Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 383-388

PLASMINOGEN ACTIVATOR INHIBITOR 2. ISOLATION AND CHARACTERIZATION OF THE PROMOTER REGION OF THE GENE

E.K.O. Kruithof and E. Cousin

Hematology Division, Department of Medicine, University Hospital, 1011 Lausanne, Switzerland

Received August 29, 1988

Summary We have isolated the promoter region and part of the coding region of the human plasminogen activator inhibitor 2 gene. The first two exons (65 and 177 base pairs, respectively) are separated by a 3.6 kilo base pair intron. More than 2 kilo base pair of the promoter region was sequenced and analyzed. The promoter region is characterized by: a) a TATAAA box located 31 base pairs upstream of the trancription initiaton site; b) a 123 base pair stretch (-598 to -720) that is homologous (>90% identity) to the inverted sequence of -1268 to -1380; c) a 24 base pair inverted repeat at -276 to -299 and three direct repeats; and d) several sequences that are homologous (up to 1 mismatch) to the cAMP responsive element or to binding sites for the transcription factors AP1 and AP2.

Plasminogen activator inhibitor 2 (PAI-2) is one of the principal inhibitors of the fibrinolytic system, an enzyme cascade system that generates localized proteolysis. Two forms of PAI-2 have been recognized: a glycosylated and a nonglycosylated form. The majority (over 90%) is nonglycosylated and intracellular, whereas only a small part is glycosylated and secreted (1,2). Release of intracellular PAI-2 by cell death in the context of wound healing or inflammation may provide a means to block local fibrinolysis. Several lines of evidence suggest that indeed PAI-2 plays a role in local inflammatory processes. Thus, PAI-2 is a major constituent of monocytes and macrophages (3-5) and is also present in endothelial cells and fibroblast like cells; in these cells, biosynthesis and release of PAI-2 is stimulated by phorbol ester, endotoxin and the inflammatory mediator tumor necrosis factor (1,5-9). Induction by phorbol ester in the promonocyte cell line U-937 or by tumor necrosis factor in fibrosarcoma cells is followed by an up to 50 fold increase in the rate of gene transcription (7,8). As a first step to understand the molecular mechanisms underlying the response of the PAI-2 gene to tumor necrosis factor or other inflammatory mediators we have isolated the gene and sequenced more than two kilobase pair of the promoter region.

MATERIALS AND METHODS

Enzymes and chemicals - Restriction endonucleases, T4 DNA polymerase and T4 polynucleotide kinase were obtained from New England Biolabs (Beverly, MA, USA) and labelled nucleotides from New England Nuclear (Boston, MA, USA).

0006-291X/88 $1.50

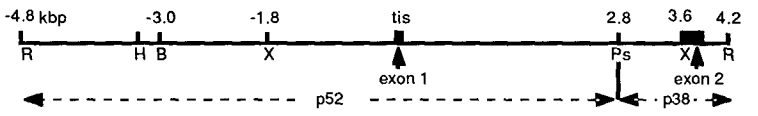
Screening of human genomic DNA library - One million recombinant phage of a human genomic Charon 4A library (10) were plated on Escherichia coli LE 392 at a density of approximately 50'000 plaque-forming units/15 cm diameter petri dish and screened by hybridization with a random primer labelled (11) insert of PAI-2 cDNA (pPAI J7, ref.7). Duplicate plaque lifts were made on nitrocellulose filters and hybridized with the labelled insert. Hybridization and washing conditions were as described (12). Phage that hybridized on both filters were replated and rescreened until all phage hybridized with the cDNA probe.

Preparation of phage DNA and subcloning in pUC 18 - Phage DNA was prepared as described (11), digested with one or more restriction enzymes, subjected to electrophoresis in agarose, transferred (13) to Gene Screen plus membranes (New England Nuclear, Boston, MA) and probed with random primer labelled fragments of PAI-2 cDNA or with end labelled oligonucleotides (11), complementary to different regions of PAI-2 cDNA. Two Eco R1/Pst 1 fragments were subcloned into the pUC18 plasmid, analyzed by restriction enzyme digestion and partially sequenced by the chemical method (14) or by the dideoxy chain termination method (15) on supercoiled plasmid DNA (16) using synthetic oligonucleotides.

