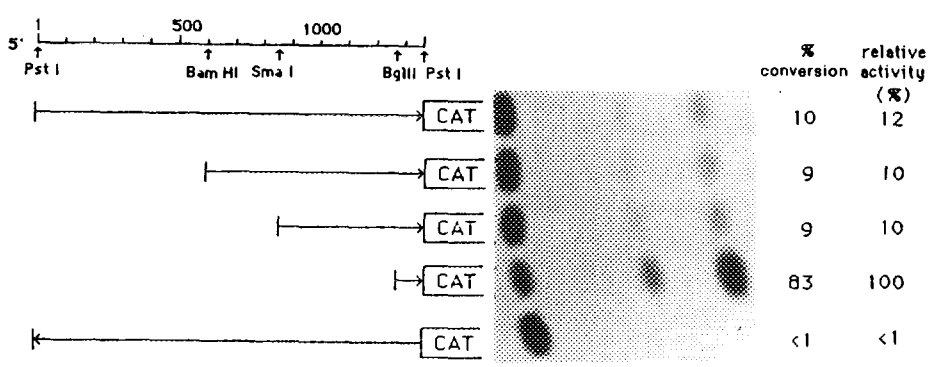

Fig. 4. Deletion analysis of the 5'-flanking sequence. Deletion plasmids pHGF(800)CAT, pHGF(600)CAT and pHGF(130)CAT as well as pHGF(1400)CAT and pHGF(1400R)CAT were transfected to KRFK cells as described in the Fig. 3 legend. pRSVZ was also co-transfected to normalize the CAT activities. Cell extracts were prepared 48 hr after the transfection and assayed for CAT activity.

of CAT expression than that with the other plasmids. This result indicates that the function of the 5-'flanking sequence is negatively regulated and that the responsive element resides within the -132 to -599 region.

DISCUSSION

As a first step in understanding the regulatory mechanism of HGF gene expression, we have cloned and determined the nucleotide sequence of the 5'-flanking region of the gene. In this region, we identified three transcriptional initiation sites that appeared to be activated by liver injury. Upstream of these initiation sites, there are putative responding elements for various factors that are believed to be involved in transcriptional regulation. Recent *in vivo* studies suggest that the expression of the HGF gene is up-regulated by IL-1, TPA, Growth hormone and IGF-1 and down-regulated by dexamethasone and TGF-β (14,15,16). Our sequencing analysis of the 5'-flanking region has revealed that there are two regions that are homologous to the IL-1 responding element. There are also two regions homologous to the TRE sequence which is assumed to respond to TPA (24). Therefore, these regions may, indeed, be involved in the up-regulation of the gene expression as observed in the *in vivo* studies. This 5'-flanking region also contains sequences homologous to the core sequence of the glucocorticoid response element (GRE) and TGF-β inhibitory element (TIE). TIE is the element to which a Fos-containing nuclear protein binds and enhances the transcriptional inhibitory activity of TGF-β (25). It is plausible that the GRE core sequence and the TIE found in the 5'-flanking region may be contributing to the down-regulation of the HGF gene expression in respond to glucocorticoid and TGF-β .

Functional analyses of the 5'-flanking region using CAT assay suggest that the expression of HGF is regulated in a tissue-specific manner. The expression of the gene was also found to be negatively regulated and the responding element resides in the -132 and -599 regions. The sequence data indicate that there is a sequence homologous to the Rb control element (RCE) in this region. Since Rb can act to suppress the expression of many genes (26), it is tempting to speculate that the negative regulation of the HGF gene by Rb is mediated by this site. It should be noted that in some tumor cells the HGF gene expression has been found to be deregulated(27). These deregulations may be due to mutation in this region or due to lack of suppression. Further mutational analysis of this region is currently underway.

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