Characterization of the 5′-flanking regions of plasminogen-related genes A and B

Masafumi Kida[a], Sadao Wakabayashi[b], Akitada Ichinose[a,b,*]

[a]*Department of Molecular Pathological Biochemistry, Yamagata University School of Medicine, Yamagata 990-23, Japan*
[b]*Department of Biochemistry, University of Washington, Seattle, WA 98195, USA*

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Abstract We examined the promoter activity of two homologous plasminogen-related genes (PRGA and PRGB) employing HepG2 cells. The 5′-flanking regions of these genes were sequenced first, then inserted into the upstream region of the CAT gene in an expression vector. CAT assays revealed that the promoter activity of PRGA was 3-fold that of plasminogen, while the activity of PRGB was 5-fold. Deletion analysis of these genes demonstrated that a region between −283 and +153 bp relative to the transcription initiation site was essential for their expression, and that there were regions with either negative or positive effects on expression farther upstream.

Key words: Plasminogen; Apolipoprotein (a); Gene family; 5′-Flanking sequence; Transcription; Expression

1. Introduction

Plasminogen (PLG) is a key proenzyme of plasmin in the fibrinolytic and thrombolytic systems. Cleavage at the Arg^{561}-Val^{562} bond in PLG by tissue PLG activator or urokinase results in the formation of a serine protease plasmin. Plasmin also appears to play an important role in processes which involve the breakdown of extracellular matrices, such as tumor cell migration, angiogenesis, and neurodevelopment [1–3].

Two genes highly homologous to PLG were found and designated PLG-related genes A (PRGA) and B (PRGB). These genes belong to the PLG-apolipoprotein (a) gene family [4,5]. The gene products of PRGA and PRGB are expected to be polypeptides of 8.8 kDa that correspond only to the preactivation peptide domain of PLG. This domain plays an essential role in conversion of the 'activation-resistant' conformation of native PLG to its 'activation-ready' form [6]. It is of interest that PRGB is expressed in the liver, and most prominently in cancer cells [7]. The regulation of these genes, however, has not yet been explored.

In order to understand the expression of PRGA and PRGB, it is essential to study the transcriptional regulation of these genes. In the present report, the sequences of the 5′-flanking regions of PRGA and PRGB are established and their transcriptional activity is demonstrated to be largely dependent on a region surrounding the transcription initiation site of PLG [8].

*Corresponding author. Fax: (81) (236) 28-5280.
E-mail: aichinos@med.id.yamagata-u.ac.jp

Abbreviations: CAT, chloramphenicol acetyltransferase; PLG, plasminogen; PRGA, plasminogen-related gene A; PRGB, plasminogen-related gene B

2. Materials and methods

2.1. Sequence analysis

Genomic clones containing the 5′-flanking region of PRGA and PRGB were obtained by screening the human fibroblast library, as reported previously [4,5]. Genomic DNA inserts were excised from the phage clones with *Eco*RI, and then subcloned into plasmid vector pUC18. The excised fragments were also subcloned into M13mp18 or mp19, and sequenced by the dideoxy termination method [9] employing [^{35}S]dATP and buffer gradient gels [10].

2.2. Construction of CAT vector

Each 1.1 kb fragment of the 5′-flanking regions of PRGA and PRGB was isolated from the subcloned vectors, and ligated into promoterless pCAT-Basic and -Enhancer vectors (Promega, Madison, WI) in front of the chloramphenicol acetyltransferase (CAT) gene. The resultant plasmids contained the 5′-flanking sequences of PRGA and PRGB spanning from positions −910 to +153 relative to the transcription start site in PLG [8]. Fragments spanning from positions −698 and −283 to +153 of either PRGA or PRGB were obtained by digestion with *Xba*I and *Hin*dIII, respectively, and religation. The resulting DNA fragments were released and 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.

The CAT plasmids containing the PLG genes constructed in a previous study [11] were also used as references.

2.3. Cell culture and transfections

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 pCAT-Basic and -Control vectors were also employed as negative and positive controls, respectively. The cells were incubated for 5 h and exposed to 15% (v/v) glycerol in HEPES-buffered saline for 2 min. After being cultured for 48 h, 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 embryonic kidney 293 cells and baby hamster kidney (BHK) cells.

