The Hematopoietic Transcription Factor PU.1 Represses Gelatinase A Transcription in Glomerular Mesangial Cells*

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The matrix metalloproteinase gelatinase A plays a key role in the evolution of glomerular injury and is a major contributing factor to the development of glomerulosclerosis. Prior studies have focused on a potent cis-acting enhancer element located in the near 5′-flanking region of the rat and human gelatinase A genes (Harendza, S., Pollock, A. S., Mertens, P. R., and Lovett, D. H. (1995) J. Biol. Chem. 270, 18286–18796; Mertens, P. R., Alfonso-Jaume, M. A., Steinmann, K., and Lovett, D. H. (1999) J. Am. Soc. Nephrol. 10, 2480–2487). Given the combinatorial nature of transcriptional regulation, we examined additional regions of the 5′-flanking region of the rat gelatinase A gene to identify further regulatory elements. In this study the identification of a silencing element located between −1903 and −1847 base pairs of the 5′-flanking region of the rat gelatinase A gene is reported. Sequence analysis, electrophoretic mobility studies, and transfection experiments demonstrate that a specific binding sequence for the hematopoietic transcription factor PU.1 is present within the silencing sequence. PU.1 activity is absolutely required for the expression of silencing activity within the context of transfected glomerular mesangial cells. Western blots identify the PU.1 protein within nuclear extracts of mesangial cells, and cotransfection with a PU.1 expression vector directly augments silencing activity. These studies underscore the complex patterns of gelatinase A transcriptional regulation and also strongly suggest that glomerular mesangial cells are ultimately derived from bone marrow cells.

Gelatinase A (also denoted MMP-2 or 72-kDa type IV collagenase) is an important member of the large family of matrix metalloproteinases that have been ascribed key roles in multiple biologic processes, including embryogenesis, wound healing, and neoplasia. As with all members of this family, gelatinase A is secreted in a latent proenzyme form, is dependent upon zinc for catalytic activity, and is inhibited by a family of low molecular weight proteins, the tissue inhibitors of metalloproteinases (4). Gelatinase A is secreted in vitro by many cells of mesenchymal or neoplastic origin and displays highly regulated expression during murine embryogenesis (5). Numerous studies have defined a critical role for gelatinase A in renal inflammatory processes affecting both the glomerulus and interstitium (6, 7). For example, enhanced gelatinase A synthesis by a proliferating glomerular mesangial cell population is characteristic of a model of immune-complex-mediated glomerulonephritis in rats (6, 8) and in chronic glomerulosclerosis in a variety of human renal diseases (9). The functional significance of enhanced gelatinase A synthesis during these processes was underscored by the observation that inhibition of gelatinase A expression in glomerular mesangial cells blocks cellular proliferation and interstitial scar collagen formation (10). Recent studies with hepatic stellate cells, a myofibroblast that closely resembles the mesangial cell, have also demonstrated that gelatinase A is a direct determinant of cellular proliferation and interstitial collagen formation (11, 12).

Given the functional significance of gelatinase A expression for multiple glomerular disease processes, our laboratories have focused on a detailed analysis of the transcriptional regulatory mechanisms that determine gelatinase A expression by the mesangial cell type. We initially identified a potent 80-bp enhancer element, denoted RE-1, located at −1342 to −1262 bp relative to the translational start site of the rat gelatinase A gene that drives in a cell-specific manner high level gene expression (1). Subsequent expression cloning studies have demonstrated the specific interaction of the highly conserved transcription factor, YB-1, with the RE-1 sequence (2). Positive transactivation by YB-1 is further augmented by cooperative interactions with the transcription factor, AP-2, leading to major increases in gelatinase A transcription and translation rates (13). An analogous sequence in the human gelatinase A gene has been recently identified and shown to also interact in a specific manner with YB-1 and AP-2 (3).

