Localization of Regulatory Elements Mediating Constitutive and Cytokine-stimulated Plasminogen Gene Expression*

Received for publication, March 14, 2002, and in revised form, July 29, 2002
Published, JBC Papers in Press, July 30, 2002, DOI 10.1074/jbc.M202509200

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The activity of plasmin, the major enzyme responsible for dissolving fibrin clots, is regulated by plasminogen activators, plasminogen activator inhibitors, α2-antiplasmin, and inflammatory mediators. Recent studies suggest that plasmin activity can be regulated also at the level of plasminogen gene expression. In this study, we characterized the murine plasminogen promoter and 5′-flanking region. The major transcription start site was identified at −83 bp relative to the ATG translational initiation codon. A series of 5′-flanking sequences up to 2400 bp upstream of the transcription initiation site were fused to the luciferase reporter gene and transfected into hepatocytic cells. A 106-bp 5′-flanking region of the murine plasminogen gene demonstrated sufficient functional promoter activity in plasminogen-expressing cells. IL-6 treatment stimulated luciferase activity driven by the 5′-flanking region and an intact consensus IL-6-responsive element at −791, was required for maximal stimulation by this cytokine. These results indicate the presence of regulatory elements in the 5′-flanking region of the murine plasminogen promoter that may regulate murine plasminogen gene expression and, hence, plasmin activity.

Plasminogen is the zymogen of the serine protease plasmin, which is the major enzyme responsible for degrading fibrin clots (1). Plasmin activity is regulated by the presence of plasminogen activators, plasminogen activator inhibitors, α2-antiplasmin, and inflammatory mediators (2, 3). Recent studies from our laboratory and others suggest that plasmin activity can be regulated also by the modulation of plasminogen gene expression (4–7).

Plasminogen is synthesized primarily in the liver (8–11) as an 810-amino acid residue polypeptide chain. Murine plasminogen contains two additional amino acid residues at positions 543 (Ser) and 587 (Gly). Like human plasminogen, murine plasminogen is converted to plasmin by cleavage of a single Arg-Val peptide bond by plasminogen activators (either tissue plasminogen activator or urokinase) (12). Murine plasmin is composed of a 562-amino acid heavy chain (derived from the amino terminus of plasminogen) that is disulfide-linked to a 231-amino acid light chain (derived from the carboxyl terminus of plasminogen). The catalytic domain contained in the 231-amino acid light chain is 84% identical when comparing murine and human plasminogens (13).

Several reports suggest that plasminogen is an acute phase reactant (14–18). In addition to its function in fibrinolysis, plasminogen participates in a variety of physiological processes including wound healing (19, 20), vascular remodeling (21, 22), and leukocyte migration (23). In recent years, the murine model has been used extensively to study both physiological and pathological processes associated with plasminogen deficiency. Pathobiological conditions associated with plasminogen deficiency that are observed in both plasminogen–/– mice and homozygous plasminogen-deficient humans include thrombotic disease (24–26) and ligneous conjunctivitis (27–29).

Previous studies conducted in our laboratory demonstrated that plasminogen mRNA expression is increased in primary murine hepatocytes treated with interleukin 6 (IL-6).1 Furthermore, mice injected with IL-6 exhibit increases in hepatic plasminogen mRNA and circulating plasminogen levels, compared with mice injected with saline (4). In the present study, we have sequenced the 5′-flanking region 2600 bp upstream of murine plasminogen exon I, delineated the transcriptional start site, and defined the minimal promoter region required for constitutive expression of the murine plasminogen gene in hepatocytic cells. Our studies demonstrate that a 106-bp fragment of the 5′-flanking region of the murine plasminogen gene is sufficient to confer transcription in plasminogen-expressing cell lines. We also show that IL-6 stimulates murine plasminogen gene expression and have identified cis-acting elements in the plasminogen promoter that may

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1 The abbreviations used are: IL-6, interleukin-6; IL6-RE, interleukin-6-responsive element; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; PLPR, plasminogen 5′-flanking region; NF, nuclear factor; C/EBPα, CCAT/enhancer binding protein β; HNF-1, hepatic nuclear factor 1; HLF, hepatic leukemia factor; AP-1, activator protein 1; Oct-1, octamer factor 1. 
provide a mechanism for IL-6-mediated functional regulation of the plasminogen gene in vivo.

