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ARTICLES

Mapping of Sp1 Regulation Sites in the Promoter of the Human α1-Proteinase Inhibitor Gene

Yasuhiro Maruyama, Yuhong Li, Yan Zhang, Xinping Wang, Joel Sugar, and Beatrice Y.J.T. Yue*

Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago College of Medicine, Chicago, Illinois

Abstract Keratoconus is a potentially blinding disease that thins the central cornea. In afflicted corneas, the level of an inhibitor, α1-proteinase inhibitor (α1-PI), is found reduced. An increased expression of transcription factor Sp1 is also demonstrated. To examine the role of Sp1 in regulation of the human α1-PI gene, a 1.4-kb (−1397/+9) 5′-flanking promoter sequence that contains 10 Sp1 sites was cloned. Previous transient transfection experiments showed that Sp1 expression indeed repressed the α1-PI promoter activity. In this study, 12 DNA segments, a series of 5′, 3′, and internal deletions of the 1.4-kb α1-PI promoter sequence, were ligated into the SEAP (secreted alkaline phosphatase) reporter gene vector and transfected into human corneal stromal cells. Co-transfection with a Sp1 expression vector pPacSp1 was also performed in parallel. The SEAP enzyme activity was assayed. A fragment with 489 bp (−480/+9) of the 3′ sequence, and three fragments with internal deletions, were found to confer a majority of the full promoter activity. Other deletions significantly abolished the promoter activity. Site-directed mutagenesis experiments further revealed that the most proximal Sp1 site (−100/−87) may be an essential element involved in the negative regulation of α1-PI promoter activity by Sp1. Interaction between the proximal and distal Sp1 sites also seemed to be important. These results provide the first in-depth characterization of the transcription mechanisms regulating the expression of α1-PI. Mapping of the Sp1 sites may help elucidate the molecular pathway leading to the alterations observed in keratoconus. J. Cell. Biochem. 85: 482–489, 2002.

Key words: cornea; α1-proteinase inhibitor; promoter; Sp1; deletion mutants; site-directed mutagenesis

Protease inhibitors are critical in preventing and controlling proteolysis. α1-Proteinase inhibitor (α1-PI) is a major protease inhibitor in human serum [Travis and Salvesen, 1983]. One of its primary physiologic roles is to protect the elastic fibers in lung alveoli from excessive digestion by neutrophil elastase [Olsen et al., 1975]. The importance of this protein was proposed based on observations that genetically α1-PI-deficient patients developed an early-onset degenerative lung [Eriksson, 1964] or liver disease [Sharp et al., 1969]. The liver is the predominant site of its synthesis [Laurell and Jeppsson, 1975]. α-PI is also found synthesized in blood monocytes, macrophage [Perlmutter et al., 1985], and other extra-hepatic sites including the cornea [Twining et al., 1994], a transparent connective tissue located at the front of the eye.

The human α1-PI gene contains seven exons: Ia, Ib, Ic, and II through V [Brantly et al., 1988]. Multiple transcription initiation sites and α1-PI transcripts that comprise different numbers of exons have been identified [Ciliberto et al., 1985; De Simone et al., 1987]. The use of different transcription start sites and the alternative splicing in different cells suggest that the gene transcription may respond to tissue- or cell-specific regulatory mechanisms [Long et al., 1984; Perlino et al., 1987; Hafeez et al., 1991].

In an ocular disease called keratoconus [Krachmer et al., 1984; Rabinowitz, 1998] that

Abbreviations used: α1-PI, α1-proteinase inhibitor; bp, base pairs; kb, kilobases; SEAP, secreted alkaline phosphatase; SV 40, simian virus 40.

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Yasuhiro Maruyama and Yuhong Li are equal contributors.

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*Correspondence to: Dr. Beatrice Y.J.T. Yue, University of Illinois at Chicago, Department of Ophthalmology and Visual Sciences, 1855 W. Taylor Street, Chicago, IL 60612. E-mail: u24184@uic.edu

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thins and distorts the central portion of the corneal stroma, a markedly reduced expression of the α1-PI gene has been found [Sawaguchi et al., 1990; Whitelock et al., 1997a]. This non-inflammatory disease affects approximately one in two to ten thousand of people in the general population and leads to visual handicap in the productive second and third decades of life [Krachmer et al., 1984; Rabinowitz, 1998]. No specific treatment of this almost universally bilateral disease exists, except to replace the corneal tissues by surgery when visual acuity is impaired beyond correction with contact lenses [Mandell, 1997]. A positive family history in approximately 10% of the cases has been described [Rabinowitz, 1998].

