# Differential Binding of the Transcription Factors Sp1, AP-1, and NFI to the Promoter of the Human α5 Integrin Gene Dictates Its Transcriptional Activity

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**Purpose.** Damage to the corneal epithelium results in the massive secretion of fibronectin (FN) shortly after injury and induces the expression of its integrin receptor α5β1. The authors reported previously that FN induces α5 expression in human corneal epithelial cells and rabbit corneal epithelial cells by altering the binding of the transcription factor (TF) Sp1 to a regulatory element from the α5 promoter that it is also flanked by binding sites for the TFs NFI and AP-1. Here, they assessed the function of NFI and AP-1 on α5 gene expression and evaluated the contribution of FN to their overall regulatory influence.

**Methods.** TF binding to the α5 promoter was evaluated in vitro by electrophoretic mobility shift assays and in vivo by ligation-mediated PCR or chromatin immunoprecipitation. TFs expression was monitored by Western blot, whereas their influence was assessed by transfection and RNAi analyses.

**Results.** Coexpression of Sp1, NFI, and AP-1 was demonstrated in all cell types, and each TF was shown to bind efficiently to the α5 promoter. Whereas both AP-1 and Sp1 activated expression directed by the α5 promoter, NFI functioned as a potent repressor of that gene. Interestingly, FN could either promote or repress α5 promoter activity in a cell density–dependent manner by differentially altering the ratio of these TFs.

**Conclusions.** These results suggest that α5 gene expression is likely dictated by subtle alterations in the nuclear ratio of TFs that either repress (NFI) or activate (Sp1 and AP-1) α5 transcription in corneal epithelial cells. (*Invest Ophthalmol Vis Sci.* 2009;50:57–67) DOI:10.1167/iovs.08-2059

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Damage to any organ induces a rapid response from the body to repair the wounded tissue. The complex cascade of events then triggered, involving cell migration and proliferation, extracellular matrix (ECM) remodeling, neovascularization, and apoptosis, is crucial to the tissue response to injury.[1] Inflammatory cells, fibroblasts, and epithelial cells migrate to the wounded area and subsequently proliferate to ensure healing of the damaged tissue.[2] Easily accessible external organs such as skin and cornea have facilitated in vitro and in vivo studies of the wound healing process.[3–11]

One of the earliest features that appears in the wound after skin injury is the production of a provisional ECM resulting from the deposition of polymeric fibrin stabilized by factor XIIIa, with subsequent deposition of vitronectin, fibronectin, and collagens. Early events in wound healing are also characterized by the migration of epithelial cells and the reconstitution of basement membrane (BM) components (collagen IV, laminin [LM], glycoproteins) that cover the granulation tissue.[12] In the cornea, this process is characterized by the massive secretion of fibronectin (FN), which peaks between 3 and 12 hours after corneal damage and then starts disappearing 1 week later.[13] Fibronectin is of particular interest because it is not a major BM constituent in cornea and skin; only traces can be detected in these supportive structures. Rather, it is expressed transitorily in response to tissue injury. In addition to the skin and cornea, FN expression has been shown to increase rapidly during the repair process of other damaged organs, such as the brain,[14] heart,[15] liver,[16] and kidneys,[17] and of the arteries.[18]

Fibronectin exerts its many biological functions by interacting with specific membrane-bound receptors that belong to the integrin family. Integrins, which are composed of noncovalently associated α and β subunits and bridge the cell cytoskeleton to the various components of the ECM, are known to play major functions in many physiologic and pathologic processes, including wound healing (for a review, see Vigneault et al.[19]). To date, 18 α- and eight β-subunits that can form 24 different heterodimers have been recognized. Although at least nine different types of integrin receptors were reported to bind FN with varying affinities, the main FN receptor remains the α5β1 integrin. Direct evidence that FN can positively alter α5β1 integrin expression at the protein and mRNA levels have been provided through FN antisense expression studies.[20,21] Examination of the human α5 promoter indicates that it lacks both TATA and CCAAT boxes and that it has a high content in GC residues and an initiator (Inr) consensus sequence.[22] In vitro analyses conducted in human keratinocytes have shown that a proximal AP-1 (−45/−51) and Sp1 (−52/−60) sites are occupied by c-Jun/c-Fos dimers and Sp1 in vitro, respectively.[23] In addition, a target site for C/EBP (−66 to −73) was also identified in the α5 promoter.[23] Studies emerging from our laboratory have shown that FN can alter the activity of the α5 promoter by altering the binding of Sp1 to a segment from the α5 promoter that we designated the FN-responsive element.[24] In addition to this promoter-proximal

site, Sp1 was found to interact with a more distantly located site (−117 and −101) from the α5 promoter.[25] Thorough examination of the α5 promoter revealed the presence of a putative binding site for the transcription factor (TF) nuclear factor I (NFI) located immediately upstream (from positions −67 to −71) of the proximal Sp1 and AP-1 sites. Although Sp1 is unquestionably recognized as a TF that positively influences gene transcription (for a review, see Zhao and Meng[26]), the picture is far more complex for NFI because the members of this family—the four isoforms NFI-A, NFI-B, NFI-C, and NFI-X—have been reported to function either as repressors[27,28] or activators[29] of gene transcription.

In this study, we examined the expression and binding of the TFs AP-1, NFI, and Sp1 to a short proximal region (SPR) from the basal promoter of the human α5 gene in corneal and skin epithelial cells and in HeLa cells. Direct interaction between these TFs and their respective α5 target sites was demonstrated in vitro and in vivo. Although Sp1 and AP-1 were found to function as activators of α5 gene transcription, NFI proved to be a potent repressor that competes with Sp1 and AP-1 for the availability of the α5 SPR.

## METHODS

All experiments were conducted in voluntary compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Laval University Animal Care and Use Committee approved all procedures. This study was also conducted in accordance with our institution's guidelines and the Declaration of Helsinki. The protocols were also approved by the institution's Committee for the Protection of Human Subjects.

### Plasmids and Oligonucleotides

The plasmids −132α5-CAT and −92α5-CAT, which bear the human α5 gene promoter extending up to 5′ positions −132 and −92, have been previously described.[30] The derivatives from −92α5-CAT, which bear mutations into the Sp1, NFI, and AP-1 sites, either individually (−92α5/Sp1m; −92α5/NFIm; −92α5/AP1m) or in combination (−92α5/Sp1m+ AP1m; −92α5/Sp1m+ NFIm; −92α5/NFIm+AP1m; −92α5/Sp1m+NFIm+AP1m), were produced through site-directed mutagenesis (QuikChange Site-Directed Mutagenesis kit, Stratagene, La Jolla, CA) using the following mutated oligonucleotides: Sp1m, 5′-GCCGGGAGTTTGGCAAACT**AAAAAA**CGCGTTGAGTCATTCGCCTCTGGGAGG-3′; NFIm, 5′-GGGCTCAGCCGGGAGTTT**AAA**AAACTCCTCCCCGCG-3′; and AP-1m, 5′-CTCCTCCCCGCGTT**AAAAA**ATTCGCCTCTGGGAGGTTTAGGAAGCGGC-3′. Mutated residues are in bold. The plasmid PXGH5, which bears a secreted version of the human growth hormone (hGH), is a kind gift of David D. Moore (Department of Molecular and Cell Biology, Baylor College of Medicine, Houston, TX).