**EXPERIMENTAL PROCEDURES**

**Murine Plasminogen Promoter and 5′-Deletional Constructs**—A DASH II 129/SvJ murine genomic library was screened by *in situ* hybridization using a 32P-labeled 580-bp EcoRI-NsiI fragment from murine plasminogen cDNA (13) containing the amino-terminal portion of the cDNA through the second kringle domain. Positive phase clones were isolated and screened by polymerase chain reaction (PCR) for the presence of exons I and II using the following exon-specific primer pairs: (a) mPLE1-5′ (5′-CCGGCTGTGGGTGCTCAGTCG-3′) and mPLE3-1′ (5′-CTGGTTCAAGGCAAGAG-3′) corresponding to nucleotides 1–21 and 73–125 of the murine plasminogen cDNA and (b) mPLE2-5′ (5′-GGGACTCAGTGGATGGCTA-3′) and mPLE2-3′ (5′-TTTACATTGGCCAAAGAC-3′) corresponding to nucleotides 78–107 and 73–136 of the murine plasminogen cDNA (13). An 11.5-kb SstI fragment of murine plasminogen DNA (data not shown) was excised from purified phage DNA by restriction enzyme digestion with *Sac I* (24). The fragment was inserted into the Bluescript II plasmid, and correct orientation, upstream of the luciferase reporter gene. All of the constructs had the anticipated DNA sequences. The expression of the experimental reporter gene was normalized to the promoterless control vector, pGL2/Basic, to monitor the background level of luciferase expression. As a positive control for transfection efficiency, separate cell cultures were transfected with the pGL2/5SV40 plasmid, which contains an SV40 promoter/enhancer and expresses high levels of luciferase activity.

To further study the cellular specificity of the murine plasminogen gene promoter responsiveness, we transfected the 106-bp mPLP CR construct in the hepatic cell line, Hepa 1–6, and nonhepatic IMR-32 (neuroblastoma) and Caco-2 (colorectal adenocarcinoma) cell lines.

In all experiments, plasminogen promoter constructs were cotransfected with the Renilla luciferase reporter, pRL-TK (Promega) at a ratio of pGL2/hPLPR to pRL-TK DNA of 4:1 (Promega) and 4:1 (Promega) and 4.9:1 (Promega). The expression of the experimental reporter gene was normalized to the activity of the Renilla luciferase reporter gene and expressed as normalized -fold change in luciferase activity relative to the activity of the pGL2/Basic control plasmid.

**Cell Lines and Cell Culture**—Hepa 1–6 murine hepatoma cells and Nor-10 murine skeletal muscle cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Biowhittaker, Walkersville, MD) containing 4 mmol/liter l-glutamine (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT). Hep G2 human hepatocellular carcinoma cells (Promega) were grown and maintained in Eagle’s minimal essential medium (Biowhittaker) supplemented with 2 mmol/liter glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), and 10% FBS. Human breast carcinoma MCF-7 cells were obtained from the ATCC and grown in RPMI 1640 medium (Biowhittaker) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 10% FBS. JAR-12 human neuroblastoma cell lines (ATCC) and Caco-2 human colorectal adenocarcinoma cells (ATCC) were grown and maintained in Dulbecco’s modified Eagle’s medium supplemented with 1.5 mmol/liter sodium bicarbonate (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1.0 mM sodium pyruvate (Invitrogen), and 10% FBS. All cell lines were grown in 162-cm² culture flasks (Corning Inc., Corning, NV) at 37 °C in a humidified atmosphere of 5% CO₂.

**RNA Isolation and Primer Extension**—Total RNA was harvested from livers of C57Bl/6 male 5-week-old mice using the guanidinium isothiocyanate procedure (32). For identification of the murine plasminogen transcription-initiation site, two oligonucleotide primers: 5′-GCAAGTCGCAATCTTGGCGCTG-3′ and 3′-TCATTGTCAGTGGTGCTGTCAGTTGCGCC-3′, complementary to nucleotides −13 to +13 of the cDNA sequence of murine plasminogen (13), were used. The methionine initiation (ATG) codon of the murine plasminogen gene was designated as nucleotide +1 (Fig. 1). The primer extension phosphorylation reaction contained, in a total volume of 10 μl, 10 pmol of primer, 50 mmol/liter Tris-HCl (pH 7.5), 10 mmol/liter dithiothreitol, 0.1 mmol/liter spermidine, 5% (v/v) [γ-32P]ATP (3000 Ci/mol (Amersham Biosciences)), and 10 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The mixture was incubated at 37 °C for 10 min, and then heated to 90 °C for 2 min to inactivate the T4 polynucleotide kinase. The primer-extension hybridization reaction contained the following in a final volume of 11 μl: 10–30 μg of total liver mRNA, 1 × 10⁵ cpm oligonucleotide primer, 10 mmol/liter Tris-HCl (pH 8.3), 50 mmol/liter KCl, 10 mmol/liter MgCl₂, 10 mmol/liter dithiothreitol, 1 mmol/liter each deoxynucleoside triphosphate, and 0.5 mmol/liter spermidine. The primer was annealed to the mRNA by heating to 65 °C for 5 min and allowed to cool slowly to 22 °C. Reaction was carried out in a final volume of 20 μl with the addition of 2 mmol/liter sodium pyrophosphate plus 1 unit of avian myeloblo-