Our initial series of studies focused on the near 5′ (up to −1686 bp)-flanking region of the human and rat gelatinase A genes, and the current investigation was designed to identify additional regulatory sequences located in the further up-stream regions of the rat gelatinase A gene. In this report we define a specific silencer element located at −1869 to −1845 bp relative to the translational start site that specifically interacts with the hematopoietic transcription factor PU.1. Overexpression of PU.1 results in highly significant silencing of gelatinase A gene transcription and further underscores the complex combinatorial nature of the regulation of this important gene.

Received for publication, February 16, 2000
Published, JBC Papers in Press, April 12, 2000, DOI 10.1074/jbc.M001322200

* This work was supported by Deutsche Forschungsgemeinschaft Grant HA 2056/3-2 (to S. H.) and National Institutes of Health Grant DK 39776 (to D. H. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); PMA, phorbol myristoyl acetate; NRK, normal rat kidney; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; PE1, polyethyleneimine; EMSA, electrophoretic mobility shift analyses; MC, mesangial cells.
In brief, 50 kDa PEI (Sigma) was prepared as a neutralized, et al. fragment extending from the second intron in the 5'-direction has been previously characterized in detail (1–3). The adjacent PatI KpnI fragment 2 is the object of the current study.

EXPERIMENTAL PROCEDURES

Cell Culture—Rat glomerular mesangial cells were isolated and characterized as reported in detail (6). Mesangial cells were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Human monocytic leukemia U937 cells and rat renal fibroblast NRK cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in the same growth medium as defined above. For this study the adjacent 5' PatI-KpnI 1311-bp fragment (see map in Fig. 1) was subcloned into pBluescript KS+ (Stratagene) and sequenced.

Luciferase Reporter Constructs—Plasmid pT4-Luc1686 consists of 1686 bp of the immediate 5'-flanking region of the rat gelatinase A gene subcloned into the promoterless luciferase expression vector, pGL2-Basic (Promega), as reported in detail (1). For these studies the 1311-bp PatI-KpnI fragment was subcloned into pT4-Luc1686 5’ to the 1686-bp insert (see diagram of constructs, Fig. 2). This construct is denoted pT4-Luc2A2997. A series of truncation constructs extending over the 1311-bp PatI-KpnI segment was prepared by polymerase chain reaction using the PatI_KpnI fragment as a template. These constructs were terminated at -2783, -2563, -2343, -2123, and -1903 bp relative to the translational start site and are denoted pT4-Luc2A2686, pT4-Luc2A2486, pT4-Luc2A2286, pT4-Luc2A2086, and pT4-Luc1A903, respectively. A second series of deletion constructs was prepared with 5’ terminations at -1947 and -1791 bp, and the constructs are denoted pT4-Luc1A1847 and pT4-Luc1A1791, respectively. The sequence between bp -1867 and -1903 was also subcloned in the reverse orientation into pT4-Luc1868 and is denoted pT4-Luc1A1903(Inv) (see diagram of constructs, Fig. 2).

The pT4-Luc1686 construct includes the strong enhancer element located between -1342 and -1262 bp (1). To assess silencing activity in the absence of this enhancer element, a deletion construct, pT4-Luc1007, which extends to -1007 bp was prepared. The sequence between -1903 and -1867 bp was subcloned into pT4-Luc1007 yielding pT4-Luc1007/1903-1687. The sequences between -1847 and -1687 bp and -1791 and -1687 bp were also subcloned into pT4-Luc1007, yielding pT4-Luc1007/1847-1687 and pT4-Luc1007/1791-1687, respectively.

A final series of constructs was designed to map further the silencer activity located between -1903 and -1792 bp. The sequence between -1903 and -1792 was subcloned into pT4-Luc1007 to yield pT4-Luc1007/1903-1792. Constructs pT4-Luc1007/1903-1847 and pT4-Luc1007/1847-1792 were prepared similarly. Finally, the consensus PU.1-binding motif 5' -GAGGAA-3' in pT4-Luc1007/1903-1847 was mutated to 5' -CTATCG-3', yielding construct pT4-Luc1007/1903-1847mu (see diagram of constructs, Fig. 2).