All double-stranded oligonucleotides were chemically synthesized (Biosearch 8700 apparatus; Millipore, Billerica, ME). They contained the human α5 promoter from positions −36 to −82 (α5.4, 5′-GATCAGCCGGGAGTTTGGCAAACTCCTCCCCGCGTTGAGTCATTCGCCTCT-3′) or its derivative, which bear mutations into the α5 Sp1 site (5′-GCCGGGAGTTTGGCAAACT***AAAAAA***CGCGTTGAGTCATTCGCCTCTGGGAGG-3′), the DNA-binding site for human NF-I (5′-GATCTTATTTTGGATTGAAGCCAATATGAG-3′), the AP-1 site from the α5 promoter and located from positions −59 to −37 (5′-GATCCCCGCGTTGAGTCATTCGCCTC-3′)[23] or its mutated derivative (AP-1m; 5′-GATCCCCGCGTT***AAAAA***ATTCGCCTC-3′), and the high-affinity binding site for Sp1 (5′-GATCATATCTGCGGGGCGGGGCAGACACAG-3′).

### Cell Culture

Human corneal epithelial cells (HCECs) were isolated from the eyes of either a 9- or a 62-year-old donor (for transfection analyses) or from either a 10-month-old or a 59-year-old donor (for ligation-mediated PCR [LMPCR]; eyes were obtained through the Eye Bank from the CHUL Research Center) and were cultured as reported.[31] Rabbit corneal epithelial cells (RCECs) were isolated and grown into supplemented hormonal epithelial medium (SHEM), as described.[32] When indicated, culture dishes were coated with human plasma FN at 8 μg/cm$^2$, as described.[24] Human skin keratinocytes (HSKs) were obtained from normal newborn foreskin specimens from a 17-day-old donor (HSKnb) or from normal adult (52-year-old donor) breast skin (HSKad) and were cultured with a feeder layer, as recently described.[33] Human HeLa cells (ATCC CCL 2) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS). All cells were grown under 5% $CO_2$ at 37°C, and gentamicin was added to all media at a final concentration of 15 μg/mL. All primary cultured cells (HCECs, HSKs, RCECs) were used at passages 1 to 5.

### Transient Transfections and CAT Assays

RCECs, HSKs, and HeLa cells were grown to 80% confluence and were transfected using the polycationic detergent Lipofectamine (Gibco BRL), as described.[24,25] Primary cultured HCECs were transfected according to the calcium phosphate precipitation procedure.[34] CAT activity was determined and normalized to secreted hGH, as described.[34] Each CAT value corresponded to the mean of at least three separate transfections performed in triplicate. Student's *t*-test was performed for comparison of the groups. Differences were considered to be statistically significant at $P < 0.05$. All data are expressed as mean ± SD.

### Nuclear Extracts, Electrophoretic Mobility Shift Assays, and Supershift Experiments

Nuclear extracts were prepared from midconfluent cells, as detailed previously.[24,25,27] EMSAs were conducted, as described,[24] by incubating nuclear proteins with 5′-end labeled, double-stranded oligonucleotides bearing the binding sites for the TFs Sp1, NFI, and AP-1 or for the entire α5 promoter region (α5.4). When indicated, unlabeled oligomers for the TFs Sp1, NFI, and AP-1 (and its mutated derivative AP-1m) were added as competitors during the assay. For the displacement experiment in EMSA, recombinant Sp1 (kindly provided by Claude Labrie, Oncology and Molecular Endocrinology Research Center, CHUL, Québec, Canada), NFI (a carboxymethyl-Sepharose enriched preparation of rat liver NFI[27]), and AP-1 (Promega/Fisher, Ontario, Canada) were also used in EMSA, in combination with the α5.4-labeled probe. Supershift experiments in EMSA were conducted by incubating 5 μg nuclear protein from RCECs in the presence of no polyclonal antibodies or 2 μL polyclonal antibodies raised against the TFs Sp1, Sp3, c-Jun (catalog nos. sc-59, sc-644, and sc-45, respectively; Santa Cruz Biotechnology, Santa Cruz, CA), NFI (a kind gift of Peter C. Van der Vliet, Laboratory for Physiological Chemistry, Utrecht, The Netherlands), and AP-1 (c-Fos [Ab5] PC38; Oncogene Research Products, San Diego, CA).

### Western Blots

Western blot analysis was conducted as described[24] using 30 μg nuclear proteins. Membranes were blotted with the following primary antibodies (all purchased from Santa Cruz Biotechnology, except when otherwise indicated): rabbit polyclonal antibodies directed against the TFs Sp1 (catalog no. sc-59, 1:5000), Sp3 (catalog no. sc-644, 1:4000), NFI (catalog no. sc-5567, 1:1200), c-jun (catalog no. sc-45, 1:3000), JunD (catalog no. sc-74, 1:2000), FosB (catalog no. sc-7203, 1:900), c-Fos (catalog no. sc-52, 1:300), Fra-1 (catalog no. sc-605, 1:1000), and Fra-2 (catalog no. sc-604, 1:1200) or mouse monoclonal antibodies against JunB (catalog no. sc-46 1:3000), and actin (catalog no. CLT 9001, 1:35,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). The blots were then incubated with a 1:1000 dilution of a peroxidase-conjugated goat secondary antibody directed against either mouse (catalog no. 115-035-003; Jackson ImmunoResearch Laboratories) or rabbit (catalog no. 111-035-003; Jackson ImmunoResearch Laboratories) IgG, and immunoreactive complexes were revealed

with the use of a Western blot detection kit (Amersham, Baie d'Urfé, Canada).

### Chromatin Immunoprecipitation Assays

ChIP analyses were conducted on HSKs grown to 80% confluence and chromatin immunoprecipitated with 1 μg (5 μg was used for Sp1) antibodies against the TFs Sp1, Sp3, NFI, JunB, c-Jun, JunD, FosB, c-Fos, Fra-1, and Fra-2, as previously reported.[35,36] The resultant DNA was analyzed by PCR using a pair of primers (ITGA5-U, 5′-CTTAGGGGTGGGGGACGC-3′; ITGA5-L, 5′-CGCCCGCTCTTCCCTGTC-3′) spanning the α5 gene promoter. As a negative control, each ChIP sample was also subjected to PCR using primers (p21-U, 5′-AATTCCTCTGAAAGCTGACTGCC3′; p21-L, 5′-AGGTTTACCTGGGGTCTTTAGA-3′) specific to a region located approximately 2 kbp upstream from the human p21 promoter. Cycle parameters were denaturation at 99°C for 10 seconds, annealing at 68°C for 20 seconds, and extension at 72°C for 20 seconds, with 38 cycles.

### Ligation-Mediated PCR

HCECs were grown to 80% cell density and treated with 0.02% dimethyl sulfate (DMS; Sigma-Aldrich Canada), as described.[37] The methylated base residues were converted to strand breaks using hot piperidine. Ligation-mediated PCR (LMPCR) analyses were carried out as described[37] using the following primer sets: LMPCR5.1, 5′-CAGACAACCGGCTTCCAGC-3′, position −236 to −218; LMPCR5.2, 5′-CTGGGGAAAGGGGGTTGGAGGGGTGC-3′, position −212 to −187; LMPCR6.1, 5′-CACGAGCAGCAACAGCAG-3′, position +121 to +104; LMPCR6.2, 5′-CAGCGGCACGAGCGGGGGTCGG-3′, position +106 to +85, allowing analysis of the α5 promoter from nt +65 to −167.