Primer extension analysis - A single stranded oligomer (TCTGAGTTGCTGTCTGAC) complementary to the 5' region of the PAI-2 cDNA (17,18) was labelled with [$^{-32}$P]dATP and polynucleotide kinase to a specific radioactivity of 10^8 cpm per nmol. Ten µg of poly A+ RNA obtained from phorbol ester stimulated U-937 cells (7) was coprecipitated with 1 pmol of the labelled primer. The pellet was dissolved in 12.5 µl of 0.5 mM EDTA, denatured at 90 °C for 3 min and 38 U (1µl) of RNAse inhibitor (RNA guard, Pharmacia, Uppsala, Sweden) and 2.5 µl of 0.5 M Tris-HCl (pH 8.3) - 0.5 M KCl - 0.05 M $MgCl_2$ - 0.4 M dithiotreitol added. After 10 min hybridization at 37 °C, 6 µl of the mRNA/primer mixture was mixed with 3 µl of 2.5 mM each of the four deoxynucleotide triphosphates and 1 µl (16 U) of reverse transcriptase (Super RT, Staehelin, Basel, Switzerland). The primer extended DNA product was extracted with phenol, ethanol precipitated and run on a 6% sequencing gel alongside a dideoxy sequencing ladder obtained on a supercoiled plasmid using the same primer.