Transient Transfections—Transient transfection of mesangial cells was performed with polyethyleneimine (PEI) according to Bousif et al. (15). In brief, 50 kDa PEI (Sigma) was prepared as a neutralized, sterile-filtered aqueous 10 mM stock. Triplicate cultures were plated at a density of 100,000 cells/well ( Falcon 6-well dishes) and cultured overnight prior to transfection. The cultures were rinsed twice with phosphate-buffered saline (PBS) and given fresh growth medium with-out serum. Purified pT4-Luc expression plasmids (2 μg/well) and a normalizing PMC-β-galactosidase plasmid (2 μg/well) were diluted in 100 μl of PBS and vortexed. In a separate tube 10.8 μl of PEI stock solution was vortexed into 100 μl of PBS. After 10 min the solutions were mixed, vortexed, and incubated for 10 min at room temperature, followed by addition to the cultures. After 4 h the medium was supplemented with 10% fetal calf serum, and the cells were harvested after an additional 18 h. Luciferase and β-galactosidase assays of cell lysates were performed as described (16, 17). All transfections were performed in triplicate for each construct, and all transfection sets were repeated at least three times. Transfection results were averaged, normalized with the β-galactosidase results, and expressed as the means (S.D less than 15%).

For cotransfection experiments, 1 μg of the PU.1 expression plasmid PU-PECE or control pECE plasmid (the kind gift of Matthew J. Fenton, Boston) was included in the transfection mixture. The effects of bacterial endotoxin (LPS) and PMA on silencer function were assessed by addition of 1 ng/ml LPS or 10⁻⁷ M PMA for the final 6 h of the transfection period.

Electrophoretic Mobility Shift Assay—Nuclear extracts from mesangial cells, phorbol-differentiated U937 cells, and NRK cells were prepared as reported in detail (1). Synthetic oligonucleotides were annealed and end-labeled with polynucleotide kinase and [γ-³²P]dATP according to standard methodology. Nuclear extracts were used at 10 μg of protein/reaction, incubated with oligonucleotides, and electrophoresed as reported in detail (1, 2). For competition experiments, unlabeled oligonucleotides were added to 50-fold molar excess to the nuclear extracts for 15 min prior to addition of labeled oligonucleotides to the reaction mixture. Antibody supershift experiments were performed by preincubation of the nuclear extracts for 1 h at 4 °C with 1–2 μg/ml rabbit polyclonal anti-mouse PU.1 or control rabbit serum (Santa Cruz Biotechnology) prior to addition of labeled oligonucleotide and electrophoresis.

Western Blot Analysis of Nuclear Extracts—Nuclear extracts (50 μg) were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose blots (Hybond ECL, Amersham Pharmacia Biotech). Membranes were blocked in 5% dried milk in wash buffer (1× PBS, 0.1% Tween 20) for 1 h at room temperature, followed by incubation for 1 h at room temperature with wash buffer containing 1 μg/ml rabbit anti-mouse PU.1 antibody. Membranes were then washed three times in wash buffer and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1 μg/mL wash buffer, Southern Biotechnology). Washed blots were then developed using the ECL system (Amersham Pharmacia Biotech) according to the manufacturer’s instructions and exposed to film for 10 min.

RESULTS

Characterization of the Transcriptional Regulatory Activity of the 5' PatI/KpnI Fragment of the Gelatinase A Gene—For our initial investigations of the transcriptional regulation of the gelatinase A gene, a 17-kb genomic clone was isolated from a rat A phase genomic library using a cDNA probe composed of exon 1 (1, 14). The KpnI_NotI fragment, which includes 1866 bp of the 5' regulatory region of the gelatinase A gene, has been extensively characterized (1, 2, 13). These studies have demonstrated a potent, cell-specific 80-bp enhancer element located between -1342 and -1262 bp (1, 2). The adjacent 1311-bp PatI/KpnI fragment (fragment 2 in Fig. 1) was subcloned into
To determine whether the silencing activity identified in sequence −1903 to −1687 was the result of direct suppression of the strong enhancer element located at −1342 to −1262 bp, a second series of deletions was subcloned into plasmid pT4-Luc1007, which does not include the enhancer sequence (Fig. 5B). As observed with the earlier set of deletion constructs, only the construct including −1903 to −1687 bp reduced the luciferase activity of construct pT4-Luc1007. These experiments indicate that the silencing activity of the −1903 to −1687-bp sequence is not dependent upon interactions with the enhancer element and is more probably the consequence of interaction with the proximal promoter.

