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Expression and Induction by IL-6 of the Normal and Variant Genes for Human Plasminogen

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We examined the promoter activity of the gene for human plasminogen (PLG) employing its 1.1 kb fragment of the 5′-flanking region inserted in front of a reporter gene. Deletion analysis revealed that a region surrounding the transcription start site was essential for the PLG expression. Since the PLG gene has three sequences for the interleukin-6 (IL-6) responsive element, we examined the effect of IL-6 on the PLG expression. IL-6 stimulation of PLG resulted in a 2.5-fold increase in its transcription. This is also true for the PLG gene of a case with dysplasminogenemia. Although the patient's gene had six mutations in the 5′-flanking region, its promoter activity was 1.8-fold that of normal PLG.

Plasminogen (PLG) is a key proenzyme in the fibrinolytic and thrombolytic systems. Cleavage at the Arg561-Val562 bond in PLG by tissue PLG activator or urokinase results in the formation of a serine protease plasmin. The proteolytic activity of plasmin contributes to digestion of the insoluble fibrin clot, by which normal recanalization and tissue repair can be accomplished (1,2). Plasmin can also serve as a broad spectrum protease capable of cleaving a wide variety of proteins and activating other enzymes, such as collagenase. Accordingly, plasmin appears to play an important role, not only in fibrinolysis, but also in processes which involve the breakdown of extracellular matrix proteins, e.g. in tumor cell migration, angiogenesis, ovulation, and development (3-5).

We previously characterized the normal (6) and variant genes (7,8) of human plasminogen (PLG) as well as homologous genes including PLG-related genes (PRGA and PRGB) (9), and the apolipoprotein(a) [apo(a)] gene and its related gene (9-11). Nucleotide sequence analysis revealed that the variant PLG gene of Case PLG NagoyaI had 6 mutations in its 5′-flanking region (7). These mutations may have some effect on the expression of PLG. It has been shown that PLG is synthesized in the liver and its transcript is detected in hepatocytes (12,13); however, its gene regulation has not been studied in detail.

In the present report, the transcriptional activity of normal and variant PLG genes has been determined by employing a 1.1 kb fragment of the 5′-flanking region for the PLG genes inserted into chloramphenicol acetyltransferase (CAT) vectors, and demonstrated to be largely dependent on a region surrounding their transcription initiation sites (13).

MATERIALS AND METHODS

Construction of CAT vectors. Genomic sequences containing the 5′-flanking region of normal and variant PLG genes were obtained by screening a human fibroblast library (9) and by *in vitro* amplification (14) of genomic DNA obtained from a patient with dysplasminogenemia, PLG NagoyaI (7), respectively. A genomic DNA insert was excised from the phage clone or the amplified product with *Eco*RI, and then subcloned into a plasmid vector, pUC18. Each 1.1 kb fragment of the 5′-flanking regions of PLG and PLG NagoyaI was isolated from the subcloned vector, and inserted into the 5′-region of promoterless pCAT-Basic and -Enhancer vectors (Promega, Madison, MI). The promoter regions of both genes span from positions −915 to +153 relative to the start site of transcription in PLG (13).

Fragments spanning from positions −703 and −283 to +153 of either PLG or PLG NagoyaI were obtained by digestion of the plasmids with *Xba*I and *Hind*III, respectively, and religation. Additional fragments, such as *Rsa*I-*Rsa*I (−670 to −317, −316 to −128) and *Rsa*I-*Bal*I (−127 to +153), were generated by digestion of the PLG plasmid. The DNA fragments were cloned into the pCAT vectors. The orientation and sequence of these constructs were verified by digestion with several restriction enzymes and the dideoxy termination method. Plasmids were prepared by double ultracentrifugation in cesium chloride density gradient.