**Transfections and Reporter Assays**—Hepa 1–6, Nor-10, Hep G2, and MCF-7 cells were transiently transfected, separately, with each of six constructs: 1) pGL2/mPLP1, 2) pGL2/mPLP2, 3) pGL2/mPLP3, 4) pGL2/hPLPR, 5) pGL2/Basic, or 6) pGL2/5SV40 (Promega). At 90% confluence, cells were transfected with 6 μg of DNA in 12-well plates using LipofectAMINE 2000 (Invitrogen) according to the instructions from the manufacturer. In a separate set of experiments, Hepa 1–6 and Nor-10 cells were transiently transfected with the murine deletional constructs (as described above). Separate cultures of cells were also transfected with the promotorless control vector, pGL2/Basic, to monitor the background level of luciferase expression. As a positive control for transfection efficiency, separate cell cultures were transfected with the pGL2/5SV40 plasmid, which contains an SV40 promoter/enhancer and expresses high levels of luciferase activity.

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**Treatment of Hepa 1–6 Cells with Murine Interleukin 6 (mIL-6)—** Hepa 1–6 cells transfected with the pGL2/mPLP constructs were grown in 12-well cluster plates containing in each well (3.8 cm²) 1.5 ml of DMEM supplemented with 10% FBS and 4 mmol/liter l-glutamine. At 75–90% confluence, cells were rinsed with phosphate-buffered saline and then grown in serum-free DMEM supplemented with 0.1% bovine serum albumin and 4 mmol/liter l-glutamine. For the murine IL-6 dose-response study, recombinant murine IL-6 (mIL-6; Sigma) was added in concentrations from 0 to 750 units/ml. Cell extracts were assayed for luciferase activity 48 h after addition of mIL-6 to the cells. At 48 h, Hepa 1–6 cells transfected with Hepa 1–6 cells were incubated with 500 units/ml mIL-6. Cell extracts were then assayed for reporter gene activity at 0 (no treatment), 24, 48, and 72 h after addition of mIL-6 (500 units/ml) to the cells. As a positive control for IL-6 stimulation, cells were transfected with a pGL2/fibrinogen construct. pGL2/fibrinogen consisted of a 200-bp fragment of the β-chain of fibrinogen cloned into the pGL2/Basic plasmid in the correct orientation, upstream of the luciferase reporter gene. The effects of IL-6 stimulation...
on the serial 5'-deletional constructs of the murine and human plasminogen promoters were examined as well.

Site-directed Mutagenesis of the Interleukin-6-responsive Element (IL6-RE)—The pGL2–1.7 kb and pGL2–2.4 kb (mPLPR–2400/multiILRE) mutated plasmids were constructed using the QuikChange site-directed mutagenesis kit according to the instructions from the manufacturer (Stratagene, La Jolla, CA). The site-specific mutated constructs were made using 25 ng of wild-type plasmid templates and the sense oligonucleotide 5'-AAACGGAATTCATTACACCTGCAAGACT3' in combination with the antisense oligonucleotide 3'--GGTTCCTGAGATTGTGAGATCTGAGAGG-5' in a final volume of 50 μL. Each construct was sequenced to confirm the incorporated mutation (Retrogen, San Diego, CA).

Statistical Analysis—All data are presented as means ± S.E. of the mean. Statistical significance (p < 0.05) in all dual luciferase reporter assays was determined via one-way analysis of variance followed by the Student-Newman-Keuls post hoc test.

RESULTS

DNA Sequence of the 5'-Flanking Region of the Murine Plasminogen Gene—The sequence of the 5'-flanking region 2600 bp upstream of murine plasminogen exon 1 was determined (Fig. 1). The TRANSFAC data base was used to search for transcription factor binding sites. Putative binding sites for the liver-enriched transcription factors: CCAAT/enhancer binding protein β (C/EBPβ or nuclear factor IL-6, NF-IL6), hepatic nuclear factor 1 (HNF-1) (34), and hepatic leukemia factor (HLF) were present in the 2600-bp murine plasminogen promoter sequence (Fig. 1). The sequence of the 2600-bp murine plasminogen gene promoter was aligned with the published sequence of the human plasminogen promoter. Comparison of 2600 bp of the 5'-flanking region of the murine gene with the corresponding sequence of the human plasminogen gene (35) showed 50% identity. In the 5'-flanking region spanning −250 bp relative to the ATG start site (designated as +1), comparison between the murine and human plasminogen 5'-flanking region showed 70% identity. As shown in Fig. 2, within this region, a number of putative regulatory elements were conserved, notably for liver-specific transcription factors C/EBPβ, HNF-1, HLF, and ubiquitous factors activator protein 1 (AP-1) and nuclear factor κB (NF-κB). These data suggest the existence of a similar regulation pathway by binding factors in these two species.

