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PEA3 and AP-1 Are Required for Constitutive IL-8 Gene Expression in Hepatoma Cells

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Interleukin-8 (IL-8) mRNA was constitutively expressed in human hepatoma cell line, HepG2 and in human hepatocellular carcinoma (HCC), which often form hypervascular tumors. The sequence 5′-AGGAAG-3′ at −137 to −132 bp of IL-8 promoter was shown to be polyomavirus enhancer A binding protein-3 (PEA3) binding site, which can cooperate with activator protein-1 (AP-1). Both PEA3 and AP-1 are essential for constitutive IL-8 expression in HepG2 cells, determined by promoter assays. Moreover, PEA3 and IL-8 proteins coexisted in HCC tissues, but not in uninvolved liver tissues. It is possible PEA3 may have important roles in tumor progression and in angiogenesis in HCC.

Key Words: **interleukin-8 (IL-8); polyomavirus enhancer A binding protein-3 (PEA3); activator protein-1 (AP-1); hepatocellular carcinoma (HCC); HepG2.**

Hepatocellular carcinoma (HCC) has been shown hypervascular tumor (1, 2). A potent angiogenic factor, interleukin-8 (IL-8) (3) is produced in some carcinoma cell lines, including the hepatoma cell line (4). The pathophysiological relevance of constitutive IL-8 production in cancer cell lines remains to be investigated.

IL-8 is transcribed and eventually IL-8 protein is rapidly produced in response to a wide variety of stimuli including lipopolysaccharide (5), interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α) (6). Moreover, evidence is accumulating that the cooperative activation of two distinct transcription factors, nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1), is essential for inducible IL-8 gene expression. Several groups and we have reported that the AP-1 protein is important in tumorigenic processes (7, 8). Thus, it is tempting to speculate that constitutive activation of AP-1 and may induce malignant transformation as well as IL-8 gene expression.

We have searched for potential additional transcription factor binding sites in the IL-8 gene and have identified the presence of a polyomavirus enhancer A binding protein-3 (PEA3) (9, 10) binding site at −137 bp to −132 bp (5′-AGGAAG-3′), localized only 6 bp upstream from the AP-1 binding site. PEA3 protein contains a conserved ETS-domain, essential for the binding to its *cis*-acting element. More than 30 transcription factors contained ETS-domain family (11, 12) are presumed to be important for tumorigenesis and developmental processes of several organs. These observations prompted us to investigate the role of PEA3 in the constitutive IL-8 gene expression in hepatoma cells.

MATERIALS AND METHODS

Cell cultures. HepG2 cells were grown and maintained in DMEM containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) (Gibco BRL, Co., New York, NY).

Transfection and CAT assay. We have previously described the methods of constructing CAT reporter plasmids for IL-8 promoter (6). For the transfections, the CAT expression constructs (5 μg) were transfected into HepG2 cells utilizing Lipofectamine Reagent (Gibco BRL), according to the manufacturer's instructions. To correct for variation in transfection efficiency, 1 μg of the pCMV-β-galactosidase DNA was cotransfected with each construct. CAT activity was determined by the method described by Gorman *et al.* (13).

Reverse transcriptase PCR. Frozen HCC tissues and uninvolved liver tissues surrounding HCC were both obtained from patients with HCC who underwent surgical hepatectomy. Total RNA (5 μg) was then extracted and cDNA was synthesized using a First-Strand cDNA Synthesis kit (Pharmacia Biotech, Tokyo, Japan), according to the manufacturer's instructions. The amplification steps involved 30 cycles of PCR (95°C, 1 min; 65°C, 1 min; 72°C, 1 min). Oligonucleotide primer pairs for β-actin and IL-8 were as follows: β-actin sense: 5′-AAGAGAGGCATCCTCACCCT-3′, β-actin antisense: 5′-TACATGGCTGGGGTGTTGAA-3′, IL-8 sense: 5′-ATGACTTCCAAGCTGGCCG-3′, IL-8 antisense: 5′-CTCAGCCCTCTTCAAAAACTT-3′