For further mapping of the silencing activity, an additional series of deletion constructs of the −1903 to −1687-bp region was prepared, using the enhancerless pT4-Luc1007 reporter construct (Fig. 5C). Deletion constructs including the most 5′ 83 and 56 bp (plasmids pT4-Luc1007/1903-1820 and pT4-Luc1007/1903-1847, respectively) had the same degree of silencing activity as obtained with the complete −1903- to −1687-bp fragment. The 27-bp sequence extending from −1847 to −1820 bp (pT4-Luc1007/1847-1820) did not demonstrate any significant silencing activity, thereby mapping the silencer to the sequence spanning −1903 to −1847 bp.

The 56-bp −1903 to −1847 sequence includes the consensus core PU.1-binding site discussed above. In order to determine the functional significance of this site, an additional construct was prepared in which the core PU.1-binding site was mutated to 5′-CTATCG-3′, according to Klemsz et al. (20), creating plasmid pT4-Luc1007/1903-1847mut). This mutated plasmid did not express silencing activity as compared with control pT4-Luc1007, indicating that an intact PU.1 core consensus binding site is required for silencing activity.

**Electrophoretic Mobility Shift Analyses (EMSA)**—EMSA using nuclear extracts from mesangial cells and a synthetic oligonucleotide extending from −1869 to −1845 bp, which includes the PU.1-binding site, was performed. In the presence of
mesangial cell nuclear extract, the radiolabeled −1869 to −1845-bp oligonucleotide showed significant mobility retardation with formation of a single major oligonucleotide-protein complex (Fig. 6, 2nd lane). The formation of the oligonucleotide-protein complex was significantly reduced when the mesangial cell nuclear extracts were preincubated with a specific rabbit anti-PU.1 IgG (Fig. 6, 3rd lane), whereas preincubation with a control rabbit anti-mouse IgG had no significant effect on the formation of the oligonucleotide-protein complex (Fig. 6, 4th lane). The specificity of the oligonucleotide-protein complex formation was further confirmed by competition experiments. A 50-molar excess of cold −1869- to −1845-bp oligonucleotide strongly competed for nuclear protein binding, resulting in the disappearance of the major shifted complex (Fig. 6, 5th lane), whereas a 50-molar excess of the cold −1875 to −1856 oligonucleotide, which lacks the PU.1 consensus binding site, did not compete for complex formation (Fig. 6, 6th lane). A synthetic oligonucleotide extending from −1869 to −1845 was prepared in which the consensus PU.1 site was mutated. Preincubation with a 50-molar excess of this oligonucleotide had no effect on nuclear protein-oligonucleotide complex formation (Fig. 6, 7th lane), providing further confirmation that the PU.1 protein is required for complex formation.

The cell-specific nuclear protein binding activity for the −1869 to −1845 oligonucleotide was investigated by comparing nuclear extracts from mesangial cells, phorbol ester-differentiated U937 monocytic leukemia cells, which express PU.1 protein, and fibroblastic NRK cells, which do not. EMSA with nuclear extracts from mesangial cells and differentiated U937 cells yielded identically retarded complexes following incubation with radiolabeled −1869 to −1845 oligonucleotide (Fig. 7A), whereas nuclear extracts from PU.1-negative NRK cells did not yield oligonucleotide-protein complexes. When a Western blot with anti-PU.1 antibody was performed with the respective nuclear extracts, specific bands of 42 kDa, consistent with the molecular mass of PU.1, were detected in the mesangial and U937 cell extracts but not with the NRK extracts. These studies provide further confirmation for the direct role of the PU.1 protein in the formation of the oligonucleotide-protein complexes.