Determination of the Transcriptional Start Site of the Murine Plasminogen Gene—The transcriptional start site of the murine plasminogen gene was determined by primer extension analysis. The end-labeled [γ-32P]ATP primer, 5'-GCACCTTGACAACTGTTTCTC-3', complementary to nucleotides −37 to −18, was used in extension reactions with total RNA from murine liver (the major site of plasminogen synthesis) and Nor-10 skeletal muscle cells (negative control). The major extension product was located 83 bp upstream from the ATG initiation codon (designated as +1) and ended at a T residue, thus identifying −83 as the major transcription initiation site in the murine liver (Fig. 3). No bands were detected when the extension reaction was performed with RNA from the negative control, Nor-10 skeletal muscle cells (data not shown). The same start site was identified when a radiolabeled 26-oligonucleotide primer, 5'-CCTATGTTCCATGTGGGATGCGCC-3', complementary to nucleotides −13 to +15 was used (data not shown).

Functional Analysis of the Murine Plasminogen Promoter and 5'-Flanking Region in Hepatocytic Cells—The ability of the 1064-bp murine plasminogen promoter and 5'-flanking region to drive expression of a luciferase reporter gene was examined and directly compared with a human plasminogen promoter of similar length that we have characterized previously, pGL2/hPLPR (4). To determine whether the murine plasminogen 5'-flanking region could confer liver-specific transcription, four cell lines (Hepa 1–6, Nor-10, Hep G2, and MCF-7) were transfected with each of six constructs: 1) 1064-bp pGL2/mPLPR, 2) 1064-bp pGL2/mPLPR', 3) 1067-bp pGL2/hPLPR, 4) 1067-bp hPLPR', 5) pGL2/Basic, and 6) pGL2/SV40. The dual luciferase reporter assay was employed for the quantitative measurement of plasminogen promoter activity. Hepa 1–6 cells transfected with the pGL2/mPLPR construct expressed luciferase activity that was 4.6-fold higher (p < 0.001) than cells transfected with the promoterless vector control, pGL2/Basic (Fig. 4A). No induction of luciferase activity relative to the activity of pGL2/Basic was observed in Hepa 1–6 cells transfected with pGL2/mPLPR' (Fig. 4A), a construct that is identical to pGL2/mPLPR except that the 1064-bp plasminogen 5'-flanking region is cloned in the reverse orientation. Luciferase expression driven by the positive control for transfection efficiency, pGL2/SV40, increased 123-fold (p < 0.05) in Hepa 1–6 cells (data not shown). To investigate cell specificity of the murine plasminogen promoter, we transfected cells of the Nor-10 murine skeletal cell line with the pGL2/mPLPR construct. (Plasminogen expression is not detectable in murine skeletal muscle (11)). As shown in Fig. 4B, there was no statistically significant difference in luciferase activity in Nor-10 murine skeletal muscle cells transfected with either pGL2/mPLPR or pGL2/mPLPR when compared with cells transfected with pGL2/Basic. Luciferase expression driven by pGL2/SV40 increased 17-fold (p < 0.05) in Nor-10 cells when compared with cells transfected with the pGL2/Basic construct (data not shown).

We also examined the activity of the murine 5'-flanking region in human Hep G2 cells, a representative hepatoma line. Hep G2 cells transfected with the pGL2/mPLPR construct exhibited luciferase activity 32-fold greater than that of cells transfected with the pGL2/Basic construct (Fig. 4C, p < 0.001). Luciferase expression by cells transfected with pGL2/mPLPR was not significantly increased compared with cells transfected with the pGL2/Basic construct (Fig. 4C). Hep G2 cells transfected with pGL2/SV40 provided a 504-fold stimulation (p < 0.05) of luciferase activity compared with pGL2/Basic (data not shown). As a control to examine cell specificity, MCF-7 (human breast carcinoma) cells were transfected with the pGL2/mPLPR construct. As shown in Fig. 4D, there was no significant difference in luciferase activity in MCF-7 cells transfected with either pGL2/mPLPR or pGL2/mPLPR' when compared with cells transfected with pGL2/Basic (p > 0.05). Luciferase expression driven by pGL2/SV40 increased 104-fold (p < 0.05) in MCF-7 cells when compared with cells transfected with the pGL2/Basic construct (data not shown). Taken together, these results suggest that 1064 bp of the 5'-flanking region of the murine plasminogen gene are sufficient to confer liver-specific transcription.