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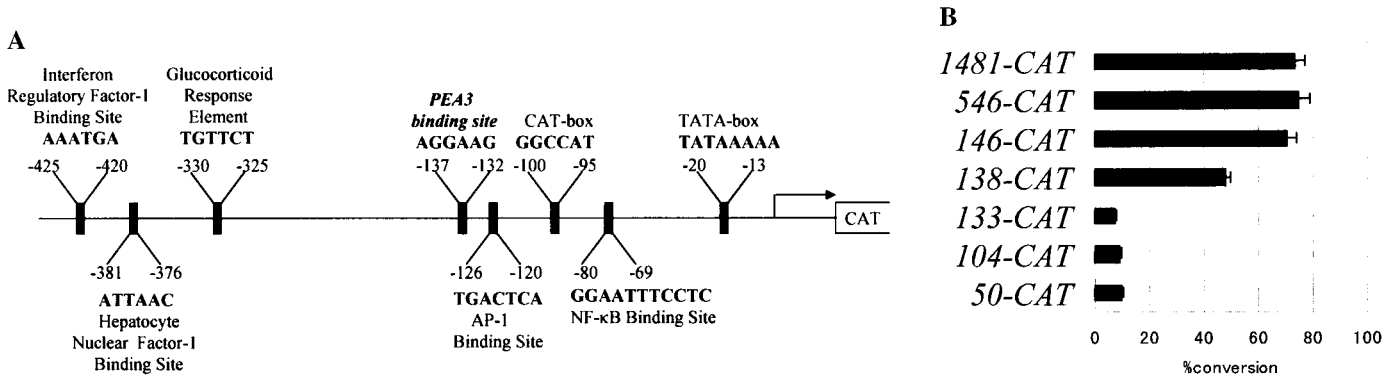

FIG. 1. (A) Potential binding sites for nuclear factors on the IL-8 promoter. In the present study, we identified a potential PEA3 binding sequence (AGGAAG) from −137 to −132 bp. (B) Relative CAT activity of 8 deletion constructs were measured in HepG2 cells as described under Materials and Methods.

(14). Expected product sizes for β-actin and IL-8 were 218- and 273-bp long, respectively.

Extraction of nuclear proteins and electrophoretic mobility shift assays (EMSA). We have previously described the methods for extracting nuclear proteins and EMSA (15). Ten microgram of nuclear protein extracted from HepG2 cells was mixed with ^{32}P-labeled oligonucleotide probe. For competition assays, the unlabeled competitor was premixed with the labeled probe at a 100-fold molar excess. For supershift assays, nuclear protein was incubated with specific antibodies, anti-PEA3 antibody, or anti-c-Jun antibody (Santa Cruz, Biotechnology, Inc., Santa Cruz, CA). The binding site oligonucleotides were as follows: PEA3: 5′-TCGAGCAGGAAGTTCGA-3′, AP-1 (c-Jun): 5′-TTCCGGCTGACTCATCAAGCG-3′ (99). The underlines are PEA3 or AP-1 consensus sequences, respectively.

Immunohistochemistry. Paraffin embedded sections of HCC tissues were immunostained with mouse monoclonal IgG antibody for PEA3 (Santa Cruz) and IL-8 (16) at dilutions of 1:5 and 1:100, respectively. And then secondary antibody, anti-mouse-horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA), was incubated. The immunocomplexes were detected with diaminobenzidine.

RESULTS

AP-1 and PEA3 binding sites required for constitutive IL-8 mRNA expression in HepG2 cells. We performed transient transfection experiments utilizing CAT reporter constructs containing deletions of the human IL-8 promoter. Based on this analysis, the location of potential *cis*-elements is shown in Fig. 1A. Deletion of the interval extending from −1481 to −138 bp had no significant effect on the constitutive promoter activity in HepG2 (Fig. 1B). Further deletion of the promoter down to −104 bp resulted in the loss of the PEA3 and AP-1 binding sites. This, in turn, led to an almost fivefold reduction in the constitutive promoter activity. Further deletion of the interval between −104 and −50 bp, which includes the NF-κB binding site, did not significantly affect the constitutive promoter activity. These results indicate that the region between −138 and −104 bp of the IL-8 gene is essential for the constitutive promoter activity in HepG2 hepatoma cells.