Transfection with PU.1 or Activation Silences Gelatinase A Gene Transcription—To assess directly the influence of PU.1 on the silencing activity, cotransfection experiments with the luciferase reporter constructs and a eukaryotic expression vector PU-pECE encoding PU.1 were performed. The results of these experiments are summarized in Fig. 8. Cotransfection of the PU-pECE plasmid with the pT4-Luc1686 construct had no significant effect on relative luciferase activity, as compared with cotransfection with a control pECE plasmid. The results of these experiments were summarized in Fig. 8. Cotransfection of the PU-pECE plasmid with the pT4-Luc1686 construct had no significant effect on relative luciferase activity, as compared with cotransfection with a control pECE plasmid. Cotransfection of PU-pECE, but not control pECE, with the pT4-Luc1903 plasmid, which includes the PU.1-binding site, fur-
ther reduced luciferase activity by more than 50%.

The phosphorylation status of the PU.1 protein has been previously shown to affect DNA binding and transcriptional regulatory activity (21, 22). By using hematopoietic cells, incubation with bacterial lipopolysaccharide (LPS) or phorbol ester (PMA) enhances PU.1 activity through protein kinase-dependent pathways (24, 25). To determine whether a similar pathway is operative for PU.1-mediated gelatinase A silencing, cultured mesangial cells were transfected with the reporter constructs pT4-Luc1686 and pT4-LucA1903. Following transfection the cultures were incubated either in control medium, 1 ng/ml LPS, or 1 × 10⁻⁷ M PMA for 6 h. The results of these experiments are means of three independent transfection experiments.

**Fig. 5.** A, transient transfection of deletion constructs of the 217-bp fragment between −1903 and −1687 bp in plasmid pT4-Luc1686 in MC. B, transient transfection with the same set of deletions of the 217-bp fragment used in A subcloned into the enhancerless plasmid pT4-Luc1007. C, transcriptional activity of the 217-bp fragment and its subfragments (hatched boxes) including mutations (black box) subcloned into pT4-Luc1007 in MC. All data are given as ratios of luciferase (LUC) versus β-galactosidase activities with construct pT4-Luc1007 assigned a value of 1. Results are means of three independent transfection experiments.
rection for transfection efficiency with expression of construct pT4-Luc1686 was given a value of 1 after conversion vector PU-pECE. The vector pECE served as an empty control plasmid. Data are given as ratios of luciferase versus β-galactosidase activities with construct pT4-Luc1686 assigned a value of 1. Results are means of three independent transfection experiments.

FIG. 8. Luciferase expression of constructs pT4-Luc1686 and pT4-LucA1903 in MC after cotransfection with the PU.1 expression vector PU-pECE. The vector pECE served as an empty control plasmid.

Displacement of PU.1-binding sequence, GAGGAA, which is flanked on the 3' aspect by GG. The GAGGAAGG sequence is present in the promoters of the FcγRI and FcγRIIIA genes and specifically binds PU.1 (23). Mutation of this sequence in the gelatinase A gene resulted in complete loss of silencing and EMSA activity. Furthermore, studies with a highly specific anti-PU.1 antibody confirmed the presence of PU.1 protein binding with the GAGGAAGG-containing oligonucleotide, whereas Western blot analysis of mesangial cell nuclear extracts specifically detected the 42-kDa PU.1 protein. Cotransfection with a PU.1 expression plasmid significantly enhanced the silencing activity of this sequence, as did incubation with bacterial endotoxin and phorbol esters. Bacterial endotoxin and phorbol esters have been demonstrated to increase the transcriptional activity of PU.1 through casein kinase II- or protein kinase C-mediated PU.1 phosphorylation (24, 25).