2.4. CAT and β-galactosidase assay

CAT activity was measured by the standard method [12], and β-galactosidase activity by the colorimetric method [13]. CAT activity was normalized to β-galactosidase activity to correct for differences in transfection efficiency and cell number.

3. Results and discussion

3.1. 5′-Flanking sequences of PRGA and PRGB

Sequences of more than 1000 bp of the 5′-flanking and 5′-untranslated regions of PRGA and PRGB were established in the present study and were compared to each other (Fig. 1). The sequences of PRGA and PRGB are 94.5% identical to that of PLG. PRGA and PRGB differ in sequence from each

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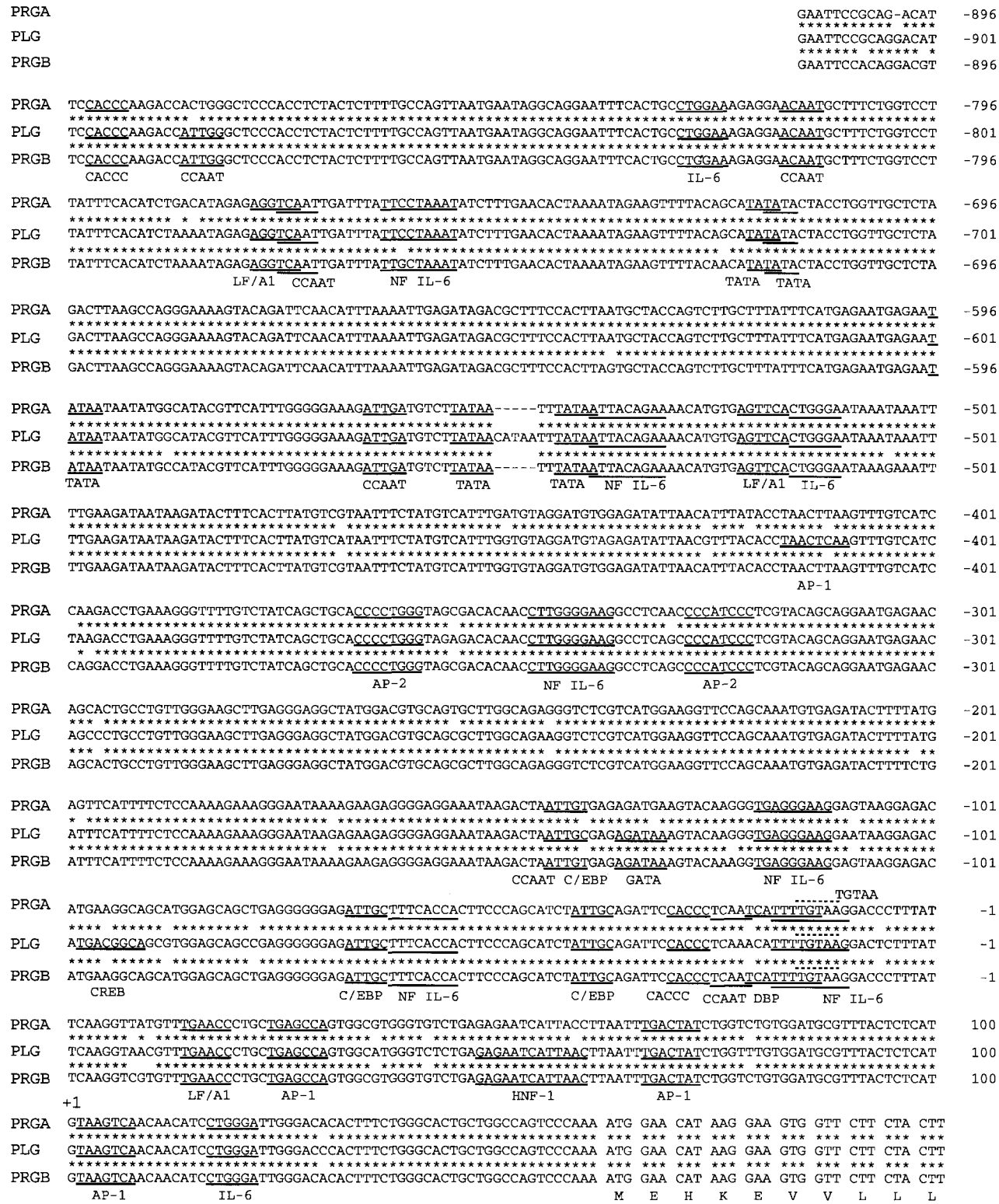

Fig. 1. Nucleotide sequences of the 5′-flanking regions of PLG, PRGA and PRGB. Nucleotide numbers are shown in the right margin. Asterisks show nucleotides identical to those of the PLG gene. Consensus sequences for transcription regulatory elements are underlined and labeled.

other by 1–2%. Both genes are missing 5 nucleotides (from −551 to −547 bp) in the PLG gene.