The exact cause of this disease is still not clear. Previous biochemical studies by our group [Yue et al., 1984] and others [Critchfield et al., 1988] have shown that the amount of total protein present in keratoconus corneas is lower than that in normal controls, while protein synthesis proceeds normally in some cases [Yue et al., 1984, 1985]. This led to the formulation of the hypothesis that the abnormality in keratoconus may lie in the degradative pathway of macromolecules [Yue et al., 1985]. Subsequent data support the degradation hypothesis, demonstrating increased levels of degradative enzymes including cathepsins B and G [Sawaguchi et al., 1989; Zhou et al., 1998] and decreased amounts of protease inhibitors including α1-PI and α2-macroglobulin [Sawaguchi et al., 1990, 1994; Whitelock et al., 1997a] in keratoconus specimens compared with those of normal and other disease controls. The up- or downregulation of these genes was noted at both protein and mRNA levels [Whitelock et al., 1997a]. In view of the multiple gene involvement and the possibility of a coordinated gene regulation mechanism, several transcription factors were examined. Among them, Sp1 was found specifically upregulated in keratoconus corneas [Whitelock et al., 1997b].

Sp1 is known to interact with GC/GT boxes in the promoter elements, acting as a positive or a negative regulator for the expression of many viral and cellular genes [Dynan and Tjian, 1983; Ogra et al., 2001]. Although it is ubiquitously expressed, Sp1 has been shown to play a role in cell type-specific regulation of genes such as MUC1 [Morria and Taylor-Papadimitriou, 2001] and involucrin [Banks et al., 1999]. To delineate the role of Sp1 in regulation of the genes affected in keratoconus corneas, we cloned and sequenced a 2.7-kilobase (kb) region of human α1-PI gene upstream of the corneal transcription start site. Transient transfection experiments showed that the 2.7-kb 5′-flanking DNA is functional in human corneal stromal cells and that the proximal 1.4-kb fragment is sufficient for full promoter activity [Li et al., 1998]. More interestingly, co-transfection of Sp1 expression vector with the 1.4-kb fragment in corneal stromal cells suppressed the α1-PI promoter activity [Li et al., 1998]. The negative regulation of Sp1 suggests that the downregulation of the α1-PI gene in keratoconus corneas may be directly related to the abnormally elevated Sp1.

Ten Sp1 binding sites (Sp1-1 to Sp1-10, Fig. 1, left panel) were identified previously in the 1.4-kb promoter region of the α1-PI gene by DNase I footprinting analyses [Li et al., 1998]. To map the elements required for the Sp1 regulation, this α1-PI promoter region was analyzed in further detail in the current study. Twelve deletion mutants of the promoter region were constructed and subcloned into the pSEAP2-Basic plasmid. The recombinants were used in transient transfections in human corneal stromal cells. Site-directed mutageneses were also performed.

MATERIALS AND METHODS

Deletion Constructs

Twelve DNA fragments with serial deletions in the α1-PI promoter region (Fig. 1, left panel), each with a varying number of Sp1 binding sites, were constructed and/or ligated into a SEAP (secreted alkaline phosphatase) reporter gene vector (pSEAP2-Basic; Clontech, Palo Alto, CA). Among 12 new constructs, three deletion constructs (F2: −480/+9, F8: −1397/−884, F9: −1397/−969) were made by PCR using pα1PI1.4SEAP+ [Li et al., 1998] as the template. Three pairs of primers (for F2, forward primer: GGTACCAGCTCCCCACTGCAATCC, reverse primer: AGATCTAGGGTTGCTGCGTGGAGGC; F8, GGTACCCAGAGGCTGCCCGAAGTG and AGATCTAGGGCCCTGCCTGGAGATG; and F9, GGTACCCAGAGGCTGCCCGAAGTG and GGGTAAGGGGGTGAGGTGTGCCAG) were selected through the computer program Vector NTI (InforMax, Inc., Oxford, UK) based on the known promoter sequences [Li et al., 1998]. Primers were synthesized by Genemed