We further investigated the region between −138 and −104 bp by using a −138 promoter construct in HepG2 cells. In these constructs, the PEA3 and AP-1 sites were individually mutated (Fig. 2). Mutation of PEA3 and AP-1 decreased the constitutive promoter activity by 77 and 83%, respectively. The promoter with mutations at both the PEA3 and AP-1 sites showed the lowest IL-8 CAT activity (90% decrease), compared to that of the −138-promoter CAT construct, in which both PEA and AP-1 sites remained intact.

Activation of PEA3 and AP-1 in nuclei from HepG2 cells. Constitutive binding of PEA3 or AP-1 protein to their cognate binding regions was observed (Fig. 3) by EMSA. Both binding reactions were inhibited by the unlabeled PEA3 (lane 2) or c-Jun (lane 7) consensus

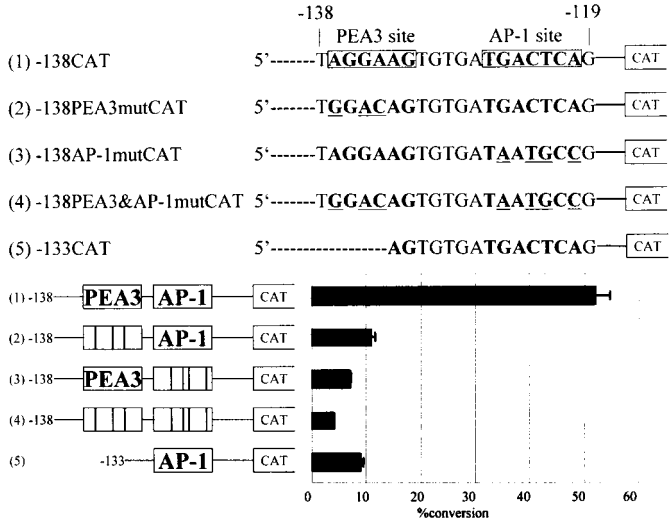

FIG. 2. Characterization of the enhancer region from the possible PEA3 binding site to the AP-1 binding site on the IL-8 promoter. (1) −138CAT, no mutation of both PEA3 and AP-1 binding sites; (2) −138 PEA3mutCAT, three mutations of the PEA3 binding site (underlined); (3) −138AP-1mutCAT, four mutations of the AP-1 binding site (underlined); (4) −138PEA3mutCAT&AP-1mutCAT, three mutations of PEA3 binding site and four mutations of the AP-1 site (underlined); (5) −133CAT, deletion of PEA3 binding sites. Relative CAT activities were measured as described under Materials and Methods.

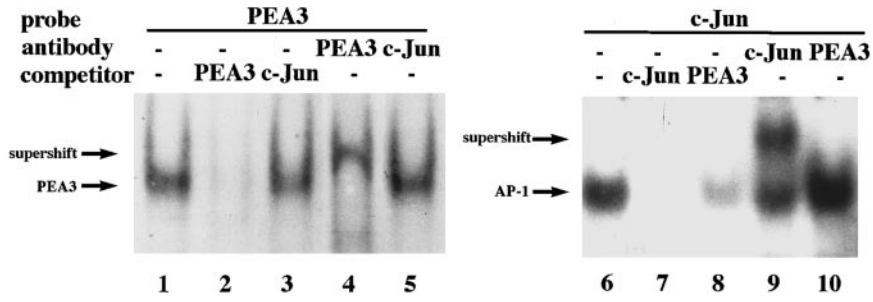

FIG. 3. Constitutive formation of PEA3 or AP-1 complexes in HepG2 cell nuclei determined by EMSAs. The nuclear extracts were incubated with labeled PEA3 probe in the absence (lane 1) or the presence of unlabeled PEA3 probe (lane 2), unlabeled c-Jun probe (lane 3), the specific antibody against PEA3 protein (lane 4), or the specific antibody against c-Jun protein (lane 5), while the nuclear extracts were incubated with labeled c-Jun probe in the absence (lane 6) or the presence of unlabeled c-Jun probe (lane 7), unlabeled PEA3 probe (lane 8), the specific antibody against c-Jun protein (lane 9), or the specific antibody against PEA3 protein (lane 10).