PU.1 is an important member of the large Ets family of transcriptional regulatory proteins. PU.1 expression is generally considered to be restricted to cells of hematopoietic lineage, including stem cells, macrophages, B-cells, and neutrophils (20, 27–29). Glomerular mesangial cells are multipotential pericytes that can execute macrophage-like functions, including phagocytosis, release of reactive oxygen species, and antigen presentation (30, 31). These properties have given rise to speculation that mesangial cells derive from the bone marrow, a speculation that has been recently confirmed using transplanted green fluorescent protein-expressing transgenic bone marrow cells (32). Hence, the observation in this report that cultured glomerular mesangial cells express PU.1 protein is consistent with a hematopoietic origin for these cells.

Most studies examining the transcriptional regulatory activity of PU.1 have demonstrated positive transactivation. For example, PU.1 enhances transcription of a large group of genes involved in myeloid differentiation, including the macrophage colony-stimulating factor receptor, the macrophage scavenger receptor, and the common β subunit of the interleukin-3, granulocyte-macrophage colony-stimulating factor, and interleukin-5 receptors (29, 33–36). PU.1 has also been shown to repress transcription of a much more limited set of genes, including CD11c integrin, the c-myb, gene and the I-Aβ gene (37–39). Thus, PU.1-mediated gelatinase A transcriptional silencing in mesangial cells may be but one component of a larger group of PU.1-regulated genes in this cell type.

Transcriptional silencers have been found in a number of genes, including vimentin, thyrotropin-β, plasminogen activator inhibitor type-2, platelet-derived growth factor, and c-fos (26, 40–43). It has been suggested that bound silencer proteins repress transcription by interfering at a distance with the core proximal promoter, by interfering with an enhancer element directly, or by interfering with enhancer-core promoter interactions (19). The behavior of the PU.1-silencing element in the gelatinase A gene is most consistent with the first model, since core proximal promoter activity was repressed in the absence of the enhancer element. A similar pattern has been demonstrated for the vimentin silencer (40).

In summary, this study has characterized a PU.1-binding silencer element in the 5'-flanking region of the rat gelatinase A gene. Recent sequence analysis of the human gelatinase A gene has detected a highly homologous sequence within the same region, suggesting that the current observations are applicable to regulation of the human gene as well.2 These studies underscore the complex patterns of gelatinase A transcriptional regulation within glomerular mesangial cells and provide further support for the macrophage-like nature of this critical inflammatory effector cell.

FIG. 9. Transient luciferase expression in MC obtained with construct pT4-Luc1686 or pT4-LucA1903 and coincubation for 6 h with either LPS (1 ng/ml) or PMA (1 × 10−7 M). The luciferase expression of construct pT4-Luc1686 was given a value of 1 after correction for transfection efficiency with β-galactosidase expression. Experiments were performed in triplicate, and data are expressed as means.

experiments are shown in Fig. 9. Treatment with either LPS or PMA had no significant effect on the transcriptional activity of the pT4-Luc1686 plasmid, whereas both reagents significantly increased the silencing activity contained with the pT4-LucA1903 plasmid, which includes the PU.1-binding site. Taken together with the PU.1 transfection experiments, these studies provide direct experimental evidence for the involvement of PU.1 in the silencing activity mapped to −1903 to −1847.

DISCUSSION

Enhanced gelatinase A expression is a critical component of the glomerular inflammatory process and is directly associated with the development of sclerosis and loss of renal function. Thus, a comprehensive understanding of the transcriptional regulation of this gene within the context of glomerular mesangial cells is an important experimental goal. In this study, we have further analyzed the 5'-flanking region of the rat gelatinase A gene and have identified the transcription factor PU.1 as a potent silencer of gelatinase A transcription. The silencer activity was localized to a 56-bp sequence located between −1903 and −1847 bp relative to the translational start site. Inspection of this sequence revealed the consensus core PU.1-binding sequence, GAGGAA, which is flanked on the 3' aspect by GG. The GAGGAAGG sequence is present in the

2 D. H. Lovett, unpublished observations.
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J. Biol. Chem. 2000, 275:19552-19559.
doi: 10.1074/jbc.M001322200 originally published online April 12, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001322200

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