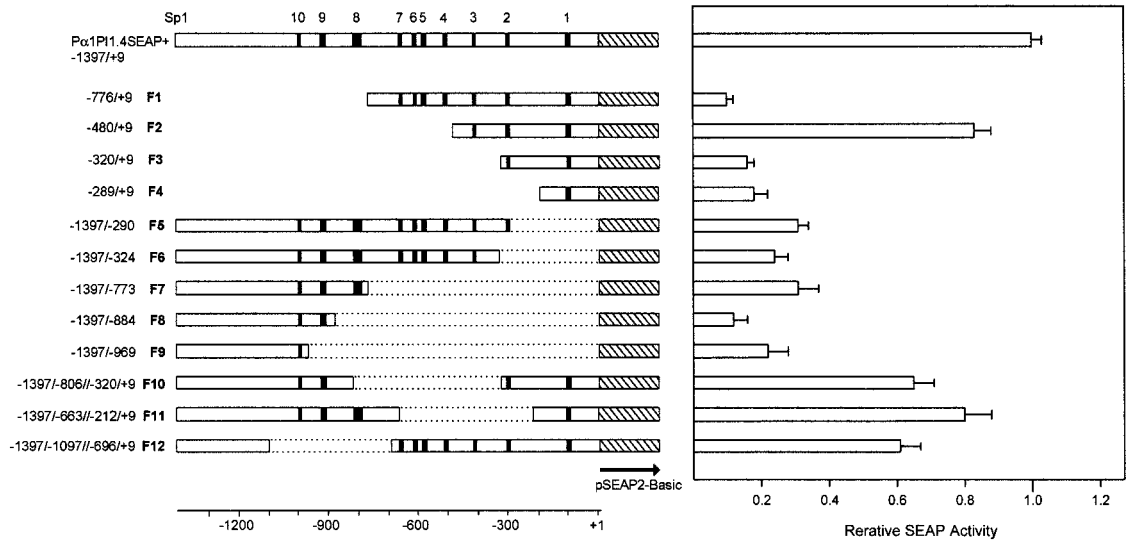

Fig. 1. Deletion analysis of 1.4-kb α1-PI promoter activity. Depicted in the left panel of the figure is a map of the deletion constructs (F1–12) from the 1.4-kb α1-PI promoter region. The 10 Sp1 binding sites (black bars) identified by DNase I foot printing [Li et al., 1998] are numbered on top with Sp1-1 being the most proximal and Sp1-10 the most distal site. The positions of the Sp1 sites are: Sp1-1, −100/−87; Sp1-2, −301/−290; Sp1-3, −411/−403; Sp1-4, −519/−498; Sp1-5, −593/−579; Sp1-6, −622/−612; Sp1-7, −669/−658; Sp1-8, −822/−794; Sp1-9, −932/−915; Sp1-10, −998/−988. Sp1-1, -2, -3, and -8 are the sites selected for mutations. The scale is shown on the bottom. +1 denotes the corneal transcription start site. The relative SEAP enzyme activities in deletion constructs are shown in the right panel of the figure. Normal human corneal stromal cells were transfected with pSEAP2-Basic, pα1PI1.4-SEAP+, or F1 to F12 vectors along with pSV-β-Galactosidase control vector. The positive control, using the pSEAP2-Control vector, yielded more than an order of magnitude higher SEAP activity. The enzyme activities of each construct were normalized against the β-galactosidase enzyme activities. Data were subtracted from the basal activity and were expressed relative to the 1.4-kb construct values. Data (mean ± SEM) presented were those averaged from four different experiments.

Biotechnologies, Inc. (South San Francisco, CA). The PCR products were inserted into pSEAP2-Basic at the multiple cloning sites to yield F2-SEAP+, F8-SEAP+, and F9-SEAP+.

The other nine constructs (F1, F3–7, and F10–12) were made from p1.4α1PI-SEAP+ with the use of restriction enzymes. The enzymes used were: Kpn I and Mfe I for F1 (−776/+9), Kpn I and PfLM I for F3 (−320/+9), Kpn I and Stu I for F4 (−289/+9), Bgl II and Stu I for F5 (−1397/−290), Bgl II and PfLM I for F6 (−1397/−324), Bgl II and Mfe I for F7 (−1397/−773), PfLM I and Mfe I for F10 (−1397/−806//−320/+9, or Δ−805/−321), Xma I for F11 (−1397/−663//−212/+9, or Δ−662/−213), and Bal I for F12 (−1397/−1097//−696/+9, or Δ−1096/−697).

These DNA plasmids, pSEAP2-Basic and pSEAP2-Control (positive control, driven by the SV40 early promoter, Clontech), along with pSV-β-Galactosidase control vector (pSV-βgal, used to control the transfection efficiency, Promega, Madison, WI) and/or Sp1 expression vector pPacSp1 (a generous gift from Dr. R. Tjian, University of California Berkeley) were used in transfection experiments.

Site-Directed Mutagenesis

Mutations in the Sp1 elements were generated using the GeneEditor site-directed mutagenesis kit (Promega) according to the manufacturer's instructions. Mutated oligonucleotides created for four or five nucleotide substitutions (underlined) in the binding motif of Sp1-1, -2, and -3 sites in the 1.4-kb α1-PI fragment were as follows: for Sp1-1 (−100/−87), CCTGTCCAAGCTCCTTTTTCCTCCCCAGCCTACTG; Sp1-2 (−301/−290), GGTCTGTGCCAGGATTTTGAGGCCTGTCATTTC; and Sp1-3 (−411/−403), CAGTGCACACAGACTTTTTTCAACTGTGGGGCTGC. The Sp1-8 site (−822/−794) in deletion construct F11 was also mutated using oligonucleotide GCATTTCCTCTGGTTTGATCAGCCCAGATGC.