oligonucleotide, indicating specificity in protein binding. The PEA3 binding signal was partially inhibited by an excess of unlabeled c-Jun oligonucleotides (lane 3), and the AP-1 binding signal was also partially inhibited by an excess of unlabeled PEA3 oligonucleotides (lane 8), suggesting a weak physical association between PEA3 and AP-1 complexes. The PEA3 protein binding band was clearly supershifted by anti-PEA3 antibody (lane 4), but not by anti-c-Jun antibody (lane 5). Similarly, the AP-1 binding band was supershifted by anti-c-Jun antibody (lane 9), but not anti-PEA3 antibody (lane 10). These results suggests that nuclear proteins from HepG2 cells contain both PEA3 and AP-1, both of which might constitutively and individually bind to their cognate *cis*-elements and synergistically enhance constitutive IL-8 gene transcription in HepG2 cells.

Coexpression of PEA3 and IL-8 in HCC. IL-8 mRNA expression was detected in three HCC tissues from three patients that we examined. All three cases showed a tumor staining upon clinical angiographies (Fig. 4, lanes 1, 2, 3). IL-8 mRNA was also expressed in unstimulated HepG2 cells (Fig. 4, lane 4). However, IL-8 mRNA was not detected in uninvolved liver tissues surrounding HCC that did not show any tumor staining (Fig. 4, lanes 5, 6, 7).

These HCC tissues (Fig. 5A) from patients with hepatoma were then examined immunohistochemically.

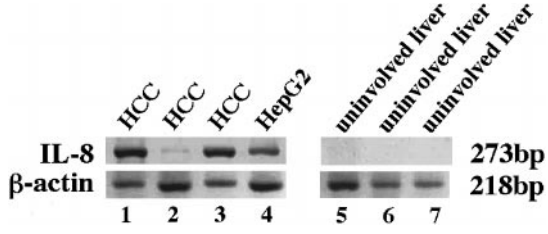

FIG. 4. IL-8 and β-actin mRNA expression in human HCC tissues, HepG2 cells, and uninvolved liver tissues by RT-PCR. Lanes 1–3, HCC tissue; lane 4, HepG2 cells; lanes 5–7, uninvolved liver tissues.

On serially cut sections, PEA3 proteins were clearly stained in the nuclei of all three HCC tissues that we examined (Fig. 5B), where IL-8 protein was detected in the cytoplasm (Fig. 5C). In contrast, hepatocytes from uninvolved liver tissues surrounding HCC did not express either the PEA3 protein (Fig. 5D) or IL-8 protein (data not shown). In order to examine the roles of IL-8 and PEA3 gene expression in tumor progression, we studied IL-8 and PEA3 expression in ten early-stage HCC, whose diameter was less than 20 mm without any tumor stain in clinical angiographies. IL-8 mRNA expression was restricted to half of them by RT-PCR, and PEA3 protein was expressed only in seven of them by immunohistochemistry (data not shown), in contrast to large HCC tissues, all of which expressed constitutively IL-8 mRNA and PEA3 protein (Figs. 4 and 5). Thus, constitutive PEA3 and IL-8 expression correlated with the progression of HCC, although their expression was observed at a relative early stage of HCC.

