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Type 1 plasminogen activator inhibitor gene: Functional analysis and glucocorticoid regulation of its promoter

(fibrinolysis/luciferase assay/transcriptional regulation)

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ABSTRACT **Plasminogen activator inhibitor type 1 is an important component of the fibrinolytic system and its biosynthesis is subject to complex regulation. To study this regulation at the level of transcription, we have identified and sequenced the promoter of the human plasminogen activator inhibitor type 1 gene. Nuclease protection experiments were performed by using endothelial cell mRNA and the transcription initiation (cap) site was established. Sequence analysis of the 5′ flanking region of the gene revealed a perfect "TATA box" at position −28 to position −23, the conserved distance from the cap site. Comparative functional studies with the firefly luciferase gene as a reporter gene showed that fragments derived from this 5′ flanking region exhibited high promoter activity when transfected into bovine aortic endothelial cells and mouse Ltk⁻ fibroblasts but were inactive when introduced into HeLa cells. These studies indicate that the fragments contain the plasminogen activator inhibitor type 1 promoter and that it is expressed in a tissue-specific manner. Although the fragments were also silent in rat FTO2B hepatoma cells, their promoter activity could be induced up to 40-fold with the synthetic glucocorticoid dexamethasone. Promoter deletion mapping experiments and studies involving the fusion of promoter fragments to a heterologous gene indicated that dexamethasone induction is mediated by a glucocorticoid responsive element with enhancer-like properties located within the region between nucleotides −305 and +75 of the plasminogen activator inhibitor type 1 gene.**

The fibrinolytic system provides a means of generating localized proteolytic activity. In addition to its role in the resolution of blood clots (1), this system also functions in a variety of other biological processes, including ovulation, embryo implantation, macrophage activation, neoplasia, breast involution, and neovascularization (2). Regulation of the fibrinolytic system is achieved primarily at the level of the synthesis and activity of plasminogen activators (1). These molecules convert the zymogen plasminogen into plasmin and thus initiate the fibrinolytic process.

Type 1 plasminogen activator inhibitor (PAI-1) is the primary inhibitor of tissue-type plasminogen activator and may thus play a key role in the control of plasmin formation (3). PAI-1 is synthesized *in vitro* by endothelial cells, hepatocytes, and a number of other cell types (3), and its biosynthesis appears to be highly regulated compared to other components of the fibrinolytic system. For example, PAI-1 synthesis can be induced in endothelial cells by serum (4), lipopolysaccharides (5–7), thrombin (8), inflammatory cytokines [such as interleukin 1 and tumor necrosis factor type α (6, 9, 10)], and growth modulators [including transforming growth factor type β and basic fibroblast growth factor (11)]. In cultured rat ovarian granulosa cells the production of PAI-1 appears to be suppressed by follicle-stimulating hormone and leuteinizing hormone (12). Enhanced PAI-1 levels in response to the synthetic glucocorticoid dexamethasone have been demonstrated in human fibroblasts (13), fibrosarcoma cells (14), and mammary carcinoma cells (15) and in rat HTC hepatoma cells (16, 17).

To understand the complex regulation of PAI-1 biosynthesis, the PAI-1 gene has been isolated and characterized (18). In the current study, we have identified the promoter of the PAI-1 gene. Its structure† is described and functional studies using the firefly luciferase gene as a reporter gene are presented. The results show that the PAI-1 promoter is a relatively strong promoter in bovine aortic endothelial (BAE) cells and mouse fibroblasts but is weak or silent in HeLa cells and rat hepatoma cells. However, the PAI-1 promoter can be induced in the hepatoma cells with dexamethasone. This induction is mediated by a glucocorticoid-responsive promoter element with enhancer-like properties.

METHODS

General Methods. Plasmid DNA was isolated by a modification of the alkaline lysis procedure followed by CsCl equilibrium centrifugation (19). Enzyme reaction conditions were as recommended by suppliers. DNA fragments were isolated from low-melting-temperature agarose gels. DNA sequencing was performed by a modification of the dideoxy chain-termination procedure (20) with a commercial Sequenase kit (United States Biochemical, Cleveland, OH), after subcloning fragments of pUCEK19 (see below) in M13mp18 and M13mp19 DNA (19, 21).

