Involvement of Sp1 Elements in the Promoter Activity of the α1-Proteinase Inhibitor Gene*

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The transcripts of the α1-proteinase inhibitor in the cornea are different from those in hepatocytes and monocytes, suggesting that α1-proteinase inhibitor gene transcription may respond to different cell-specific regulatory mechanisms. Although information on α1-proteinase inhibitor gene structure has been obtained, little is known regarding the cis- and trans-acting factors that regulate its expression. In this study, we cloned and sequenced a 2.7-kilobase 5′-flanking region upstream from the corneal transcription initiation site of the gene, demonstrated functional promoter activity, and identified the regulatory elements. Sequencing revealed that the 5′-flanking element was highly G/C-rich in regions proximal to the corneal transcription start site. DNase I footprinting located 10 potential Sp1-binding sites between nucleotides −1319 and +44. The putative promoter was functional in human corneal stromal cells, but not in human skin, scleral, and conjunctival fibroblasts, suggesting that the promoter may be corneal cell-specific. The promoter activity in the corneal cells was repressed when Sp1 was coexpressed. In the cornea-thinning disease keratoconus, down-regulation of the α1-proteinase inhibitor gene and increased Sp1 expression have both been demonstrated. The current results suggest that down-regulation of the inhibitor in keratoconus corneas may be related directly to overexpression of the Sp1 gene. This information may help elucidate the molecular pathways leading to the altered α1-proteinase inhibitor expression in keratoconus.

Proteinase inhibitors are critical in preventing and controlling proteolysis. The α1-proteinase inhibitor (α1-PI) is a major protease inhibitor in human serum (1). One of its primary physiologic roles is to protect the elastic fibers in lung alveoli from excessive digestion by neutrophil elastase (2, 3). The importance of this protein was proposed in the 1960s based on observations that genetically α1-PI-deficient patients developed an early-onset degenerative lung disease (4) or a liver disease (5). The liver is the predominant site of α1-PI synthesis (1). This protein is also found synthesized in blood monocytes and macrophages (6), alveolar macrophages (7), intestinal epithelial cells (8), and human breast carcinoma (9). Recently, the synthesis of α1-PI by human corneal cells has been reported (10).

The cornea, located in the anterior portion of the eye, is a transparent connective tissue made up of epithelial, stromal, and endothelial layers. The balance between proteinases and proteinase inhibitors is believed to play a significant role in maintaining normal cellular contents and normal function of the cornea (10). A proper level of α1-PI may protect the cornea from degradation by neutrophil elastase during inflammation. Additionally, α1-PI may function as a backup inhibitor for other serine proteinases such as plasmin and cathepsin G in the cornea.

In an ocular disease called keratoconus, α1-PI expression is reduced to one-third to one-fifth of the normal level, and the expression of degradative enzymes is increased (11, 12). This disease is a noninflammatory disorder that progressively thins and distorts the central portion of the cornea and leads to visual impairment (13). The etiology is unclear, although one hypothesis is that the abnormality in keratoconus may lie in the degradative pathway of macromolecular constituents in the cornea (14). A reduction in the α1-PI level certainly would have a direct impact on the degradation processes, contributing to keratoconus conditions.

The α1-PI gene has been localized on chromosome 14. It contains seven exons: Ia, Ib, Ic, and II–V (15). α1-PI transcripts that comprise different numbers of exons have been identified. The α1-PI mRNA from the liver and intestinal epithelium contains five exons (Ic and II–V), with a single transcription start site in the middle of exon Ic (16). The major promoter elements for this transcript are found in exon Ic and in the intron between exons Ib and Ic. In blood monocytes, multiple transcripts exist; all seven exons can be expressed, or exons Ib and Ic can be spliced out (17). In addition, three transcription initiation sites (Fig. 1) were noted: two in exon Ia and the third in exon Ib (16). The promoter region upstream from exon Ia has not previously been studied.

In the cornea, alternatively spliced forms have been found by reverse transcription-polymerase chain reaction (PCR). One of these forms is similar in size to that found in monocytes that has exons Ib and Ic spliced out, and the other involves an alternative splicing between exons Ib and Ic. The transcription initiation site for the cornea-specific form (Fig. 1) is ~2 kb upstream from the hepatocyte site between the two macrophage sites in exon Ia. The translation start site, ~7.4 kb

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‡ The abbreviations used are: α1-PI, α1-proteinase inhibitor; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s); SEAP, secreted alkaline phosphatase.

