An Involucrin Promoter AP1 Transcription Factor Binding Site Is Required for Expression of Involucrin in the Corneal Epithelium In Vivo

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Purpose. Cell division of corneal limbal stem cells gives rise to transient amplifying cells that ultimately differentiate to form the multilayered corneal epithelium. The mechanisms that regulate changes in gene expression during this process are not well understood. In the present study, the involucrin gene was used as a model to study this regulation.

Methods. Regulation of human involucrin gene expression and promoter activity was assessed using in vivo transgenic mouse models and cultured primary human corneal epithelial cells.

Results. Human involucrin (hINV) is a structural protein that is selectively expressed in differentiating corneal epithelial cells. The results reveal that an activator protein one (AP1) DNA-binding site is essential for appropriate basal and stimulus-dependent hINV promoter activity. Mutation of this site, AP1-5, results in a loss of hINV gene expression in the corneal epithelium in vivo and in cultured corneal epithelial cells. A gel mobility supershift analysis revealed interaction of the AP1 factors, Fra-1 and JunB, with this element. Inhibition of AP1 function with a dominant-negative form of AP1 also inhibited expression. Treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activator, increased hINV gene expression, a response that correlates with increased AP1 factor (Fra-1 and JunB) binding to the hINV gene AP1-5 response element.

Conclusions. These findings point to an essential role for AP1 transcription factors, acting through a distal regulatory region AP1-5 element, in the regulation of involucrin gene expression during corneal epithelial cell differentiation. (*Invest Ophthalmol Vis Sci.* 2005;46:1219–1227) DOI:10.1167/iovs.04-1285

The corneal epithelium undergoes a programmed process in which stem cells, located in the limbus, divide to give rise to transient amplifying daughter cells.[1–4] Transient amplifying cells undergo additional cell division before undergoing cell differentiation to produce mature cells.[5–9] Identifying the mechanisms that regulate gene expression during the transition from stem cells to transient amplifying cells to terminally differentiated cells is an area of active investigation. Several genes are known to be regulated during this transition.[10,11] These include the gene encoding involucrin (hINV).[12,13] However, information regarding expression of these genes is limited. In the present study, we examined the mechanisms that regulate hINV gene expression in the corneal epithelium.

hINV is an essential surface epithelia structural protein.[14,15] Immunoblot analysis reveals its presence in the corneal epithelium in mice and humans,[13,16] and immunohistology reveals suprabasal expression in the human corneal epithelium.[16] It is a 46-kDa, α-helical, rod-shaped protein.[17] Its amino acid sequence consists of highly homologous repeats of 10 amino acids.[15] Each repeat encodes glutamine and lysine residues that serve as reactive sites for the formation of ϵ-(γ-glutamyl)lysine isopeptide bonds.[18,19] Its function has been most extensively studied in human epidermis and in cultured human epidermal keratinocytes. It is initially produced as an soluble cytosolic protein.[18] In differentiated epidermal keratinocytes, it is used as a substrate by transglutaminase and is covalently cross-linked to other proteins to form an insoluble scaffolding that is attached to the inner surface of the plasma membrane. Further cross-linking adds other proteins to this structure.[20] This cross-linked structure provides a protective surface to resist environmental damage. Type I transglutaminase, the enzyme that catalyzes formation of the interprotein ϵ-(γ-glutamyl)lysine bonds,[21] is responsible for assembly of this structure. Both hINV and type I transglutaminase are expressed in corneal epithelial cells, suggesting that assembly of a covalently cross-linked structure is likely to play a role during the corneal epithelial cell life cycle.[12,13,16] Indeed, transglutaminase activity acts to stabilize corneal tissue, and increased transglutaminase activity is detected in corneal wounds.[22–24]

hINV expression has been reported to be regulated by activator protein one (AP1) and lens epithelium-derived growth factor in SV40-immortalized human corneal epithelial cells[25,26]; however, many fundamental questions regarding expression remain to be addressed. In the present study we show that a specific AP1 transcription factor–binding site is required for hINV expression in differentiating normal human corneal epithelial cells. Activation of protein kinase C (PKC), using 12-*O*-tetradecanoylphorbol-13-acetate (TPA), results in hINV promoter activation, and this response is eliminated by mutation of a specific AP1 transcription factor–binding site, AP1-5. In addition, a dominant-negative form of AP1 inhibits hINV promoter activation. Finally, we confirm that mutation of the hINV promoter AP1-5 site results in a loss of hINV expression in the corneal epithelium of transgenic mice; thus, confirming in a stringent in vivo system that AP1 factors and the AP1-5 site play a central role in regulating hINV gene expression.

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Materials and Methods

Chemicals and Reagents

Keratinocyte serum-free medium (KSFM), trypsin, Hanks' balanced salt solution, and gentamicin were purchased from Invitrogen (Carlsbad, CA). Phorbol ester (12-*O*-tetradeconylphorbol-13-acetate; TPA) and dimethyl sulfoxide were obtained from Sigma-Aldrich (St. Louis, MO). pGL2-Basic plasmid and chemiluminescent luciferase assay system were from Promega (Madison, WI), and chemiluminescence was measured with a luminometer (Berthold, Wildbad, Germany). [γ-^{32}P] adenosine triphosphate (ATP) was purchased from Perkin Elmer Life Sciences (Boston, MA). The hINV-specific polyclonal antibody was generated by injecting rabbits with recombinant hINV. This antibody detects hINV, but does not cross-react with mouse hINV.[27] Transcription factor-selective rabbit polyclonal antibodies specifying c-fos (sc-52X), fosB (sc-48X), Fra-1 (sc-605X), Fra-2 (sc-171X), JunB (sc-46X), c-Jun (sc-1694X), and JunD (sc-74X) were from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody specific for β-actin was purchased from Sigma-Aldrich and used for immunoblot at a dilution of 1:3000. Peroxidase-conjugated goat anti-mouse IgG (sc-2005) and anti-rabbit IgG were obtained from Santa Cruz Biotechnology and diluted 1:5000. hINV promoter reporter plasmids, containing various lengths of hINV upstream promoter sequence, with or without mutations at specific sites, were constructed in pGL2-basic.[28,29] pRSVα-TAM67 encodes dominant-negative c-Jun.[30,31]

Immunoblot Method

For immunologic analysis, equivalent amounts of protein were electrophoresed on denaturing and reducing 6% polyacrylamide gels and transferred to nitrocellulose membrane. The membrane was blocked by 5% nonfat dry milk and then incubated with the appropriate primary and secondary antibodies. Secondary antibody binding was visualized using chemiluminescence detection technology.

