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ISOLATION AND EXPRESSION OF cDNA FOR DIFFERENT FORMS OF HEPATOCYTE GROWTH FACTOR FROM HUMAN LEUKOCYTE

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SUMMARY: Human leukocyte cDNA library was screened to isolate cDNA clones coding for hepatocyte growth factor using cDNA from human liver as a probe. Nucleotide and deduced amino acid sequences were analyzed for two of four clones obtained. One of them contained an open reading frame coding for a polypeptide chain of 728 amino acid residues like that of cDNA clone derived from human liver. In another clone a spontaneous deletion of 15 base pairs was found within the coding sequence. When expressed transiently using COS-1 cells both clones produced protein with similar biological activity against rat hepatocyte *in vitro*.

A potent hepatotrophic factor acting as a trigger for liver regeneration events after partial hepatectomy or injury, designated as hepatocyte growth factor (HGF), was first purified and characterized from rat serum (1) and was subsequently detected (2) and purified (3,4) from rat platelets. Purification of HGF from serum of human (5,6) and rabbit (7) has been also reported. HGF has been shown to consist of two polypeptide chains with molecular weight of 65-70 kD and 30-35 kD respectively, and exhibit growth stimulatory effect on mature parenchymal hepatocyte in primary culture.

Molecular cloning of cDNA for human and rat HGF has shown that both heavy and light chains (named α- and β-chains respectively) are encoded in a single open reading frame of highly coserved 728 amino acid residues with a characteristic feature containing four kringle modules and serine protease-like domain lacking active centers (8-10).

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Abbreviation: HGF, hepatocyte growth factor.

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In this report we describe the isolation of a deleted form of cDNA clone for HGF from human leukocyte cDNA library. Alternative splicing for the human HGF gene is discussed.

MATERIALS AND METHODS

Northern blot analysis: 2 μg of human poly(A) RNAs purchased from Clontech were electrophoresed in formaldehyde agarose gel, blotted onto nylon membrane and hybridized with ^{32}P-labelled *Bam*HI-*Kpn*I 2.2 kb fragment of human liver HGF cDNA as described previously (8).

cDNA cloning and DNA sequencing: Double-stranded cDNA was synthesized from 3 μg of human leukocyte poly(A) RNA using cDNA synthesis system plus (Amersham) primed from phosphorylated oligonucleotide 5'-ACATTCTCTGAAATCTTCAT-3' designed after the nucleotide sequence of anti-sense strand of liver HGF cDNA sequence 50-70 downstream from the termination codon. Result cDNA was ligated to *Eco*RI adaptor (Pharmacia), introduced into dephosphorylated λgt10 and packed with Giga Pack Gold (Stratagene). Plaques on *Escherichia coli* NM514 were screened with 0.3 kb fragment of liver cDNA containing 5'-flanking region and coding region for the first 40 amino acids labelled with [α-^{32}P]dCTP using multiprime DNA labelling system (Amersham). DNA fragments were subcloned into M13mp18 and M13mp19 and sequenced by the dideoxy chain termination method (11) using Sequenase (US Biochemicals).

Transient expression and in vitro assay of HGF activity: 2.2 kb *Bam*HI-*Kpn*I fragment of cDNAs from human liver clone and leukocyte clone HLC2 and HLC3 were joined to synthetic *Kpn*I-*Sal*I adaptor, 5'-CACAGTCATAGCTGTTAACCCGGG-3', 5'-TCGACCCGGGTTAACAGCTATGACTGTGGTAC-3', which codes carboxy-terminal amino acids and termination codon, treated with T4 DNA polymerase and inserted into expression vector CDM8 (12) which has been digested with *Bst*XI and filled-in with T4 DNA polymerase. The constructed expression plasmid vectors CDM (hHGF) for liver cDNA, CDM (dLeHGF) for HLC2 and CDM (LeHGF) for HLC3 were transfected with COS-1 cells by DEAE-dextran method (8) modified as follows: amount of plasmid DNA was decreased to 1 μg, concentration of DEAE-dextran was increased to 1000 μg/ml and concentration of chloroquine in DMEM containing 10% FCS was changed to 1 mM. After incubated for 7 days, HGF activity in culture media was measured by incorporation of ^{125}I-labelled deoxyuridine into rat hepatocyte in primary culture (8).