These mutagenic oligonucleotides were phosphorylated at the 5′ end and were annealed, along with the same strand selection oligonucleotide for the β-lactamase gene, to

denatured double-stranded p1.4-SEAP+ or F11 to yield respectively α1PI1.4mut1, α1PI1.4-mut2, α1PI1.4mut3, and F11mutD3. This selection oligonucleotide gives an additional resistance for GeneEditor antibiotics selection mix to the mutant. Synthesis of mutant strands was performed by T4 polymerase and T4 ligase, and competent BMH 71–18 *mut* S bacteria were transformed with the extended products. The efficiency of mutagenesis was enhanced by transforming BMH 71–18 *mut* S, because this bacteria is a repair minus strain. Transformed bacteria were selected by growth overnight in liquid medium containing the GeneEditor antibiotic selection mix. The plasmids were isolated and transformed into JM109 bacteria. The transformed bacteria were selected with both ampicillin (125 μg/ml) and GeneEditor antibiotic selection mix. Resistant clones were picked and Sp1 binding-site mutations were confirmed by sequencing.

Transient Cell Transfection

Corneal stromal cells were cultured from normal human corneas (donor ages; 7, 10, 44, and 57 years) obtained from the Illinois Eye Bank, Chicago. They were grown and maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 4 mM glutamine, 10% fetal calf serum, 0.1 mM non-essential and essential amino acids, 1.2 μg/ml amphotericin B, and 10 μg/ml gentamicin [Yue and Baum, 1981; Li et al., 1998]. The tenets of the Declaration of Helsinki for research involving human subjects were followed, and the research was approved by the University of Illinois Institutional Review Board.

For transfection, stromal cells were plated at 4.0×10^4 cells/well on 24-well plates 24 h before DNA transfection. They received fresh medium and were transfected 2 h later using the Fugene 6 transfection reagent (Roche, Indianapolis, IN). In brief, 0.75 μg of the test plasmid and 0.2 μg of pSV-βgal were mixed with 2.85 μl of Fugene 6. Another series of cells also received 0.1875 μg of Sp1 expression vector pPacSp1. None of the test plasmids were added to cells serving as negative controls. The medium and cell lysate were collected 48 h later for SEAP, β-galactosidase, and protein assay.

For SEAP assay, 100 μl of medium was mixed with 300 μl of 1 × dilution buffer. After a 30-min incubation at 65°C, 100 μl of aliquot was mixed with 100 μl each of assay buffer (mixture of phosphatase inhibitors) and reaction buffer (chemiluminescent substrate with luminescence enhancer) according to the manufacturer's protocol (Tropix, Inc., Bedford, MA). The enzyme activity, represented by the light emission, was read for 5 s on a Luminometer (MGM instruments, Inc., Hamden, CT). For β-galactosidase assays, cells were harvested, washed with phosphate-buffered saline, lysed with 70 μl of 100 mM potassium phosphate, 0.2% Triton X-100 and 0.5 mM dithiothreitol, and centrifuged at 16,000*g* for 2 min to pellet cell debris. The supernatant was mixed with chemiluminescent substrate (Galacton-plus, Tropix). Light emission accelerator (Accelerator-II) was injected to each sample 2 s before the measurement. The β-galactosidase activity was used to normalize the SEAP enzyme activity. Assays were performed in triplicate, and each experiment was repeated at least four times. Total protein content in lysates was determined using the BCA protein assay kit (Pierce, Rockford, IL) to control the cell number in each well. Two-tailed student's *t*-tests were used to analyze the significance of the data.

RESULTS

Activity of Deletion Constructs

Twelve DNA segments, containing a series of deletions of the 1.4-kb α1-PI (−1397/+9) promoter sequence, were made. These constructs, together with α1PI1.4-SEAP+, were tested in transient transfection assays. The 1.4-kb promoter fragment was approximately 5.5 times more active at driving the SEAP reporter gene expression than the promoterless pSEAP2-Basic vector used to detect basal activity. As shown in the right panel of Figure 1, F1 (−776/+9), with 761 base pairs (bp) of the 5′ sequence deleted, displayed little promoter activity. A shorter 489-bp (−480/+9) F2 fragment however conferred more than 80% of the full 1.4 kb promoter activity. Fragments F3 (−320/+9) and F4 (−289/+9), with further deletions from the 5′ end, and the F5 to F9 fragments, with a series of the 3′-end sequence deletions, were not sufficient to exhibit much promoter activity. DNA fragments F10, F11, and F12, with internal truncations and containing sequences from both 3′- and 5′-regions, on the other hand, retained most of the promoter activity.