Human Corneal Epithelial Cell Culture

Human eyes, unsuitable for corneal transplantation, were obtained from the Cleveland Eye Bank at 5 to 10 hours after death from donors 40 to 75 years of age. The tissue procurement protocol was approved by the Case Western Reserve/University Hospitals of Cleveland Institutional Review Board. Eyes were obtained from subjects who had agreed to donate these organs, and all procedures adhered to the principles articulated in the Declaration of Helsinki. The globes were transferred to the laboratory in keratinocyte serum-free medium. Bulbar conjunctival tissue was removed from the corneal epithelial surface by scarping with a sterile scalpel. The cornea was then excised by a circumferential incision. The isolated cornea was then placed, corneal epithelial surface down, in 4 mL sterile Hanks' balanced salt solution containing 10 mg/mL dispase and 5 μg/mL gentamicin for 15 hours at 4°C. The corneal epithelium was then collected by gentle scraping, treated with 5 mL 0.25% trypsin for 5 minutes at 37°C, gently pipetted to create a single cell suspension, and transferred to stop medium containing (2.5 mL of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/mL penicillin, and 10 μg/mL streptomycin). The epithelial cells from each cornea were then collected by centrifugation and resuspended in KSFM, containing epidermal growth factor and bovine pituitary extract without antibiotics and distributed into two 9.5-cm^2 surface area dishes in 4 mL of culture medium. For passage, four near-confluent, 9.5-cm^2 cultures were harvested with 0.25% trypsin and transferred to one 50-cm^2 dish. When confluent, the cells were harvested and transferred to four 50-cm^2 dishes. The medium was changed every fourth day and the cultures were confluent after 6 to 8 days. Fifty to 70% confluent cultures were used for experimentation. We routinely passage at a split ratio of 1:3 in early cultures and at 1:5 as the cells expanded.

hINV Promoter Activity

For hINV promoter studies, 6 μL transfection reagent (Fugene-6; Roche Molecular Biochemicals, Indianapolis, IN) was mixed with 94 μL KSFM, and incubated at 25°C 10 minutes. This mixture was then added to 2 μg of hINV promoter reporter plasmid and incubated at 25°C for 20 minutes followed by direct addition to cultures containing 2 mL of KSFM. For cotransfection experiments, 1 μg of hINV plasmid and 1 μg of TAM67 expression plasmid were used. The final DNA concentration in all groups was maintained constant by addition of empty expression vector. At 24 hours after transfection, 2 mL of fresh medium was added containing 0 or 50 ng/mL TPA. After an additional 24 hours, the cells were washed with phosphate-buffered saline (pH 7.5; PBS) and scraped into 200 μL of cell lysis buffer,[29] and luciferase activity was assayed immediately. All assays were performed in triplicate, and each experiment was repeated a minimum of three times. Luciferase activity was normalized per microgram of protein, as described previously.[29]

Production and Identification of hINV Gene-Positive Mice

Mouse embryos from a B6CBA x B6CBA mating were injected with each hINV transgene and implanted into surrogate mothers, as previously described.[32,33] In each construct, the authentic relationship between the promoter and upstream regulatory region and the structural gene is maintained. Founder animals were identified by tail blot analysis of DNA with a hINV-specific probe. Expression of the transgene is monitored in tissue by assaying for the presence of hINV protein using a hINV-specific antibody that does not cross-react with mouse hINV.[27] All studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

hINV Expression in Transgenic Mouse Cornea

The central corneal epithelium was excised by using a microscope and fine scalpel, and eye fluid was removed by scraping. The corneal epithelium was then dissected into small pieces and homogenized in buffer A (200 μL; 1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM sodium ortho vanadate, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) at 4°C. After a 30-minute incubation on ice, the lysate was centrifuged at 12,000 rpm, and the supernatant was stored (−80°C). hINV protein was detected by electrophoresis on an 8% gel and transfer to nitrocellulose for immunoblot analysis. The hINV level was monitored by immunoblot.[27] It was also monitored in central corneal sections. Sections were fixed and then incubated with hINV-specific antibody followed by peroxidase-linked secondary antibody.[32]

Nuclear Extract Preparation and Gel Mobility Shift Assay

Sixty percent confluent human corneal epithelial cells, growing in KSFM, were incubated with 0 or 50 ng/mL TPA for 24 hours. The cells were then washed with PBS, and total cell extract was prepared in the presence of proteinase inhibitors, as previously described.[29] Nuclear extracts were prepared according to the method of Schreiber et al.,[34] in the presence of 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 1 mM PMSF. Protein content was measured by using a protein assay reagent (DC; Bio-Rad, Hercules, CA).

Identification of transcription factors binding to the hINV promoter AP1-5 site was achieved by electrophoretic mobility shift assay.[28] Three micrograms of nuclear extract was incubated for 25 minutes at room temperature in a total volume of 20 μL containing 20 mM HEPES (pH 7.5), 10% glycerol, 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 1 μg/mL poly(dI-dC), 0.1 mg/mL bovine serum albumin, and 50,000 cpm of radioactive double-stranded, ^{32}P-labeled AP1-5 site oligonucleotide (5′-GGCTCTTATTATGCCG**TGAGTCAG**AGGGCGGGAGGCA). The hINV AP1-5 binding site is indicted in bold. The AP1 5-mm oligonucleotide encodes a mutated AP1-5 site (5′-GGCTCTTATTATG CCG***A*GC*TC*CAG**AGGGCGGGAGGCA). The mutated AP1-5 site is indicated in bold with the altered nucleotides in bold italic. The Sp1c oligonucleotide encodes a Sp1 consensus site (5′-ATTCGATCG**GGGCGGG**GCGAGC). The Sp1site is indicated in bold. For competition studies, radio-inert competitor oligonucleotide was