### RNAi Assays and RT-PCR Analyses

Total RNA was isolated from midconfluent HSKad transfected either with a negative control (Silencer; Ambion, Austin, TX) or with predesigned siRNA duplexes (Silencer; Ambion) against the NFI (NFIA, NFIB, NFIC, and NFIX) or AP-1 (c-Jun, JunB and JunD) family of TFs and was reverse transcribed as described.[25] The resultant first-strand cDNAs were used for PCR amplification of the α5 transcript (5′ primer, GGCAGCTATGGCGTCCCACTGTGG-bp; 3′ primer, GGCATCAGAGGTGGCTGGAGGCTT-bp; PCR product, 171 bp) and the 18S ribosomal RNA (Quantum RNA 18S Internal Standards kit; Ambion). Cycle parameters were the same for all primers used (denaturation at 95°C for 30 seconds; annealing at 58°C for 30 seconds; extension at 72°C for 30 seconds). To eliminate the possibility that saturation of the PCR reaction was reached, amplifications were performed at 20, 22, 23, 24, 25, and 27 cycles. PCR amplification of the α5 product was linear from 22 to 25 cycles of amplification (data not shown).

## Results

### Transcription Factors Sp1, NFI, and AP-1 Expressed by Primary Cultured Cells and Established Cell Lines

We previously demonstrated the binding of Sp1 to two discrete sites along the promoter of the α5 gene.[24,25] Interestingly, sequence examination of the proximal Sp1 site revealed that it is flanked by an NFI site on its 5′ side and an AP-1 site on its 3′ end (Fig. 1A). We therefore examined whether primary cultures of epithelial cells from either the skin (HSKs) or the cornea (RCECs and HCECs) could sustain DNA-binding activities for these TFs. As shown in Figure 1, nuclear extracts from each cell type could efficiently sustain DNA binding of Sp1, NFI, and AP-1 in EMSA (Fig. 1B, C+). Similar results were obtained with HeLa cells (data not shown). The specificity for the formation of these complexes was established by the fact that either a 50- or a 250-fold molar excess of unlabeled oligonucleotides for Sp1, AP-1, and NFI strongly impaired or prevented the formation of their specific complexes in the EMSA, whereas their mutated derivatives (Sp1m and AP-1m for Sp1 and AP-1, respectively) or an oligomer bearing the unrelated Sp1 target site (for NFI) could not.

The expression of Sp1 (and its related family member, Sp3), NFI, and AP-1 was next monitored at the protein level in nuclear extracts prepared from HSKnb, RCECs, and HeLa cells. Sp1, Sp3, and NFI were expressed to varying levels in all types of cells (Fig. 1C). Because many isoforms exist for the AP-1–constituting subunits, antibodies against the Jun subunits JunB, c-Jun, and JunD and against the Fos subunits FosB, c-Fos, Fra-1, and Fra-2 were selected for analysis. As shown in Figure 1C, HeLa, HSKnb, and RCECs expressed the three Jun isoforms, though at different levels. Similarly, HeLa cells expressed the Fos subunits FosB and c-Fos, whereas HSKnb predominantly expressed the Fra-1 and Fra-2 Fos subunits. RCECs predominantly expressed Fra-2 (and, to some extent, Fra-1).

### Binding of Sp1, NFI, and AP-1 to the SPR from the Human α5 Gene Promoter In Vitro and In Vivo

To assess whether Sp1, NFI, and AP-1 can bind the α5 SPR in vitro, EMSAs were conducted by incubating nuclear extracts from RCECs with a DNA-labeled probe (α5.4) that constitutes all three TF sites from the α5 promoter (Fig. 2A). Incubation of the α5.4 probe with the extract from RCECs yielded a number of DNA-protein complexes on gel (Fig. 2B, lane 1) whose formation was competed off by the addition of an excess of unlabeled α5.4 (lane 5). A similar excess of the unlabeled Sp1 oligomer substantially reduced the formation of these complexes (lane 2), yet some of them could still be detected under this condition. Unlabeled AP-1 and NFI oligomers had little, if any, influence on the formation of these complexes (lanes 3 and 4). Considering that the DNA-protein complexes corresponding to Sp1/Sp3 might have electrophoretic mobility similar to that of the NFI and AP-1 complexes on native gels, thereby masking their detection in EMSA, we conducted supershift experiments with polyclonal antibodies directed against NFI or AP-1 on the DNA-protein complexes that remained detectable with the addition of the Sp1-unlabeled oligonucleotide. As shown on Figure 2C, the addition of an excess of the unlabeled Sp1 oligonucleotide again could not prevent the formation of DNA-protein complexes in EMSA because at least three shifted bands could still be detected under this condition (compare lanes 1 and 2). Interestingly, the addition of an NFI antibody when the unlabeled Sp1 competitor was present prevented the formation of the strong, slow-migrating complex (Fig. 2C, lane 3; NFI) and increased the formation and the resolution of a faster migrating complex that otherwise appeared as a faint band in lane 2. On the other hand, adding an AP-1 antibody strongly impaired the formation of this complex and yielded a supershifted complex on the gel (Fig. 2C, lane 4; AP-1). A similar supershift experiment was conducted for Sp1 but in the presence of the unlabeled AP-1 and NFI oligonucleotides to prevent any interference by both these TFs on the recognition of the α5.4-labeled probe by Sp1. The addition of the Sp1 antibody strongly reduced the formation of the slow-migrating complex but had a modest influence on the fast-migrating one (Fig. 2C, compare lanes 5 and 6), whereas adding Sp3 antibody considerably reduced and prevented the slow- and fast-migrating complexes, respectively (lane 7). Incubation in the presence of the Sp1 and Sp3 antibodies prevented the formation of all complexes and yielded a supershifted band that corresponded to DNA-bound Sp1 or Sp3 complexed with the Sp1 and Sp3 antibodies (lane 8). Therefore, Sp1/Sp3, NFI, and AP-1 all individually had the ability to bind SPR from the α5 promoter in vitro.

We then sought to determine whether Sp1, NFI, and AP-1 would also bind to their corresponding target site in the α5 SPR