Regulation of Deletion Constructs by Sp1

To examine the Sp1 regulation of the promoter activity of the various deletion constructs, half of the corneal stromal cells were co-transfected with Sp1 expression vector pPacSp1 during transient transfection experiments. Consistent with the results demonstrated previously [Li et al., 1998], co-transfection of pPacSp1 significantly ($P < 0.0001$) reduced the 1.4-kb α1-PI promoter activity. Three other fragments, F2, F11, and F12, that showed positive promoter activities, were likewise found to be negatively regulated by Sp1 co-expression ($P < 0.007$, Table I). The fold of repression varied from 1.9 to 3.6. These Sp1 responsive fragments all contain the most proximal Sp1 binding site (Sp1-1). Other common Sp1 sites included Sp1-2, -3, -8, -9, and -10. One other fragment, F10, also had positive promoter activity. Unlike F2, F11, or F12, however, Sp1 co-expression did not significantly reduce its activity. This F10 construct contains Sp1-1, -2, -9, and -10 sites, but lacks Sp1-3 or -8.

Constructs F1 and F3–F9, all with low promoter activity, were not significantly repressed by Sp1. In the case such as F5, addition of Sp1 even appeared to enhance the activity.

Effects of Sp1 Site Mutations on the α1-PI Promoter Activity

Four different sites (Sp1-1, -2, -3, and -8) were selected for mutation. Vectors α1PI1.4mut1, α1PI1.4mut2, and α1PI1.4mut3 each contained mutations in the Sp1-1, -2, and -3 sites of the 1.4-kb fragment and had promoter activity not significantly different from that of the wild type. While the activity of the 1.4-kb wild type fragment was repressed by co-expression with pPacSp1 (Fig. 2), α1PI1.4mut1 was not (Fig. 2). The Sp1 sensitivity of the 1.4 kb wild type thus appeared to be abolished by mutations in the core of Sp1-1 (CCCGCCCC converted to CTTTTTCC). Constructs α1PI1.4mut2 and α1PI1.4mut3, with mutations in Sp1-2 (AGGGCGAGG to ATTTTGAGG) and Sp1-3 (CCCGCCC to CTTTTTC), respectively, remained negatively regulated by Sp1. Mutations at Sp1-8 (GGGGGA to GTTTGA) in the F11 fragment did not affect much of the F11 promoter activity nor its Sp1 responsiveness (Fig. 3).

DISCUSSION

The current study maps further the promoter regions of the human α1-PI gene. A series of deletions from the 1.4-kb (−1397/+9) corneal promoter regions were made. Transient transfection assays indicate that both the 3′- and the 5′-end sequences are of importance for the promoter activity. When the 5′ sequence is not present, a minimal 3′-end region containing approximately 0.5 kb (−480/+9, F2) sequence upstream of the corneal transcription start site is still sufficient for a majority of the promoter activity.

It is unclear why F1, a fragment (−776/+9) with a longer 3′-end sequence than F2, exhibited

TABLE I. Regulation of Promoter Activities by Sp1

Constructs	SEAP activity with Sp1 relative to that without Sp1 co-transfection	Fold repression	Number of Sp1 sites
pα1PI1.4-SEAP+	0.31 ± 0.03, $P < 0.0001$	3.2	10*
F1	0.80 ± 0.10, NS	—	7*
F2	0.28 ± 0.04, $P < 0.0001$	3.6	3*
F3	0.88 ± 0.06, NS	—	2*
F4	0.61 ± 0.06, NS	—	1*
F5	1.26 ± 0.06, NS	—	9
F6	1.00 ± 0.08, NS	—	8
F7	1.19 ± 0.06, NS	—	3
F8	1.00 ± 0.05, NS	—	2
F9	0.82 ± 0.05, NS	—	1
F10	0.89 ± 0.05, NS	—	4*
F11	0.53 ± 0.06, $P < 0.007$	1.9	4*
F12	0.46 ± 0.02, $P < 0.002$	2.2	7*

Normal corneal stromal cells were transfected with pα1PI1.4-SEAP+, or F1 to F12 vectors along with pSV-β-Galactosidase control vector in the absence or presence of Sp1 expression vector pPacSp1. The SEAP activity with Sp1 co-transfection relative to that without pPacSp1 was presented (mean ± SEM, n = 4). Two-tailed student's *t*-tests were used to analyze the significance of the data. Asterisks denote constructs that contain the most proximal Sp1-1 site. Among the various constructs examined, pα1PI1.4-SEAP+, F2, F10, F11, and F12 showed positive promoter activity.