Cell culture and transfection. HepG2 cells, a human hepatoma cell line (Japanese Cancer Research Resources Bank, Tokyo), were cultured in Dulbecco's-modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (penicillin, streptomycin, and neomycin). 10 μg of test plasmids and 5 μg of pSV-β-Galactosidase plasmid (Promega) as an internal control were cotransfected into cultured cells by the calcium phosphate precipitation method. Both

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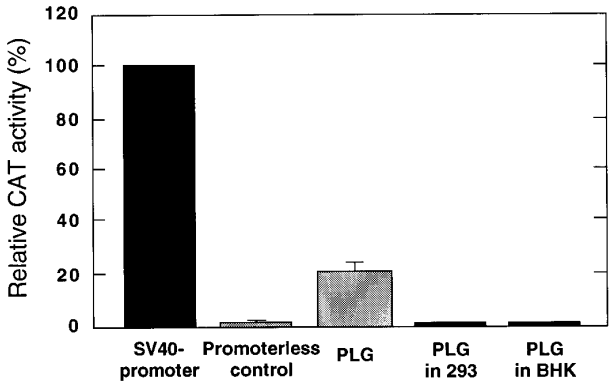

FIG. 1. Transient expression of CAT activity by reference constructs transfected into HepG2, 293, or BHK cells. The CAT activity of the control SV40 early promoter vector was defined as 100% in HepG2 cells to normalize the CAT activity of other constructs including the original 1.1 kb PLG plasmid and the promoterless CAT vector.

pCAT-Basic and -Control vectors (Promega) were also employed as negative and positive controls, respectively. The cells were incubated for 5 hrs and exposed to 15% (v/v) glycerol in Hepes-buffer saline for 2 min. After being cultured for 48 hrs, the cells were harvested for analysis. Each transfection was repeated at least four times and the average results are presented.

These experiments were also carried out employing human 293 cells and baby hamster kidney (BHK) cells.

CAT and β-galactosidase assay. CAT activity was measured by the standard method (15), and β-galactosidase activity by the colorimetric method (16). CAT activity was normalized to β-galactosidase activity to correct for differences in transfection efficiency and cell number.

IL-6 stimulation. Cells transfected with the 1.1 kb fragment of PLG were induced 24 hrs after the transfection with 100, 200, 400, 1000, and 2000 U/ml of human recombinant IL-6 (the kind gift of Kirin Co., Tokyo), then cultured for an additional 24 hrs in serum-free medium, and harvested for the expression assay.

RESULTS AND DISCUSSION

Liver-specific expression of the PLG gene. When the PLG vector containing 1.1 kb of its 5′-flanking region was transfected into HepG2 cells, significant levels of CAT activity were produced: About 9-fold higher than that obtained with a reference promoterless CAT vector. When presented as a percentage of the control SV40 early promoter gene (100%), the relative promoter activity of the PLG vector was 22% (Figure 1). Only background levels of activity were observed with this vector in 293 and BHK cells. These results indicate that the 5′-flanking sequence of 1.1 kb in the PLG gene is sufficient to support basal liver-specific gene expression. It is consistent with the finding that the PLG mRNA is detected most abundantly in hepatocytes and HepG2 cells (12,13).

Deletion analysis. A series of deletion constructs were then generated and tested for activity in HepG2 cells (Figure 2). A deletion from −915 to −703 bp caused an 80% increase in activity, suggesting the presence of a *cis*-acting silencer element in this region. A further deletion from −702 to −283 bp resulted in a 270% increase in expression, indicating that an additional strong silencer is present in this region. An AP-1 site at position −418 (Figure 3) is a good candidate for the putative silencer since AP-1 functions as a repressor as well as an activator (17,18).

A deletion from −282 to −128 bp caused a 330% reduction in activity, indicating the presence of a strong enhancer in this region. A C/EBP site at −145 bp may act as an enhancer. It was of interest that the segment of −282 to −128 bp demonstrated considerable CAT activity, while other upstream fragments did not show any activity (Figure 2). The C/EBP and GATA sequences in this region may support the initiation of PLG gene expression from another site. However, this hypothesis is unlikely since a single transcription initiation site was identified universally in all cell types including HepG2 cells and hepatocytes (13).