DISCUSSION

Here we demonstrate that the expression of IL-8 mRNA is constitutively expressed in a hepatoma cell line, HepG2 and human HCC tissues, which frequently exhibit hypervascularity. Moreover, in HepG2 cells, the PEA3 and AP-1 sites of the IL-8 promoter were essential for constitutive IL-8 gene expression. Furthermore, HepG2 cells constitutively produced and secreted a significant amount of IL-8 sufficient to induce angiogenesis *in vitro* (1309 ± 151 pg/ml in 1×10^6 cells for 48 h incubation). Thus, it is tempting to speculate that malignant transformation of HepG2 cell might be associated with constitutive activation of PEA3 and AP-1, which results in the constitutive production of a potent angiogenic factor, IL-8. Several lines of evidence suggest potential roles of IL-8 in tumor angiogenesis. We observed that the cell lines derived from pancreatic tumors, a representative hypovascular tumor, produced much lower levels of IL-8 constitutively (less than 50 pg/ml under confluent state), compared with

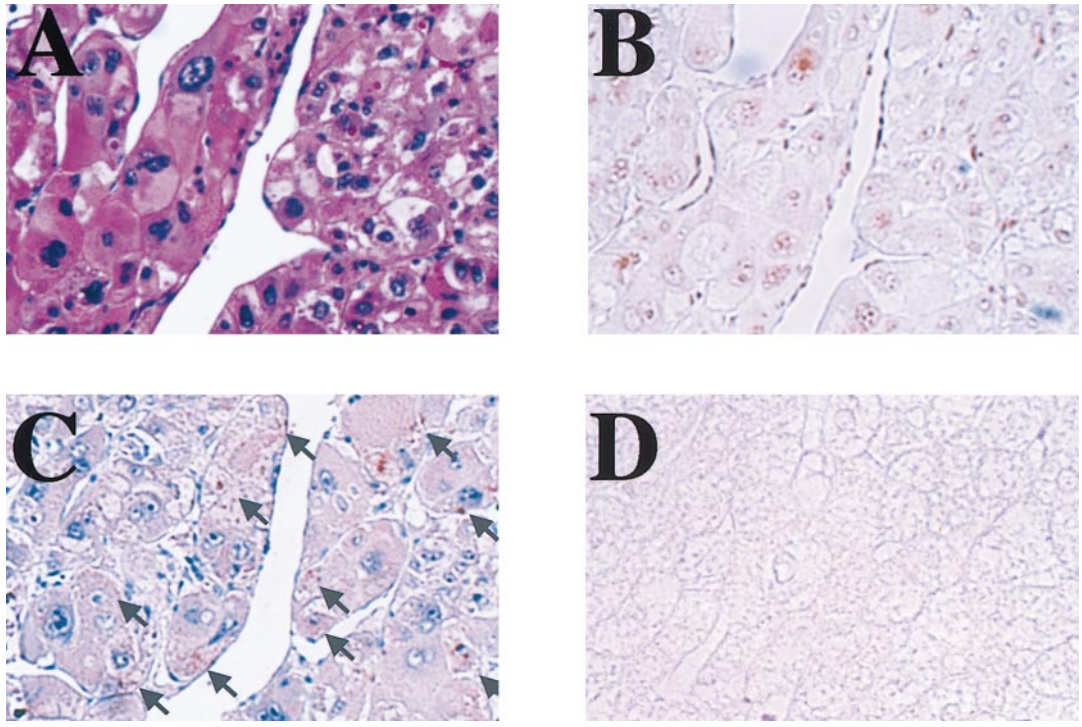

FIG. 5. Immunohistochemical PEA3 and IL-8 protein expression in HCC tissues, endothelium cells at tumor sites, and uninvolved liver tissue surrounding HCC. (A, B, and C) Serial sections of a HCC tissue were stained with hematoxylin and eosin (A), an anti-PEA3 antibody (B), and an anti-IL-8 (C, arrows) as described under Materials and Methods. (D) Uninvolved tissues from HCC were stained with the anti-PEA3 antibody. The sections immunostained with an anti-IL-8 antibody were also stained with hematoxylin additionally.