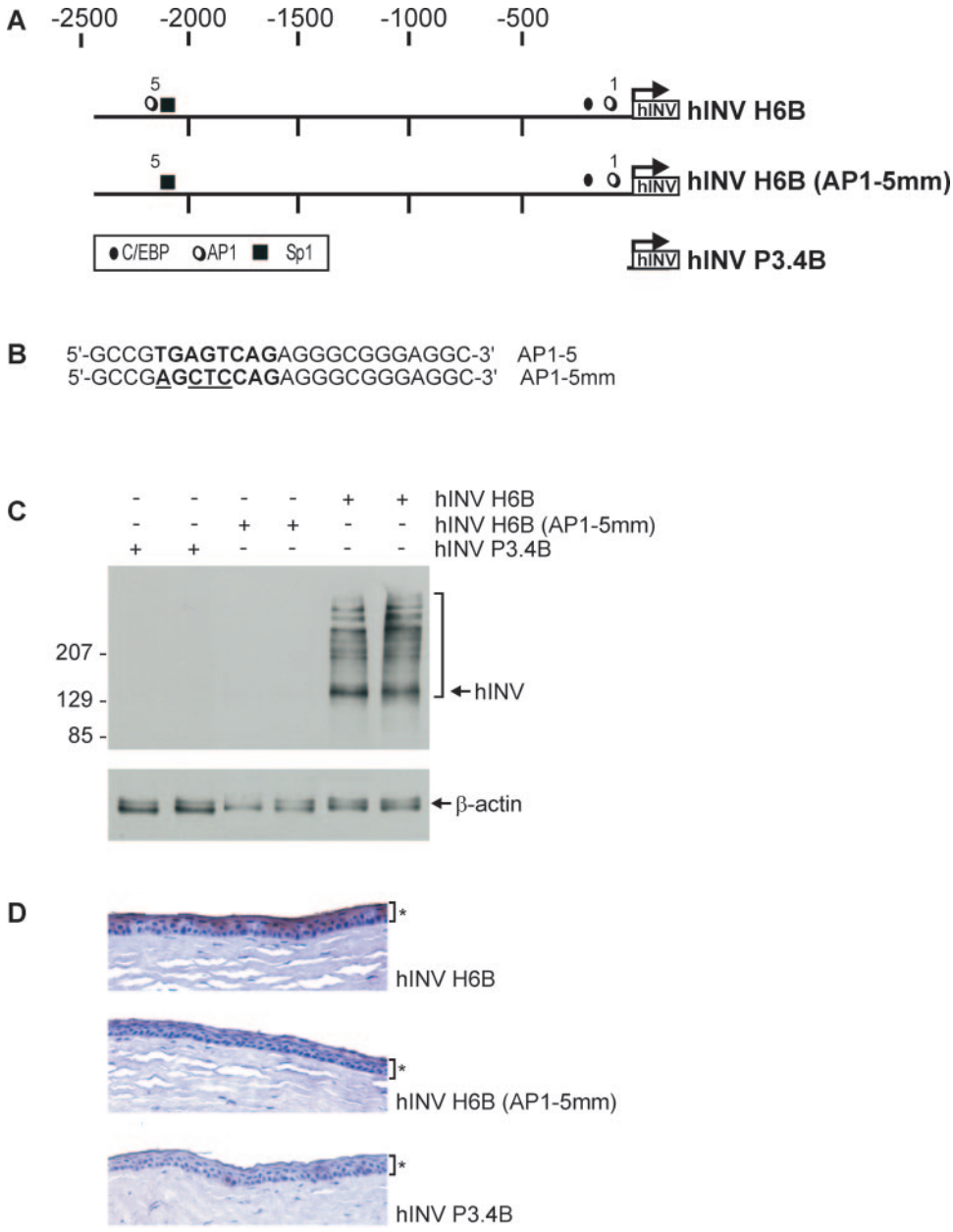

FIGURE 1. The AP1-5 site is essential for in vivo corneal epithelial expression of hINV in the context of the full-length upstream regulatory region. (**A**) Mouse embryos were injected with each of the three transgenes. hINV H6B encodes the full-length upstream regulatory region. hINV H6B(AP1-5mm) is identical, except that the AP1-5 site is mutated. hINV P3.4B, which lacks all upstream sequence except the minimal promoter (−41/−1), serves as a control. The hINV *box* indicates the hINV protein coding sequence; the *arrow* defines the start site and direction of transcription. (**B**) Sequence of the hINV AP1-5 site mutations. Sequence of the native AP1-5 site with surrounding sequence and sequence of AP1-5 site mutation present in hINV H6B(AP1-5mm). (**C**) Detection of hINV protein expression in the corneal epithelium. Central corneal epithelium was harvested from each transgenic mouse line. Total cell extracts were prepared in sample buffer and electrophoresed on an 8% denaturing and reducing polyacrylamide gel, transferred to nitrocellulose, and incubated with an antibody that specifically detects hINV. Antibody binding was visualized with chemiluminescence detection reagents. Six independently transgenic lines were derived for each construct. Expression in two representative lines from each construct is shown; however, all six lines displayed similar patterns of expression. In addition, no signal was detected in nontransgenic mice (not shown). β-Actin level was monitored as a loading control. (**D**) Immunohistochemical localization of hINV protein in sections derived from the central corneal epithelium of hINV transgenic mice. Sections were prepared from central corneal epithelium and then incubated with anti-hINV followed by peroxidase-conjugated goat anti-rabbit IgG.

added to the DNA-binding reaction. For the gel mobility supershift assay, AP-1 factor–specific antibodies (2 μg) were added to the reaction mixture and incubated at 4°C for 45 minutes. The ^{32}P-labeled probe was then added to the mixture and incubated for an additional 20 minutes at room temperature. Protein-DNA complexes were subsequently separated in nondenaturing 6% polyacrylamide gels, and the position of the complex was determined by autoradiography.