RESULTS AND DISCUSSION

Isolation of PAI-2 genomic clones - Two recombinant phages were isolated from the Charon 4A human gene library and plaque purified. The DNA of both phages was subjected to restriction enzyme digestion and probed with labelled oligonucleotide probes complementary to ten different regions of the cDNA. Only two probes, representing the 5' end of the coding region, reacted with the DNA. The rfestriction enzyme pattern of both phages was identical. Screening of a second library (human leukocyte genomic in EMBL3, Clontech Laboratories, Palo Alto, CA, USA) did not yield additional positive clones. One of the Charon 4A clones was analyzed in detail. A 9 kbp EcoR1 fragment of its DNA reacted with the two probes complementary to the 5' end of the cDNA. This fragment was additionally cut with Pst 1 and the resulting two subfragments of 7.6 and 1.4 kilo base pair inserted into pUC 18, yielding two plasmids: pPAI-2/52 and pPAI-2/38, respectively. DNA sequencing and restriction enzyme analysis of both plasmids revealed that pPAI-2/52 contains the first (65 base pair) exon, 4.8 kilo base pair of upstream sequences and 2.8 kilo base pair of the first intron and that pPAI-2/38 contains the second (177 base pair) exon, 0.8 kilo base pair of the first intron and 0.4 kilo base pair of the second intron (Fig. 1). The sequence of the two exons was




```
-2080 CTAAGGGCTC TCTGAATTAA TACCCGAAGA AAATTAGGAA GAAGGGTAGC AGAAAGGCAT TGATTGGGGT AGGCAATCAA
-2000 CAGTACCAGC TGTATAGAAT TATTAGAAAA TTAGAAGTTA GAGTAGTTGA GAAATACGTG CAAGATTATA CAACTAGTAA
-1920 ATGGCAAAAA GAAGATTCTG AGTCTGAGAC TTTTCTGAGT CTCTCAGTAT TCATATCCTT GTTCACCACA GTATAGGGCC
-1840 TCTCTAGAGA GTTTTAGCAT GTTACTCCAC CCATTAAAAA TATTTGGTAA GAATTTGCTT GATGCTCACC ACTGTTCTTG
-1760 CTGCTCAAGG GAAACCAAGT ATATATTTGT GGATAGATCC TAACTCAGAT GATACTGTCA GAATATATAA GATTCCTATA
-1680 CCACATCCTG AACTCTGAAA GTTGCAGTTC TACGTAGAAG TTCACTGAGG GTTGTAAGAG TCAGAATGGA CTCCATGGAA
-1600 GTTATGGGGT GTGAATCAAA CCTCACAGGT GAGTCAGTGG GGAGAAAGAA GCATGACATT CCTCTTGAGA ATAACTGGAA
-1520 TGAAAATAAT ACGGTGAGAA AGAGTAGCAC CTGAAAAGTG AGTGAAGGAA AGGGTGGTGG AAGTTCTAGG TACATTGATA
-1440 TCTGTCCAAC TTGTTGAGCT GTCAATGTGT GGTTAAAAAA CAGTTAAACA GGCCAGGCCC GGTGGCTCAC ACCTGTAATC
-1360 CCAGCACTTT GGGAGGCCGA GGTGGGCGGA TTGCCTGAGG TCAGGAGTTT GAGACCAGCC TGGCCAACAT GGTGAAACCC
-1280 CGTCTCTACT AAAAATACCA AAAATTAGCC AGTCGTAGTG GTTGGGCACC TGTAATCCCA GCTATTCAGG AGGCTGAGGC
-1200 AGGAGGATCA CTTGAACCCA AGAGGCGGGA GTTGCAGTGA GCAGAGATCA CGCCATTGCA CCCCAGCCTG GGCAACAAGA
-1120 GTGAAACTCG ATCTCAAAAA AACAAAAACA AAAACAAAAA ACGTTAAACG TGGTTGGTCA CATAGACATT ACCAGAGGGG
-1040 TCTTCTTGGA CAGAGGCTGA AAAGAAAGAA GGGACTTAGC TCACCACCAG ACTCCTCAGC ACTCACGGGA GAAGAGCACT
 -960 AAGGACAAAG GGGCACTTAA CACGTGTAAA AGGGCAATGG CTCCTAATAA AGGATAAGAT CTGTTTTCTT TTTATTTTAT
 -880 TTTTTGTTTT TGAGATGGAG TCTCACTCTG TCACCCAGGC TGGAGTGCAG TGGCACAGTC TCAGCTCAAT GCAACCTCCA
 -800 CCTCCCAGGT TCAAGTGATT CTTCTGCCTC AGCCTCTCAA GTAGCTGGAA CTGGAGATGT GTGCCACCAC GTGCAGCTAA
 -720 TTTTTGTATT TTTAGTAGTG ACAGGGTTTC ACCATGCTGG CCAGGCTGGT CTCAAACTCC TGACCTCAAG TGATCCTCCC
 -640 GCCTCGGCCT CCCAAAGTGC TGGGATTACA TGCATGAGCC ACCACACCCG GCCATAAGAT CTATTTTATA ATCAAGAGAA
 -560 GATGAAAAAT TCTTCTTGTG TCAGGATATT AGGGTAGAAT CCGAGTTATT TCGAAAATTA CAGCTTGAGA CAATGTAAGA
 -480 CAGATTAAAA TAGAGAATTT CTAAGACAGA AAATGGATTT TTTCTTCCCT TCCCATGCCC TAAGCATCGC TTGGGTCTCC
 -400 CTAGGCTCAA AAGAGAGAGT AAATCTCATT ATATTTATAT ATTGGAAGAA GCCCACAGGC CATGGTTGTC CAAAGCTAGA
 -320 AGAAGTATTG TAGAATTTGA TACTATCCTT GAGCACAAGT ATAGTGGGGG AAATCTTGAA ACTGGGGCTG ACAGATTTTA
 -240 GCTAACTTTC ACAGGCATGT GTAGATATTG ACAAATTCTT AAGTTTCAGA GTGACCTCAT CCTCCCCCAA AATTTCTTAA
 -160 ACCATGCCTT TACAAACAAG TATTTCTATT CTAATGATGT CTTCCTGAGT AAAATGTTGA ATCACTCAAA GGACACAGAT
  -80 CAAAAGACAG AGGGAGAAAA AAAAATGCCA TGTGGGAGGG GCAAAGCTGT ATAAAACCAG TCATTACCAT GTCTGAACTG
tis TAACAACTCT CAGAGGAGCA TTGCCCGTCA GACAGCAACT CAGAGAATAA CCAGAGAACA ACCAGgtatt tcaatgattt
    ccatgccatg ......intron A (3.6 kbp)......ttcttcctctcttgcttctagATTGAAACA ATG GAG GAT CTT
    TGT GTG GCA AAC ACA CTC TTT GCC CTC AAT TTA TTC AAG CAT CTG GCA AAA GCA AGC CCC ACC CAG
    AAC CTC TTC CTC TCC CCA TGG AGC ATC TCG TCC ACC ATG GCC ATG GTC TAC ATG GGC TCC AGG GGC
    AGC ACC GAA GAC CAG ATG GCC AAG gtgagtttgagctgagctcacatttgggcgagtag .....intron B......
```