Plasmids. A 1.5-kilobase (kb) *Kpn* I–*Eco*RI fragment from a cosmid containing the entire PAI-1 gene (18) was cloned into the *Kpn* I and *Eco*RI sites of pUC19 to create pUCEK19. For promoter deletion analysis, a series of PAI-1 promoter fragments were isolated from pUCEK19 and the PAI-1 cosmid and cloned into the multiple cloning site of p19LUC (see Fig. 4). All fragments had an identical 3′ end (i.e., the *Eco*RI site at position +71) and a different 5′ end. Thus the following sites were used: for p187LUC, the *Rsa* I site at position −189; for p305LUC, the *Hin*dII site at position −308; for p549LUC, the *Sph* I site at position −549; for p800LUC, the *Hin*dIII site at position −800; for p1.5kLUC, the *Kpn* I site at −1.5 kb; for p3.4kLUC, the *Xba* I site at −3.4 kb, and for p6.4kLUC, the *Sma* I site at −6.4 kb. The following approach was employed to derive plasmids pSV232ACATA, pSV232ACATB, pSV232ACATC, and pSV232ACATD: two PAI-1 promoter fragments, *Hin*dII

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Abbreviations: PAI-1, plasminogen activator inhibitor type 1; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; SV40, simian virus 40; GRE, glucocorticoid responsive element.

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†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03836).

(position −719) to *Hin*dII (position −308) and *Hin*dII (position −308) to *Eco*RI (filled in with *Escherichia coli* DNA polymerase I, large fragment) (position +71), were isolated. They were then cloned in both orientations into the *Bam*HI site of plasmid pSV232ACAT (22), directly upstream of the chloramphenicol acetyltransferase (CAT) gene by using *Bam*HI linkers (pCGGATCCG, New England Biolabs). The Rous sarcoma virus (RSV) long terminal repeat promoter vector, pRSVL, has been described (23).

Mung Bean Nuclease Analysis. Mung bean nuclease protection analysis was as described (24) and used a 380-base-pair (bp) *Hin*dII–*Eco*RI fragment (positions −305 to +75) from pUCEK19, ^{32}P-end-labeled with polynucleotide kinase at position +75. Hybridization was at 50°C for 6 hr with 20 μg of total RNA isolated from human umbilical cord vein-derived endothelial cells (4). Mung bean nuclease incubations were performed with mung bean nuclease (1250 units/ml) for 1 hr at 37°C. Protected fragments were separated on 8% polyacrylamide gels containing 8 M urea.

Cell Culture. FTO2B rat hepatoma cells were cultured as described (25). BAE cells (clone A) and human umbilical vein endothelial cells were isolated, cloned, and subcultured as described (26). HeLa (human epithelioid carcinoma) and Ltk$^-$ (mouse fibroblast) cell lines were obtained from the American Type Culture Collection and cultured as instructed.

Transfection Experiments. Semiconfluent cells in 6-well (10 cm^2 per well) tissue culture plates (Corning) were transfected with various DNA constructs by the DEAE-dextran method coupled with a dimethyl sulfoxide shock treatment (27). For dexamethasone induction studies, 2 μM dexamethasone (Ascot Pharmaceutical, Deerfield, IL) was added to wells immediately after shock treatment, and 48 hr later the cells were washed and collected by scraping with a rubber policeman.

Luciferase and CAT Assays. Cell extracts were prepared and assayed for luciferase activity exactly as described (23) by using a Monolight 2001 luminometer (Analytical Luminescence Laboratory, San Diego, CA). CAT activity was determined as described (28).