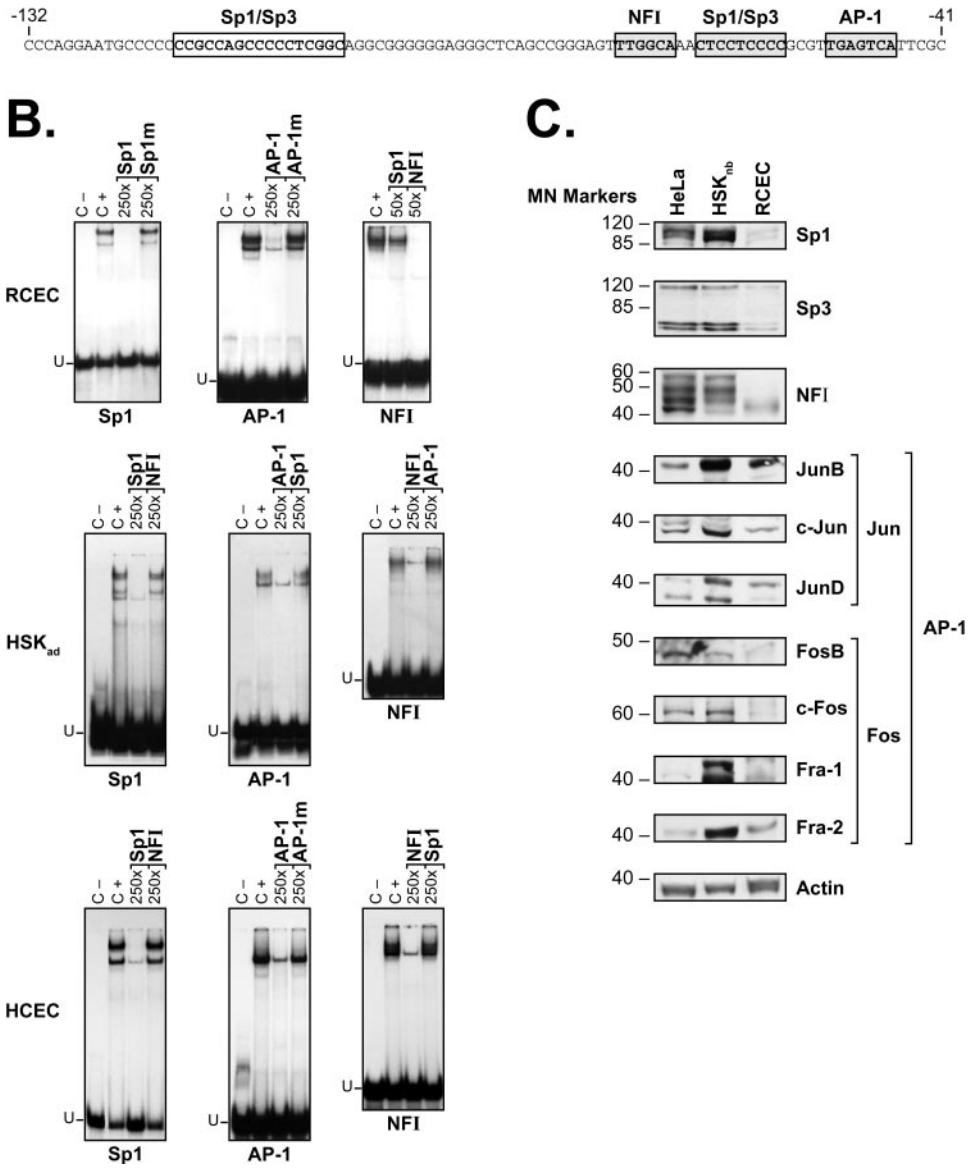


**FIGURE 1.** Expression of Sp1, NFI, and AP-1 in primary cultured cells. (**A**) Target sites identified for Sp1, NFI, and AP-1 are shown along the DNA sequence of the $\alpha$5 promoter. (**B**) DNA-labeled probes bearing the binding sites for the TFs Sp1 and NFI or for AP-1 in the $\alpha$5 promoter were incubated with nuclear proteins (7.5 $\mu$g each) from midconfluent RCECs, HSKs, and HCECs in the presence of no (C+) or 50- and 250-fold molar excesses of unlabeled competitors (Sp1, Sp1m, AP-1, AP-1m, NFI). Formation of DNA/protein complexes was then monitored by EMSA. C−, labeled probe alone; C+, probe with proteins but without competitor; U, free probe. (**C**) Nuclear extracts from RCECs, HSKnb, and HeLa cells were Western blotted with antibodies against the TFs Sp1, Sp3, NFI, JunB, c-Jun, JunD, c-Fos, FosB, Fra-1, and Fra-2. Actin expression was also monitored as a normalization control.

in living cells using in vivo LMPCR. As shown on Figures 3A and 3B (also summarized in Fig. 3C), comparison of the band intensity between the in vivo (v) and in vitro (t) treatments revealed that many G residues are either partially or totally protected in the area from the $\alpha$5 promoter which also bears the Sp1, NFI, and AP-1 sites (one G residue was protected on the bottom strand in the NFI site, seven residues were protected on the bottom strand in the Sp1 site, and three residues [two on the top and one on the bottom strand] were protected in the AP-1 site).

The binding of Sp1, Sp3, NFI, and AP-1 to the $\alpha$5 SPR was further examined in vivo by ChIP assays. Because conducting this experiment on that number of TFs required a large quantity of cells, we selected HSKs over HCECs primarily because skin biopsy samples are easier to obtain than human corneas. Antibodies against Sp1, Sp3, and NFI, and those directed against all the AP-1 Jun and Fos subunits, enriched the $\alpha$5 promoter sequence in HSKs, indicating that this genomic area is bound in vivo by these TFs (Fig. 3D). In contrast, none of these antibodies could enrich a region located approximately 2 kbp upstream from the p21 promoter that is used as a negative control for the ChIP assay.[35]

## Regulatory Influence of Sp1, AP-1, and NFI on the $\alpha$5 Promoter-Driven Transcription

The plasmid −92$\alpha$5-CAT, which bears the $\alpha$5 promoter from position −92 to +23 and therefore comprises the intact Sp1, NFI, and AP-1 sites from the SPR, or derivatives mutated into each of these sites, was transfected into primary cultured RCECs, HCECs, and HSKs or in HeLa cells. Mutating the Sp1 site (in −92$\alpha$5/Sp1m) had only a marginal effect on the activity directed by the $\alpha$5 promoter in RCECs and HSKs but unexpectedly resulted in a near fivefold increase in HeLa cells (Fig. 4A). On the other hand, mutations that prevented the binding of AP-1 (in −92$\alpha$5/AP-1m) considerably impaired $\alpha$5 promoter activity in RCECs and HeLa cells but resulted in a weak but significant 2.3-fold increase in HSKs. Mutating the NFI site (in