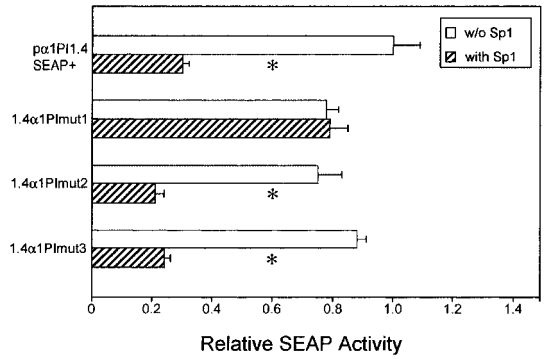

Fig. 2. Effect of mutations of the core of the Sp1 site at −100/−87, −301/−290, and −411/−403 on the activity and Sp1 sensitivity of the 1.4-kb α1-PI promoter. Human corneal stromal cells were transfected with the α1PI1.4-SEAP+ vector, or mutant constructs α1-PImut1, α1-PImut2, or α1-PImut3, along with pSV-β-Galactosidase control vector in the absence or presence of Sp1 expression vector pPacSp1. The SEAP activity was normalized by the β-galactosidase enzyme activity. Data were subtracted from the basal SEAP activity and were expressed relative to the 1.4-kb wild type construct (without pPacSp1) values. Data (mean ± SEM) presented were those averaged from four different experiments. Two-tailed student's *t*-tests were used to analyze the significance of the data. Asterisks denote data significantly different from those obtained from cells transfected without Sp1 co-expression ($P < 0.0008$).

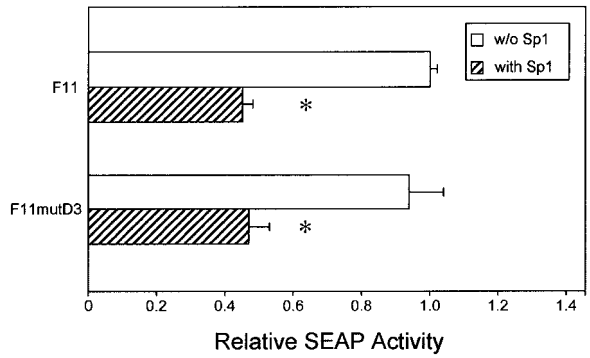

Fig. 3. Effect of mutations of the core of the Sp1 site at −822/−794 on the activity and Sp1 sensitivity of F11 construct. Human corneal stromal cells were transfected with F11, or mutant construct F11mutD3 vector, along with pSV-β-Galactosidase control vector without or with pPacSp1. The SEAP activity was normalized by the β-galactosidase enzyme activity. Data were subtracted from the basal SEAP activity and were expressed relative to the F11 construct (without pPacSp1) values. Data (mean ± SEM) presented were those averaged from four different experiments. Two-tailed student's *t*-tests were used to analyze the significance of the data. Asterisks denote data significantly different from those obtained from cells transfected without Sp1 co-expression ($P < 0.007$).

little promoter activity. From the F1 and F2 data, it seems reasonable to suggest that the sequence between −776 and −480 may contain a silencing or negative element. This conjecture however may be oversimplified. Results from F11 and F12 constructs indicate a more complicated scenario, as the former contains the sequence −776 to −663 and the latter contains −696 to −480, and yet both are active promoter fragments. Additional studies are needed for clarifications.

The current study does provide evidence that Sp1 is a major regulator of the promoter activity of the human α1-PI gene. The promoter activity of the 1.4-kb fragment and three deletion constructs, F2, F11, and F12, were all suppressed by Sp1 over-expression. All these fragments contain the most proximal Sp1-1 site, suggesting that it may be of key importance. F2 and F12 in addition share the Sp1-2 and Sp1-3 sites and F11 contains the three most distal Sp1 binding sites (Sp1-8, -9, and -10). Somewhat surprisingly, another fragment, F10, with promoter activity and containing Sp1-1, does not exhibit Sp1 sensitivity. In addition to Sp1-1, F10 also possesses Sp1-2, -9, and -10, but not Sp1-3 or -8.

Site-directed mutagenesis to create mutations on four Sp1 sites (Sp1-1, -2, -3, and -8) was performed. The data (Fig. 2) reveal that mutations at Sp1-1 site virtually eliminated the Sp1 downregulation of the 1.4-kb α1-PI promoter, confirming that this most proximal site is an essential element for the Sp1 action. Such a finding is consistent with the notion that Sp1 is in general a protein interacting with elements in the proximity of the transcription start site [Blake et al., 1990; Mastrangelo et al., 1991]. Neither mutations in the Sp1-2 nor those in the Sp1-3 site abrogated the 1.4-kb wild type Sp1 sensitivity. These two sites thus may not be crucial for the Sp1 regulation. Data from mutations of Sp1-8 (Fig. 3) in F11 fragment also indicate that this site is not a key element for the F11 repression by Sp1.