RESULTS

Role of the AP1-5 Transcriptional Element in In Vivo Corneal Epithelial hINV Expression

The mechanisms that regulate gene expression during the transition from stem cell to transient amplifying cell to differentiated cell are not well understood. hINV expression is increased during this transition. A major goal of this study was to assess the role of AP1 factors in this regulation. Studies have shown that the hINV promoter distal regulatory region (DRR) encodes several potential transcriptional regulatory elements.[28] These include an AP1 factor–binding site, AP1-5. We began by performing experiments designed to assess the in vivo role of the AP1-5 site. For these studies, hINV promoter transgenic mice, including hINV H6B, hINV H6B(AP1-5mm) and hINV P3.4B, were constructed. hINV H6B encodes the full-length hINV promoter linked in its native context to the hINV protein coding sequences.[35] H6B(AP1-5mm) is identical with H6B, except that the AP1-5 site is mutated (Fig. 1A). P3.4B encodes the hINV gene minimal promoter. The authentic and mutated AP1-5 sequences are indicated in Figure 1B. Each construct retains the authentic relationship between the upstream regulatory region (nucleotides −2473/−1), the hINV transcription start site (Fig. 1A, arrow), and the hINV gene coding sequence (hINV box). Six independent transgenic lines were established for each construct. Figure 1C shows an immunoblot analysis of hINV protein content in punch biopsy tissue derived from the central corneal epithelium of mice representing two representative transgenic lines for each con-

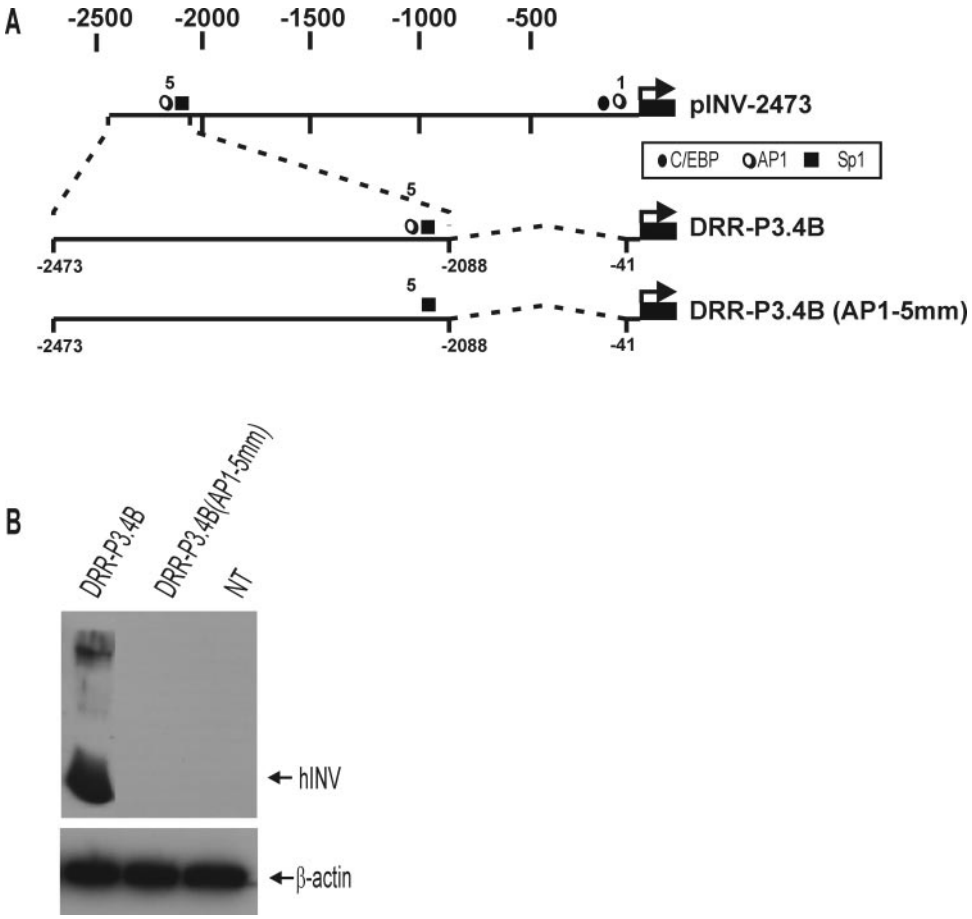

FIGURE 2. The AP1-5 site is required for in vivo corneal epithelial expression of hINV in the context of the isolated DRR segment. (**A**) Mouse embryos were injected with each of the indicated transgenes. DRR-P3.4B encodes a segment of the distal regulatory region fused to the hINV minimal promoter. DRR-P3.4B(AP1-5mm) is identical, except that the AP1-5 site is inactivated by mutation. The AP1-5 site mutation is shown in Figure 1B. (**B**) Detection of hINV protein expression in the corneal epithelium. Central corneal epithelium was harvested from each transgenic mouse line. Total cell extracts were prepared in sample buffer and electrophoresed on an 8% denaturing and reducing polyacrylamide gel, transferred to nitrocellulose and incubated with an antibody that specifically detects human hINV. Antibody binding was visualized using chemiluminescence detection reagents. Six independently transgenic lines were derived for each construct. Expression in two representative lines from each construct are shown; however, all six lines displayed similar patterns of expression. β-actin level was monitored as a loading control.

struct. Transgenic mice harboring H6B, the full-length transgene, express high levels of hINV in the central corneal epithelium. In contrast, no hINV was detected in mice harboring hINV H6B(AP1-5mm), in which the AP1-5 site was mutated. As a control, we also monitored corneal epithelial hINV content in mice harboring hINV P3.4B, a transgene that encodes only the hINV minimal promoter (nucleotides −41/−1). No expression was detected. To assess the status of expression as a function of corneal epithelial differentiation, we treated sections derived from central cornea epithelium with anti-hINV. As shown in Figure 1D, hINV was expressed in the central cornea upper epithelial layers and also in selected basal cells. In contrast, and consistent with the immunoblot data (Fig. 1C), no hINV was detected in comparable sections prepared from hINV H6B(AP1-5mm) or hINV P3.4B mice.

The hINV promoter encodes several AP1 sites that are functional.[28,29] To provide an independent characterization of the AP1-5 site in the absence of AP1-1, we cloned nucleotides −2473/−2088, which encodes a major segment of the distal regulatory region (DRR), at a position immediately upstream of the hINV minimal promoter (Fig. 2A). A parallel construct, encoding an AP1-5 site mutant, was also constructed. As shown in Figure 2B, mutation of the AP1-5 site in the context of the isolated DRR results in a complete loss of expression in the corneal epithelium, thus providing additional evidence of an essential role for the AP1-5 site.