When we compared the activities of the pGL2/mPLPR with a construct containing the proximal 1067 bp of the human plasminogen 5'-flanking region (pGL2/hPLPR), luciferase expression driven by the murine and human constructs differed by less than 2-fold in Hepa 1–6 cells (Fig. 4A). There was no statistical difference in the induction of luciferase activity between Hep G2 cells transfected with either the 1064-bp pGL2/mPLPR or the 1067-bp pGL2/hPLPR (Fig. 4C). Hepa 1–6 and Hep G2 cells transiently transfected with the 1067-bp pGL2/hPLPR also showed no statistical difference in the induction of luciferase activity compared with the pGL2/Basic control (Fig. 4, A and C). The results indicate that the 5'-flanking regions of the murine and human plasminogen genes contain sequences that control the expression of these genes, and that constitutive promoter function is orientation-dependent. These data also suggest that the 1064-bp murine and 1067-bp human plasminogen promoter regions exhibit similar activities and, because
the overall level of induction was much greater in Hep G2 cells than in Hepa 1–6 cells, that levels of transcription factors in the two cell lines, Hepa 1–6 and Hep G2, may differ.

Transient Transfections with Deletional Constructs of the Murine Plasminogen Gene 5′-Flanking Region—Luciferase expression plasmids containing a series of murine 5′-flanking region.

**FIG. 1.** Nucleotide sequence and putative regulatory elements of the 5′-flanking region of the murine plasminogen gene. The coding sequence of the first exon is indicated by a box. Putative regulatory elements identified by a TRANSFAC database search are designated by either overlining or underlining the sequence. Numbers refer to the nucleotide position relative to the ATG translational initiation codon (designated as +1).
sequences immediately upstream of the ATG translational initiation site were constructed, and their abilities to drive luciferase expression were compared in both Hepa 1–6 and Nor-10 cells. The 5′-flanking constructs ranged in size from 106 to 2400 bp. The construct containing the first 106 bp of the 5′-flanking region of the murine plasminogen gene drove luciferase expression in Hepa 1–6 cells that was not statistically different from the luciferase expression driven by the 2400-bp construct (Fig. 5A). Thus, minimal promoter activity was contained in the first 106 bp upstream from the transcription initiation site. A deletion from −699 to −500 bp resulted in a 2-fold increase in luciferase activity, suggesting the presence of a repressor element in this region. Further deletion from −499 bp to −403 bp led to a 2-fold decrease in promoter activity, suggesting the presence of an enhancer element within this region. In negative controls, none of the constructs exhibited increased luciferase expression compared with the pGL2/Basic construct, when transfected into Nor-10 cells (data not shown).

The results show that the 106-bp minimal mPLPR construct was sufficient to increase luciferase activity in Hepa 1–6 cells. The data also suggest that there are negative and positive cis-acting regulatory elements within 2400-bp of the 5′-flanking region of the murine plasminogen gene.

We also examined promoter activities of serial 5′-deletional human plasminogen promoter (hPLPR) constructs transfected into Hep 3B cells (Fig. 5B). A sequence consisting of the first 189 bp upstream of exon I of the human plasminogen gene, exhibited minimal promoter activity. A deletion from −708 bp to −515 bp resulted in a modest increase in luciferase activity, suggesting the presence of a repressor element within this region. Further deletion of sequences from −514 bp to −290 bp led to a 1.5-fold decrease in promoter activity suggesting the presence of an enhancer element within this region. Luciferase activity of MCF-7 cells transfected with the hPLPR deletional constructs did not significantly differ from the reporter gene activity of cells transfected with the pGL2/Basic construct (data not shown). These results suggest that the murine and human plasminogen 5′-flanking regions contain similar positive and negative cis-acting regulatory elements involved in liver-specific transcriptional activity of the plasminogen promoter.

To examine whether the 106-bp minimal promoter region of the murine plasminogen gene confers liver specificity, we transfected the 106-bp mPLPR construct into two nonhepatic plasminogen-expressing cell lines, IMR-32 (neuroblastoma) and Caco-2 (colorectal adenocarcinoma) (36) and compared luciferase expression with transfected Hepa 1–6 cells. As shown in Fig. 6, luciferase expression by cells transfected with the construct containing the first 106 bp (relative to the transcription initiation site) of the 5′-flanking region of the murine plasminogen gene was significantly increased (p < 0.05) compared with cells transfected with the pGL2/Basic construct in all three cell lines consistent with plasminogen expression by these cells. In addition, the minimal 106-bp mPLPR construct drove luciferase expression in each of the three plasminogen-expressing cell lines that was not statistically different from luciferase expression driven by the mPLPR-2400 construct (p > 0.05). These data suggest that a 106-bp fragment of the 5′-flanking region of the murine plasminogen gene is sufficient to direct transcription in plasminogen-expressing cells but sequences within this region do not confer liver specificity of plasminogen expression.