RESULTS

Identification of the Transcription Initiation Site. We have extended the restriction map of the human PAI-1 gene (18) ≈10 kb upstream of the first exon. To determine the cap site of this gene, we performed a mung bean nuclease protection assay. A 380-bp fragment spanning the region from the *Eco*RI site in the first exon to the first upstream *Hin*dII site was ^{32}P-labeled at the 5′ protruding end generated by *Eco*RI and hybridized to total endothelial cell RNA (Fig. 1). After incubating the formed heteroduplexes with mung bean nuclease, the protected fragments were electrophoresed on a 8% polyacrylamide gel containing 8 M urea. A number of protected fragments, not found in the control lane, were detected by autoradiography. These were 70–77 nucleotides long (Fig. 2). The same set of protected fragments was found in a number of mung bean protection assays performed either with different endothelial cell RNA preparations or with various nuclease incubation conditions (data not shown). These results indicate that the transcription initiation site is heterogeneous. The major cap site appears to be an adenosine 145 nucleotides upstream of the methionine initiation codon.

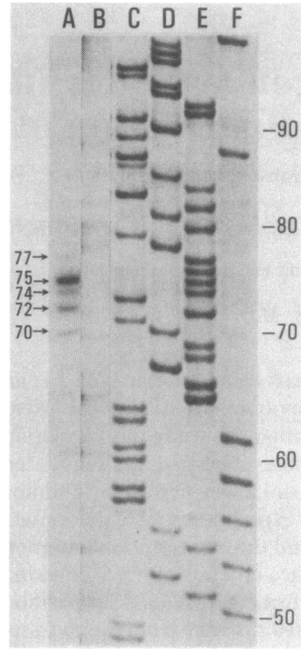

FIG. 2. Mung bean nuclease protection mapping of the 5′ end of the PAI-1 mRNA. Total endothelial cell RNA was hybridized to a 380-bp 5′ ^{32}P-labeled probe spanning the immediate 5′ flanking region of the PAI-1 gene. The resulting heteroduplexes were incubated with mung bean nuclease and the protected fragments were analyzed on a standard sequence gel. The autoradiogram was exposed for 4 days. Lanes: A, protected fragments after hybridization of probe with 20 μg of total endothelial cell RNA; B, control performed with 20 μg of calf liver tRNA; C–F, sequence lanes used as size markers. Arrows, protected fragments that were not found in the control lane.

DNA Sequence Analysis of the 5′ Flanking Region. The 874-bp *Eco*RI–*Hin*dIII fragment containing the immediate 5′ flanking region of the PAI-1 gene (Fig. 1) was subcloned and sequenced (Fig. 3). The adenosine representing the major transcription initiation site was designated position +1. The sequence data indicate that the PAI-1 gene resembles the majority of eukaryotic genes in that transcription initiates with an adenosine that is part of, or in close proximity to, a CA (positions +3 and +4 and positions +5 and +6) sequence (29, 30). In addition, a perfect "TATA box" (positions −28 to −23) was identified, and it was located at the conserved distance from the cap site (30). Computer comparison of the sequence with consensus sequences of known regulatory elements revealed the presence of a possible AP-1 site (positions −152 to −145). This regulatory element has been found to mediate the induction of transcription of several genes by phorbol esters such as phorbol 12-myristate 13-acetate (31). Another potentially interesting feature was the presence of a number of sequences with the *in vitro* potential of assuming a Z-DNA conformation (ref. 32; e.g., $[CACA]_5$ and $[CACA]_2$) that were detected between nucleotides −186 and −152. Similar sequences have been shown to enhance the activity of the simian virus 40 (SV40) early promoter by as much as 10-fold (37).

