Involucrin Expression in the Corneal Epithelium: An Essential Role for Sp1 Transcription Factors

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Purpose. Identifying the mechanism(s) that regulate gene expression during the transition of the limbal stem cell to a differentiated superficial cell is an important area of interest in the corneal epithelium.

Methods. However, the factors that regulate gene expression during this process are not well understood. In the present study, the human involucrin (hINV) gene was used as a model to study gene expression in the corneal epithelium. Expression was studied in normal human corneal epithelial cell cultures and hINV promoter transgenic mice.

Results. Studies in cultured cells revealed that an Sp transcription factor–binding site, located in the upstream regulatory region of the hINV promoter, is essential for optimal hINV gene expression. Mutation of this site reduces promoter activity. Expression of Sp1 results in an Sp1-dependent increase in activity, whereas expression of dominant-negative Sp1 inhibits promoter activity. Gel mobility shift analysis showed the interaction of Sp1 and Sp3 with the Sp DNA element. Treatment of the corneal epithelial cells with 12-*O*-tetradecanoylphorbol-13-acetate increased hINV gene expression and this response is associated with increased nuclear factor binding of Sp1 and Sp3 to the Sp DNA response element. Promoter mutagenesis studies in transgenic mice confirmed the importance of the Sp site, as removal of this site by promoter truncation or point mutation resulted in a complete loss of in vivo corneal epithelial cell gene expression.

Conclusions. These studies provide in vivo evidence that Sp transcription factor input is absolutely necessary for activation of involucrin gene expression in the differentiating corneal epithelium. (*Invest Ophthalmol Vis Sci.* 2005;46:3109–3120) DOI:10.1167/iovs.05-0053

During the process of corneal epithelial cell differentiation, proliferating stem cells, located in the limbus of the eye, give rise to transient amplifying cells and superficial cells that populate the multilayered corneal epithelium.[1–3] Ultimately, the superficial cells are lost from the corneal surface.[4] This process is associated with specific changes in the expression of genes that are necessary to maintain corneal epithelial cell integrity and function. Involucrin is a marker of the transition from proliferation to differentiation.[5] Activation of involucrin expression is associated with the exit of the stem cell from the limbal compartment, and involucrin is maximally expressed in the differentiated, superficial cell layer. There is intense interest in elucidating the mechanisms that regulate the transition from stem cell to transient amplifying cell to differentiated cell and the mechanisms that regulate gene expression during this process. However, there is only limited information regarding the mechanisms that regulate gene expression during this process. We have developed the involucrin gene as a model to monitor this process, with a goal of providing understanding regarding the transcriptional mechanisms that regulate gene expression during corneal epithelial cell maturation.

Involucrin is a rod-shaped, α-helical structural protein[6] that serves as a substrate for the formation of covalent interprotein ϵ-(γ-glutamyl)lysine isopeptide bonds.[7,8] It is produced as a soluble cytosolic protein.[7] However, during the terminal stages of differentiation in surface epithelial cells, it becomes covalently cross-linked at sites on the inner face of the plasma membrane, where it serves as a scaffold protein to stabilize cell structure.[6,9,10] Type I transglutaminase,[11] which catalyzes formation of interprotein ϵ-(γ-glutamyl)lysine bonds, catalyzes the formation of interprotein covalent cross-links between involucrin and other proteins.[12,13] Both involucrin and type I transglutaminase are present in corneal epithelial cells, and assembly of covalently cross-linked structures most likely plays a role during the corneal epithelial cell life cycle.[5,14]

In the present study, we used two models as part of our effort to identify mechanisms that regulate the transition during corneal epithelial cell differentiation: primary human corneal epithelial cultures, for in situ analysis of promoter function, and a series of human involucrin (hINV) promoter truncation and point mutation transgenic mice, to confirm the in vivo physiologic relevance of the cell-culture–based studies. Studies with cultured human corneal epithelial cells showed that Sp1 and Sp3 interact with the involucrin Sp response element to regulate involucrin gene expression. Moreover, mutation of the Sp site, or expression of dominant-negative Sp1 confirmed that involucrin expression is Sp1 factor dependent. Parallel studies in transgenic mice confirmed that the involucrin promoter distal regulatory region (DRR), which contains the Sp site, is absolutely necessary for corneal expression. Additional studies showed that mutation of the DRR Sp site eliminated involucrin gene expression in the corneal epithelium but not in other surface epithelia. These studies implicate Sp transcription factors as key regulators of corneal epithelial cell gene expression during the transition from stem cell to superficial cell in the corneal epithelium.

Materials and Methods

Chemicals and Reagents

Keratinocyte serum-free medium (KSFM), trypsin, Hanks' balanced salt solution, and gentamicin were purchased from Invitrogen (Carlsbad,

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CA); phorbol ester (12-*O*-tetradeconylphorbol-13-acetate [TPA]) and dimethyl sulfoxide from Sigma-Aldrich (St. Louis, MO); the pGL2-Basic plasmid and a chemiluminescent luciferase assay system from Promega (Madison, WI); and [γ-^{32}P]ATP from Perkin Elmer Life Sciences (Boston, MA). The hINV-specific polyclonal antibody was generated as previously described.[13] Transcription factor-selective rabbit polyclonal antibodies specifying Sp1 (SC-59, diluted 1:1000 for immunoblot) and Sp3 (SC-644, diluted 1:1000 for immunoblot) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), as were blocking peptides for each antibody (SC-59P, SC-644P). The mouse monoclonal β-actin antibody was from Sigma-Aldrich and was used diluted 1:3000 for immunoblot assays. Secondary antibodies include HRP-conjugated goat anti-mouse IgG, and HRP-conjugated goat anti-rabbit IgG, both from Santa Cruz Biotechnology. The hINV promoter reporter plasmids were constructed in pGL2-Basic, as previously described.[15,16] Recombinant human involucrin (rhINV) was produced by cloning the hINV coding sequence into pRSET-B to yield phINV(1-585). This vector was used to produce histidine-tagged hINV in bacterial strain BL21. The recombinant protein (rhINV) was then purified with a nickel metal affinity column and used to produce a rabbit polyclonal antibody that detects hINV but not murine (m)INV.[13] Recombinant murine involucrin (rmINV) was produced with a similar system.

Immunoblot Method

For immunoblot, an identical number of protein equivalents were electrophoresed on denaturing and reducing 6% polyacrylamide gels and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk and then incubated with the appropriate antibodies. Antibody binding was visualized with chemiluminescence detection technology.

Plasmids

Construction of the involucrin promoter luciferase reporter plasmids has been described.[17,18] The human Sp1-encoding plasmid was provided by Jon Horowitz (Duke Medical Center, Durham, NC).[19] pcDNA3-HA-dnhSp1 (HA-tagged dominant-negative human Sp1) was constructed by fusing an HA epitope upstream of amino acids 619 to 785 of human Sp1 (GenBank accession no. BC062539; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) by polymerase chain reaction and then cloning the resultant *Bam*HI/*Eco*RI fragment into pcDNA3. Amino acids 619 to 785 encode the three C-terminal zinc fingers of Sp1 but lack the amino terminal transcriptional activation domains.[20,21]

Human Corneal Epithelial Cell Culture

Human eyes 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 Institutional Review Board. Eyes were obtained from subjects who had agreed to donate these organs, and all procedures followed the principles articulated in the Helsinki Declaration. The methods for obtaining corneal epithelial cells and culturing these cells has been described.[22] Cells were routinely passaged at a split ratio of 1:3 in early cultures and at 1:5 in established cultures. Cultures that were 50% to 70% confluent were used for experimentation.

hINV Promoter Activity

For hINV promoter studies, 6 μL of a transfection reagent (Fugene-6; Roche Diagnostics, Indianapolis, IN) was mixed with 94 μL of KSFM and incubated at 25°C for 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, the involucrin reporter plasmid and Sp1[19] and Sp3 expression plasmids were used at the levels indicated in the Figure 6. The final DNA concentration in all groups was maintained constant by addition of empty expression vector. In some experiments, at 24 hours after transfection, 2 mL of fresh media was added containing 0 or 50 ng TPA per milliliter. After an additional 24 hours, the cells were washed with phosphate-buffered saline (pH 7.5) and scraped into 200 μL of cell lysis buffer,[16] and luciferase activity was assayed immediately. All assays were performed in triplicate, and each experiment was repeated a minimum of three times.