Stimulation of Plasminogen Promoter Activity in Hepa 1–6 Cells by Murine IL-6—We have demonstrated previously that interleukin 6 increases plasminogen mRNA levels in primary murine hepatocytes (4). In addition, mice injected with IL-6 exhibit increases in hepatic plasminogen mRNA and circulating plasminogen levels compared with mice injected with saline (4). To examine whether the murine pGL2/mPLPR construct behaved as the endogenous gene, we tested whether cells transfected with the 1064-bp pGL2/mPLPR construct could respond to mIL-6 (murine IL-6). Hepa 1–6 cells were transfected with the 1064-bp pGL2/mPLPR, 1064-bp mPLPR, or pGL2/Basic and grown in the presence of increasing concentrations of mIL-6 for 48 h. The maximal increase in luciferase activity expressed by cells transfected with the 1064-bp pGL2/mPLPR construct was achieved with 500 units/ml mIL-6 (2.2-fold) compared with untreated cells (Fig. 7A). As a positive control, Hepa 1–6 cells transfected with the pGL2/fibrinogen construct and incubated with 500 units/ml mIL-6 for 48 h exhibited an 2.3-fold increase in luciferase activity compared with untreated cells (data not shown). The maximal concentration of 500 units/ml mIL-6 is similar to the concentration at which maximal stimulation of human plasminogen mRNA expression is achieved in primary murine hepatocytes with human IL-6 (4). In a separate set of experiments, a time-dependent increase in murine plasminogen promoter activity was also observed in Hepa 1–6 cells in response to mIL-6 treatment. As shown in Fig. 7B, Hepa 1–6 cells transfected with the 1064-bp mPLPR construct and incubated with 500 units/ml mIL-6 exhibited maximal stimulation of luciferase activity compared with untreated cells (4.8-fold) at 48 h (p < 0.05). Hepa 1–6 cells transfected with the positive control, pGL2/fibrinogen, and incubated with 500 units/ml mIL-6 for 48 h exhibited a significant 7.7-fold stimulation (p < 0.05) in luciferase activity compared with untreated cells (data not shown). These results show that the 1064-bp 5′-flanking region of the murine plasminogen promoter...
minogen promoter behaves as the endogenous gene, with regard to the response to IL-6 treatment of the cells. Experiments were then performed to localize the region(s) in the murine plasminogen promoter that mediate IL-6-dependent stimulation in Hepa 1–6 cells. The location of putative IL-6-responsive elements present in the murine plasminogen promoter region are depicted in Fig. 8A. Hepa 1–6 cells were transfected with the series of mPLPR 5'/H11032-deletional constructs and then incubated for 48 h in the presence of 500 units/ml mIL-6 prior to measuring luciferase activity. One region in the murine plasminogen gene appeared to predominantly regulate the increased gene expression in response to IL-6 treatment. The level of IL-6-dependent stimulation fell from 3.4- to 1.5-fold when the region from 1063 to 700 was deleted. This region contains a C/EBP(NF-IL6) consensus sequence beginning at 791 bp (relative to the ATG codon). The 106-bp mPLPR construct, which contains two IL-6-responsive elements, positioned at 139 and 173 bp, respectively, did not significantly respond to IL-6 stimulation compared with the response of the promoterless vector (p > 0.05) (Fig. 8B). In addition, the presence of 144 bp upstream of the 106-bp minimal promoter (250-bp construct containing an additional consensus IL-6-responsive element positioned at 206 bp) did not significantly alter IL-6 responsiveness compared with the promoterless vector. These results suggest that the three putative IL-6-responsive elements present in the region from 250 to 83 relative to the ATG codon may not play a major role in the IL-6 inducibility of the murine plasminogen gene in Hepa 1–6 cells.

To further investigate whether the IL6-RE motif at −791 plays a role in the induction of murine plasminogen gene expression by interleukin-6, Hepa 1–6 cells were transfected with either the wild-type (intact IL6-RE) 1712-bp mPLPR or 2400-bp mPLPR or mutant (containing a 3-bp mutated IL6-RE binding site) mPLPR-1712/mutIL6RE or mPLPR-2400/mutIL6RE constructs or the promoterless control vector, pGL2/Basic. The mutation within the putative IL6-RE binding motif is shown in Fig. 9A. IL-6-treated Hepa 1–6 cells transfected with either the mPLPR-1712/mutIL6RE or mPLPR-2400/mutIL6RE constructs did not exhibit increased luciferase activity compared with promoterless control vector, pGL2/Basic.
Results are shown with murine plasminogen (Panel A) and human plasminogen (Panel B) promoter deletions. The measurement of luciferase activity between Hepa 1–6 and Hep 3B cells, respectively. Promoter activities were compared with that of the promoterless control vector, pGL2/Basic. Results represent mean ± S.E. (n = 4–11 transfections). *, p < 0.05, compared with the pGL2/Basic plasmid.