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downstream from the corneal transcription initiation site (18), is the same for hepatocytes, monocytes, and the cornea. The use of different transcription start sites for the α1-PI gene and the alternative splicing in different cells suggest that the gene transcription may respond to cell-specific regulatory mechanisms (17–20). Although liver-specific α1-PI gene regulation has been intensively studied (19, 20), there is relatively little information regarding the cis- and trans-acting factors directing α1-PI gene expression in extrahepatic cell types.

In this study, a 2.7-kb 5′-flanking DNA upstream from the corneal transcription initiation site of the α1-PI gene was sequenced; its functional activity in corneal cells was demonstrated; and the regulatory elements of this putative promoter were investigated. Emphasis was placed on the binding sites for transcription factor Sp1 and gene regulation by Sp1 because the Sp1 level has been found to be increased 10–15-fold in keratocous corneas (21). Our results suggest that the 5′-flanking element identified may be cornea-specific, that Sp1 appears to be involved in regulation of the promoter activity, and that the down-regulation of the α1-PI gene observed in keratocous corneas may be directly related to the overexpression of Sp1. This information may help elucidate the molecular pathways leading to the altered α1-PI expression in keratocous.

EXPERIMENTAL PROCEDURES

Materials

Corneal stromal cells were cultured from normal human corneas obtained from the Illinois Eye Bank. The corneal cells, skin fibroblasts derived from skin tissues, and scleral and conjunctival fibroblasts derived from donor eyes were grown and maintained in Dulbecco’s modified Eagle’s minimum essential medium supplemented with glutamine, 10% (v/v) fetal calf serum, nonessential and essential amino acids, and antibiotics as described previously (22).

For PromoterFinder experiments, a pair of sense strand PCR primers, gene-specific for α1-PI (Gsp1 and Gsp2), were selected through the computer program Oligo Version 4.1 (National Biosciences, Plymouth, MN) from the known human exon 1a genomic DNA sequence of α1-PI (17, 19) and were used to amplify the upstream region. Primer sequences were as follows: Gsp1, GTAGACTTCGGGTGGAGGCAGT; and Gsp2, GGGGAGCTTGGAGCGAGGAGT. Primers were synthesized by Genemed Biotechnologies, Inc. (South San Francisco, CA).

Methods

PromoterFinder, Cloning, and Sequencing—The PromoterFinder DNA Walking kit (CLONTECH, Palo Alto, CA) was used to amplify the upstream region. This kit provides five human genomic libraries, each exhaustively digested with one of five restriction enzymes and manipulated to have specific known sequences attached to either end of all digested fragments. A pair of primers specific for these attached sequences (Ap1 and Ap2) were used in conjunction with the α1-PI gene-specific primers (Gsp1 and Gsp2) in five long PCR reactions (one for each library) using the Expand Long Template PCR system (Boehringer Mannheim). The primary round of PCR was carried out with the outer primers (Ap1 and Gsp1) with seven cycles at 94 °C for 2 s and 72 °C for 3 min, 32 cycles at 94 °C for 2 s and 67 °C for 3 min, and 1 cycle at 67 °C for 4 min using the GeneAmp 2400 Cycler (Perkin-Elmer). One microliter of a 1:50 dilution of the primary PCRs was subjected to a second nested long PCR using the inner primers (Ap2 and Gsp2) with five cycles at 94 °C for 2 s and 72 °C for 3 min, 20 cycles at 94 °C for 2 s and 67 °C for 3 min, and 1 cycle at 67 °C for 4 min. The PCR products were analyzed on a 1.2% agarose gel and cloned into the pGEM-T Easy vector (Promega, Madison, WI). DNA sequencing was performed using the Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp.) and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). The sequences were analyzed by the MacVector computer program.

Southern Blot and Restriction Digestion Analysis—A 2712-bp (−2703 to +9) α1-PI 5′-flanking region obtained from a long PCR was ligated into the multiple cloning sites of the pSEAP2-Basic vector. This plasmid was digested with restriction enzymes and separated on a 1% agarose gel. Southern blotting was performed according to the Boehringer-Mannheim nonradioactive detection protocol using Hybond N membranes (Amersham Pharmacia Biotech). The two DNA probes (probes 1 and 2) were selected and prepared by PCRs using genomic DNA as the template. The DNA sequence of probe 1 (−2695 to −1977) was according to the sequence we identified, and that of probe 2 (−172 to +44) was from the sequence published in the literature (17). Probes 1 and 2 were randomly labeled using a nonradioactive labeling kit. Restriction digests using endonucleases Apol, SmaI, and PstI were performed to further prove the identity of the α1-PI 5′-flanking sequence we identified.