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 phosphate-buffered saline, and total cell extract was prepared in the presence of proteinase inhibitors,[16] and nuclear extracts were prepared according to Schreiber et al.[23] in buffer containing 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein content was measured with a protein assay reagent (DC; Bio-Rad, Hercules, CA). Identification of binding to the hINV promoter Sp site was detected with an electrophoretic mobility shift assay.[15] Nuclear extract (3 μg) 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 radioactive, double-stranded, ^{32}P-labeled Sp site oligonucleotide (5′-ATTCGATCG*GGGGCGGG*GCGAGC). The Sp-binding site is italic. For competition studies, nonradioactive competitor oligonucleotide was added to the DNA-binding reaction. For the gel mobility supershift assay, Sp 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, and the incubation was continued for an additional 20 minutes at room temperature. Protein-DNA complexes were then resolved in nondenaturing 6% polyacrylamide gels, and the position of the complex was determined by autoradiography.

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.[24–26] 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 blotting of DNA using an hINV-specific probe. Expression of the transgene is monitored in tissue by assaying for the presence of hINV protein using an hINV-specific antibody that does not cross-react with mouse involucrin.[13] A minimum of four separate transgenic lines was examined for each DNA construct.

Involucrin Expression in Transgenic Mouse Cornea

Mice were killed, and the central corneal epithelium was excised by a circumferential cut with fine scalpel under a microscope. Internal eye fluid was removed by scraping and the corneal epithelium was minced into small pieces and homogenized in 200 μL of buffer A (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 0.1 mM sodium orthovanadate, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) at 4°C. The lysate was incubated for 30 minutes on ice and centrifuged at 12,000 rpm, and the supernatant was stored at −80°C. To detect hINV protein, equivalent quantities of protein from each sample were electrophoresed on an 8% gel and transferred to nitrocellulose for immunoblot assays. The hINV level was monitored by immunoblot.[13] hINV expression was also measured by immunohistology of sections derived from the central cornea. The sections were fixed and then incubated with hINV-specific antibody followed by peroxidase-linked secondary antibody.[25] β-Actin was used as a control for normalization of the amount of protein in each lane.

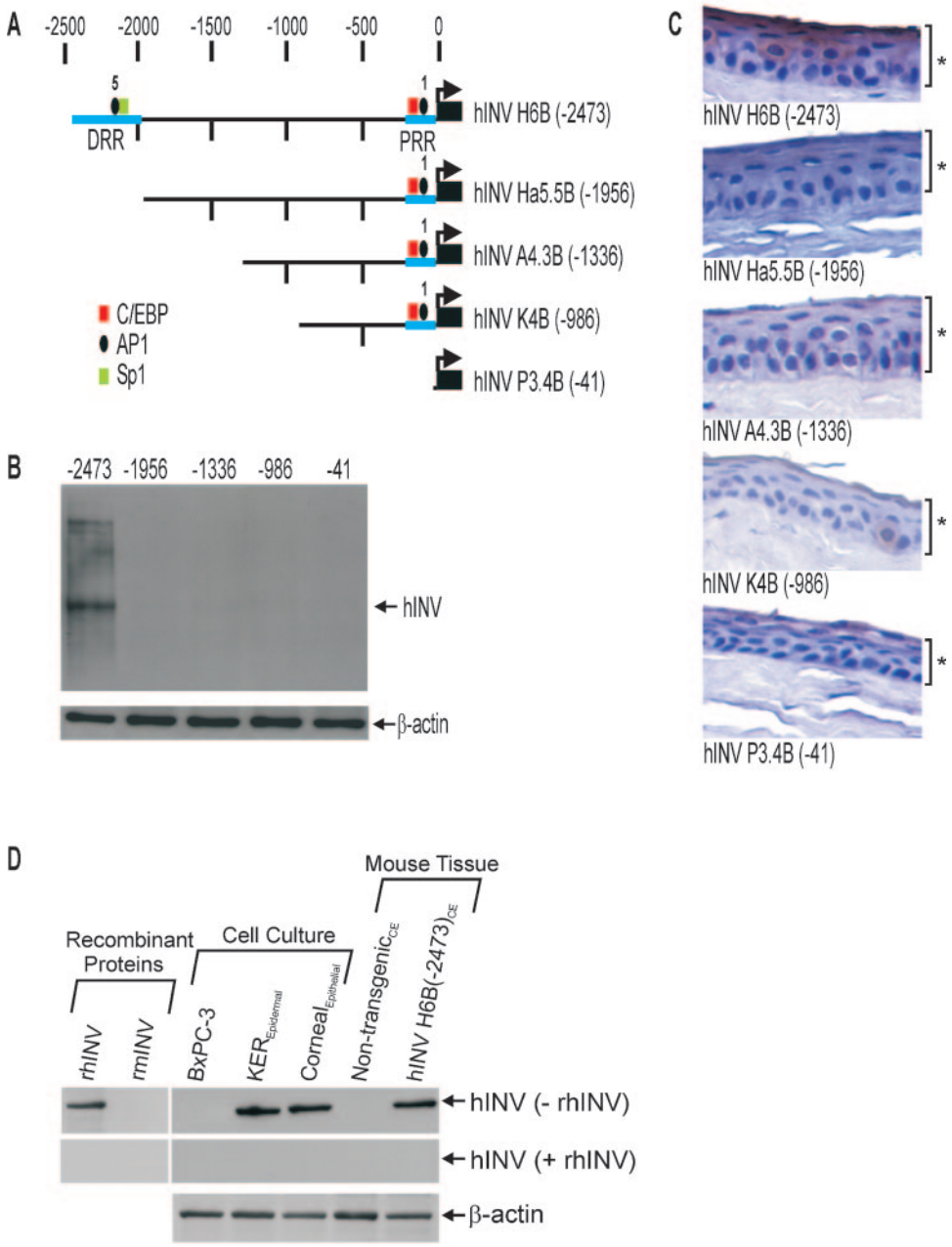

FIGURE 1. The DRR is necessary for in vivo corneal epithelial expression of involucrin. (**A**) Mouse embryos were injected with each of the indicated transgenes. hINV H6B encodes the full-length (2473 nucleotides) upstream regulatory region. Constructs Ha5.5B, A4.3B, K4B, and P3.4B encode 1956, 1336, 986, and 41 nucleotides of the upstream regulatory region, respectively. hINV P3.4B, which lacks all upstream sequence except the minimal promoter (−41/−1), encodes only the basal promoter and serves as a negative control. *Black rectangle*: involucrin protein coding sequence; *arrow*: start site and direction of transcription. The distal (DRR) and proximal (PRR) regulatory regions are indicated, as are functionally important transcription-factor–binding sites that interact with AP1, Sp1, and C/EBP transcription factors.[15] (**B**) Detection of hINV transgene expression in the corneal epithelium. Central corneal epithelium was harvested from individual transgenic mouse lines. 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 using chemiluminescence detection reagents. Four to 10 independently derived transgenic lines were tested for each construct with identical results. No signal was detected in nontransgenic mice (not shown). The β-actin level was monitored as a loading control. (**C**) 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[27] followed by peroxidase-conjugated goat anti-rabbit IgG. *Asterisk*: the extent of the corneal epithelium. (**D**) The hINV-specific antibody does not detect murine involucrin (mINV). rhINV and rmINV (500 ng per lane), extracts prepared from BxPC-3 cells (a human-derived pancreatic cancer cell line that does not express involucrin), normal epidermal keratinocytes, and normal corneal epithelial cells and extracts from the central corneal epithelium harvested from mice that are nontransgenic, nontransgenic$_{CE}$, or transgenic, hINV H6B(−2473)$_{CE}$, for hINV. Cell and tissue extracts were electrophoresed at 20 μg protein/lane on a 6% acrylamide gel and then incubated with anti-hINV antibody[13,27] (*top*). The primary anti-hINV antibody was incubated in the absence (− rhINV) or presence (+ rhINV) of 2 μg/mL recombinant hINV as a specific antibody competitor. A parallel blot was incubated with anti-β-actin to confirm appropriate loading.