Functional Analysis and Tissue Specificity of the PAI-1 Promoter. To establish the functional activity of the putative PAI-1 promoter region, we have performed transfection experiments employing plasmid constructs that contain fragments of the 5′ flanking region of the PAI-1 gene cloned

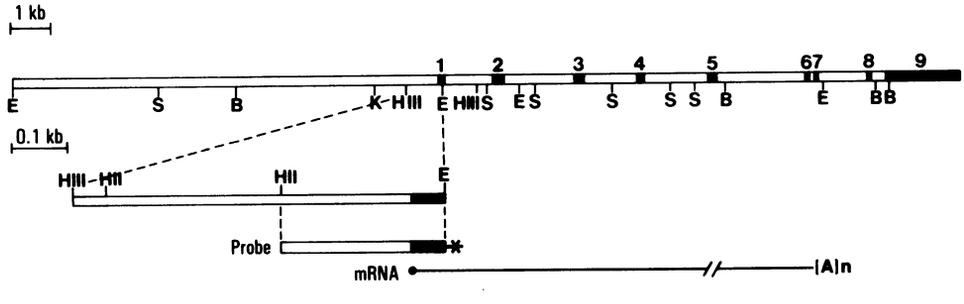

FIG. 1. Strategy for the mapping of the 5′ end of PAI-1 mRNA. Partial restriction map of the PAI-1 gene including ≈10 kb of 5′ flanking DNA. Exons are indicated by the solid boxes and are numbered 1–9. *, ^{32}P-labeled end of the probe. B, *Bgl* II; E, *Eco*RI; HII, *Hin*dII; HIII, *Hin*dIII.

upstream of a reporter gene. A sensitive reporter gene system has been developed based on the firefly luciferase gene (23). To facilitate the insertion and analysis of DNA fragments upstream of the luciferase reporter gene, we have constructed the promoter assay plasmid p19LUC (Fig. 4). This modification of plasmid pSVOAL-AΔ5′ (23) contains a pUC19-derived multiple cloning site directly upstream of the luciferase gene and downstream of two SV40-derived polyadenylylation sites that function to eliminate any cryptic transcriptional activity from the remainder of the plasmid. A series of overlapping fragments with identical 3′ ends (i.e., the *Eco*RI site in the 5′ noncoding region of the cDNA) were prepared. These fragments, containing from 100 bp to 6.4 kb of DNA immediately upstream of the cap site, were cloned in the multiple cloning site of p19LUC (Fig. 5*A*). Two of the plasmid preparations, p187LUC and p1.5kLUC, were then transfected into four mammalian cell types to establish their relative promoter activity. In each transfection experiment, a control plasmid (pRSVCAT; ref. 33) containing the CAT gene under transcriptional control of the neutral RSV long terminal repeat promoter was cotransfected with the luciferase construct. In this way, we were able to correct for differences in DNA uptake by the cells. Cell lysates were prepared 48 hr after transfection, and both luciferase and CAT activities were assayed. Table 1 summarizes the activity of the PAI-1 promoter constructs relative to the activity of the neutral non-tissue-specific RSV promoter construct. The promoterless reporter gene plasmid (p19LUC) produced barely detectable luciferase activity in all of the cell types, whereas p187LUC and p1.5kLUC exhibited similar and strong promoter activity in mouse Ltk$^-$ fibroblasts and in BAE cells. The strong promoter activity in both of these cell types agrees with the observations that these cells express PAI-1 (26, 34). HeLa cells do not express significant amounts of PAI-1 (35), and very little PAI-1 promoter activity was detected in transfection experiments with these cells. Rat hepatoma cells can be induced to synthesize high amounts of PAI-1 by the synthetic glucocorticoid dexamethasone (16). We have thus performed transfection experiments to investigate