**Fig. 5.** Comparison of promoter activities of murine and human plasminogen constructs. Results are shown with murine plasminogen (A) and human plasminogen (B) promoter deletions. The murine and human deletional constructs were transiently transfected into Hepa 1–6 and Hep 3B cells, respectively. Promoter activities were compared with that of the promoterless control vector, pGL2/Basic. Results in panels A and B are given as mean ± S.E. (n = 3–15 transient transfections).

Basic (Fig. 9B). Under these conditions, IL-6-treated Hepa 1–6 cells transfected with either the wild-type mPLPR-1712 or mPLPR-2400 constructs exhibited significantly (p < 0.05) increased luciferase expression (1.8- and 2.2-fold, respectively) compared with cells transfected with the control vector alone (Fig. 9B) similar to the extent of stimulation at 24 h as shown in Fig. 7B. There was no statistical difference in the induction of luciferase activity between Hepa 1–6 cells transfected with either mPLPR-1712 or mPLPR-2400 constructs. These results suggest that the interleukin-6-responsive element positioned at −791 bp is essential for stimulation of plasminogen gene expression in response to IL-6.

**Discussion**

An emerging area of research has demonstrated that the presence and regulation of plasminogen gene expression in various tissue and cell types plays a critical role in numerous physiologic and pathologic processes (19–29). Therefore, the elucidation of the molecular mechanisms involved in the modulation of plasminogen gene expression requires the identification and characterization of the transcriptional regulatory regions of the plasminogen gene. In addition, the recent characterizations of mice deficient in plasminogen (24, 25) provided an impetus for the study of the structure and function of the murine plasminogen promoter, to assess the applicability of the murine model to the human system. In the present study, we have determined the sequence 2.6 kb upstream from exon I of the murine plasminogen gene, identified the transcription initiation site, demonstrated cis-regulatory elements sufficient to direct tissue-specific regulation of the gene, and localized the minimal promoter region required by plasminogen-expressing cells. In addition, we demonstrated that expression of the murine 5′-flanking region was increased in response to IL-6 treatment, mimicking the function of the endogenous gene in vivo. Furthermore, we have localized a major region in the murine 5′-flanking sequence that is predominantly responsible for mediating the response to IL-6.

There is a distinct tissue-specific pattern of expression of the plasminogen gene with the liver being the predominant site of plasminogen synthesis (8–11). We found that a 1064-bp murine plasminogen 5′-fragment cloned upstream of the luciferase reporter gene drove luciferase expression in the murine hepatoma cell line, Hepa 1–6, and the human hepatoblastoma cell lines, Hep G2 and Hep 3B. In controls, luciferase expression was not increased compared with the vector alone in cells that do not express plasminogen, murine Nor-10 skeletal muscle cells and human breast carcinoma MCF-7 cells. Furthermore, we demonstrated that the murine plasminogen minimal promoter was active in plasminogen-expressing cell lines IMR-32 and Caco-2 (36). Thus, the ability of the 5′-flanking region of the murine plasminogen promoter to drive luciferase expression is consistent with the known tissue expression of the plasminogen gene.

The promoter functions of the 1064-bp murine plasminogen 5′-fragment and a 1067-bp human plasminogen 5′-fragment (previously described from our laboratory (Ref. 4)) were similar. Compared with the promoterless vector, the luciferase activities driven by both murine and human 5′-flanking regions were both ~32-fold in human Hep G2 cells; ~6- and ~13-fold, respectively, in human Hep 3B cells (data not shown); and ~5- and ~9-fold, respectively, in murine Hepa 1–6 cells. Thus, interspecies promoter strengths were similar, although differences in the stimulating activities of the hepatocytic cells were observed. A single transcription start site for
the murine plasminogen gene was identified 83 bp upstream of
the ATG initiation codon. This result is similar to the utiliza-
tion of a single transcription start site in the human plasmin-
ogen gene (35). Minimal murine plasminogen promoter activity
was contained within the first 106 bp upstream of the tran-
scription initiation site in Hepa 1–6 cells as well as the plas-
minogen-expressing cell lines Caco-2 and IMR-32 (36). Thus,
this region is sufficient to direct plasminogen transcription in
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basal and inducible transcription of a variety of genes associ-
ated with the acute phase response (37–39).