Although a segment of −127 to +153 bp demonstrated essentially the same activity as the original 1.1 kb construct of the PLG gene, no activity was observed when this segment was inserted in reverse orientation. Thus, it can be concluded that the promoter acts unidirectionally. The CAT activity was also completely abolished when a segment of −282 to +153 bp was removed. These results suggest that this region is functionally responsible and sufficient for transcription as the promoter of the PLG gene. There was no canonical TATA sequence around the transcription initiation site; instead, the PLG gene has a TATA-like sequence,

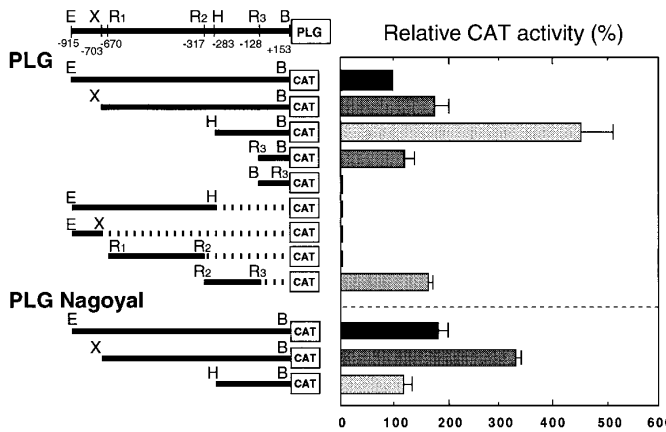

FIG. 2. CAT activity of deletion constructs. (Left) A series of PLG-CAT fusion constructs containing varying lengths of the PLG gene 5′-flanking sequences was generated. Capital letters B, E, H, R, and X stand for restriction sites of *Bal*I, *Eco*RI, *Hind*III, *Rsa*I, and *Xba*I, respectively. Broken lines indicate deleted portions. (Right) Relative CAT activity expressed by deletion constructs is shown. The CAT activity of the original 1.1 kb PLG vector was arbitrarily defined as 100% in HepG2 cells and used as a reference to normalize the CAT activity of other constructs.

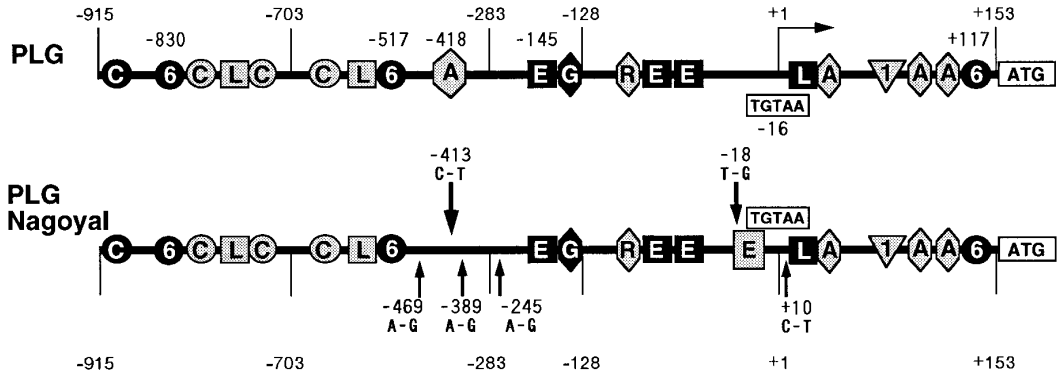

FIG. 3. Schematic comparison of the putative regulatory transcription sites present in the normal and variant PLG genes. Each element is labeled according to its corresponding transcription factor: 1, HNF-1; 6, IL-6; A, AP-1; C, CCAAT; D, DBP; E, C/EBP; G, GATA; L, LF/A-1; R, CREB. Arrows with base numbers indicate locations of mutations found in PLG NagoyaI.