As stated above, deletion construct F10, despite the presence of Sp1-1, is not suppressible by Sp1 co-transfection. It thus appears that Sp1-1, while required, may not be sufficient to confer the Sp1 sensitivity. Additional upstream Sp1 element somewhat distant from Sp1-1 (e.g., Sp1-3 as in F2 and F12 and Sp1-8 as in F11, both lacking in F10), may also need to be present to allow regulation by Sp1. The crosstalk of the

proximal and the distal sites, a speculation, is in line with the looping model first proposed by Ptashne [1986] to be a mechanism of long-range interaction between physically separated regulatory regions. As supported by electron microscopic and other data [Mastrangelo et al., 1991; Pascal and Tjian, 1991; Vergeer et al., 2000], the looping does allow formation of higher-order complexes to mediate interactions with the transcription apparatus. Further of note is the result that mutations in Sp1-3 or Sp1-8 did not seem to affect the Sp1 responsiveness of the α1-PI fragments. Perhaps a tight binding at the distal site with the transcription factor is not necessary for the assembly of the Sp1-promoter complex or a stable loop structure and interaction is sufficiently preserved even with mutations.

Taken together, using a series of 5′, 3′, and internal deletion constructs, we have determined that both the 5′- and 3′-end sequences of the 1.4-kb α1-PI fragment are important for the promoter activity. The promoter activity can be negatively regulated by Sp1 and Sp1-1 that binds Sp1 between residues −100 and −87 is a crucial site. Disruption of the nucleotide sequence of this 14-bp element abolishes the Sp1 responsiveness of the promoter. The current study clearly demonstrates the involvement of Sp1-binding sites in regulation of the α1-PI promoter and provides the first in-depth characterization of the transcription mechanisms regulating the expression of α1-PI in corneal cells. The identification of cis-acting elements in the promoter of the α1-PI gene that are targets of Sp1 regulation may permit a better understanding of the complex mechanisms that modulate the transcriptional activity of α1-PI gene during physiological processes of embryogenesis and repair. This information may also make it possible for the development of novel therapeutic approaches for disease conditions such as keratoconus characterized by decreased expression of the α1-PI gene. For example, antisense oligonucleotides designed to target the proximal Sp1 element may be employed to restore the production of α1-PI in keratoconus.

REFERENCES

Banks EB, Crish JF, Eckert RL. 1999. Transcription factor Sp1 activates involucrin promoter activity in non-epithelial cell. Biochem J 337:507–512.

Blake MC, Jambou RC, Swick AG, Kahn JW, Azizkhan JC. 1990. Transcriptional initiation is controlled by upstream GC-box interactions in a TATA-less promoter. Mol Cell Biol 12:6632–6641.

Brantly M, Nukiawa T, Crystal RG. 1988. Molecular basis of α1-antitrypsin deficiency. Am J Med 84:13–31.

Ciliberto G, Dente L, Cortese R. 1985. Cell-specific expression of a transfected human α1-antitrypsin gene. Cell 41: 531–540.

Critchfield JW, Calandra AJ, Nesburn AB, Kenney MC. 1988. Keratoconus: I. biochemical studies of normal and keratoconus corneas. Exp Eye Res 46:953–963.

De Simone V, Ciliberto G, Hardon E, Paonessa G, Palla F, Lundberg L, Cortese R. 1987. Cis- and trans-acting elements responsible for the cell-specific expression of the human α1-antitrypsin gene. EMBO J 6:2759–2766.

Dynan WS, Tjian R. 1983. Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. Cell 32:669–680.

Eriksson S. 1964. Pulmonary emphysema and α1-antitrypsin deficiency. Acta Med Scand 175:197–205.

Hafeez W, Ciliberto G, Perlmutter DH. 1991. Constitutive and modulated expression of the human α1 antitrypsin gene. J Clin Invest 89:1214–1222.

Krachmer JH, Feder RS, Belin MW. 1984. Keratoconus and related noninflammatory corneal disorders. Surv Ophthalmol 28:293–322.

Laurell CB, Jeppsson JD. 1975. Protease inhibitors in plasma. In: Putnam FW, editor. The plasma proteins. 2nd edn (Vol. 1). New York: Academic Press. p 229–264.

Li Y, Zhou L, Twining SS, Sugar J, Yue BYJT. 1998. Involvement of Sp1 elements in the promoter activity of the α1-proteinase inhibitor gene. J Biol Chem 273:9959–9965.