We next examined the role of the AP1-5 site using cultured human corneal epithelial cells. The distal regulatory region (nucleotides −2473/−2088) was cloned adjacent to the hINV minimal promoter upstream of the luciferase reporter gene to create pINV(−2473/−2088; Fig. 3A), and this construct was transfected into normal corneal epithelial cells. Figure 4B shows that pINV(−2473/−2088) is active and is regulated by TPA, an agent that is known to regulate hINV promoter activity in corneal epithelial cells.[26] Moreover, mutation of the AP1-5 site, pINV(−2473/−2088)AP1-5mm, results in a complete loss of both basal promoter activity and the promoter response to TPA. As a second method of assessing the role of AP1 factors, we monitored promoter activity in the presence and absence of TAM67. TAM67 is a dominant-negative form of c-Jun that binds to and inhibits the activity of all AP1 factors.[36] As shown in Figure 4, TAM67 completely suppressed pINV(−2473/−2088) activity, confirming that AP1 transcription factor interaction with the promoter is necessary for activation.

AP1 Factor Binding to the hINV Promoter AP1 Site

These findings indicate that the AP1-5 site, located within the hINV upstream regulatory region, is absolutely necessary for promoter function in vivo and in cultured corneal epithelial cells. To gain insight into the mechanism of regulation and the transcription factors involved, we performed gel mobility shift experiments. An oligonucleotide, ^{32}P-AP1-5, encoding the hINV promoter AP1-5 site and surrounding sequence (Fig. 5A), was incubated with nuclear extract prepared from TPA-treated and nontreated corneal epithelial cells. As shown in Figure 5B, a single slow mobility band indicated by the arrow (AP1), was observed when nuclear extracts were incubated with ^{32}P-AP1-5, before nondenaturing gel electrophoresis. As is evidenced in Figure 5B, and confirmed by gel scanning (not shown), the level of binding increased fourfold after TPA treatment. Moreover, this binding was specific, as a marked reduction in band intensity was observed when the reaction mixture

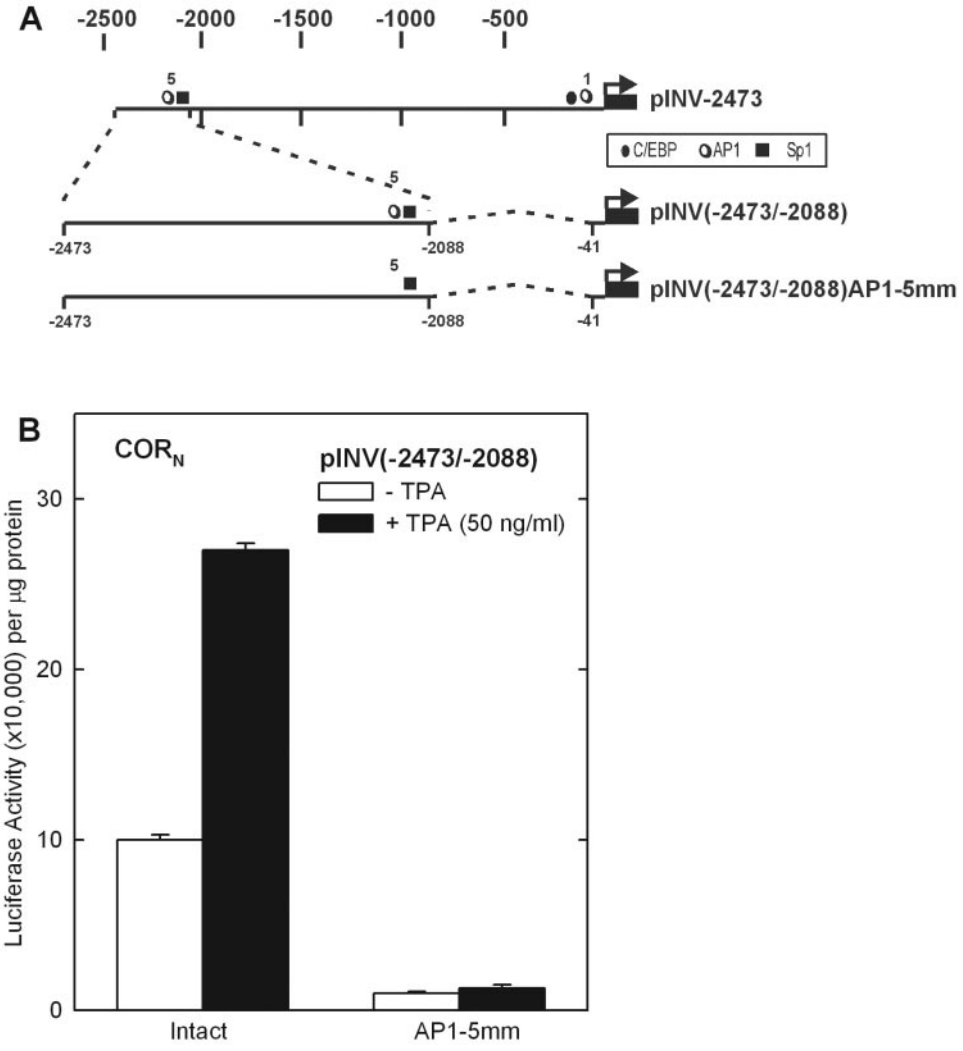

FIGURE 3. The AP1-5 site is essential for hINV promoter activity when tested in cultured human corneal epithelial cells. (**A**) Structure of hINV luciferase reporter constructs encoding the −2473/−2088 segment of the hINV upstream regulatory region. The structure of the full-length upstream regulatory region (*top line*) is provided for reference. The AP1-1, AP1-5, C/EBP, and Sp1 sites are indicated.[33] The other construct encodes the −2473/−2088 segment encoding an AP1-5 site mutation (AP1-5mm). The *box* indicates the luciferase reporter gene and the *arrow* the direction of transcription. (**B**) Corneal epithelial cells were transfected with 1 μg of each construct, and after 24 hours the cells were further treated with 50 ng TPA/mL for 24 hours. Cell extracts were then assayed for luciferase activity. The results are expressed as the mean ± SEM, $n = 4$.

included a 10- or 100-fold molar excess of radio-inert AP1-5 oligonucleotide. In addition, as a further proof of specificity (Fig. 5C), neither AP1-5mm, an oligonucleotide in which the AP1-5 site is mutated such that it does not bind AP1 factors (Fig. 5A),[37] nor Sp1c, which encodes a consensus site for Sp1 transcription factors (Fig. 5A),[38] competes with ^{32}P-AP1-5 for this binding. In addition, the TPA-dependent increase in intensity of the AP1 band in the gel mobility shift assay suggests increased transcription factor–binding at the site.