Fig. 1 **Structure of the 5' end of the PAI-2 gene.** A: restriction map of the cloned region (R: EcoRI; H: Hind III; B: Bam HI; X: XbaI; P: PstI). The positions are given in kilo base pair with respect to the transcription initiation site (tis). B: DNA sequence of the promoter region of the PAI-2 and of the first two exons with their flanking sequences. Note the tataaa box at position -31 to -26, a long inverted repeat starting at -1258 and -598, a short inverted repeat between-299 to -276 and two direct repeats at -1219 and -1356 and at -1124 and -1292 (underlined with arrows). In the region up to -600 are also marked the sequences that are homologous to binding sites (maximum 1 mismatch) for the transcription factors AP1 (////) and AP2 (\\\\), as well as the cAMP responsive element (==). DNA sequences coding for the PAI-2 protein are given as triplets. Intron sequences are given in lower case.

identical with that of the cDNA (7,17,18). The 3.6 kilo base pair first intron is located 9 base pairs upstream of the start codon of PAI-2, in a region where all known members of the serpin family of proteins have an intron. The second intron is located after a secondary structure element (Helix C) where PAI-2 has, compared to the other serpins, an additional 23 aminoacids inserted in the loop structure between helices C and D. To determine the size of the second intron two oligonucleotides were prepared

complementary to the cDNA sequence just downstream of the second exon, but no hybridization of these probes with phage DNA was obtained.

Determination of the transcription initiation site - To locate the transcription initiation site a labelled 18-mer complementary to the 5' end of the PAI-2 cDNA was hybridized to mRNA isolated from phorbol ester stimulated U-937 cells and extended as described in the methods section. One major extended DNA product was observed (Fig. 2). Alignment with the DNA sequence run alongside revealed the most likely position of the transcription initiation site. As expected for an eukaryotic gene, a TATAAA consensus sequence is situated at 26 to 31 base pair upstream (Fig.1).

Sequence and analysis of the promoter region of the PAI-2 gene - As a first step to the identification of structural elements involved in the regulation of the PAI-2 gene we have sequenced and analyzed more than 2 kilo base pair of the promoter region. Several repeats and inverted repeats were noted (Fig. 1). Particularly noteworthy is the 123 base pair stretch from -598 to -720 that is almost identical (> 90% identity) to the inverted sequence of -1258 to -1380. Interestingly, two direct repeats (a 16 base pair repeat at -1219 and -1356; and a 15 base pair repeat, 1 mismatch, at -1124 and -1292) are located within the 123 base pair inverted repeat region -1258 to -1380. An almost perfect (1 mismatch) contigouous inverted repeat is located at position -276 to -299.

In recent years a number of transcription factors have been identified. We therefore searched the promoter region for homologies with sequences known to interact with

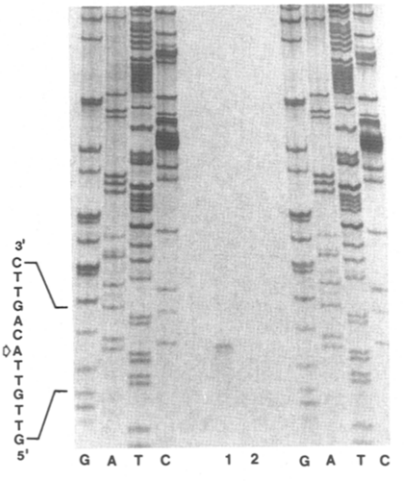


Fig. 2 Primer extension analysis of the 5' end of the human PAI-2 gene. An 18 base pair synthetic oligomer (TCTGAGTTGCTGTCTGAC) was end-labelled, hybridized to 10 μg of poly (A^+) mRNA from phorbol ester stimulated U-937 cells and extended on the mRNA as described in the methods section (lane 1). The reaction product and the labelled primer alone (lane 2) were electrophoresed between the products of dideoxy chain termination sequencing reactions performed on the plasmid containing the transcription initiation site (pPAI 2-52) using the same primer. The sequence at the right of the figure represents the complement of the coding strand sequence: GAACTGTAACAAC; underlined is the most likely position of the transcription initiation site.