The sequences surrounding the transcription start site (numbered +1) and translation initiation site of PLG [8] are identical to those in PRGA and PRGB. This spatial arrangement was also assigned in PRGA and PRGB. Although three TATA boxes were present between −600 and −550 bp in all three genes, there was no canonical TATA sequence around

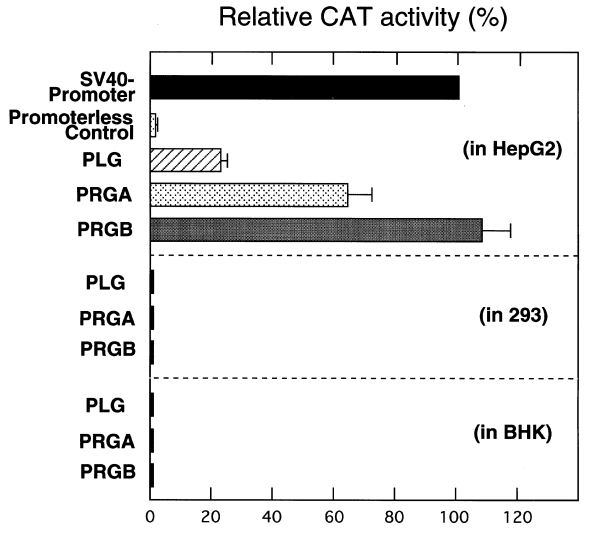

Fig. 2. Transient expression of CAT activity of reference constructs transfected into HepG2 (top), 293 cells (middle), and BHK cells (bottom). The CAT activity of the control SV40 early promoter vector was defined as 100% in HepG2 cells to normalize the CAT activity of other constructs including the original 1.1 kb PLG plasmid and the promoterless CAT vector.

the transcription initiation site. Instead, these genes have a TATA-like sequence, TGTAA, at position −16 (Fig. 2). This sequence corresponds exactly to the TATAA box of the homologous apolipoprotein (a) gene [14,15], which is located 31 bp upstream from its transcription start site [16]. Accordingly, the TGTAA sequence is very likely involved in the expression of the TATA-less promoter in these three genes.

Various consensus sequences for binding sites of transcription factors were found by a computer-assisted search employing a directory [17]; these include sites for AP-1, C/EBP, CREB, HNF-1, LF/A-1, NF-IL6, etc. (Fig. 1). Since PLG and PRGB are synthesized in the liver, it was of interest to examine the related genes for consensus sequences of liver-specific and liver-enriched transcription factors. A sequence homologous to the HNF-1 site (GTTAATNATTAAC [17]) was identified in PLG and PRGB, while PRGA lacks this site because of a single mismatch at position +59. There were two sites of the consensus sequence for C/EBP (GTGGT/AT/AT/AG or ATTGC [17]) in PRGA and PRGB which were common among all three genes, while one at −145 bp exists only in PLG. There were also three sites for LF/A-1 (TGG/AA/CCC [18]) in all three genes, and a DBP site (TGATTTTGT [17]) in PRGA and PRGB. Among these sites, those for C/EBP, CCAAT, DBP, LF/A-1, HNF-1, and AP-1 were located within 50 bp from the transcription initiation site of PLG [8,11]. It is common for a transcription initiation site to be surrounded by many regulatory elements.

It is of particular interest that three IL-6 responsive elements (or acute-phase response factor, APRF, sites) (CTGGG/AA [19,20]) are also located in the 5′-flanking regions of these genes. IL-6 stimulation of HepG2 cells transfected with a CAT construct for PLG resulted in a 2.5-fold increase in the reporter expression [11]. Thus, these three genes might be up-regulated by IL-6 during the acute-phase response.