RESULTS

Involucrin Expression: A Critical Role for an Sp Factor DNA-Binding Site

To begin examining the mechanisms regulating hINV expression during corneal epithelial cell maturation, we developed transgenic mice that encode selected segments of the hINV promoter. Each transgene encodes a segment of the promoter upstream regulatory region linked to the hINV protein coding region (Fig. 1A). Previously identified functional domains, including the distal (DRR) and proximal (PRR) regulatory regions, are indicated for each construct.[15] Transgene expression in corneal epithelia was monitored by immunoblot or immunohistology using an hINV-specific antibody.[15,24] Four to 10 independent transgenic mouse lines were tested for each construct. To monitor for transgene expression, total cell extracts were prepared from the central corneal epithelium of mice harboring each transgene, and samples were electrophoresed for immunoblot. Figure 1B shows that deletion of the DRR (nucleotides −2473/−1956)[15] results in a complete loss of hINV expression in the corneal epithelium. Moreover, further truncation of the promoter to nucleotide positions −1956, −1336, −986, or −41 does not restore the lost expression. P3.4B (−41) is a basal promoter construct that encodes only the hINV promoter transcription start site.[24–26] Figure 1C shows immunohistochemical staining of corneal

epithelial sections with anti-hINV. In mice encoding the full-length hINV promoter H6B (−2474), hINV is detected in the murine corneal epithelium. Consistent with the immunoblot findings, no corneal epithelial expression was observed for constructs −1956, −1336, −986, or −41. The present study relied on the ability to detect hINV transgene expression in mice that express murine involucrin. For this purpose, we generated an antibody that specifically detects hINV.[13] As shown in Figure 1D, the antibody detected recombinant hINV (hINV), in extracts from cultured human corneal and epidermal epithelial cells and in tissue extracts of corneal epithelium derived from the central corneal epithelium of the hINV transgenic mouse line, hINV H6B(−2473). In contrast, no signal was detected in cells that did not express involucrin (including BxPC-3 cells, an involucrin-negative human pancreatic cancer cell line) or in extracts prepared from central corneal epithelium derived from mice lacking the involucrin transgene (non-transgenic$_{CE}$). Moreover, the antibody does not detect recombinant murine involucrin (rmINV). Finally, supplementation of the primary antibody incubation mixture with an excess of recombinant hINV (+rhINV) efficiently eliminates detection. Thus, the antibody detects human but not murine involucrin, making it an appropriate reagent for detection of hINV in the murine corneal epithelium.

The above results suggest that the DRR, nucleotides −2473/−1956, is necessary for corneal epithelial hINV gene expression. However, these findings do not demonstrate whether the DRR alone is sufficient for this expression. To assess this possibility, we compared expression of P3.4B, the basal promoter construct with DRR-P3.4B. As shown in Figure 2A, in DRR-P3.4B, the DRR is cloned immediately upstream of the basal promoter at position −41. Figure 2B shows that the P3.4B construct does not drive expression. In contrast, DRR-P3.4B drives expression, which is comparable to that observed for the full-length promoter construct H6B (−2473). This finding suggests that the DRR is both necessary and sufficient to drive corneal epithelial expression in vivo.

We next examined the role of specific nucleotide motifs within the DRR. An analysis of the DRR sequence[24] reveals the presence of an Sp factor–binding site.[15,18,29] Because Sp factors have been shown to be essential in regulating cytokeratin K3 expression in cultured rabbit corneal epithelial cells,[30] we designed a transgene to determine whether mutation of the hINV promoter Sp-binding site results in altered gene expression. For this purpose, we generated multiple transgenic mouse lines encoding the DRR(Sp1m)-P3.4B construct and compared the expression with that observed for DRR-P3.4B (Fig. 3A). The sequence of the Sp site mutation is shown in Figure 3B (underscored). Figure 3C confirms that DRR-P3.4B drives appropriate corneal epithelial involucrin expression. In contrast, mutation of the Sp site resulted in a complete loss of transgene expression. As expected, no expression was observed in nontransgenic (NT) mice. Figure 3D shows a comparison of the pattern of expression detected by immunohistologic methods. This analysis indicated that DRR-P3.4B drives normal differentiation-dependent corneal epithelial expression and showed that no staining is observed in DRR(Sp1m)-P3.4B mice or nontransgenic mice. A remarkable feature of this regulation was the complete absence of expression of the DRR(Sp1m) construct, suggesting a strict requirement for the Sp site for involucrin expression in the corneal epithelium in vivo.

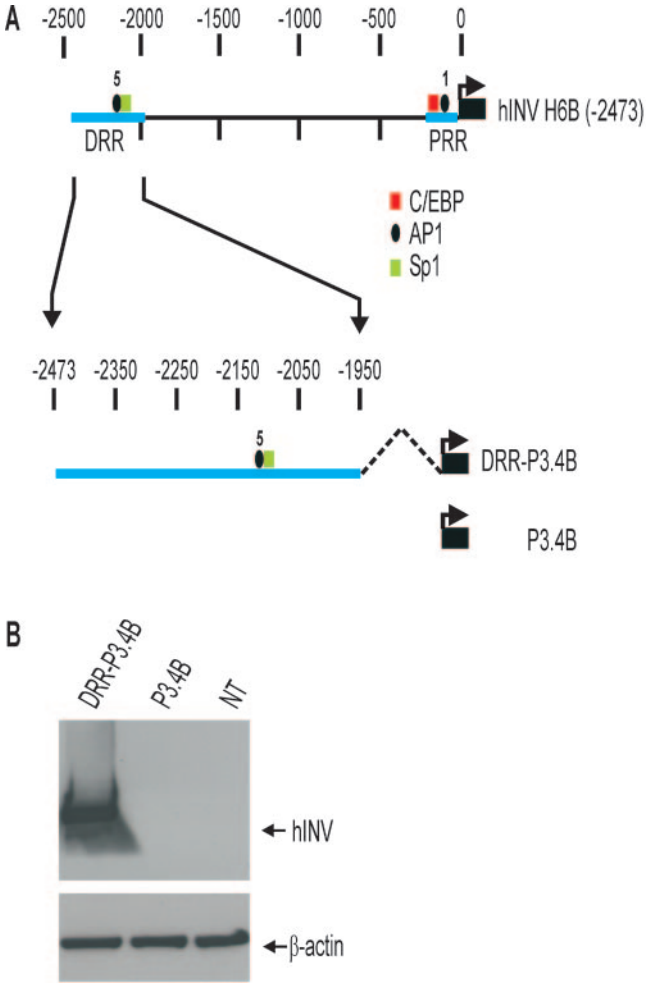

Figure 2. The hINV promoter DRR is necessary for in vivo corneal epithelial cell involucrin expression. (**A**) Mouse embryos were injected with each of the indicated transgenes, and a minimum of six individual transgenic lines were tested for each construct. DRR-P3.4B encodes nucleotides −2473/−1950 of the hINV upstream regulatory region. This segment is linked directly to the minimal hINV promoter. P3.4B encodes only the hINV gene minimal promoter. The positions of the DRR and PRR and C/EBP, Sp1, and AP1 sites are indicated.[15,28] (**B**) Detection of hINV protein expression in the corneal epithelium. Central corneal epithelium was harvested from each independently derived 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 hINV-specific antibody as in Figure 1. The β-actin level was monitored as a loading control.

Sp Factors in the Mouse Corneal Epithelium

The above in vivo studies strongly support the hypothesis that involucrin expression requires Sp factors to be present in the corneal epithelium. We therefore tested for the presence of Sp1 and Sp3, two Sp family members that are expressed in many tissues. Tissue samples from the mouse central corneal epithelium were prepared for monitoring Sp1 and Sp3 content by immunoblot. For comparison, parallel samples were prepared from mouse liver. As shown in Figure 4, both Sp1 and Sp3 were detected by the corresponding specific antibodies, and in each case the signal was efficiently competed by addition of antibody-specific blocking peptide (Fig. 4, BP). The Sp3 blocking peptide only partially blocked binding to the major Sp3 band (asterisk), presumably because of the abundance of this band. In addition, several individual Sp1- and Sp3-immunoreactive bands were detected. This result is as expected, as the single Sp1 isoform is known to be phosphorylated, glycosylated, acetylated, and proteolytically processed, and Sp3 exists as four isoforms that are each differentially acetylated and sumoylated.[31–34]