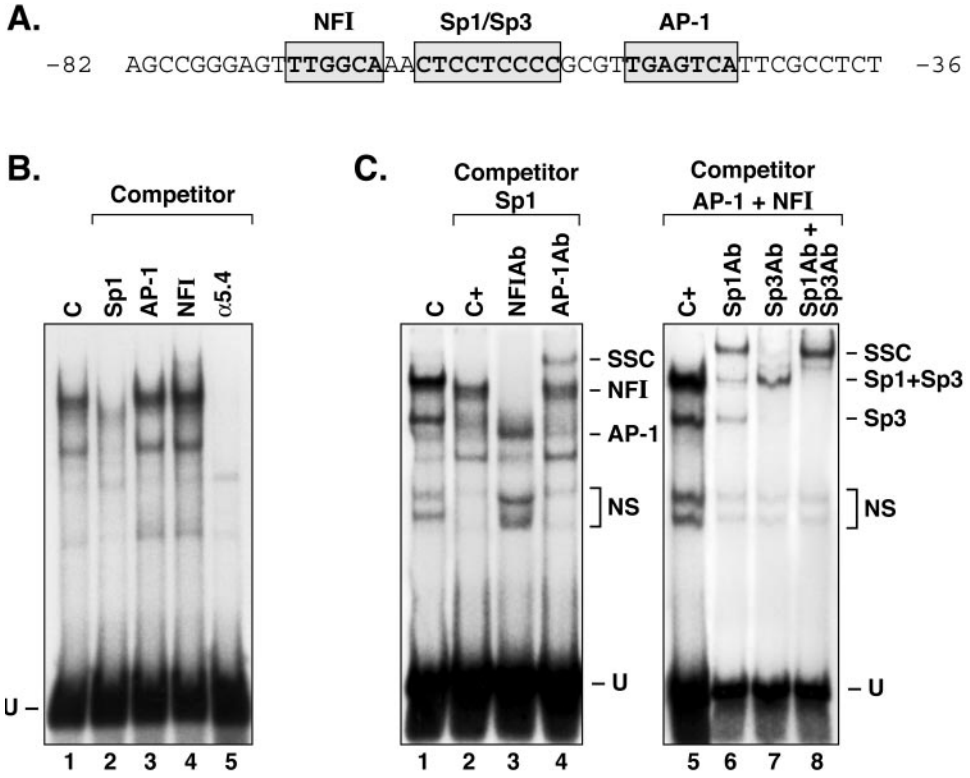


**FIGURE 2.** EMSA competition and supershift analyses of Sp1, AP-1, and NFI binding to the α5 basal promoter in vitro. (**A**) Schematic representation of the α5.4 oligonucleotide used as the probe. (**B**) The α5.4 oligonucleotide was labeled and incubated with nuclear proteins (7.5 μg) from midconfluent RCECs either alone (C, *lane 1*) or in the presence of a 100-fold molar excess of unlabeled competitors (Sp1, AP-1, NFI, or α5.4; *lanes 2–5*, respectively). Formation of DNA/protein complexes was monitored by EMSA on a 6% native gel. (**C**) The α5.4 probe was incubated with nuclear proteins (7.5 μg) from RCECs grown to 80% cell density either alone (C and C+; *lanes 1, 2, 5*) or with antibodies against AP-1 (2 μL; *lane 4*), Sp1 (1 μL; *lanes 6, 8*), Sp3 (1 μL; *lanes 7, 8*), or NFI (2 μL; *lane 3*). This experiment was conducted with or without 500-fold molar excess of unlabeled competitors (either Sp1 [*lanes 2, 3*] or a combination of AP-1 and NFI [*lanes 6–8*]). C, probe with proteins but without competitor; C+, probe with proteins and competitors; SSC, supershifted complexes; NS, nonspecific complexes; U, free probe.

−92α5/NFIm) caused a dramatic increase in α5 promoter activity in all types of cells (ranging from 5- to 16-fold increases), indicating that NFI is a potent repressor of α5 transcription.

We next compared the activity directed by double TF mutants with that of the promoter construct in which all three sites are mutated (−92α5/Sp1+NFI+AP-1m). Double-mutating both the NFI and the AP-1 sites (−92α5/ NFI+AP-1m), thereby leaving only the Sp1 site intact, resulted in 2.9- and 2.4-fold increases in CAT activity in RCECs and HSKnb, respectively, but yielded no change in HeLa cells when compared with the activity encoded by the triple mutant construct (Fig. 4B). On the other hand, the −92α5/Sp1+NFIm construct resulted in a substantial (5-fold to 8-fold) increase in α5 promoter activity, indicating that most of the positive regulatory influence of the α5 SPR is determined by AP-1 and not by Sp1 in RCECs and HeLa cells, whereas both TFs contribute, though to different levels, to the transcription of the α5 gene in HSKnb cells. Preserving only the NFI site in the double-mutant −92α5/Sp1+AP-1m again resulted in a dramatic repression of α5 promoter function in all types of cells (8-fold to 25-fold).

Evidence that NFI represses α5 expression was further examined through the suppression of the endogenous NFI transcripts by RNAi. Preliminary experiments indicated that primarily NFI-B and NFI-C, but not NFI-A or NFI-X, accounted for the repression of the activity directed by the −92α5 construct (data not shown). Indeed, suppression of NFI-B and NFI-C by RNAi in HSKad cells translated into a substantial increase in the expression of the endogenous α5 mRNA transcript (Fig. 5A; left). Examination of NFI DNA-binding properties in NFI-B+C siRNA-transfected cells revealed a strong increase in the DNA binding of the NFI isoforms with lower electrophoretic mobility (most likely the consequence of the remaining NFI-A and NFI-X proteins), whereas fast-migrating NFI isoforms (likely NFI-B and NFI-C) completely disappeared (Fig. 5B; left). Consistent with the weak influence of Sp1 observed in transfection, suppression of Sp1 through RNAi had no significant influence on the transcription of the α5 gene (Fig. 5A; middle), despite that Sp1 siRNAs were particularly effective in suppressing Sp1 expression in HSKs (as revealed through EMSA analysis; Fig. 5B; middle). Given that all three Jun subunits from the transcription factor AP-1 are expressed in HSKad cells, we simultaneously suppressed the expression of JunB, c-Jun, and JunD through RNAi to achieve efficient knockdown of AP-1 expression in these cells. Unlike that of Sp1, RNAi inhibition of AP-1 translated into a dramatic reduction in the expression of the endogenous α5 mRNA transcript (Fig. 5A; right), consistent with a decrease in the DNA binding of AP-1 in RNAi-treated cells (Fig. 5B; right). Therefore, AP-1 is a strong activator and NFI is a strong repressor of α5 promoter activity, whereas Sp1 only functions as a weak activator.

## Competition of NFI with Sp1 but Not AP-1 for Availability of α5 SPR

Because the binding sites for Sp1, AP-1, and NFI are located close to each other on the α5 basal promoter, we next determined whether these TFs compete with each other for the availability of this regulatory element. As shown on Figure 6A, increasing the concentration of Sp1 while keeping that of NFI constant resulted in near complete abolition of NFI binding to the α5 SPR element and in the progressive appearance of the Sp1 complex (identical results were also obtained when Sp1 was kept constant while NFI was increased; data not shown). However, no additional complex with an electrophoretic mobility distinct from that yielded by each TF was produced, indicating that Sp1 and NFI could not bind simultaneously but rather competed with each other for the availability of the SPR.

Increasing AP-1 while keeping Sp1 constant resulted in the formation of a new, slower migrating DNA-protein complex (Fig. 6B, Ω) whose intensity increased as the amount of AP-1 was increased (identical results were obtained when AP-1 was kept constant while increasing Sp1; data not shown). This result suggested that AP-1 does not compete with Sp1 but