HepG2 cells (17). Kitadai and his colleagues observed a good correlation of IL-8 expression with vascularity in gastric carcinomas (18). Moreover, in collaboration with Kitadai and his colleagues, we observed that the transfection of IL-8 gene increased angiogenesis of human gastric carcinoma cells in nude mice (19). Collectively, it is likely that IL-8 might play angiogenetic roles in the progression of hypervascular tumors.

The ETS-domain protein family has been implicated in tumorigenesis and developmental several organs, such as ocular (20) and tracheal (21). Moreover, PEA3 has been associated with several tumorigenic processes and shows differential expression in a range of breast cancer cells (22, 23). Several lines of evidence indicate the involvement of PEA3 in gene expression of urokinase (24, 25) and matrix metalloproteinase (26, 27), both of which are presumed to be responsible for tumor cell infiltration. Thus, PEA3 may have important dual roles in tumor progression; induction of tumor cell infiltration by promoting urokinase or matrix metalloproteinase expression and angiogenesis by enhancing constitutive production of a potent angiogenic factor, IL-8. Besides IL-8, vascular endothelial growth factor (VEGF) is presumed to be important in the hypervascularity observed in HCC (28–30). Several lines of evidence suggest that ETS-domain proteins are involved in VEGF-induced tumor angiogenesis (31, 32). Thus, it is tempting to speculate that the activation of ETS-domain proteins such as PEA3, may induce simultaneously the expression of potent angiogenic factors, VEGF and IL-8 in the case of hypervascular HCC.

The sequences of the PEA3 and AP-1 sites have been previously reported to be cooperatively activating the expression of several distinct types of genes (9, 10). The constitutive IL-8 promoter activity was markedly reduced in HepG2 cells when the deletion or the mutation was introduced to the region between −137 and −132 bp. Moreover, the mutation in AP-1 binding site also markedly reduced the constitutive promoter activity. These observations would indicate that the cooperative activation of PEA3 and AP-1 binding site is responsible for the constitutive IL-8 gene transcription in HepG2 cells. The proximity between the PEA3 site (from −137 to −132 bp) and the AP-1 site (−126 to −120 bp) may be responsible for the cooperation of these two distinct types of transcription factors in the constitutive IL-8 gene transcription. Several independent groups claimed that the spacing between both elements is critical for their synergistic effect (26, 27). As the distance between the two sites in IL-8 promoter is only 6 nucleotides, it may be short enough to induce constitutive IL-8 gene expression.

EMSA analysis demonstrated that PEA3 and AP-1 complexes bound constitutively and individually to their corresponding binding sites of the IL-8 promoter. As PEA3 binding affinity is the same irrespective of

whether AP-1 is bound (27), PEA3 and AP-1 did not directly affect their partner's DNA binding activity. To explain these observations, Ptashne (33) proposed that two transcription factors can work synergistically not because they interact with each other, but rather because they simultaneously can associate with a third protein. This notion may be supported by our observation that AP-1 complex formation was weakly reduced by an excess amount of the unlabeled PEA3 binding motif.

In the present study, we demonstrated the expression of IL-8 in HCC tissues, but not in uninvolved liver tissues. Moreover, PEA3 was detected immunohistochemically only in cells expressing IL-8. Furthermore, several lines of evidence indicated the presence of IL-8 receptors on endothelium cells at tumor sites (24, 34). Thus, it is tempting to speculate that constitutive activation of PEA3 can enhance the production of a potent angiogenic factor, IL-8, thereby inducing angiogenesis through paracrine mechanism, and eventually tumor growth. If so, PEA3 protein or the PEA3 binding promoter site might be a novel target of anti-cancer therapy to inhibit angiogenesis, an important step for tumor progression.

In conclusion, IL-8 and PEA3 are expressed constitutively in hepatoma cells. Moreover, both the PEA3 and the AP-1 sites on IL-8 promoter are independently essential for constitutive IL-8 expression in a hepatoma cell line, HepG2. Thus, the cooperation of these two sites may play an important role in hepatoma tumorigenesis.

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