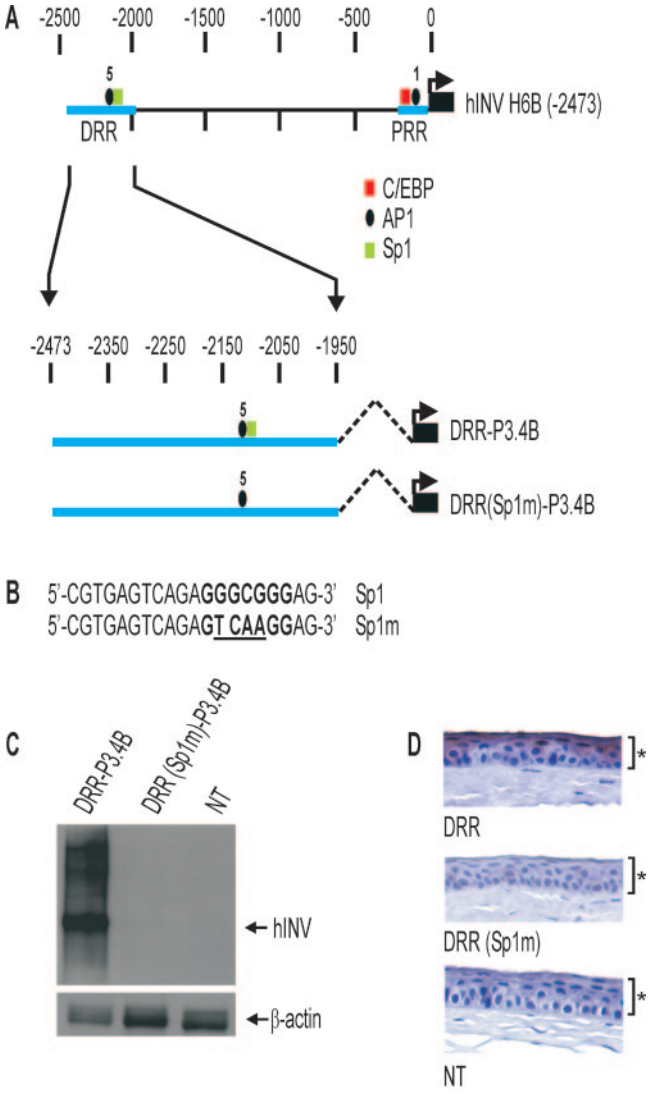

FIGURE 3. The DRR Sp1 site was shown to be essential for in vivo corneal epithelial cell involucrin expression. (**A**) Mouse embryos were injected with each of the indicated transgenes. DRR-P3.4B encodes nucleotides −2473/−1950 of the hINV upstream regulatory region. This segment is linked directly to the minimal hINV promoter. DRR(Sp1m)-P3.4B is identical, except that the Sp1 site is mutated. The positions of the DRR, PRR, and transcription-factor–binding sites are indicated.[15,28] (**B**) The authentic (Sp1) and mutated (Sp1m) Sp1 sites encoded by the DRR-P3.4B and DRR(Sp1m)-P3.4B constructs, respectively. The Sp1 site is indicated in bold[24,26] and altered residues in Sp1m are *underscored*. (**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 as in Figure 1. Six independently transgenic lines were tested for each construct with identical results. No signal was detected in nontransgenic mice (NT). The 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.[24]

hINV Expression in Cultured Human Corneal Epithelial Cells: Sp Site-Dependent Promoter Activity

To gain additional insights regarding the Sp-related mechanisms that regulate involucrin expression, we used human corneal epithelial cell cultures. hINV promoter activity is known to be TPA-dependent in cultured human corneal epithelial cells.[22] To examine the role of the DRR and the Sp site in this regulation, normal human corneal epithelial cells were transfected with pINV(−2473/−2088), which encodes the hINV promoter DRR segment linked to luciferase, and then treated with TPA. The DRR is the DNA segment that is necessary for hINV transgene expression in mice (see Fig. 2). TPA treatment resulted in a threefold increase in hINV promoter activity (Fig. 5). In contrast, mutation of the Sp site resulted in substantially reduced basal promoter activity and reduced activation in response to TPA.

Sp1 and Sp3 Regulation of hINV Promoter Activity in Cultured Corneal Epithelial Cells

To gain insight into the role of individual Sp factors, cultured human corneal epithelial cells were transfected with the full-length hINV promoter-luciferase reporter construct in the presence of Sp1 or Sp3. We examined the effect of Sp1 and amino terminal-truncated Sp3 (ΔN-Sp3), since these are known to

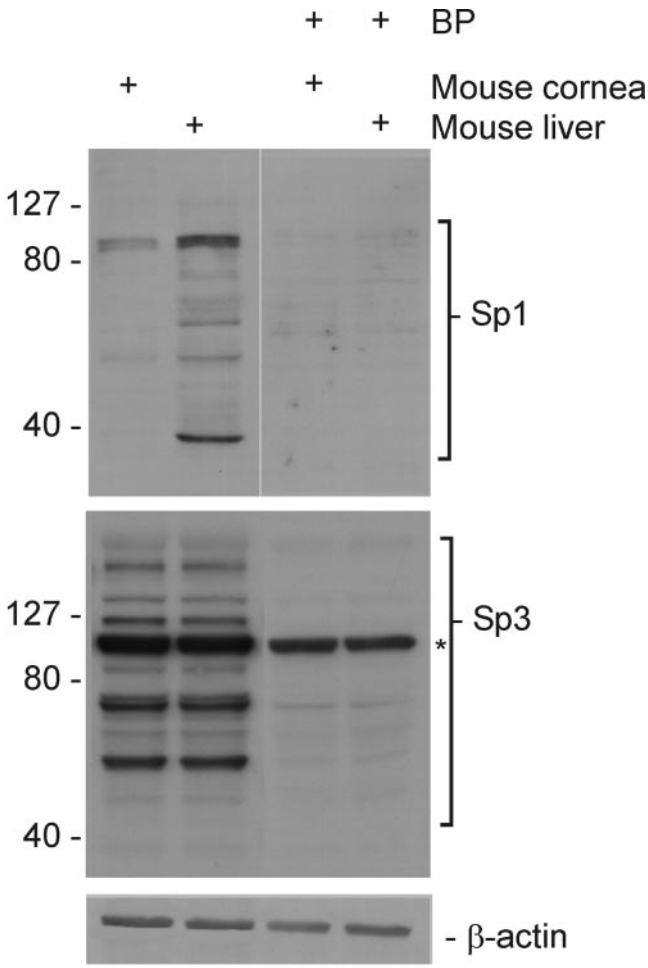

FIGURE 4. Sp1 and Sp3 are expressed in murine corneal epithelial cells. Total cell extracts were prepared from murine corneal epithelium and from murine liver. Equivalent quantities of protein were electrophoresed in parallel sets of lanes and transferred to nitrocellulose for immunoblot with anti-Sp1 or anti-Sp3. Sp1 or Sp3 blocking peptide (BP) was included in the antibody incubations as indicated. The antibody (0.5 μg) was preincubated with 2.5 μg of blocking peptide. *Asterisk*: an abundant band, with only partial competition from the blocking peptide. The β-actin was blotted as a loading control.

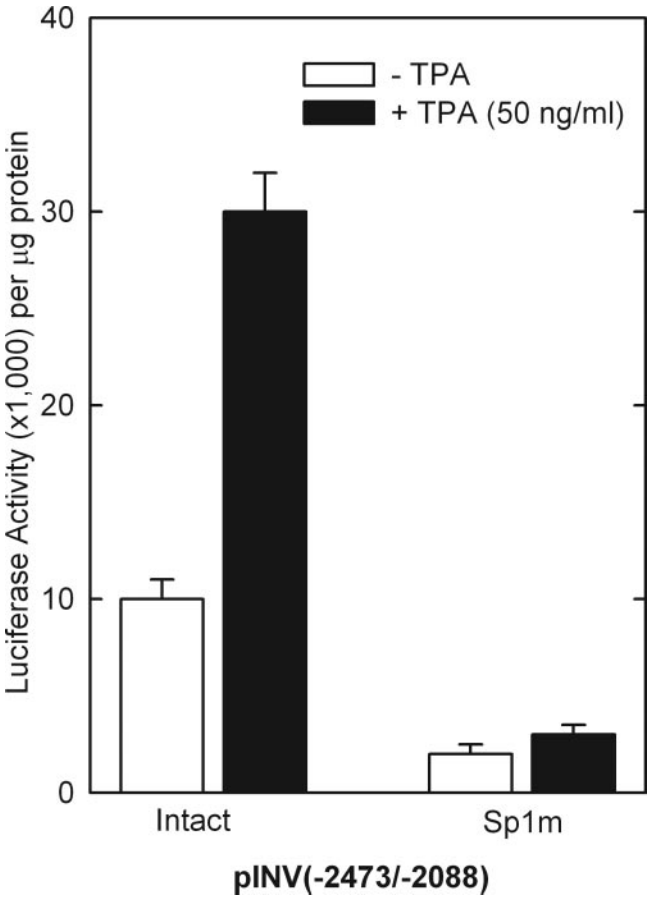

FIGURE 5. Regulation of hINV promoter activity in human corneal epithelial cells. The hINV promoter DRR luciferase reporter construct, pINV(−2473/−2088), encoding an intact or mutant Sp1 site, was transfected into 40% confluent cultures of normal human corneal epithelial cells. After 24 hours, the cells were treated with 50 ng/mL TPA. After an additional 24 hours, the cells were harvested and assayed for luciferase activity with a fluorometer. Error bars, ±SEM. Data are the mean of results in four separate experiments.

have opposing regulatory actions in several systems.[35] Figure 6A shows that Sp1, an Sp factor that is known to function as a transcriptional activator,[35–37] increased hINV promoter activity. In contrast, expression of ΔN-Sp3 reduced promoter activity (Fig. 6B). Moreover, increasing concentrations of amino-terminal Sp3 reduced Sp1-dependent promoter activation (Fig. 6C). In addition, parallel experiments indicated that ΔN-Sp3 inhibited the TPA-dependent increase in promoter activity (not shown), suggesting that the TPA-dependent increase is induced by Sp1.