Alignment between the 5′-flanking regions spanning 250 bp
from the ATG start site of the murine and human plasminogen
genomes showed a high overall degree of identity (70%). Within
this region and upstream of −106, a number of putative regu-

FIG. 7. Dose response and time courses for the effect of mIL-6
on mPLPR activity in Hepa 1–6 cells. A, cells were transiently
transfected, separately, with each of three promoter constructs (●,
1064-bp mPLPR, ○, 1064-bp mPLPR; ■, pGL2/Basic) and incubated in
the presence of increasing concentrations of mIL-6. Luciferase activity
was measured after 48-h treatment with mIL-6. Results are expressed
as mean ± S.E. relative to the pGL2/Basic control. B, Hepa 1–6 cells
were treated with 500 units/ml mIL-6 and incubated for 0 (no treat-
ment), 24, 48, or 72 h. Results in panels A and B are given as mean ±
S.E. (n = 5–11 transient transfections). *, p < 0.05, compared with the
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this region and upstream of −106, a number of putative regu-
latory elements were conserved, notably for liver-specific tran-
scription factors C/EBP, HLF, and HNF-1 (34). Further stud-
ies are needed to determine whether binding of these
transcription factors plays a role in the high level of plasmin-
genon expression in hepatic versus nonhepatic tissues (11).

Results obtained with transfection studies using murine
plasminogen promoter deletion constructs provided insight
into the regions involved in plasminogen gene expression, and
it is of interest to compare these results with those obtained
with the human promoter. Deletional analysis revealed that
the murine plasminogen promoter contains a negative (−699 to
−500 bp) cis-regulatory element within 2400 bp of the 5′-
flanking region of the murine plasminogen gene. Two possible
candidates for transcriptional repressors are octamer factor 1
(Oct-1) and activator protein 1 (AP-1). Oct-1 and AP-1 are
regions. Similar structure/function relationships observed for both the murine and human plasminogen promoter may suggest broad applicability of murine models to further investigate plasminogen transcriptional regulation and its potential role in human physiology and pathophysiology.

Plasminogen gene regulation in response to inflammatory mediators and cytokines has not been addressed in detail in the literature. However, several reports suggest that plasminogen behaves as an acute phase reactant (14–18, 47, 48). The acute phase mediator, IL-6, is induced following induction of the acute phase response (49, 50). We have shown previously that mice injected with IL-6 exhibit increases in hepatic plasminogen mRNA; consequently, circulating plasminogen levels are significantly higher in mice injected with IL-6, compared with mice injected with saline (4). Furthermore, primary murine hepatocytes treated with IL-6 also increase plasminogen mRNA expression (4).

Using reporter gene functional analysis, we characterized the IL-6-responsive elements of the murine plasminogen gene. A 1064-bp mPLPR construct conferred the strongest response to IL-6 stimulation in transfected murine hepatoma Hepa 1–6 cells. The experimental data correlate with the observations in the human plasminogen promoter wherein IL-6 stimulation results in a 4.5-fold increase in 1067-bp hPLPR expression in human hepatocarcinoma Hep 3B cells (4). The level of IL-6 induction of murine plasminogen gene expression was significantly decreased upon deletion of the region from −1063 to −700 bp, suggesting the presence of a functional IL-6-responsive element in this region. Mutation of the putative NF-IL6 consensus sequence positioned at −791 bp to −783 bp, relative to the ATG codon, abolished responsiveness of the murine plasminogen 5′-flanking region to IL-6, suggesting that the wild-type sequence is necessary for IL-6-stimulated plasminogen gene expression.

Recently we conducted a tissue survey for plasminogen mRNA expression in mice (11) and found that plasminogen mRNA is expressed broadly extrahepatically at low levels. Plasminogen mRNA is present in adrenal, kidney, brain, testis, heart, lung, uterus, spleen, thymus, and gut (11). The brain, testis, and the thymus cortex are separated from the circulation by anatomic barriers so that plasminogen synthesis within these tissues should provide an exclusive source of plasminogen. Two recent reports have demonstrated the regulation of plasminogen expression in such extrahepatic tissues not exposed to circulating plasminogen. Kainic acid stimulates plasminogen mRNA and protein levels in rodent hippocampal neurons (5, 6), and interleukins-1α and -1β increase levels of plasminogen mRNA and protein in the cornea (7). Thus, regulation of the plasminogen gene may be particularly important at these sites of extrahepatic plasminogen synthesis.

Analysis of regulatory elements within the murine plasminogen 5′-flanking region that modulate both extrahepatic constitutive plasminogen synthesis and stimulation of plasminogen synthesis by inflammatory mediators in both liver and in extrahepatic cells is a promising new area of investigation that should provide key insights into the physiologic and pathophysiologic functions of plasminogen.

**Acknowledgments**—We thank Drs. Nicholas M. Andronicos, Neill Gingles, and Michael C. Bannach for helpful discussions.

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