PRGA and PRGB have three sites for a ubiquitous transcription factor AP-1 (TGAG/CTC/AA [17]), while PLG has four, due to one nucleotide difference at position −413.

3.2. Promoter activity of the homologous genes

To examine whether PRGA and PRGB are functionally active, each 1.1 kb DNA fragment carrying the 5′-flanking region of one of the three genes was linked to a promoterless CAT reporter plasmid. Low but detectable levels of CAT activity were produced in HepG2 cells, e.g. a plasmid containing the 1.1 kb fragment of PLG had CAT activity about 9-

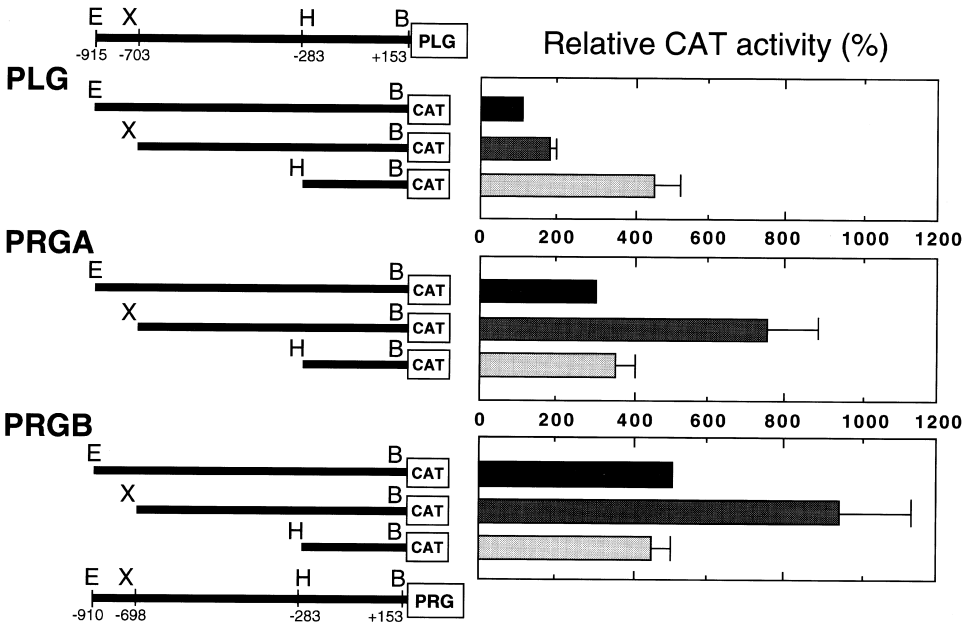

Fig. 3. CAT activity by deletion constructs transfected into HepG2 cells. Left: A series of PRGA- or PRGB-CAT fusion constructs containing varying lengths of their 5′-flanking sequences. Right: CAT activity expressed by deletion constructs. The CAT activity of the original 1.1 kb PLG vector was arbitrarily defined as 100% in HepG2 cells and used as a reference to normalize the CAT activity of other genes.