RESULTS

cDNA cloning and nucleotide sequence determination

To analyze the distribution of HGF transcripts and determine the source for cDNA cloning, Northern blot hybridization was carried out using commercially available human poly(A) RNAs. As shown in Fig. 1, mRNA isolated from leukocyte and placenta as well as liver RNA which we used in previous work (8) represented distinct bands of about 6 kilobases. cDNA library was constructed using human leukocyte mRNA, which was positive in Northern blotting experiment, and screened for HGF cDNA with ^{32}P-labelled

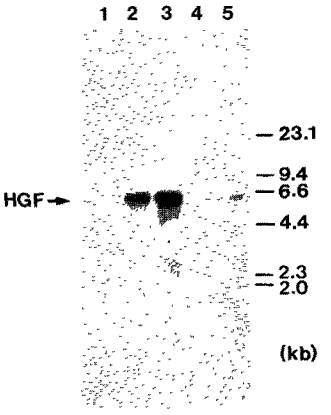

Fig. 1. Northern blot analysis of human RNA

2 μg per lane of human poly(A) RNAs were tested for the presence of HGF transcripts. Lanes 1, brain; 2, placenta; 3, leukocyte; 4, lung; 5, liver. The numbers on the right indicate the length of λ DNA digested with *Hind*III used as molecular weight marker.

human liver HGF cDNA as described in MATERIALS AND METHODS. Four positive clones were obtained and the inserts had shown similar restriction patterns (data not shown). Nucleotide sequence of two of them, HLC2 and HLC3, were determined (Fig. 2). In HLC3 cDNA an open reading frame of 728 amino acids were observed like the liver-derived clone, but among the cDNA isolates from different sources, mismatch of 39 nucleotides were found within the coding region resulting in replacement of 14 amino acid residues as shown in Fig. 2. The sequence of leukocyte cDNA matched completely with that of HGF cDNA isolated from human placenta (9). Nucleotide sequence of the other clone HLC2 agreed completely with that of HLC3 except for a deletion of 15 base pairs (nucleotide 483-497) as shown in Fig. 2.

Transient expression of leukocyte HGF cDNA

To demonstrate that the newly isolated clones may produce functional protein, cDNA inserts of HLC2, HLC3 and the liver cDNA was introduced into expression vector CDM8 then transfered into COS-1 cells by DEAE-dextran method. Medium of 7 days incubation was recovered and used for HGF activity assay against rat hepatocyte in primary culture. The result illustrated in Fig. 3 shows that the leukocyte-derived cDNA clones HLC2 and HLC3 as well as cDNA from liver exhibited significant incorporation of ^{125}I-labelled deoxyuridine. This indicates that the difference in amino acid sequence or deletion of 5 amino acid residues does not affect the activity of gene product *in vitro*.