DNAse I Footprinting—Four DNA fragments covering 1563 bp (−1519 to +44) of the α1-PI promoter region were prepared from genomic DNA by PCR (fragment 1, −338 to +44; fragment 2, −782 to −321; fragment 3, −1200 to −731; and fragment 4, −1519 to −1115). They were 5′-end-labeled with [γ-32P]ATP using T4 poly nucleotide kinase. Footprinting was performed using the labeled DNA fragments (30 ng) and human Sp1 transcription factor from Promega (4 or 2 footprinting units) according to the Promega core footprinting protocol. In the competitive footprinting assay, 50- and 100-fold molar excesses of unlabeled Sp1 oligonucleotide competitors (Promega) were included in the reaction mixture. Purine sequence ladders of DNA probes were prepared (23) and subjected to gel electrophoresis on urea sequencing gels adjacent to footprint reactions of the same probe to localize binding site sequences.

Promoter Activity Analysis—The series of secreted alkaline phosphatase (SEAP) vectors available from CLONTECH were used for promoter-reporter plasmid constructions. A 2712-bp (−2703 to +9) α1-PI promoter fragment and a 1406-bp (−1397 to +9) fragment obtained from a long PCR were ligated into the KpnI and BglII multiple cloning sites of the pSEAP2-Basic vector, yielding the p2.7SEAP vector. The plasmids p2.7SEAP−, p1.4SEAP−, pSEAP2-Basic, and pSEAP2-Control (positive control, driven by the SV40 early promoter) and pSV-βgal (Promega) used in cell transfections were purified by Qiagen ion-exchange columns and were partially sequenced or restriction-digested to confirm their identity and orientation. Human corneal stromal cells and skin, scleral, and conjunctival fibroblasts were plated at 0.9 × 10^6 cells/well on six-well plates 24 h before DNA transfection. Three hours before transfection, the dishes received fresh medium. Cells were transfected by the calcium phosphate method using the CalPhos Maximizer transfection kit from CLONTECH. In brief, 20 μg of the test plasmid (p2.7SEAP−, p1.4SEAP−, pSEAP2-Basic, or pSEAP2-Control) along with 2 μg of pSV-βgal vector, used to control transfection efficiency, were mixed with 40 μl of CalPhos Maximizer as recommended by the manufacturer. Some cells also received 2 or 4 μg of Sp1 expression vector pAcpSp1. None of the test plasmids were added to cells serving as negative controls. After incubation with the DNA
mixture for 4 h, fresh culture medium was added to the cells, and the medium was collected 72 h later for SEAP assay. Cells that received no DNA were used as negative controls. For SEAP activity, 15 ml of cell medium was mixed with 45 ml of 1:3 dilution buffer and 60 ml of assay buffer according to the manufacturer's protocol (CLONTECH). For the enzyme activity, the absorbance was read for Luminometer (Wallac, Gaithersburg, MD). For β-galactosidase assays, cells were harvested, washed in phosphate-buffered saline, resuspended in 150 ml of 0.25 M Tris (pH 8.0), lyzed by freezing and thawing, and centrifuged at 14,000 × g for 20 min at 4 °C. The extract was mixed with Galacto-Lysis solution (Tropix Inc., Bedford, MA) as recommended by the manufacturer. Ten microliters of lysate were used for the Bradford protein assay (24). The β-galactosidase activity was used to normalize the SEAP enzyme activity. Assays were performed in triplicate, and each experiment was repeated at least three times. Two-tailed Student's t tests were used to analyze the significance of the data.