This regulation predicts that Sp1 and Sp3 should be expressed in cultured primary corneal epithelial cell cultures. To confirm expression, we prepared total cell extracts and monitored Sp1 and Sp3 level by immunoblot. Endogenous Sp1 and Sp3 were present in cultured human corneal epithelial cells, and the level of each was increased by transfection with the corresponding expression vector (Fig. 6D). Sp1 and Sp3 are known to be posttranslationally modified in tissues; thus, multiple immunoreactive bands are present.[37] The band that displayed the most obvious increase in each case is indicated by an asterisk. Since approximately 20% of cells were transfected in these experiments (not shown), the vectors delivered substantial levels of Sp1 and Sp3 to the transfected cells.

Dominant-Negative Sp1 Inhibition of the TPA- and Sp1-Dependent Increase in hINV Promoter Activity

As demonstrated in Figure 6, human corneal epithelial cells expressed substantial levels of endogenous Sp1 and Sp3. We next initiated experiments designed to manipulate the impact of endogenous Sp factors. We predicted that interfering with the function of these factors would reduce hINV promoter activity and provide additional evidence of a role for Sp factors. Our initial approach was to construct and use a vector encoding dominant-negative Sp1. Previous studies indicate that the amino terminal region of Sp1 encodes the activation domain and that the carboxyl terminus encodes three zinc fingers involved in DNA binding.[20,37] Expression of the Sp1 fragment encoding only the zinc finger region does not activate transcription[31]; moreover, this fragment functions as a dominant-negative inhibitor of Sp1-dependent transcription.[38] To assess the role of endogenous Sp factors in regulating involucrin promoter activity, we transfected cells with pINV−2473 or pINV(−2473/−2088) in the presence or absence of dominant-negative human Sp1 (dnhSp1). Expression of dnhSp1 produced a concentration-dependent reduction in promoter activity (Fig. 7A), suggesting that input from endogenous Sp1 is necessary for promoter activity. However, it is also clear that dnhSp1 does not completely suppress activity, making it likely that Sp factors are not the only transcription regulator involved in maintaining hINV expression.

As a second method of assessing the role of endogenous Sp factors, we used mithramycin A (MMA). MMA is an agent that inhibits protein binding to G/C-rich motifs, such as Sp elements, and is a well-characterized inhibitor of Sp factor action.[39] We therefore treated keratinocytes with MMA and monitored the effects on hINV promoter activity. Keratinocytes were transfected with pINV−2473 or pINV(−2473/−2088) and then treated in the presence or absence of 200 nM MMA. After 24 hours, the cells were harvested, and extracts were prepared for luciferase assay. MMA treatment reduced activity of both pINV−2473 and pINV(−2473/−1088) by 40% to 50% (Fig. 7B). In addition, mutation of the Sp1 site resulted in a reduction in overall activity and a loss of MMA-dependent suppression. These findings are consistent with a role for the DRR Sp-binding site in mediating the effects of Sp1. Moreover, it should be noted that MMA treatment produced a 30% to 50% reduction in endogenous hINV protein and mRNA levels, indicating that expression of the endogenous gene was also only partially suppressed by this treatment (not shown).

Sp1 and Sp3 Binding to the hINV Promoter Sp Site

The results of functional studies described herein suggest that an increase in Sp1 factor level leads to increased transcription and predicts that this may be due to increased Sp factor binding to the hINV promoter Sp1 element. Because TPA increases hINV gene expression in corneal epithelial cells,[22] nuclear extracts were prepared from cells grown for 24 hours in the absence or presence of TPA. We hypothesized that the increased expression may be associated with increased Sp factor binding to the Sp DNA element. To assess the status of Sp factor binding to the Sp site, we incubated a ^{32}P-labeled oligonucleotide encoding an Sp consensus binding site (Sp1c; Fig. 8A) with nuclear extracts prepared from nontreated and TPA-treated human corneal epithelial cells. Electrophoresis of these mixtures on nondenaturing gels revealed the presence of shifted bands (Sp; Fig. 8B). The intensity of these bands increased in cells treated with TPA. Moreover, competition for Sp binding was induced by addition of a 10- or 100-fold molar excess of nonradioactive Sp1c oligonucleotide. Non-Sp se-

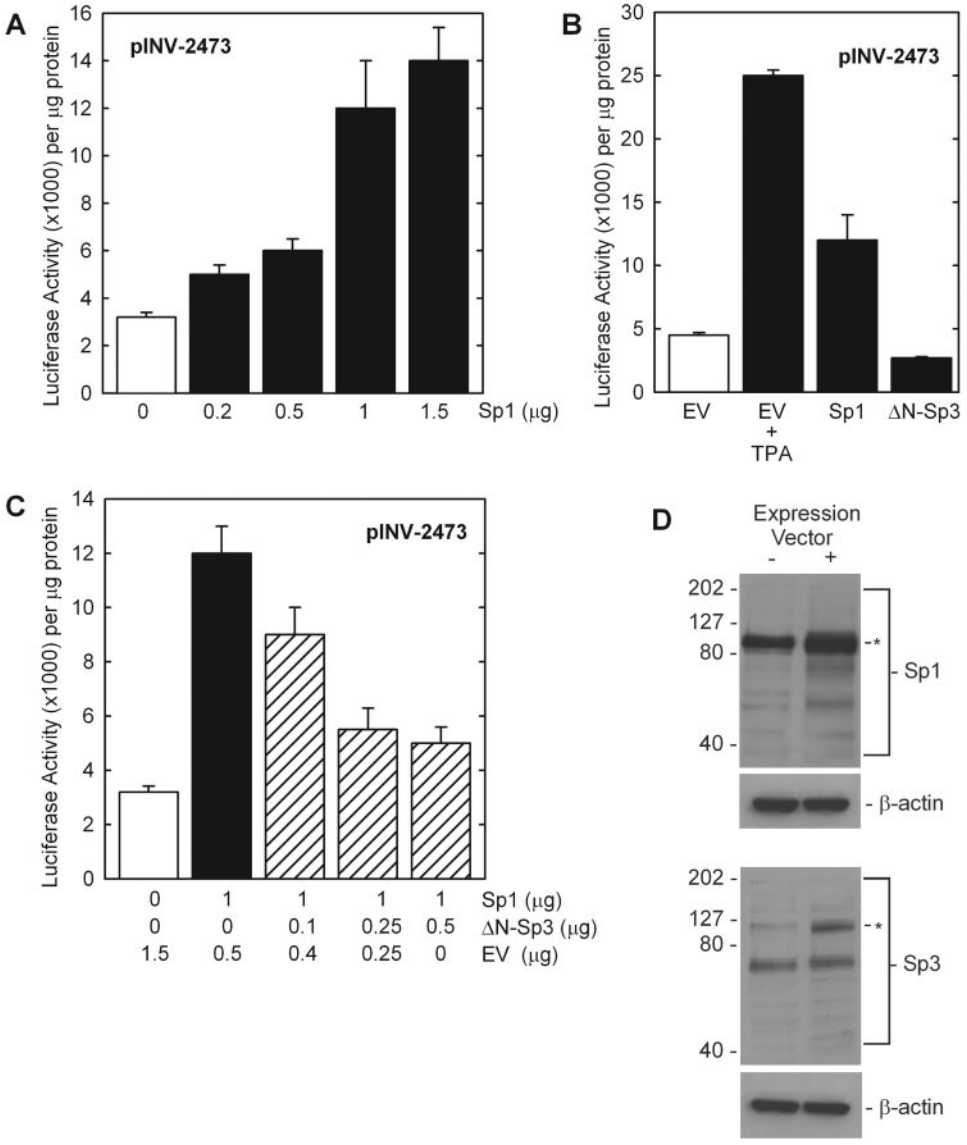

FIGURE 6. Sp1 and ΔN-Sp3 regulation of hINV promoter activity. (**A**) Sp1 increased hINV promoter activity. Cells were transfected with 0.5 μg of pINV−2473 in the presence of increasing levels of Sp1 expression vector. The total plasmid content of the transfection was equalized to 2 μg total DNA by addition of empty vector (EV). (**B**) Differential regulation by Sp1 and Sp3. Corneal epithelial cells were transfected with 1 μg of pINV−2473 in the presence of 1.0 μg of EV or the Sp1 or ΔN-Sp3 expression vector. After 24 hours, the indicated groups were treated with 50 ng TPA/mL; and, after an additional 24 hours, the cells were harvested and assayed for luciferase activity. Data are the mean ± SEM of results in four separate experiments. (**C**) Opposing action of Sp1 and ΔN-Sp3. Cells were transfected with 0.5 μg of pINV−2473 and the indicated level of EV or Sp1 or ΔN-Sp3-encoding expression vector. After 24 hours, the cells were harvested and assayed for promoter-dependent luciferase activity. Error bars, ±SEM. Data are the mean ± SEM of results in three separate experiments. (**D**) Sp1 and Sp3 immunoblots. Cells were transfected with empty expression vector (−) or expression vector encoding Sp1 or ΔN-Sp3 (+). After 24 hours, the cells were harvested for preparation of total extracts. The extracts were immunoblotted with rabbit anti-Sp1 (dilution = 1:1000) or rabbit anti-Sp3 (dilution = 1:1000). Parallel blots were incubated with anti-β-actin as a gel-loading control. *Asterisks*: band displaying the most obvious increase in Sp1- or Sp3-transfected cells.