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-800  AAGCTTACCATGGTAACCCCTGGTCCCGTTCAGCCACCACCACCCCACCCAGCA
-746  CACCTCCAACCTCAGCCAGACAAGGTTGTTGACACAAGAGAGCCCTCAGGGGCA
-692  CAGAGAGAGTCTGGACACGTGGGGAGTCAGCCGTGTATCATCGGAGGCGGCCGG
-638  GCACATGGCAGGGATGAGGGAAAGACCAAGAGTCCTCTGTTGGGCCCAAGTCCT
-584  AGACAGACAAAACCTAGACAATCACGTGGCTGGCTGCATGCCTGTGGCTGTTGG
-530  GCTGGGCAGGAGGAGGGAGGGGCGCTCTTTCCTGGAGGTGGTCCAGAGCACCGG
-476  GTGGACAGCCCTGGGGGAAAACTTCCACGTTTTGATGGAGGTTATCTTTGATAA
-422  CTCCACAGTGACCTGGTTCGCCAAAGGAAAAGCAGGCAACGTGAGCTGTTTTTT
-368  TTTTCTCCAAGCTGAACACTAGGGGTCCTAGGCTTTTTGGGTCACCCGGCATGG
-314  CAGACAGTCAACCTGGCAGGACATCCGGGAGAGACAGACACAGGCAGAGGGCAG
-260  AAAGGTCAAGGGAGGTTCTCAGGCCAAGGCTATTGGGGTTTGCTCAATTGTTCC
-206  TGAATGCTCTTACACACGTACACACACAGAGCAGCACACACACACACACACA
-152  TGCCTCAGCAAGTCCCAGAGAGGGAGGTGTCGAGGGGGACCCGCTGGCTGTTCA
 -98  GACGGACTCCCAGAGCCAGTGAGTGGGTGGGGCTGGAACATGAGTTCATCTATT
 -44  TCCTGCCCACATCTGGTATAAAAGGAGGCAGTGGCCCACAGAGGAGCACAGCTG
  11  TGTTTGGCTGCAGGGCCAAGAGCGCTGTCAAGAAGACCCACACGCCCCCCTCCA
  65  GCAGCTGAATTCCTGCAGCTCAGCAGCCGCCGCCAGAGCAGGACGAACCGCCAA
 119  TCGCAAGGCACCTCTGAGAACTTCAGGATGCAGATGTCTCCAGCCCTCACCTGC
                                  MetGlnMetSerProAlaLeuThrCys
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FIG. 3. DNA sequence of the 5′ end of the human PAI-1 gene. The sequence is shown from positions −800 to +220. The amino acid sequence of the coding region is shown under the DNA sequence. The arrow at position +1 indicates the major site of transcription initiation. The TATA box is underlined. The two arrows underline 8-bp direct repeats. A broken line overlines the potential AP-1 site and underlines stretches of simple repetitive DNA.