Specific Transcription Factor Interaction with the AP1-5 Site

Because AP1 factors comprise a large family of transcriptional regulators that frequently function in combination, it is important to evaluate which family members interact with the hINV AP1-5 site in corneal epithelial cell nuclear extracts. We therefore used gel mobility supershift assays to identify potential AP1 proteins that interact at the AP1-5 site. Nuclear extract, prepared from TPA-treated cells, was incubated with ^{32}P-AP1-5 in the presence of antibodies that detect specific AP1 factors. As shown in Figure 6A, in the absence of nuclear extract, the AP1-5-P^{32} oligonucleotide "free probe" migrated at the bottom of the gel. Addition of nuclear extract resulted in the appearance of a slow-mobility band (AP1). Addition of anti-pan-fos, an antibody that reacts with all fos family members, resulted in a strong supershift of the slow mobility band (migrating adjacent to the asterisk), indicating fos factor binding at the AP1-5 site. Treatment with anti-Fra-1 resulted in a similar supershift (migrating adjacent to the asterisk) indicating the presence of Fra-1. In contrast, no supershift was observed when the mixture was incubated with anti-Fra-2 or anti-c-fos. A similar analysis of binding of Jun family factors revealed a strong reduction in binding when extract was incubated with anti-JunB, indicating the presence of JunB in the complex (Fig. 6B). However, only a slight change in band intensity was observed after treatment with anti-c-Jun or anti-JunD.

DISCUSSION

The corneal epithelial stem cells exist in a specialized environment called the limbus.[1,2,39–42] These cells proliferate and give rise to transiently amplifying cells that migrate away from the limbus toward the central cornea. These migrating cells ultimately cease proliferation and undergo terminal differentiation to complete the corneal epithelial cell life cycle. Differentiation-dependent changes in expression of several genes in the corneal epithelia have been described.[11,43,44] Thus, the genes encoding cytokeratin K3,[2,45] keratin-12,[46–48] calcium-linked epithelial differentiation protein (CLED protein),[44] transglutaminase type I,[22] and hINV[16] all increase during this progression. However, the mechanisms that regulate gene expression during this process are not well understood. Nor has gene

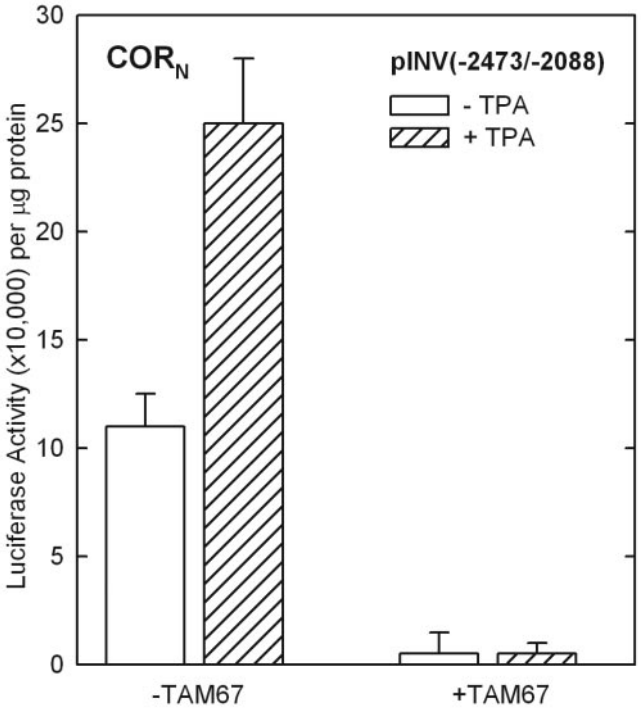

FIGURE 4. TAM67 suppresses pINV(−2473/−2088) activity. Human corneal epithelial cells were transfected with 1 μg pINV(−2473/−2088) in the presence of 1 μg of empty expression vector (pRSV2, control, −TAM67) or 1 μg of TAM67-encoding expression vector. After 24 hours, the cells were or were not treated with TPA (50 ng/mL) for 24 hours and harvested, and luciferase activity was monitored. The data are expressed as the mean ± SEM; $n = 3$.

expression been studied using stringent in vivo transgenic animal models to confirm the role of regulatory elements. To gain insights regarding this regulation, we examined the role of the hINV promoter AP1-5 site and the role of AP1 transcription factors in the regulation of hINV gene expression using both transgenic and corneal epithelial cell culture models. Both hINV and type I transglutaminase, the enzyme responsible for covalent cross-link formation in surface epithelia, are expressed in corneal epithelial cells and covalently cross-linked structure is likely to play a role during the normal corneal epithelial cell life cycle[12,13,16] and during corneal wound healing.[22–24] Thus, it is important to understand this regulation.

Role of hINV Promoter AP1-5 Site in HINV Expression in the Corneal Epithelium

AP1 factors bind to at least two potentially important AP1 sites within the hINV upstream regulatory region: AP1-1 and AP1-5.[49] A previous study examined the ability of the AP1-1 and AP1-5 sites, in the context of the full-length hINV promoter (nucleotides −2473/−1), to drive promoter activity in cultured corneal epithelial cells.[26] When tested in this context, mutation of the hINV promoter AP1-5 site produces a minimal impact on transcriptional activity, while mutation of AP1-1 markedly reduces promoter activity. The lack of effect of the AP1-5 site (nucleotides −2122/−2114) mutation is presumably because the AP1-1 site (nucleotides −125/−117), which is required for expression in cell culture,[26] masks the impact of this mutation. Thus, to examine the role of the AP1-5 site in cultured human corneal cells, we used a segment of the promoter comprising most of the distal regulatory region.[28] This segment encodes the AP1-5 site and surrounding sequence, but not the AP1-1 site.[28,32] This DNA segment was cloned adjacent to the minimal promoter to create pINV(−2473/−2088). Our studies show that this plasmid drives high-level transcription in cultured human corneal epithelial cells and that transcriptional activity is increased by treatment with TPA. Moreover, mutation of the AP1-5 site, to create pINV(−2473/−2088)AP1-5mm, results in a complete loss of basal and TPA-stimulated hINV promoter activity. To further confirm a role for AP1 factors, we monitored the effects of TAM67 on transcriptional activity. TAM67 is a dominant-negative form of c-Jun that blocks the activity of AP1 factors.[36] The presence of TAM67 completely blocks basal and TPA-stimulated promoter activity. This suggests that inhibiting AP1 factor interaction with the AP1-5 site inhibits transcription.