Fig. 3. Sequence of the α1-PI 5′-flanking element upstream from the corneal transcription start site. The cytidine (boldface) of CAG (corneal transcription initiation site) is designated as position +1. The underlined sequence shares the same 390-bp sequence published by Perlino et al. (17). The eight boxed DNA segments indicate the potential Sp1 transcription factor-binding sites identified by MacVector. Ten Sp1-binding sites identified by Dnase I footprinting are marked with asterisks. The positions of the Dnase I mapped Sp1 sites are determined from data shown in Fig. 5.
RESULTS

Isolation and Sequencing of the Corneal α1-PI 5'-Flanking Element—The corneal 5'-flanking region of the human α1-PI gene was isolated using the PromoterFinder kit (Fig. 2). Three products (2.6, 2.0, and 0.8 kb) were obtained from the EcoRV library. Two PCR fragments (0.6 and 0.28 kb) were obtained from the ScaI and PvuII libraries, respectively. From the SspI library, three PCR fragments (1.3, 1.0, and 0.7 kb) resulted. No product was observed in the DraI library.

All the PCR products were ligated into the pGEM-T Easy vector for sequencing. A total of 2725 base pairs of the 5'-flanking region from the corneal transcription initiation site was sequenced (Fig. 3). The cytidine of CAG (corneal transcription start site) was designated as position +1. The sequence underlined in Fig. 3 shared the same 390-bp sequence published by Perlino et al. (17). An equal distribution of G + C (51%) and A + T pairs (49%) was found in the entire 2725-bp sequence. However, high G + C content (61%) was noted in sequence +522 to +157 proximal to the corneal transcription initiation site. No consensus TATA box was present, but a TATA-like sequence (TCTA) was located at position +259. Eight potential binding sites for transcription factor Sp1 (boxed in Fig. 3), 57 for AP-1, 31 for NF-kB, 59 for the insulin response element, and numerous potential binding sites for other transcription factors were identified in the promoter sequence by the MacVector computer program.

For Southern blot analyses (Fig. 4A), probes 1 and 2 were made from genomic DNA according to the 5'-flanking sequence we identified and the known exon sequence (17, 19), respectively. Both probes recognized a 2.7-kb band, confirming that the DNA sequence we identified was indeed a portion of the α1-PI gene sequence. This was further proved by restriction analysis using endonucleases ApaI, SmaI, and PstI (Fig. 4B, C). The sizes of the bands matched those expected from the α1-PI 5'-flanking sequence we identified and the sequence reported in the literature.
digestion of the p2.7SEAP\textsuperscript{+} plasmid. The recognition sites for ApaI are nucleotides –1408, –868, and –145; for SmaI, nucleotides –661 and –211; and for PstI, nucleotides –1715, –955, and –346 (Fig. 4B). One of the ApaI sites (nucleotide –145) and one of the SmaI sites (nucleotide –211) have been reported previously in the literature (17). The sizes of bands resulting from enzyme digestions (Fig. 4C) matched those expected from the restriction map deduced from the identified sequence.

**Identification of the Sp1-binding Sites**—Sp1 binding to the 5′-flanking element of the α\textsubscript{1}-PI gene was further examined by DNase I footprint analysis. Ten regions in the 1563-bp (–1519 to +44) portion of the 5′-flanking DNA were found to be protected by Sp1 against DNase I digestion (Fig. 5, A–D); regions 1–10 covered from nucleotides –100 to –87; –301 to –290; –409 to –403; –519 to –498; –583 to –579; –622 to –612; –672 to –666; –819 to –793; –932 to –915; and –998 to –987, respectively. The 10 binding sites are marked by asterisks in the sequence shown in Fig. 3. Five of the regions (boxed and marked by asterisks) correlated with the Sp1-binding sites identified by MacVector. The remaining five (marked by asterisks) were G/C-rich regions. The Sp1 binding was specific because the degree of DNase I protection was reduced in the presence of a competing Sp1 oligonucleotide.

**Functional Analysis of the Corneal α\textsubscript{1}-PI 5′-Flanking Element**—The activity of this putative α\textsubscript{1}-PI gene promoter in normal human corneal stromal cells and in skin, scleral, and conjunctival fibroblasts was investigated in transient transfection assays. For corneal stromal cells, DNA fragments containing 1406 bp (p1.4SEAP\textsuperscript{+}) and 2712 bp (p2.7SEAP\textsuperscript{+}) of the 5′-flanking sequence were approximately six times more active at driving SEAP reporter gene expression than the pSEAP2-Basic vector (Fig. 6). No such activity was found, indicating that neither segment of the 5′-flanking DNA was functional in skin, scleral, and conjunctival fibroblasts. When corneal stromal cells were cotransfected with pPacSp1, the level of SEAP expression was markedly reduced (Fig. 7).