TGTAA, at position −16 (Figure 3). This sequence corresponds exactly to the TATAA box of the homologous apo(a) gene (9,10), which is located 31 bp upstream from its transcription start site (19). Accordingly, the TGTAA sequence is very likely involved in the expression of the TATA-less promoter in the PLG gene.

Induction of PLG expression by IL-6. It is known that PLG in plasma increases during the acute phase. Since the PLG gene has three sequences for the IL-6 responsive element (21,22), at positions −830, −518 and +117 in the 5′-flanking region, we examined the effect of IL-6 on the CAT activity of the PLG vector in HepG2 cells. The addition of 100 U/ml of IL-6 resulted in a 1.5-fold increase in CAT activity, and 200 U/ml caused a 2.1-fold increase (Figure 4). A plateau level was reached at around 400 U/ml, and the maximum level of induction was 2.5-fold of that obtained in the absence of IL-6. These results suggest that PLG would be up-regulated by IL-6 during the acute phase response.

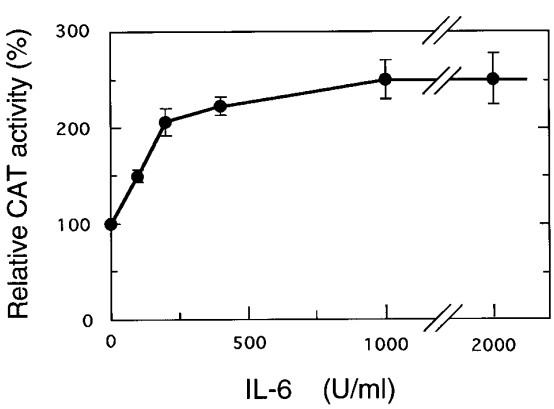

FIG. 4. Expression of CAT activity in transfected HepG2 cells in the absence and presence of IL-6. The CAT activity in the absence of IL-6 was arbitrarily defined as 100%.

Expression of variant PLG gene. In PLG NagoyaI, a Val355 amino acid was replaced by a Phe residue (7). Recently, we have detected three new unrelated families with this mutation (8); thus, it is not as rare a mutation as had been thought. Sequence analysis revealed that the variant PLG gene of PLG NagoyaI had 6 mutations in its 5′-flanking region: From A to G at position −469, C to T at −413, A to G at −389, A to G at −245, T to G at −18, and C to T at +10 (Figure 3). The substitution of C-T at position −413 destroys an existing AP-1 site, while the substitution of T-G at −18 creates a new C/EBP site. Other nucleotide changes in this gene are not located within consensus sequences for regulatory elements.

When the plasmid containing 1.1 kb of the 5′-flanking region of PLG NagoyaI was transfected into HepG2 cells, CAT activity 80% higher than normal PLG was produced (Figure 2), indicating that the basal expression level of the variant gene is increased. This is also true after a deletion of −915 to −703 bp. The variant gene may partially compensate for the decreased enzyme activity of the mutant by increasing its expression. This is likely due to the abolishment of a strong silencer (the AP-1 site discussed above). A deletion from −702 to −283 bp containing this mutation resulted in a 2.8-fold reduction in activity, indicating that there is an enhancer(s) rather than a silencer in the PLG gene. This region may have retained a positive regulatory effect since the region has a CCAAT box and LF/A-1 sequence. Alternatively, three mutations located in the remaining region from −282 to +153 bp may have introduced new unknown negative elements.

None of the 6 mutations affected the consensus sequences for IL-6 and the responsiveness of the gene for PLG NagoyaI to IL-6, since its expression was induced by IL-6 2.0-fold at 100 U/ml and 3.1-fold at 500 U/ml (data not shown). This induction occurred in a dose-dependent manner, which is similar to that for the normal PLG gene (Figure 4).

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