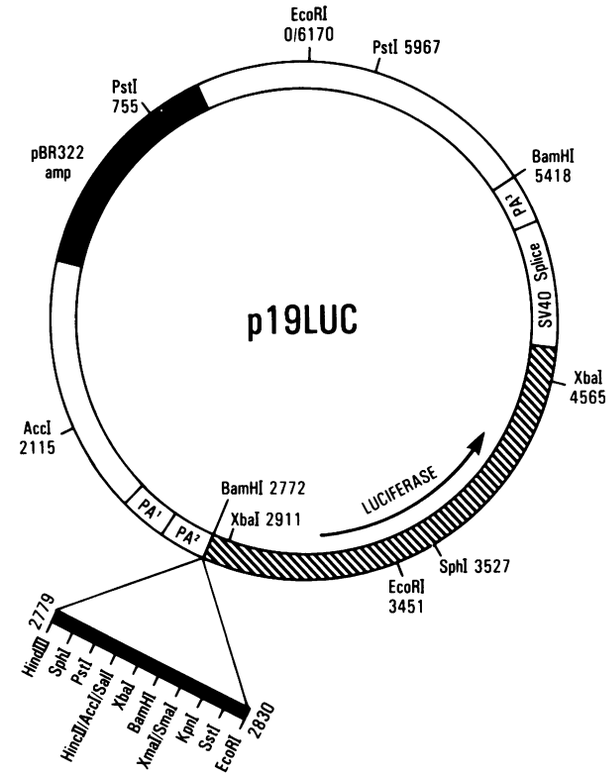

FIG. 4. p19LUC. Plasmid used to monitor promoter activity with the firefly luciferase gene fused to a SV40 splice and polyadenylylation site (22) as a reporter gene. It harbors a multiple cloning site (positions 2779–2830) preceded by two SV40-derived polyadenylylation sites (PA1 and PA2). p19LUC was derived as follows: pSVOAL-AΔ5′ (23) was linearized with *Hin*dIII. One portion of the plasmid was made blunt-ended by filling-in the *Hin*dIII sites with *Escherichia coli* DNA polymerase I large fragment (Klenow), ligated to phosphorylated *Eco*RI-linkers (pGGAATTCC, New England Biolabs), and cleaved with *Eco*RI. Two of the resulting fragments, the 621-bp fragment containing the 5′ end of the luciferase gene and the 2718-bp fragment originally located on the 5′ side of this fragment, were isolated. A second portion of *Hin*dIII-cleaved pSVOAL-AΔ5′ was ligated to a 55-bp polylinker and cleaved with *Eco*RI. The resulting 2831-bp fragment containing the multiple cloning site and the *amp* gene was isolated. These fragments were ligated to create a plasmid (p19LUC) that contained the three fragments in their original orientation but with the multiple cloning site in the original *Hin*dIII site.

the effect of dexamethasone on the PAI-1 promoter activity of a rat hepatoma cell line, FTO2B (25). In the absence of dexamethasone, little PAI-1 promoter activity was observed. However, in the presence of dexamethasone the activities of p187LUC and p1.5kLUC were induced ≈8-fold and 27-fold, respectively. The activity of the RSV promoter was induced only 1.6-fold by dexamethasone. These results indicate the presence of a strong and relatively tissue-specific promoter within the first 187 bp of the 5′ flanking region of the PAI-1 gene. This promoter is inducible by dexamethasone in rat FTO2B hepatoma cells.

The PAI-1 Promoter Contains a Glucocorticoid-Dependent Enhancer. Experiments were performed to more specifically localize the region in the promoter that mediates its dexamethasone inducibility in FTO2B cells. Rat FTO2B cells were transfected with constructs containing overlapping PAI-1 promoter fragments of various lengths. The cells were then incubated for 48 hr in the presence or absence of 2 μM dexamethasone, and cell lysates were prepared and assayed for luciferase activity. As can be seen in Fig. 5*A*, each of the promoter fragments exhibited dexamethasone-inducible promoter activity. In control experiments, both primer extension