quence oligonucleotides or oligonucleotides in which the Sp site is scrambled (Sp1c-m) do not compete for binding (Fig. 8B). To demonstrate interaction of Sp1 and Sp3 with the Sp-binding element, we performed a gel mobility supershift assay. Addition of antibody specific for Sp1 resulted in a complete loss of binding to DNA, whereas addition of anti-Sp3 produced a supershifted band (asterisk; Fig. 8C), results that provide compelling evidence for an interaction of Sp1 and Sp3 with the hINV promoter Sp factor–binding site. The positions of the bands that presumably correspond to Sp1 and Sp3, based on the gel supershift analysis, are indicated in Figure 9C. Incubation with anti-IgG did not supershift bands, indicating that the anti-Sp1- and anti-Sp3-dependent changes in band mobility were specific.

The increased binding to the Sp element in response to TPA treatment could be due to enhanced expression of Sp1 and Sp3 or to enhanced movement of Sp1 to the nucleus. To assess this possibility, we performed immunoblots with total cell and nuclear extracts prepared from TPA-treated and nontreated corneal epithelial cells. Figure 9A shows that TPA treatment for 24 hours did not alter the total cellular level of Sp1 or Sp3. However, this treatment did produce a change in cytosolic and nuclear Sp1 and Sp3 levels. The cytosolic level of these factors was decreased in TPA-treated cells, and the nuclear level of these factors, which was relatively low in the absence of TPA treatment, increased in TPA-treated cells (Figs. 9B, 9C). The level of Sp1 and Sp3 in the cytosol was substantially greater, on a per cell basis, than in the nucleus; therefore, a modest apparent reduction in cytoplasmic Sp1 and Sp3 level resulted in a substantial increase in nuclear level. These blots further show that numerous Sp1 and Sp3 immunoreactive bands were detected, a finding that is consistent with observations in other systems.[40]

Effect of the Mutation of the Sp1 Site on hINV Expression in Other Surface Epithelia

Involucrin is expressed in a wide range of surface epithelia.[14] To assess whether the hINV promoter Sp-binding site is uniquely necessary for corneal epithelial hINV expression, we examined the effects of the Sp-binding site mutation on hINV expression in other surface epithelia. Epithelial sections of footpad, cervix, epidermis, and esophagus were prepared from DRR(Sp1m)-P3.4B transgenic mice. The structure of the DRR(Sp1m)-P3.4B construct used to produce these mice is shown in Figure 3A. Involucrin expression was monitored by immunoblot and by immunohistology. Figure 10A shows that involucrin was detected in the epithelial cells derived from footpad, epidermis, and esophagus of DRR(Sp1m)-P3.4B mice, as measured by immunoblot. Analysis of cervical tissue by

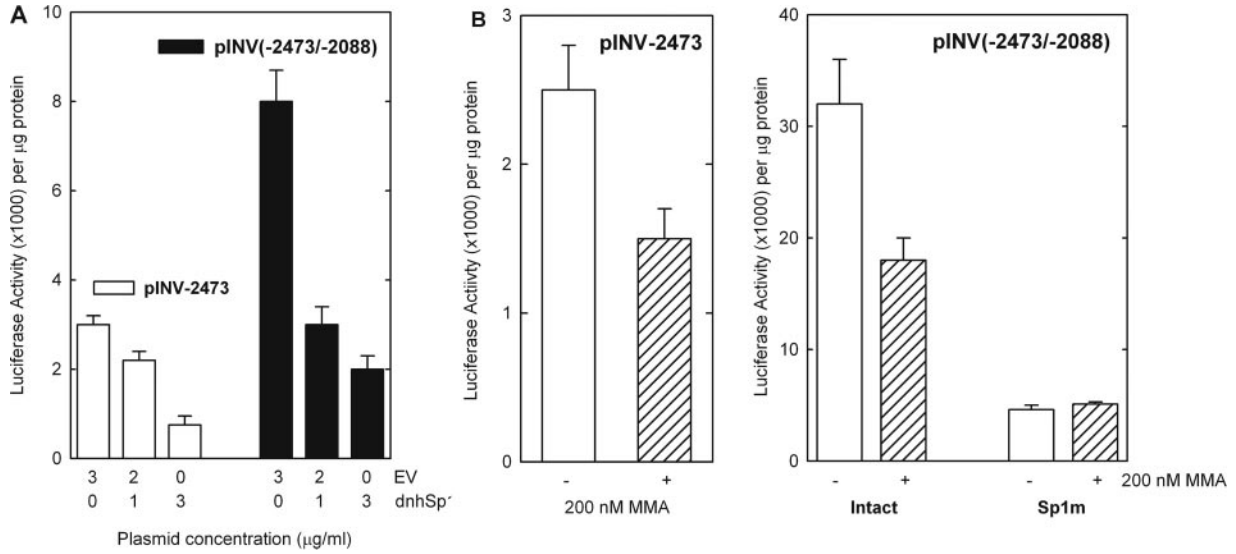

FIGURE 7. Dominant-negative Sp1 and mithramycin treatment reduce hINV promoter activity. (**A**) The hINV promoter full-length promoter construct, pINV−2473 (0.25 μg), and the DRR luciferase reporter construct, pINV(−2473/−2088; 0.25 μg) were transfected into 40% confluent cultures of normal human corneal epithelial cells in the presence of the indicated number of micrograms of human dominant-negative Sp1-encoding plasmid (dnhSP1). After 24 hours, the cells were harvested and assayed for luciferase activity. Error bars, ±SEM. Data are the mean of results in four separate experiments. (**B**) Mithramycin A treatment reduces hINV promoter activity. The hINV promoter full-length promoter construct, pINV−2473, and the DRR luciferase reporter constructs, pINV(−2473/−2088) and pINV(−2473/−2088), encoding an intact or mutant Sp1 site (Sp1m), were transfected into 40% confluent cultures of normal human corneal epithelial cells. After 24 hours, the cells were treated with 200 nM MMA. After an additional 24 hours, the cells were harvested and assayed for luciferase activity using a fluorometer. Error bars, ±SEM. Data are the mean results in four separate experiments.

immunoblot was not feasible because of the small amount of tissue. Figure 10B confirms that the pattern of expression was appropriate in each of the four tissues. That is, involucrin expression was detected in the suprabasal layers in each epithelium. Thus, in contrast to the response in the corneal epithelium, mutation of the Sp-binding site does not have as