The above studies suggest that the AP1-5 site has an important role in driving expression in cultured corneal epithelial cells, but only when the AP1-1 site is not present. Because cell culture models do not always reflect the conditions observed in vivo, we examined the effect of the AP1-5 mutation on expression by using transgenic mice. To do this, we generated mice encoding the full-length hINV promoter and compared the pattern of expression of an identical construct in which the AP1-5 site was mutated. These studies show that mutation of AP1-5, in the context of the full-length promoter upstream regulatory region, results in a complete loss of transgene expression in the murine corneal epithelium. Moreover, this finding was confirmed for a transgenic construct encoding only the DRR segment. Taken together, these findings suggest that an intact AP1-5 site is absolutely required for expression in the corneal epithelium in vivo. However, for reasons that are not presently understood, the role of the AP1-5 site cannot be demonstrated in cultured corneal epithelial cells in the context of the full-length gene. It may be that the impact of AP1-5 is masked by the AP1-1 site, which is present in the proximal regulatory region near the transcription start site.[26] In any case, these findings suggest that cell culture findings do not always reflect the observations made when using more stringent in vivo models.

Interaction of the AP1-5 Element with Selected AP1 Factors

To gain insight regarding the factors responsible for the regulation, we examined the ability of various AP1 transcription factors to interact with the AP1-5 site. The activator protein 1 family of transcription factors includes c-Jun, JunB, JunD, c-Fos, Fra-1, and Fra-2.[50–52] These factors regulate a variety of processes in different tissues and have a prominent role in regulating differentiation in surface epithelia.[37,53] AP1 factors are known to be expressed in the corneal epithelium,[54–56] and some have been shown to be expressed in specific locations. For example, in rat corneal epithelia, JunB is reported to be constitutively expressed in the superficial layers.[54] We performed mobility shift experiments to identify AP1 factors that have the potential to interact with the AP1-5 site. These studies indicate that only selected members of this family are able to bind to the AP1-5 site: Fra-1, Fra-2, and JunB. These findings are consistent with a previous study of hINV promoter function in corneal epithelial cells that suggested that Fra-1, Fra-2, and JunB interact with the hINV promoter AP1-1 site.[26] In addition, treatment with TPA results in an increase in the binding to the AP1-5 DNA element. This increase is associated with increased transcriptional activity, suggesting that increased binding may be essential for increased gene expression.

Transcriptional Regulation in the Corneal Epithelium

Several transcription factor families have been described as important in regulating corneal epithelial gene expression.

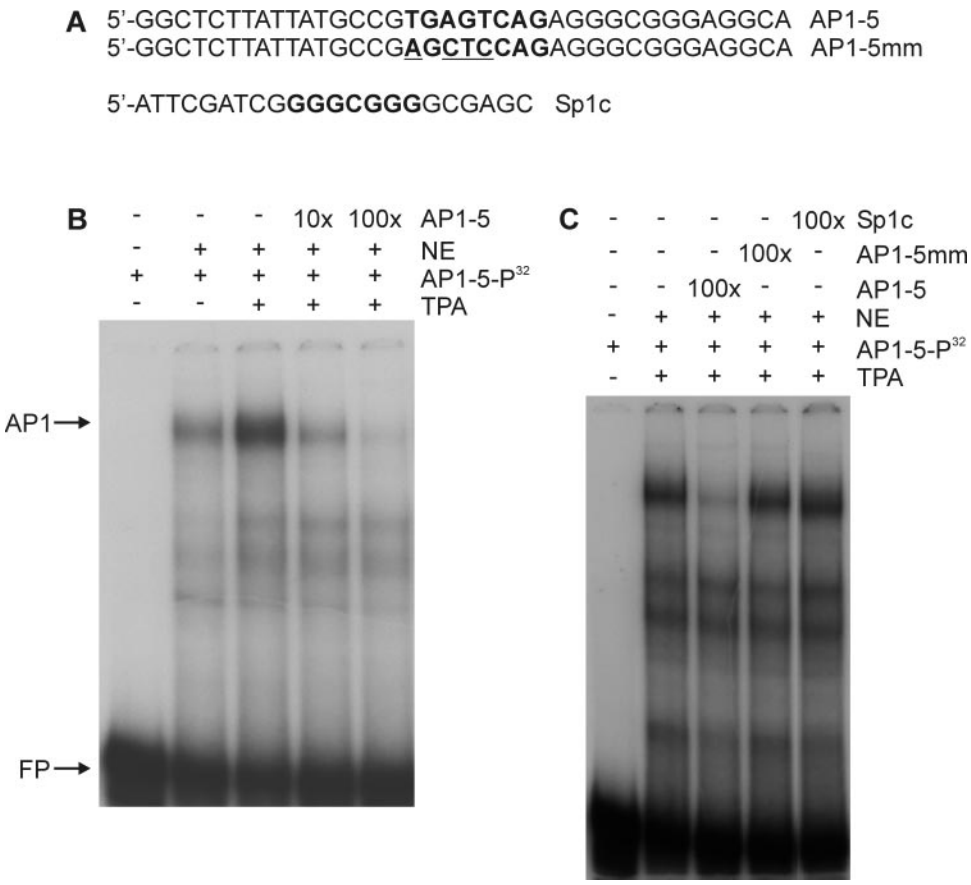

FIGURE 5. Protein/DNA complex formation at the hINV promoter AP1-5 site. (**A**) The oligonucleotides used for gel mobility shift assays include AP1-5, AP1-5mm, and Sp1c. *Bold* nucleotides: native and mutated AP1-5 sites in the AP1-5 and AP1-5mm constructs; *underscore*: mutated nucleotides in the AP1-5mm oligonucleotide. Sp1c encodes a consensus Sp1 site. (**B**) Nuclear extract (NE) was prepared from human corneal epithelial cells treated in the presence or absence of TPA (50 ng/mL) for 24 hours. Nuclear extract, prepared from each group of cells, was incubated with double-stranded ^{32}P-end-labeled AP1-5-P^{32} for 25 minutes at room temperature. Some reactions were supplemented with a 10- or 100-fold molar excess of radio-inert AP1-5. The reactions were then fractionated on nondenaturing gels, and bands were visualized by autoradiography. AP1 *arrow*: migration of the AP1/DNA complex; FP *arrow*: migration of noncomplexed free probe. (**C**) Complex formation is not inhibited by competition with AP1-5mm or Sp1c. Nuclear extract was prepared from TPA-treated and nontreated cells and then incubated with AP1-5-P^{32} in the presence of a 100-fold molar excess of radio-inert AP1-5, AP1-5mm, or Sp1c. The complexes were then gel fractionated as above, the migration of the complex was visualized by autoradiography.