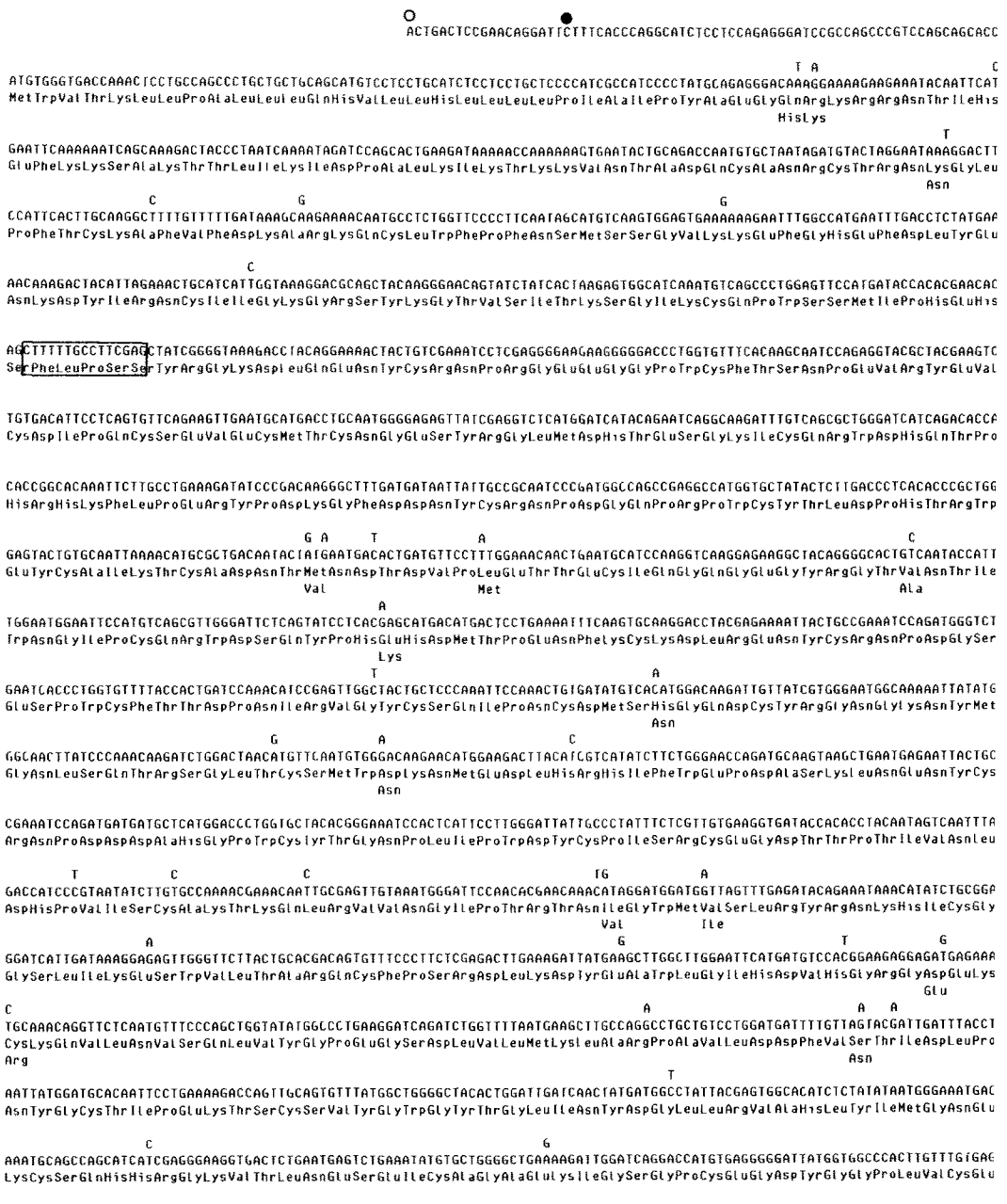

Fig. 2. Nucleotide and deduced amino acid sequence of human leukocyte HGF cDNA

The numbers beside the upper and lower lines indicate the nucleotide and amino acid residues from the first letter of the initiation codon ATG and methionine respectively. Symbols in the figure indicate 5'end of HLC2 (o), 5'end of HLC3(●), 3'end of HLC2(Δ) and 3'end of HLC3(▲). Deletion found in HLC2 is boxed. Sequence of the extension primer is underlined. Residues on the top and bottom indicate the difference from the clone isolated from human liver.

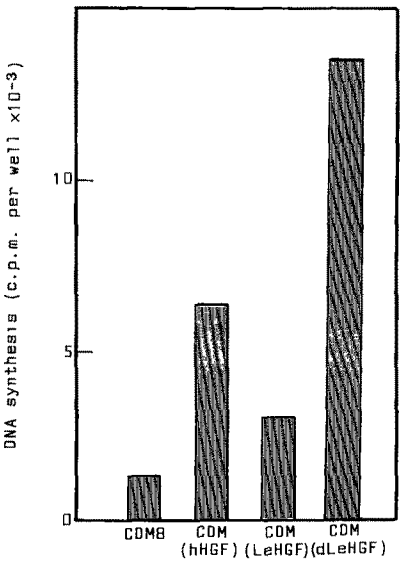

Fig. 3. Growth promoting activity of the expressed HGF cDNA
Aliquots of culture medium of COS-1 cells transfected with CDM8, CDM(hHGF) (liver clone), CDM(LeHGF) (leukocyte clone HLC3) and CDM(dLeHGF) (leukocyte clone HLC2) were assayed for HGF activity. When used mouse EGF (20 ng/ml) and saline as controls incorporation of ^{125}I-labelled deoxyuridine were 29,000 and 1,700 c.p.m. respectively.

DISCUSSION

cDNAs coding for HGF have been isolated from human leukocyte cDNA library. Northern analysis of human poly(A) RNAs has shown that the HGF gene is expressed in placenta and leukocyte as well as liver, while no positive result was obtained for brain and lung. In rat the HGF transcripts were observed in liver, kidney, lung and brain (10). The difference in the distribution of the transcripts may be due to species specificity. Actually HGF activity was detected in platelet of rat (2-4) although no similar activity was found in human platelet (6).

Determined nucleotide sequences of the cDNAs from human leukocyte library derived from one person (Clontech, personal communication) matched with that of cDNA obtained from human placenta (9), but differed from that of the liver-derived clone (8). We have characterized genomic DNA for the human HGF gene (submitted for publification) and found that the genomic sequence matches completely with those of clones derived from leukocyte and placenta. These results suggest that the representative nucleotide sequence for human HGF is that of the one reported in this paper. The difference observed with the liver-derived clone may be due to existence of polymorphism or a kind of RNA editing (13,14).

In one of the cDNA clones isolated in-frame deletion of 15 base pairs were observed. Transient expression experiment using COS-1 cells revealed that the structural difference causes no effect on biological activity in vitro. The amount of the gene product in the culture media was measured using anti-human HGF antibody and the specific activity of the smaller molecule was estimated to be almost equal to that of the larger one (data not shown). This deleted sequence locates within the region corresponding the first kringle module of the HGF molecule. Kringles are considered to act as a protein binding modules (15). In tissue-type plasminogen activator, one of the kringle-containing proteins, only the second of the two kringles is considered to be necessary for the activity (16). For HGF the first kringle may have little contribution for the growth promotion activity. Our sequence data of the human HGF gene (submitted for publification) has shown that the deleted sequence locates at the 5'-end of the fifth exon. The lost sequence CTTTTTGCCTTCGAG is similar to the conserved 3' acceptor sequence at the splice junction $(T/C)_n$ N(T/C)AG (17). So the spontaneous deletion observed in HLC2 is thought to have generated during splicing event. Similar alternative use of splice junction sequence was reported for the human granulocyte colony-stimulating factor gene (18). To prove the hypothesis we are now analyzing the human HGF transcripts for the presence of two types of spliced products.

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