**FIG. 5. DNase I protection assay.** Footprinting (A–D, fragments 1–4, respectively) was performed in the absence of human Sp1 transcription factor (lane 1) and in the presence of 4 footprinting units of Sp1 (lane 2), 2 footprinting units of Sp1 and a 50-fold molar excess of unlabeled Sp1 oligonucleotide competitors (lane 3), and 2 footprinting units of Sp1 and a 100-fold molar excess of unlabeled Sp1 oligonucleotide competitors (lane 4). Note the regions (blank areas, boxed) protected by Sp1. Two Sp1 sites were found in A, five in B, and three in C. No Sp1 protection was observed in D.

**DISCUSSION**

This study provides the first comprehensive structural and functional analysis of the 5′-flanking element of the human α\textsubscript{1}-PI gene upstream from the corneal transcription start site. The 2.7-kb region sequenced is characterized by multiple binding sites for transcription factor Sp1 and the absence of a consensus TATA box. Transient transfection experiments showed that the 2.7-kb 5′-flanking DNA is functional in human corneal stromal cells and that the proximal 1400 base pairs are sufficient for full promoter activity. No promoter activity was found for either the 2.7- or 1.4-kb segment in human skin, scleral, and conjunctival fibroblasts, suggesting that the 5′-flanking element we identified may be specific for corneal cells. Consistent with this notion, the cornea-specific alternatively spliced form of α\textsubscript{1}-PI was not found in the skin dermis or the sclera, although the form similar to that identified in monocytes was expressed (data not shown).

Multiple start sites of transcription by primer extension have been found in the human α\textsubscript{1}-PI gene (25). The α\textsubscript{1}-PI gene appears to belong to a class of eukaryotic genes (namely, housekeeping genes) that are constitutively expressed at a basal level. The promoters of these genes are characterized by a high G/C content, by multiple binding sites for Sp1, by the absence of a consensus TATA box, and by the feature that they initiate transcription from multiple sites spread over a fairly large region (26).

Several studies have shown that alternative promoters are used for different α\textsubscript{1}-PI transcripts in hepatocytes and macrophages (17, 27). For hepatocytes, the minimal promoter element required for liver-specific basal expression of human α\textsubscript{1}-PI is confined within 261 nucleotides from the transcription start site (19, 20). Binding sites for transcription factors such as LF-A1/HNF2 (28), LF-B1/HNF1 (29, 30), CCAAT/enhancer-binding protein (20, 30, 31), HNF3 (32), and Sp1 (33) have been noted within this segment, and the first two factors were found
The regulation of the α₁-PI promoter activity in corneal cells was repressed by overexpression of Sp1. This indicates that the Sp1 sites are involved in the regulation of the α₁-PI gene. In this regard, it is of interest to note that in keratoconus corneas, in which gene expression of α₁-PI is reduced, Sp1 expression is found increased in both epithelial and stromal layers. Sp1 binding activity has also been shown to be markedly enhanced in nuclear extracts from the epithelium of keratoconus corneas. The expression of four other transcription factors studied, AP-1, AP-2, cAMP-responsive element-binding protein, and NF-κB, remains unaltered (21). The Sp1 abnormality is also cornea-specific and is not found in either the conjunctiva or the skin. Sp1 is a specific factor originally described as required for SV40 transcription. It interacts with GC boxes in the promoter elements and plays an important role in the expression of many viral and cellular genes (36–38). Recent investigations have shown that the activity and synthesis of Sp1 are subject to a variety of regulations. For example, Sp1 expression is increased during SV40 infection of CV1 cells (39). Although ubiquitously expressed, the level of Sp1 protein expression varies widely among different cell types in the mouse, and increased Sp1 expression has been associated with late stages of differentiation (38). Elevated levels of Sp1 expression have also been noted in gastric carcinoma cells (40), but keratoconus is the only human disease known to exhibit altered Sp1 expression.

Our demonstration that the α₁-PI promoter activity is suppressed by expression of Sp1 in corneal cells suggests that Sp1 may play a significant role in the regulation of the α₁-PI gene in the cornea during development, normal homeostasis, or under pathologic conditions. In the cornea-thinning disease keratoconus, the reduction of the α₁-PI level may be directly related to increased Sp1 expression.

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3 L. Zhou, I. Maruyama, Y. Li, J. Sugar, and B. Y. J. T. Yue, unpublished results.
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