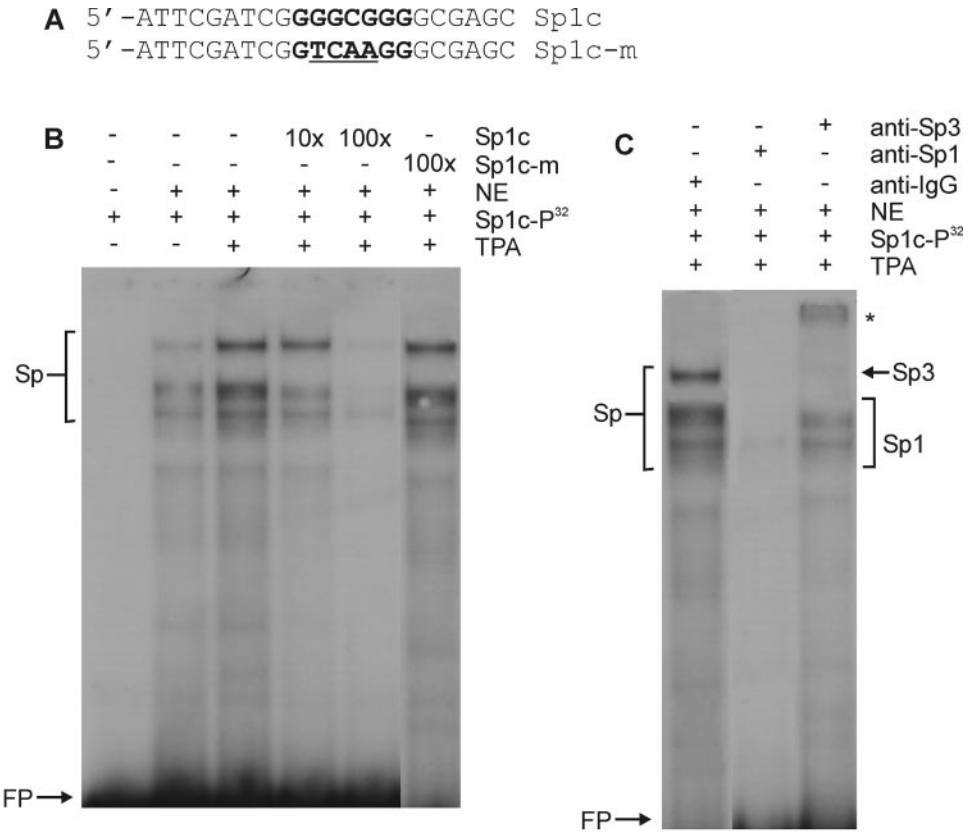

FIGURE 8. Complex formation at the hINV promoter Sp1 site. (**A**) The Sp1c and Sp1c-m oligonucleotides used for gel mobility shift assay. The Sp1 site is indicated in *bold* and matches the hINV Sp1 site.[18,29] (**B**) Nuclear extract (NE) was prepared from human corneal epithelial cells treated in the presence and 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 Sp1c for 25 minutes at room temperature. Some reactions were supplemented with a 10- or 100-fold molar excess of nonradioactive Sp1c oligonucleotide. The reaction mixtures were then fractionated on nondenaturing gels and bands were visualized by autoradiography. Sp indicates migration of the putative Sp factor complexes and FP indicates migration of ^{32}P-end-labeled Sp1c free probe. An oligonucleotide encoding a mutated Sp1 binding site 5′-ATTCGATCG**GTCAAGG**GCGAGC (Sp1 site *underscored*, mutated residues in *bold*) did not compete for binding. (**C**) Supershift analysis reveals the presence of Sp1 and Sp3 at the Sp binding site. Extracts, prepared as just described, were incubated with Sp1c-P^{32} in the presence of anti-IgG, anti-Sp1, or anti-Sp3. The complexes were then electrophoresed on a nondenaturing gel and band mobility was visualized by autoradiography. Similar results were observed in each of four separate experiments. *Asterisk*: migration of the Sp3 supershifted band.

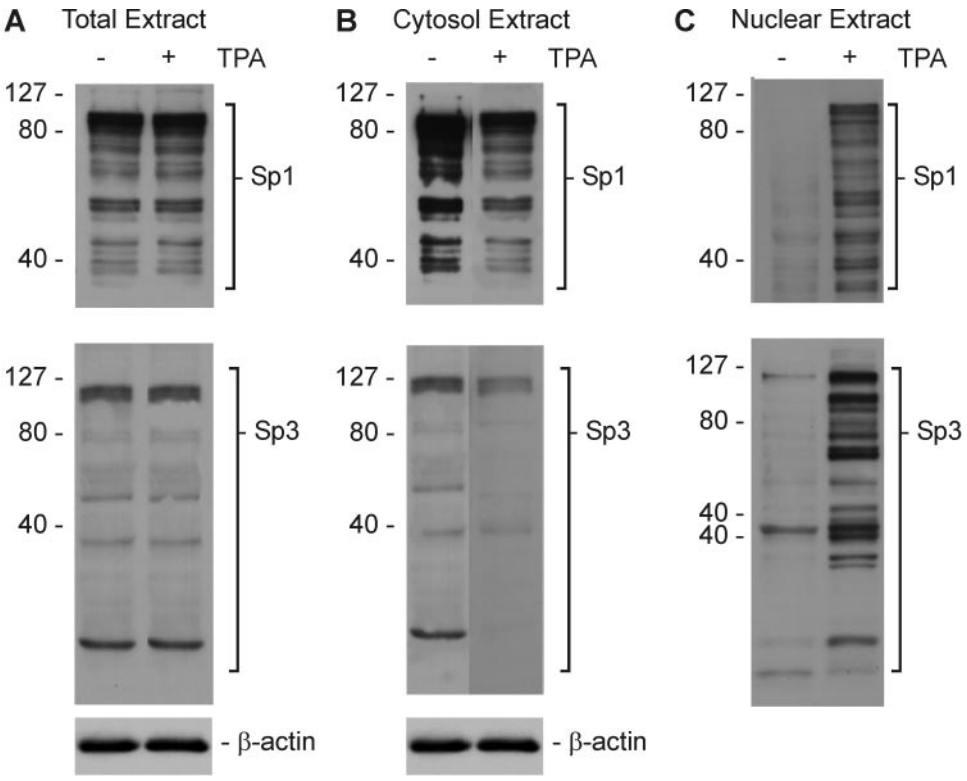

FIGURE 9. TPA treatment alters nuclear Sp factor levels. (**A**, **B**) Total cell and cytosolic extracts were prepared from cells treated for 24 hours with 50 ng/mL TPA. The extracts were then electrophoresed on an 8% acrylamide gel and transferred to nitrocellulose for immunoblot with anti-Sp1, anti-Sp3, or anti-β-actin. Complexes were then visualized by incubation with the appropriate secondary antibody and chemiluminescence detection. (**C**) Nuclear extracts were prepared from cells treated for 24 hours with 50 ng/mL TPA. The extracts were then electrophoresed on 8% acrylamide denaturing gel and transferred to nitrocellulose for immunoblot. The blots were then incubated with anti-Sp1 or anti-Sp3. Complexes were then visualized by incubation with the appropriate secondary antibody and chemiluminescence detection. For optimal visualization, the nuclear extract was exposed to film several times longer than the total and cytosolic extracts. This reflects the overall lower abundance of these factors in the nucleus.

dramatic an impact on hINV expression in other surface epithelia.

DISCUSSION

Role of the Involucrin Promoter Distal Regulatory Region Sp Factor–Binding Site in Involucrin Expression in the Corneal Epithelium

Identifying mechanisms that govern tissue-specific and differentiation-appropriate gene expression in the corneal epithelium is an important area of interest. In this epithelium, stem cells give rise to daughter cells that then differentiate to form the superficial layers of the tissue. This process is marked by profound changes in cell morphologic and biochemistry. Several genes have been described that are markers of this process. These include cytokeratins K3 and K12,[30,41,42] MUC1 and MUC4,[43] lactate dehydrogenase,[44] and involucrin.[22] Knowledge regarding the mechanisms that guide expression of these genes is limited and is confined to conclusions derived from the use of cell-culture–based models that have not been confirmed in vivo. Thus, a central goal of the present study was to gain information regarding the mechanisms that drive involucrin expression using both in vivo and cell-culture–based systems.

The most effective method of confirming cell-culture–based studies is the use of transgenic mouse models. Previous studies have shown that this method can be successfully applied to the study of involucrin gene expression.[24–26] To identify elements responsible for cornea epithelial gene expression, transgenic mice were generated encoding the intact full-length involucrin promoter and various promoter truncation and point mutants. These studies revealed that deletion of the promoter segment spanning nucleotides −2473/−1953 results in a complete absence of corneal epithelial involucrin gene expression. To assess whether the sequences that reside in this segment are sufficient for corneal expression, the −2473/−1953 DRR segment was isolated and linked directly to the involucrin minimal promoter in the absence of other regulatory elements. Analysis of these mice revealed a normal level and pattern of involucrin expression, suggesting that sequences within this segment are not only necessary, but are also sufficient to drive appropriate involucrin gene expression in the corneal epithelium.

Analysis of the sequence of the −2473/−1953 segment, the so called DRR,[17] identified several candidate transcription-factor–binding sites that could participate in this regulation, including an Sp-factor–binding site.[24] Previous studies, using cell culture models, suggest a role for Sp factors in regulation of corneal epithelial gene expression. For example, Sp factors play a role in regulation of expression of cytokeratin K3,[45] lactate dehydrogenase,[44] α5 integrin,[46] and cytokeratin 4.[47] We hypothesized that the Sp-factor–binding site within the hINV promoter DRR may be necessary for involucrin expression in the corneal epithelium. Indeed, mutation of this site resulted in a complete loss of in vivo corneal epithelial expression, suggesting that Sp factor input is necessary for in vivo involucrin expression. To our knowledge this is the first in vivo confirmation that Sp factor input is essential for expression of a gene in the corneal epithelium.

Sp1: A Selective Role in the Corneal Epithelium?