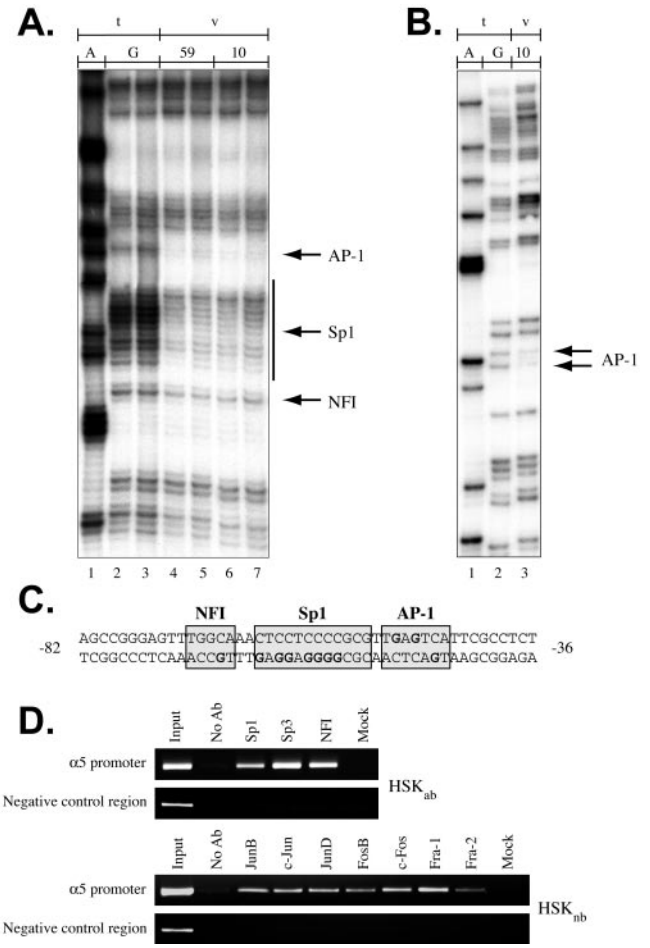


**FIGURE 3.** Analysis of Sp1, NFI, and AP-1 binding to the α5 promoter in vivo. (**A**) Binding of nuclear proteins from HCECs to the α5 promoter was examined in vivo by LMPCR on either the bottom (**A**) or the top (**B**) strand. *Lane 1*: DNA treated with $K_2PdCl_4$ to reveal the position of the adenine (A) residues. *Lanes 2* and *3*: naked DNA, purified from primary fibroblasts treated in vitro (t) with DMS to reveal the position of the guanine (G) residues. *Lanes 4–7*: DNA purified from HCECs isolated from two different donors and then treated in vivo (v) with DMS. (**B**) *Lane 1*: DNA treated with $K_2PdCl_4$. *Lane 2*: naked DNA, purified from fibroblasts treated in vitro (t) with DMS. *Lane 3*: DNA from HCECs grown to 80% cell density and treated in vivo (v) with DMS. The positions of the footprinted G residues within each of the TFs binding site (Sp1, NFI and AP-1) is indicated with *arrows*. (**C**) The G residues found to be protected in vivo are shown (*bold*) along the α5 promoter sequence. (**D**) ChIP analysis of Sp1, AP-1, and NFI binding to the α5 promoter in adult (HSKad) or newborn (HSKnb) HSKs. Cross-linked DNA-protein complexes were immunoprecipitated with antibodies to Sp1, Sp3, NFI, JunB, c-Jun, JunD, FosB, c-fos, Fra-1 and Fra-2. A "no antibody" (No Ab) control that contains chromatin but no antibody, an "input" sample corresponding to 0.2% of the total input chromatin, and a "mock" sample that does not contain chromatin were analyzed by PCR using primers specific to the α5 promoter (−262/+23). PCR amplification of a gene segment located approximately 2000 bp upstream from the p21 promoter was also conducted on the same sample as a negative control.

rather binds simultaneously with this TF to the labeled probe, therefore lowering its electrophoretic mobility on gel. Adding antibodies against Sp1 (Fig. 6C, lane 7) or AP-1 (Fig. 6C, lane 8), either individually or in combination (Fig. 6C, lane 9), reduced or prevented the formation of the Ω complex and led to the formation of supershifted complexes (SCs), therefore demonstrating that both proteins are indeed constituents of this slow-migrating complex (Fig. 6C).

We then repeated the experiment on AP-1 and NFI. Again, increasing the amount of NFI while keeping that of AP-1 constant eliminated the band corresponding to the AP-1/labeled probe complex but yielded a new slower-migrating complex on gel (Fig. 6D, Ø; also compare lanes 4 and 5 in Fig. 6C). That both AP-1 and NFI are constituents of the Ø complex was again demonstrated by supershift analysis because the NFI and AP-1 antibodies, added individually (Fig. 6E, lanes 4 and 5) or in combination (Fig. 6E, lane 6), could strongly impair the formation of this new complex and yield supershifted complexes, indicating that both TFs can bind simultaneously to the α5 SPR.

Given that NFI is a repressor but that both Sp1 and AP-1 are activators of α5 gene transcription, we next examined how increasing the amount of NFI would alter the occupation of the α5 SPR by Sp1 and AP-1. As Figure 6F indicates, incubation of the labeled probe with Sp1 and AP-1 again resulted in the formation of the Ω Sp1/AP-1/DNA complex (lane 5). However, as NFI was added (lanes 6–8), formation of the Ω complex progressively decreased while that of the NFI/AP-1 Ø complex appeared on the gel. No complex corresponding to the recognition of the probe only by AP-1 was visible, indicating that all AP-1 proteins were sequestered into the Ø NFI/AP-1 multiprotein-DNA complex. Therefore, we concluded that AP-1 could occupy the α5 promoter SPR simultaneously with Sp1 or NFI, whereas the binding of Sp1 and NFI did not occur in vitro.

## Alteration of the Expression and DNA-Binding Properties of Sp1, NFI, and AP-1 in a Cell Density–Dependent Manner by Fibronectin

We have shown previously that reaching cell quiescence by maintaining keratinocytes and RCECs at postconfluence for more than 2 days also translates to a dramatic reduction in the expression of Sp1.[25] No such experiments have been conducted for NFI and AP-1. Therefore, we cultured RCECs to varying confluences and assessed the DNA-binding properties of Sp1, NFI, and AP-1 under these culture conditions. As shown on Figure 7A, binding of all three TFs was severely reduced in the absence of FN as RCECs reached 5 days postconfluence (C5d). Indeed, no Sp1 binding could be observed at C5d, whereas alternative, fast-migrating complexes likely resulting from protein degradation were clearly visible for AP-1 and NFI, which also coincided with the disappearance of their typical, slow migrating complexes in EMSA. Unlike the detrimental influence that cell density exerts on Sp1 expression, FN causes a substantial increase in its DNA-binding properties without significantly altering Sp1 expression at the protein level, when cells are at high confluence.[24] Because FN is massively secreted in tissue during wound healing, we evaluated whether it might have any influence on the cell density–dependent extinction of Sp1, NFI, and AP-1 in RCECs. At 50% confluence, FN caused a dramatic increase in the DNA-binding properties of all three TFs, particularly AP-1 (Fig. 7A). Interestingly, at 80% confluence, FN acted positively on the DNA-binding properties of Sp1 and NFI, but not on that of AP-1. In addition, FN significantly reduced the rate of Sp1 and NFI degradation as RCECs reached C5d but had only a marginal influence on the extinction of AP-1. Besides reducing the degradation of NFI at lower cell densities, FN unexpectedly increased its DNA-binding properties at C5d, well above the level observed at midconfluence (Fig. 7A).