these factors. From their complete or almost complete (1 mismatch) identity with such sequences several putative binding sites for the transcription factors AP1 and AP2 as well as cAMP responsive elements were identified (Fig. 1). Some of these elements may be involved in PAI-2 gene regulation. Thus one or more of the putative binding sites for AP1, also known as the phorbol ester responsive element (19,20) may be implicated in the induction by phorbol ester of of PAI-2 gene transcription in U-937 cells (7). The presence of several potential AP2 binding sites, that mediate induction by protein kinase C dependent or cAMP induced signal transduction pathways (21,22) as well of two putative binding sites for the cAMP responsive element binding factor (23) render it worthwile to investigate whether PAI-2 gene transcription can be induced also via cAMP dependent pathways. Deletion and mutation studies need to be undertaken to determine which of the potential regulatory elements are implicated in the regulation of PAI-2 gene transcription.

The isolation of the promoter region of the PAI-2 gene will allow a detailed analysis of the molecular mechanisms underlying the regulation of this inhibitor. As PAI-2 is strongly transcriptionally induced by tumor necrosis factor (8), the PAI-2 promoter region will provide a model system to study the elements involved in gene regulation by this cytokine.

ACKNOWLEDGMENTS

This work was supported by a grant from the Swiss National Fund for Scientific Research (No. 3.387-0.86) and by the Foundation for Research on Atherosclerosis and Thrombosis (FRAT). We thank E. Ayala and J.P. Vögeli for synthesizing the oligonucleotides, Dr. R.L. Medcalf for providing the mRNA from phorbol ester stimulated U-937 cells and Drs. E. Kawashima, D. Jenne and W.D. Schleuning for helpful discussions.

REFERENCES

1. Genton, C., Kruithof, E.K.O., and Schleuning, W.D. (1987) J. Cell Biol. 104, 705 - 712
2. Wohlwend, A., Belin, D., and Vassalli, J.D. (1987) J. Exp. Med.165, 320 - 339
3. Golder, J.P., and Stephens, R.W. (1983) Eur. J. Biochem. 136, 517 - 522
4. Vassalli, J.D., Dayer, J.M., Wohlwend, A., and Belin, D. (1984) J. Exp. Med. 159, 1653 - 1688
5. Chapman, H.A., and Stone, O.L. (1985) Am. Rev. Respir. Dis. 132, 569 - 575
6. Kruithof, E.K.O., Vassalli, J.D., Schleuning, W.D., Mattaliano, R.J., and Bachmann, F. (1986) J. Biol. Chem. 261, 11207 - 11213
7. Schleuning, W.D., Medcalf, R.L., Hession, C., Rothenbühler, R., Shaw, A., Kruithof, E.K.O. (1987) Mol. Cell. Biol. 7, 4564 - 4567
8. Medcalf, R.L., Kruithof, E.K.O., and Schleuning, W.D. (1988) J. Exp. Med. (in press).
9. Sadler, J.E., Ye, R.D., Wun, T.C., and Scarpati, E.M. (1988) FASEB J. 2, A1736, (abstr.)
10. Lawn, R.M., Fritsch, E.F., Parker, R.C., Blake, G., and Maniatis, T. (1978) Cell 15, 1157 - 1174
11. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory.
12. Benton, W.D., and Davis, R.W. (1977) Science 196, 180 - 182

13. Southern, E. (1975) J. Mol. Biol. 98, 503 - 517
14. Maxam, A.M., and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560 - 564
15. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463 - 5467
16. Chen, E.Y., and Seeburg, P.H. (1985) DNA 4, 165 -170
17. Ye, R.D., Wun, T.C., and Sadler, J.E. (1987) J. Biol. Chem. 262, 3718 - 3725
18. Webb, A.C., Collins, K.L., Snyder, S.E., Alexander, S.J., Rosenwasser, L.J., Eddy, R.L., Shows, T.B., and Auron, P.E. (1987) J. Exp. Med. 166, 77 - 94
19. Lee, W., Mitchell, P., Tjian, R. (1987) Cell 49, 741 - 752
20. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P., and Karin, M. (1987) Cell 49, 729 - 739
21. Imagawa, M., Chiu, R., and Karin, M. (1987) Cell 51, 251 - 260
22. Mitchell, P.J., Wang, C., and Tjian, R. (1987) Cell 50, 847 - 861
23. Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G., and Goodman, R.H. (1986) Proc. Natl. Acad. Sci. USA 83, 6682 - 6686