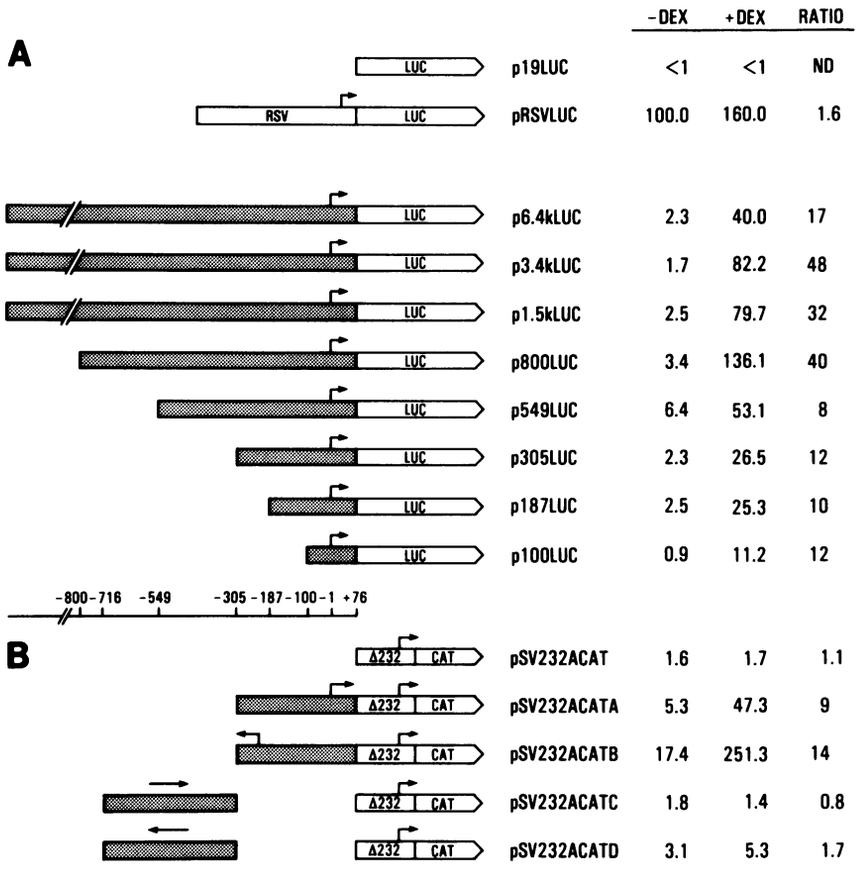

FIG. 5. Promoter deletion mapping and CAT constructs. (*A*) Luciferase constructs used for promoter deletion mapping. A series of overlapping fragments of the 5′ flanking region of the PAI-1 gene were assayed based on their capacity to promote luciferase expression in the absence and presence of 2 μM dexamethasone. Luciferase activity for each construct is expressed as a percentage of the luciferase activity of the RSV promoter construct. (*B*) Effect of PAI-1 promoter fragments on a heterologous promoter assayed with CAT constructs. Two PAI-1 promoter fragments (positions −305 to +75 and positions −716 to −305) were cloned in both orientations in a *Bam*HI site directly upstream of an enhancerless SV40 early promoter fused to the CAT gene (pSV232-ACAT) (24). The CAT activity is expressed as the percentage of the CAT activity of the RSV promoter construct. Bent arrows indicate the location and direction of transcription initiation.

and mung bean nuclease protection analyses showed that the RNA transcripts derived from construct p6.4kLUC in the FTO2B cells initiate at the same position as the PAI-1 mRNA transcripts in human endothelial cells (data not shown). Fragments containing upstream sequences from 100 to 549 bp were induced ≈10-fold by dexamethasone. A further increase in fragment length to 800 bp increased the level of induction to ≈40-fold. This ratio was maintained up to a fragment length of 3.4 kb, after which the ratio for the longest fragment (6.4 kb) dropped to 17-fold. These data show that there are two regions in the promoter that mediate a dexamethasone-dependent increase in promoter efficiency. The first, located between nucleotides −100 and +75, increases promoter activity ≈10-fold. The second is located between nucleotides −800 and −549 and mediates an additional 4-fold increase in promoter efficiency. Regions that appear to contribute to the general promoter strength (i.e., dexamethasone-independent activity) were also identified. The nucleotides −187 to −100 may be one such region, since its absence reduces the promoter strength by a factor of ≈2 (compare p100LUC to p187LUC).

Table 1. Relative activity of PAI-1 promoter constructs in Ltk⁻, BAE, HeLa, and FTO2B cells

	Relative activity of PAI-1 promoter constructs				
Plasmid	Ltk⁻	BAE	HeLa	FTO2B	FTO2B/Dex
p19LUC	<1	<1	<1	<1	<1
pRSVLUC	100	100	100	100	160
p187LUC	93	191	7	3	25
p1.5kLUC	57	111	4	3	80

Promoter activities of the PAI-1 promoter luciferase constructs are expressed as the percentage of the luciferase activity expressed under control of the RSV promoter. The promoter activity data were corrected for possible differences in DNA uptake by normalizing to an internal control (pRSVCAT, CAT assay). Each number represents the average value of at least six independent transfection experiments. Dex, dexamethasone.

The region located at positions −549 to −305 mediates a similar effect.