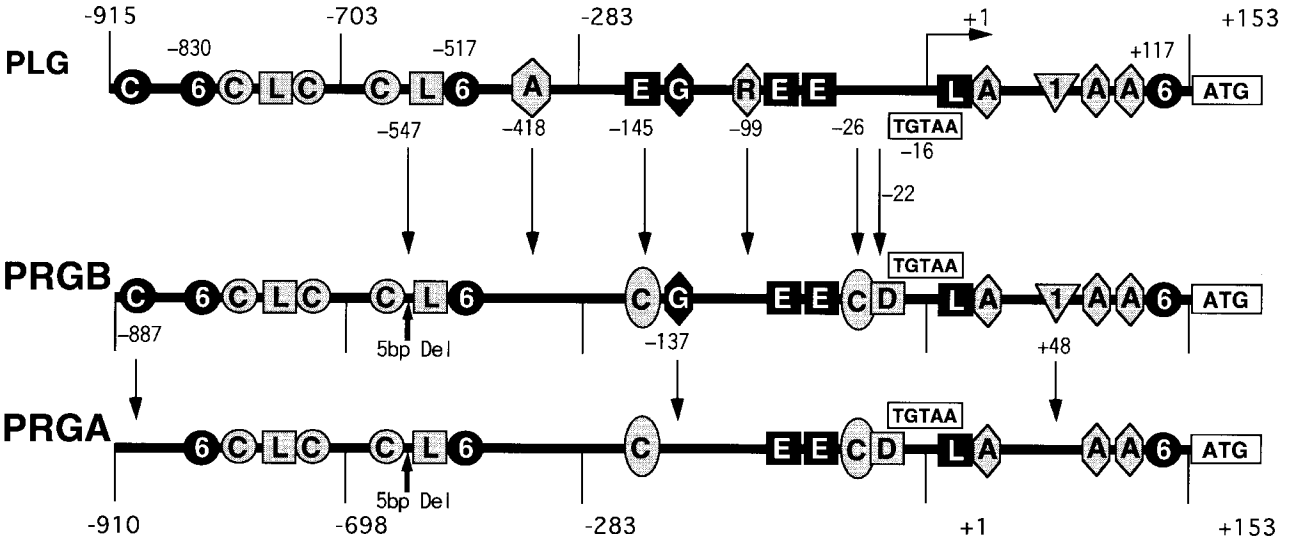

Fig. 4. Schematic comparison of the putative transcription regulatory sites present in PRGA and PRGB and those of PLG. Each element is labeled according to its corresponding transcription factor: 1, HNF-1; 6, IL-6; A, AP-1; C, CCAAT; D, DBP; E, C/EBP; G, GATA; L, LF/A-1; R, CREB.

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 was 22.0% for PLG, 65.3% for PRGA and 109% for PRGB (Fig. 2). Thus, the relative promoter activity of these two highly homologous genes was much higher than that of PLG. Similar but somewhat different results were reported by other investigators [16], probably due to their employing a 1.3 kb fragment containing all of exon I and about 150 bp of intron A. Since the coding sequence of exon I was included in their construct, the difference in expression activity might be attributable to translational efficiency rather than transcriptional efficiency, as reported in the apolipoprotein (a) gene [21].

Essentially no CAT activity was observed with the 1.1 kb fragments in 293 cells nor in BHK cells. These results indicate that the 1.1 kb 5′-flanking sequence is sufficient to direct basal liver-specific gene expression of these genes.

3.3. Deletion analysis of CAT vectors

Stepwise deletion constructs were generated from the 1.1 kb fragments of the three genes, and tested for activity in HepG2 cells (Fig. 3). A deletion from −910 to −698 bp resulted in a 2.5-fold increase in activity in PRGA and a 1.9-fold increase in PRGB, while a deletion from −915 to −703 bp caused a 1.8-fold increase in PLG [11]. These results suggest the presence of a *cis*-acting negative element in this region of each gene. The possible silencer sequence(s) is, however, not yet clear.

A further deletion from −698 to −283 bp caused a 2.1-fold reduction in activity in both PRGA and PRGB, while a deletion from −703 to −283 bp resulted in a 3.7-fold increase in PLG. These results indicate that an additional negative element is present in PLG and that there is a positive element in this region of PRGA and PRGB. As described above, PLG has a unique AP-1 site at −418 bp, while PRGA and PRGB lack this site (Figs. 1 and 4). Since all the remaining consensus sequences are common in these three genes, it is likely that the putative AP-1 site in PLG acts as a silencer [22].

Since the sequence from −283 to +153 bp supported significant transcriptional activity, it is concluded that the promoter is present in this region, and the sequences close to the transcription initiation site play an essential role in the regulation of these genes.

Expression of these genes may be modulated under particular conditions, such as malignant transformation. Indeed, in contrast to mRNA for PRGA, mRNA for PRGB has been detected in larger amounts in several cancer cells than in the corresponding normal cells ([7]; Tateno and Ichinose, unpublished data). The preactivation peptide domain of PLG plays an essential role in conversion of the 'activation-resistant' conformation to the 'activation-ready' form [6]. Accordingly, increased levels of the gene product of PRGB composed of the preactivation peptide domain alone may affect the activation of PLG to plasmin, and may play a role in invasion and metastasis of cancer cells, such as the extracellular matrix destruction [2,3]. This assumption is analogous to the fact that a 38 kDa fragment of PLG named angiostatin, but not intact PLG, potently inhibits neovascularization, growth of tumors and their metastases [23].

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