Because NFI is a repressor of α5 promoter function, we tested whether this shift toward NFI binding at postconfluence combined with the extinction of AP-1 would also translate into alteration in the activity directed by the α5 promoter in transfected cells grown on FN at varying densities. As shown on Figure 7B, FN had little influence on the α5 promoter when cells were seeded at a low density ($1.5 \times 10^4$ cells/cm$^2$), a

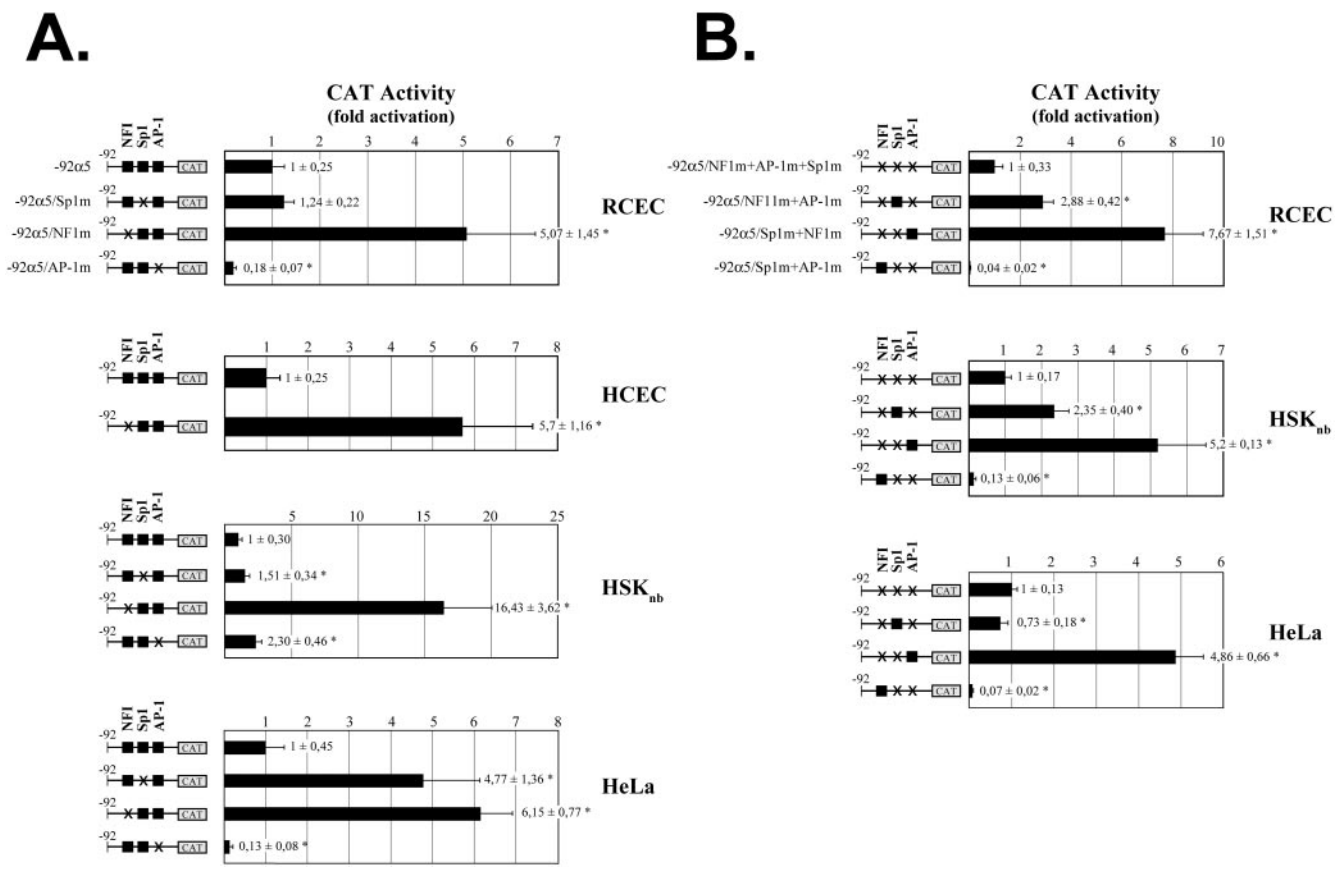


**FIGURE 4.** Transfection of the mutated α5 promoter constructs. The −92α5 construct or derivatives that bear mutations (indicated by "X") in the AP-1, Sp1, and NFI sites, either individually (**A**) or in combination (**B**), were transfected into RCECs, HCECs, HSKs, and HeLa cells. Cells were harvested 72 hours after transfection, and CAT activities (expressed as (%CAT/4 h/100 μg proteins)/ng hGH) were determined and expressed relative to the level directed by either the −92α5 (**A**) or −92α5/Sp1m+AP-1m+NFIm (**B**) construct. *CAT activities statistically different from those measured with either −92α5 (**A**) or −92α5/Sp1m+AP-1m+NFIm (**B**). $P < 0.05$; paired samples, *t*-test.

condition that yielded less than 50% coverage of the culture plate at the time of cell harvesting. However, increasing the cell density further to $2.5 \times 10^4$ cells/cm$^2$ (yielding near 70% coverage of the plate [SC]) resulted in a 10-fold increase in α5 promoter activity. FN responsiveness reached a peak at $4.5 \times 10^4$ cells/cm$^2$, a condition that yielded nearly 100% coverage of the culture plate (C). Seeding RCECs at a density of $6.5 \times 10^4$ cells/cm$^2$ almost entirely abolished cell responsiveness to FN. Interestingly, the strong positive influence that FN exerted on the α5 promoter activity turned into a negative function (3-fold repression) when cells were seeded at $1 \times 10^5$ cells/cm$^2$, a condition that maintained the cells at postconfluence for 72 hours before their isolation (C3d). We therefore conclude that FN has the ability to promote or suppress expression directed by the α5 gene promoter in a cell density–dependent manner.

## DISCUSSION

Tremendous progress has been achieved in the past decade in the understanding of the molecular events that characterize wound healing of damaged tissues. To properly heal the damaged epithelium of any given tissue, cells must first release themselves from the BM through hemidesmosome (HD) dissociation and then reorganize their cell-substrate contacts to allow migration. The heterodimeric transmembrane cell surface receptors that belong to the integrin family happen to be critical for modulating HD and focal contacts because they can bind ECM proteins through their extracellular portion and interact with cytoskeletal structures through their intracellular regions (for a review, see Vigneault et al.[19]). As HDs are disassembled and the intact BM progressively degraded, migrating epithelial cells use a provisional matrix in the wound bed that is particularly rich in FN.[38] While this is occurring, a rapid change in the pattern of integrin expression is observed in the epithelial cells that border the wounded area. One of the most affected receptors is the FN-binding integrin α5β1, whose expression, nearly undetectable in intact epithelia, has been reported to dramatically increase in corneal[13,39] and skin[40] epithelial cells in response to the massive secretion of FN that accompanies wound repair. Given that appropriate transcription of the α5 gene is critical to ensure proper wound healing of injured tissues, we investigated in further detail the basal core promoter of that gene to decipher which TFs actually contribute to direct expression of this integrin subunit gene during that process. The basal α5 promoter was found to bear a proximal small regulatory element (SPR) that binds three distinct TFs—Sp1, NFI, and AP-1—under in vivo and in vitro conditions. Primary cultured RCECs, HCECs, and HSKs and tissue culture HeLa cancer cells were found to express varying levels of these TFs. We demonstrated that Sp1 and AP-1 are activators of α5 gene transcription, whereas NFI is a potent repressor of that gene. Because of the proximity of their respective target site, these TFs could not bind simultaneously to the α5 basal promoter but only bound the SPR as pairs (AP-1/Sp1 or AP-1/NFI but not Sp1/NFI). Most of all, the stability of