An important issue is the particular role of Sp1 in the corneal epithelium. Involucrin is expressed in several surface epithelia, including the cervix, epidermis, oral cavity, and esophagus.[14] It is possible that removing the Sp binding site could eliminate involucrin expression in all these epithelia. Previous studies have shown that the involucrin transgenes encoding the full-length involucrin upstream regulatory region (nucleotides −2473/−1) are expressed in all murine surface epithelia in a pattern that mirrors the expression pattern of endogenous hINV in human tissues.[24–26] We therefore assayed whether elimination of the Sp-binding site resulted in a selective loss of transgene expression only in the corneal epithelium, or if this mutation produced a generalized loss of expression. Analysis of the pattern of expression of the transgene encoding a mutated Sp-binding site shows that the Sp site mutation selectively

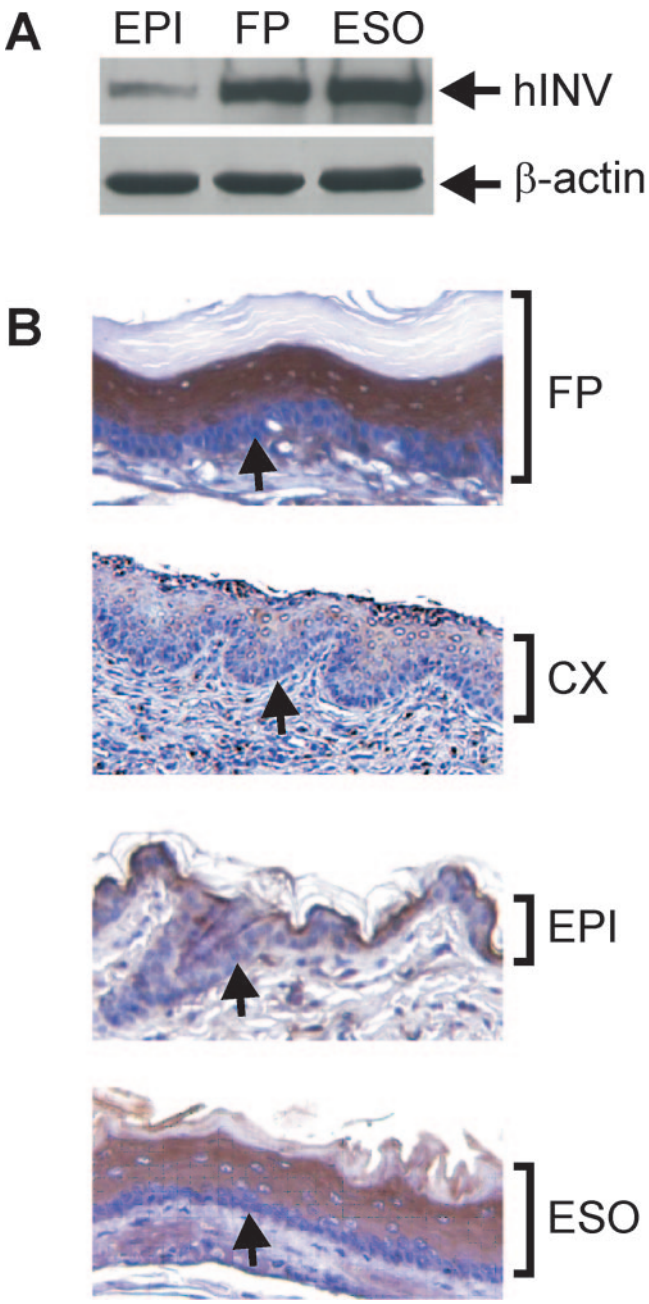

FIGURE 10. The hINV promoter Sp1 site has a unique role in the corneal epithelium. Mouse embryos were injected with the DRR(Sp1m)-P3.4B transgene. The structure of the construct is shown in Figure 3A. Expression of the transgene was monitored by immunoblot and/or immunohistology using a hINV-specific antibody. Surface epithelium from footpad, epidermis, and esophagus were harvested, and total cell extracts were prepared in sample buffer. Samples were electrophoresed on 8% denaturing and reducing polyacrylamide gels, transferred to nitrocellulose, and incubated with hINV-specific antibody. For immunohistochemical localization, sections were prepared and then incubated with anti-hINV followed by peroxidase-conjugated goat anti-rabbit IgG.[24] The samples were derived from the same six independently derived transgenic mouse lines used in Figure 3.

eliminates transgene expression in the cornea and not in other surface epithelia (i.e., cervix, epidermis, and esophagus). This finding indicates that Sp transcription factors are uniquely necessary for involucrin expression in the corneal epithelium. The effect of mutating the Sp-binding site is in contrast to the impact of mutating the AP1-5 site. AP1-5 is an AP1 transcription-factor (junB, junD, Fra-1, and Fra-2)–binding site located in the DRR, adjacent to and immediately upstream of the Sp-factor–binding site.[15,24] Previous studies have shown that mutation of AP1-5 results in a loss of transgene expression in epidermis, esophagus, and cervix.[25] Our recent study indicates that mutation of the AP1-5 site also abolishes expression in the corneal epithelium.[48] Taken together, these findings suggest that AP1 transcription factor input is essential for expression of involucrin in many surface epithelia, but that Sp factors are uniquely necessary for expression in the corneal epithelium.

Regulatory Role of Sp Factors

The Sp-factor–related regulation of involucrin gene expression in corneal epithelium is likely to be complicated. Our in vivo transgenic studies showed that mutation of the Sp site within the hINV promoter DRR results in a complete loss of in vivo corneal epithelial hINV expression. Additional studies, using cultured normal human corneal epithelial cells, showed that mutation of the Sp site causes a reduction in both basal and TPA-stimulated promoter activity. TPA treatment is known to increase involucrin promoter activity in cultured corneal epithelial cells.[22] Thus, Sp factor function is necessary for both basal and regulated involucrin promoter activity. In addition, expression of a dominant-negative form of Sp1[38] suppresses Sp1- and TPA-dependent promoter activation, and treatment with mithramycin A, an agent that selectively binds to G/C-rich DNA and prevents Sp factor binding, reduced Sp1- and TPA-dependent promoter activity and also reduced endogenous involucrin gene expression. Moreover, treatment with TPA resulted in an increased nuclear Sp factor level and increased binding of Sp factors to the hINV promoter Sp-binding site, responses that are correlated with increased hINV promoter activity. The finding that Sp factor level can alter involucrin expression is consistent with a previous study showing that overexpression of Sp1 activates expression of the endogenous involucrin gene in fibroblasts, a cell type that does not normally express involucrin in vivo.[29] Based on these findings, one could hypothesize that the absolute level of Sp1 present in the tissue directly influences the level of involucrin expression. However, this is clearly not the case. In fact, as shown in the present study, mouse liver, a tissue that does not express involucrin, expressed more Sp1 than the corneal epithelium. Moreover, other surface epithelial tissues also expressed high levels of Sp1,[18] yet mutation of the Sp binding site within the hINV promoter did not eliminate expression in these tissues (Fig. 10).

These results suggest that the unique role of Sp factors in the corneal epithelium must be explained by other mechanisms. Potential levels of differential regulation may include differences in the covalent posttranslational modification of Sp1 and Sp3. These covalent modifications, which include phosphorylation (Sp1), glycosylation (Sp1), acetylation (Sp1, Sp3), and sumoylation (Sp3), are known to influence Sp factor activity.[40] Parallel studies indicate that the Sp1, present in corneal extracts, is extensively phosphorylated and glycosylated (not shown); however, the impact of these modifications on function is not presently known. In addition, differential interaction with coregulators may play an important role. Sp1 is known to interact with a wide range of regulators in other tissues.[33,49] The precise nature of the Sp1 and Sp3 interaction with the Sp DNA element may be important, as gel mobility supershift studies suggested that Sp1 may anchor Sp3 to the Sp binding site (i.e., addition of an Sp1-specific antibody to a gel shift mixture resuled in a loss of both Sp1 and Sp3 binding, but, in contrast, addition of an anti-Sp3 antibody resulted in a loss of the putative Sp3 band, but only minor changes in the apparent level of bound Sp1). Sp1 and Sp3 are known to regulate differentially the expression of other target genes[50] and our present study suggests that Sp1 and an amino-terminal trun-

cated form of Sp3[40] differentially regulate involucrin promoter activity. This antagonistic relationship between Sp1 and this truncated form of Sp3 has been noted in other systems.[50–52] The mechanism responsible for this inverse regulatory relationship is not known. However, Sp1 and Sp3 factors can form heterodimers[37]; thus, it is possible that an Sp1/Sp3 heterodimer has altered activity.[41,53] Additional studies are under way to assess these possibilities.

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