Glucocorticoids have been shown to regulate the transcription of a variety of viral and eukaryotic genes through a direct interaction of the hormone—receptor complex with specific high-affinity DNA-binding sites in or near the promoters of the regulated genes (36). These DNA-binding sites or glucocorticoid responsive elements (GREs) behave like inducible enhancers since they confer glucocorticoid inducibility to genes, independent of their position or orientation. Experiments were performed to determine whether the PAI-1 promoter also contains a glucocorticoid-dependent enhancer. PAI-1 promoter fragments (nucleotides −305 to +75 and nucleotides −716 to −306) were cloned in both orientations in the *Bam*HI site upstream of an enhancerless SV40 promoter fused to the CAT gene (pSV232ACAT). The functional properties of these promoter constructs were then determined by measuring the effect of dexamethasone on CAT expression in transfected FTO2B cells (Fig. 5*B*). As expected, when the first 305 bp of the PAI-1 promoter was cloned in the syn conformation (pSV232ACATA), CAT expression of the double promoter became dexamethasone inducible. CAT expression from the construct that contains the same fragment in the anti orientation was also induced by dexamethasone. Thus, dexamethasone inducibility was conferred to the enhancerless SV40 promoter by this fragment. The upstream fragment (positions −716 to −306) did not appear to contain a glucocorticoid-dependent enhancer, since this fragment was unable to confer dexamethasone inducibility to the heterologous promoter in either orientation (pSV232ACATC and pSV232ACATD).

DISCUSSION

Mung bean nuclease protection experiments and sequence analysis of the 5′ noncoding region of the PAI-1 gene identified the site(s) of transcription initiation (Fig. 2) and indi-

cated a perfect TATA box (Fig. 3) at a conserved distance from the cap site (30). Fragments containing these sites harbored strong promoter activity and displayed tissue specificity similar to that of the endogenous PAI-1 genes in four cell types (Table 1). These data indicate that we have isolated and characterized the PAI-1 promoter and exclude the possibility of the presence of an additional exon at the 5′ end of the gene.

The induction of PAI-1 biosynthesis in rat hepatoma cells (HTC cells) by glucocorticoids has been shown to require concomitant RNA and protein synthesis (16). In addition, it was found that the PAI-1 response was identical to that observed for the glucocorticoid receptor-mediated induction of tyrosine aminotransferase biosynthesis (16). These observations suggested that the induction of PAI-1 in HTC cells was also mediated by the glucocorticoid receptor. The increase in PAI-1 mRNA in response to dexamethasone was not affected by the presence of cycloheximide (14), suggesting that the induction of PAI-1 mRNA may have resulted from a direct effect of the glucocorticoid–receptor complex itself on the PAI-1 gene (i.e., without the prior induction of other regulatory proteins). In the current study, we have provided further information concerning the nature of the DNA sequences involved in this interaction. A fragment containing the first 305 bp of the PAI-1 promoter, in both orientations (pSV232ACATA and pSV232ACATB, Fig. 5*B*), rendered the heterologous enhancerless SV40 promoter dexamethasone-inducible. This result clearly shows the presence of a glucocorticoid-dependent enhancer element in the PAI-1 promoter fragment. Although not proven, we assume that the same element is responsible for the dexamethasone induction of the small promoter fragment present in plasmid p100LUC. One observation in favor of this assumption is that the promoter activities of plasmids pSV232ACATA, pSV232-ACATB, and p100LUC were induced to about the same extent by dexamethasone (Fig. 5). In spite of the good evidence for the presence of a GRE between positions −100 and +75 of the PAI-1 promoter, no good homology with the GRE consensus sequence (i.e., 5′-GGTWCAMNNT-GTYCT-3′, where W is adenine or thymine, M is adenine or cytosine, Y is a pyrimidine, and N is any base) (38) was detected in this region. The best homology to this partially palindromic GRE sequence within the first 100 bp of the promoter was the sequence 5′-GGAACA-3′ (positions −64 to −59) whose complementary strand (5′-TGTTCC-3′) resembles one-half of a GRE. Thus, direct binding studies will have to be performed to determine the exact location of the GRE. In addition to the GRE in the region between positions −100 and +75, a second region in the promoter located between nucleotides −800 and −549 was observed to raise the dexamethasone-induction ratio of the promoter another 4-fold. The mechanism by which this region increases the dexamethasone induction of the promoter is still unclear since no GRE activity was detected in the region between nucleotides −716 and −306 (Fig. 5*B*). These results raise the possibility that a second GRE, or at least a part of it, is present between nucleotides −800 and −717. Alternatively, a mechanism that does not involve direct binding of the glucocorticoid receptor may be responsible for this extra induction.

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