Long GL, Chandra T, Woo SL, Davie EW, Kurachi K. 1984. Complete sequence of the cDNA for human α1-antitrypsin and the gene for the S variant. Biochemistry 23: 4828–4837.

Mandell RB. 1997. Contemporary management of keratoconus. Int Contact Lens Clin 24:43–58.

Mastrangelo IA, Courey AJ, Wall JS, Jackson SP, Hough PV. 1991. DNA looping and Sp1 multimer links: a mechanism for transcriptional synergism and enhancement. Proc Natl Acad Sci USA 88:5670–5674.

Morria JR, Taylor-Papadimitriou J. 2001. The Sp1 transcription factor regulates cell type-specific transcription of MUC1. DNA Cell Biol 20:133–139.

Ogra Y, Suzuki K, Gong P, Otsuka F, Koizumi S. 2001. Negative regulatory role of Sp1 in metal responsive element-mediated transcriptional activation. J Biol Chem 276:16534–16539.

Olsen GN, Harris JO, Castle JR, Waldman RH, Karmgard HJ. 1975. α1-Antitrypsin content in the serum, alveolar macrophages, and alveolar lavage fluid of smoking and nonsmoking normal subjects. J Clin Invest 55:427–430.

Pascal E, Tjian R. 1991. Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev 5:1646–1656.

Perlino E, Cortese R, Ciliberto G. 1987. The human α1-antitrypsin gene is transcribed from two different promoters in macrophages and hepatocytes. EMBO J 6: 2759–2766.

Perlmutter DH, Cole FS, Kilbridge P, Rossing TH, Colten HR. 1985. Expression of the α1-proteinase inhibitor gene

in human monocytes and macrophages. Proc Natl Acad Sci USA 82:795–799.

Ptashne M. 1986. Gne regulation by proteins acting nearby and at a distance. Nature 322:697–701.

Rabinowitz YS. 1998. Keratoconus. Surv Ophthalmol 42: 297–319.

Sawaguchi S, Yue BYJT, Sugar J, Gilboy JE. 1989. Lysosomal enzyme abnormalities in keratoconus. Arch Ophthalmol 107:1507–1510.

Sawaguchi S, Twining SS, Yue BYJT, Wilson PM, Sugar J, Chan S-K. 1990. α1-Proteinase inhibitor levels in keratoconus. Exp Eye Res 50:549–554.

Sawaguchi S, Twining SS, Yue BYJT, Chang SHL, Zhou X, Loushin G, Sugar J, Feder RS. 1994. α2-Macroglobulin levels in normal human and keratoconus corneas. Invest Ophthalmol Vis Sci 35:4008–4014.

Sharp HL, Bridges RA, Krivit W, Freier ER. 1969. Cirrhosis associated with α1-antitrypsin deficiency: a previously unrecognized inherited disorder. J Lab Clin Med 73:934–939.

Travis J, Salvesen GS. 1983. Human plasma protease inhibitors. Annu Rev Biochem 52:655–709.

Twining SS, Fukuchi T, Yue BYJT, Wilson PM, Boskovic G. 1994. Corneal synthesis of α1-proteinase inhibitor (α1-antitrypsin). Invest Ophthalmol Vis Sci 35:458–462.

Vergeer WP, Sogo JM, Pretorius PJ, de Vries WN. 2000. Interaction of Ap1, Ap2, and Sp1 with the regulatory regions of the human pro-α1(I) collagen gene. Arch Biochem Biophys 377:69–79.

Whitelock RB, Fukuchi T, Zhou L. 1997a. Cathepsin G, acid phosphatase, and α1-proteinase inhibitor messenger RNA levels in keratoconus corneas. Invest Ophthalmol Vis Sci 38:529–534.

Whitelock RB, Li Y, Zhou L, Sugar J, Yue BYJT. 1997b. Expression of transcription factors in keratoconus, a cornea-thinning disease. Biochem Biophys Res Commun 235:253–258.

Yue BYJT, Baum JL. 1981. Studies of corneas in vivo and in vitro. Vis Res 21:41–43.

Yue BYJT, Sugar J, Benveniste K. 1984. Heterogeneity in keratoconus: possible biochemical basis. Proc Soc Exp Biol Med 175:336–341.

Yue BYJT, Sugar J, Benveniste K. 1985. RNA metabolism in cultures of corneal stromal cells from patients with keratoconus. Proc Soc Exp Biol Med 178:126–132.

Zhou L, Sawaguchi S, Twining SS, Sugar J, Feder RS, Yue BYJT. 1998. Expression of degradative enzymes and protease inhibitors in corneas with keratoconus. Invest Ophthalmol Vis Sci 39:1117–1123.