Chen et al.[43] reported that Sp1 and AP2 play a role in regulating cytokeratin K3 expression in cultured rabbit corneal epithelial cells. Lactate dehydrogenase, which is increased during corneal epithelial cell differentiation, is also regulated by Sp1 and AP2 factors, suggesting a shared mode of regulation with cytokeratin K3.[11] Sp1 has also been implicated by cotransfection studies as a suppressor of α1-proteinase inhibitor expression in corneal epithelial cells[57] and is involved in regulation of other corneal epithelial genes, including the α5-integrin subunit[58] and cytokeratin 4.[59] The ESE-1 transcription factor has been reported to be expressed selectively in differentiated cells and thus may have a role in regulating differentiation.[60]

AP1 factors have been shown to be important in the regulation of hINV expression in cultured corneal epithelial cells through action at the AP1-1 site.[26] The present experiments extend these studies and show that the AP1-5 site binds a

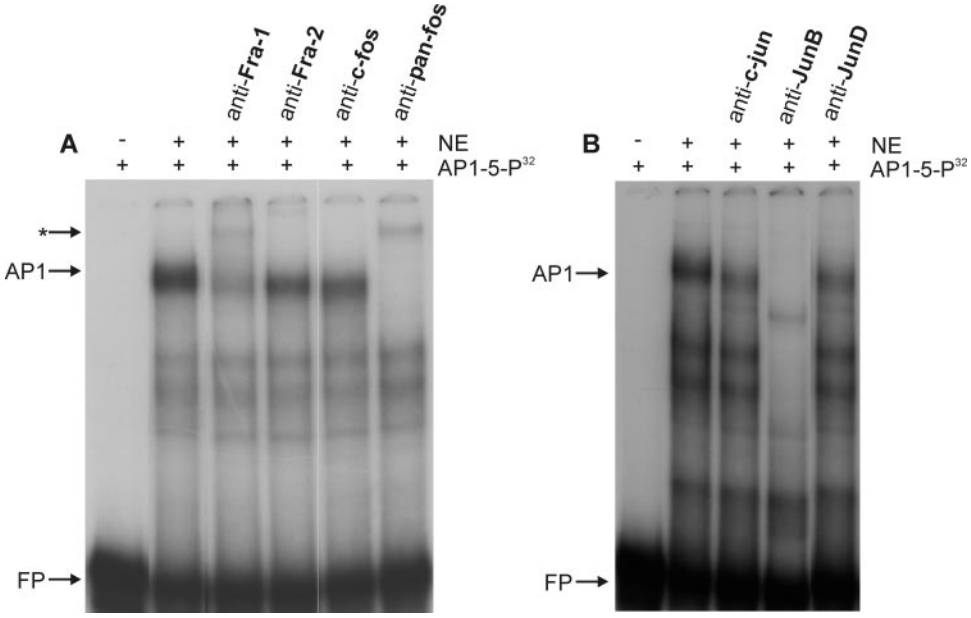

FIGURE 6. Selective AP1 transcription factor binding at the AP1-5 site. Nuclear extract, prepared from TPA-treated corneal cells, was incubated with the indicated antibody for 45 minutes at 4°C. Tracer amounts of AP1-5-P^{32} were then added to each reaction. After an additional 20 minutes at room temperature, the reactions were electrophoresed, and bands were visualized by autoradiography. *Asterisk*: supershifted complex. FP indicates migration of noncomplexed free AP1-5-P^{32}.

subset of AP1 factors similar to those that bind at the AP1-1 site. In addition, the present study shows that the AP1-5 site is absolutely required for in vivo hINV expression in the corneal epithelium. It is interesting that JunB, which binds to the hINV AP1-5 and AP1-1 sites, has been detected in the superficial layers in rat cornea.[54] This suggests that JunB may have a physiologic role in activating and/or maintaining hINV expression in the corneal superficial cell layers. Moreover, Fra-1 is also expressed in human corneal epithelium.[61] Thus, Fra-1 and JunB are viable candidates as regulators of hINV gene expression. In contrast, c-Fos, Fra-2, and FosB are not detected.[61] The finding that only selected members of the AP1 factor family directly participate in regulation of hINV gene expression is consistent with previous reports indicating selectivity in this process.[28]

Overall Role of the DRR in Regulating hINV Expression in the Corneal Epithelium

The hINV protein is known to be expressed in a range of surface epithelia, including the cervix, vagina, epidermis, esophagus, tongue, and buccal mucosa.[16] Thus, an important question is the relative role of the DRR AP1-5 site in driving hINV expression in the corneal epithelium versus these other epithelia. In addition to the AP1-5 site, the DRR encodes a Sp1 site that is located immediately downstream and separated by one nucleotide from the AP1-5 site.[33] Our recent studies indicate that this Sp1 site is important for expression of the hINV gene in the corneal epithelium. These studies demonstrate that mutation of the Sp1 site results in a complete loss of hINV gene expression in the corneal epithelium, but that expression is minimally changed in other surface epithelia (Adhikary G, Eckert RL, manuscript in preparation). Thus, Sp1 has a selective role in the corneal epithelium. The present study indicates that the AP1-5 site is also required for hINV expression in the corneal epithelium; however, unlike the Sp1 mutation, the mutation of AP1-5 results in a loss of expression in all surface epithelia.[32] On balance, these findings suggest that adjacent DRR-based DNA elements, the Sp1 and AP1-5 sites, must act together to drive corneal epithelial hINV expression and that both Sp1 and AP1 factors are required for response. In contrast, the Sp1 factor binding site and perhaps Sp1 factors may have a lesser role in other epithelia.

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