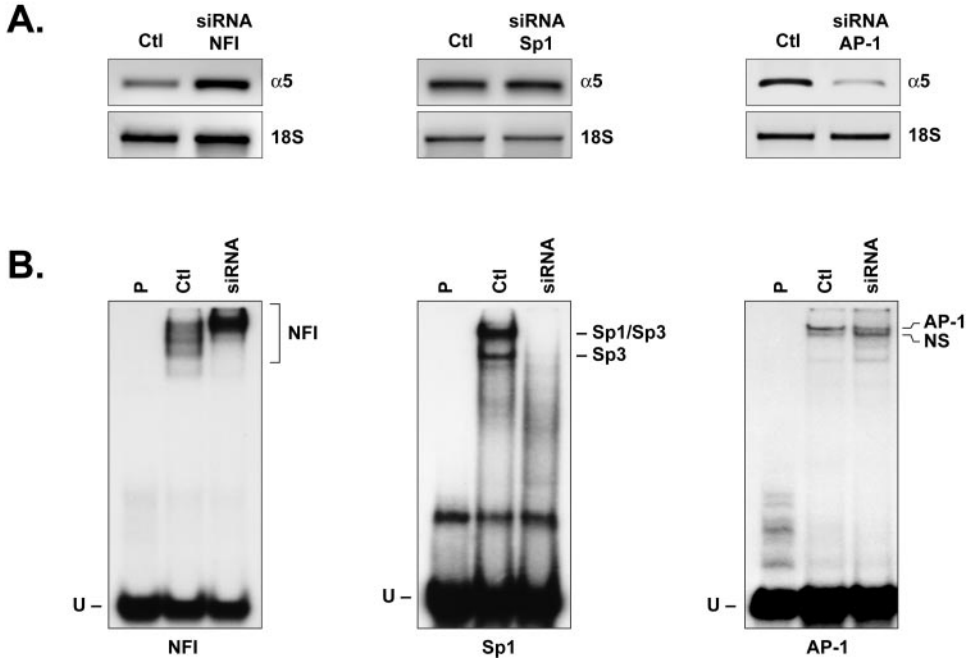


**FIGURE 5.** Influence of RNAi suppression of Sp1, NFI, and AP-1 on expression of the endogenous α5 gene. (**A**) Total RNAs extracted from HSKad cells transfected with the siRNA silencer negative control (Ctl) or with the siRNA duplexes directed against the NFI (*left*), Sp1 (*middle*) or AP-1 (*right*) transcript were PCR amplified using primers specific to the α5 and 18S ribosomal RNAs. (**B**) Nuclear extracts (10 μg) from transfected HSKad (**A**) were incubated with 5′-end labeled NFI (*left*), Sp1 (*middle*), or AP-1 (*right*) probes. Formation of Sp1/Sp3, AP-1 (the specific AP-1 signal [AP-1] and a nonspecific band [NS]), and NFI complexes was then examined by EMSA.

these TFs was found to be considerably improved in corneal epithelial cells grown in the presence of FN.

The presence of closely located target sites for the TFs Sp1, AP-1, and C/EBP in the basal promoter from the human α5 gene was reported a few years ago by Corbi et al.[23] Both the Sp1 and the AP-1 sites, which have been mapped at the same positions as those reported here, correspond to high-affinity target sites for these TFs. However, that reported for C/EBP, which overlaps the NFI site identified in the present study, diverges from the consensus because it only preserves 6 of 10 residues from the C/EBP prototypical sequence[23] and is therefore likely to be a low-affinity target site for this TF. In addition, no direct demonstration that any of these TFs was truly interacting with their respective target site in vivo was provided. Most important, mutations that disrupted the α5 C/EBP-binding site could not prevent in any way the repression of the α5 promoter by C/EBP,[23] suggesting that C/EBP must interact with the α5 promoter through a target site different from that identified by Corbi et al. Interestingly, a study recently published by Koria and Andreadis[41] provided evidence that the −65 to −75 area from the α5 promoter, which also bears the NFI-C/EBP binding sites, accounts for the keratinocyte growth factor (KGF)-dependent upregulation of α5 gene expression because mutations that disrupt NFI binding also abolished this regulatory effect of KGF, suggesting that members from the NFI family may account, at least in part, for the regulatory influences of KGF.

The structural integrity of the α5 Sp1/NFI/AP-1 SPR is not unique to the α5 promoter. Indeed, the presence of these three TFs sites has been reported in the basal promoter from the human tissue-type plasminogen activator (t-PA),[42] though they are scattered on a much longer segment of the t-PA promoter (79 bp compared with 30 bp for the α5 promoter). In addition, the overall structure of the basal promoter from the human α2 and α5 integrin subunit genes was also reported to be similar in that they share the same set of TF target sites (Sp1, AP-1, and C/EBP) in the same position and distance from the mRNA start site.[23] Adjacent or overlapping AP-1 and NFI sites are also frequently encountered in regulatory regions of viral genes but have been observed in many eukaryotic gene systems, including the α1-antichymotrypsin gene.[43] The proximity of these two binding sites suggests the opportunity for interaction between NF-1 and AP-1 proteins. Indeed, NFI-X was recently reported to bind to c-Jun independently of their interaction with their respective target site in DNA.[44] Sp1 has been reported to interact with at least 16 transcription-associated proteins, including, among others, TBP, P53, E2F, YY1, NF-κB, and TAF1 (for a review, see Li et al.[45]). Similarly, interaction of the c-Jun subunit of AP-1 with other TFs has been demonstrated for the glucocorticoid receptor, estrogen receptor, Ets family proteins, the SMAD family, and the Rel family members NF-κB and NF-AT, among others (for a review, see Ozanne et al.[46]). This extended array of protein partners offers a mechanism by which both the Sp1 and the AP-1 TF families can exert a finely tuned regulation of large sets of genes in response to a wide variety of extracellular stimuli and cellular contexts. Although the interaction of Sp1 and AP-1 with other TFs is a well-documented fact, the array of TFs with which NFI can interact is, on the other hand, much more restricted. NFI was reported to physically interact with the human estrogen receptor,[47] the TF YY1,[48] and TBP in yeast.[49] Most interesting, NFI was recently demonstrated to block the positive regulatory influence of Sp1 on the activity directed by the platelet-derived growth factor-A promoter by directly interacting with Sp1 through its subtype-specific domain.[50]

Despite the fact that AP-1 clearly plays a major role in α5 gene transcription, the nature of the Jun and Fos subunits that constitute the AP-1 heterodimer in the various cell types examined remains elusive. Results from the ChIP analysis indicated that in HSK cells, all Jun and Fos isoforms (JunB, c-Jun, JunD, FosB, c-Fos, Fra-1, Fra-2) were part of the AP-1 complex. HCECs were previously shown to express the AP-1 subunits Fra-1, Fra-2, JunD, JunB, and c-Jun.[51] Although many studies have attempted to functionally organize the various AP-1 heterodimers into strong (e.g., c-Fos, FosB, c-Jun) and weak (e.g., Fra-1, Fra-2, JunD) transactivators, this task proved to be more difficult than expected because the activity of any given AP-1 protein can also be dramatically affected by the nature of the gene promoter that bears the AP-1 target site.[52] The same difficulty is encountered with members of the NFI family because most of them have the ability to function as activators or repressors of gene transcription (for a review, see Murtagh et al.[53]), but whether their regulatory influence will be positive or

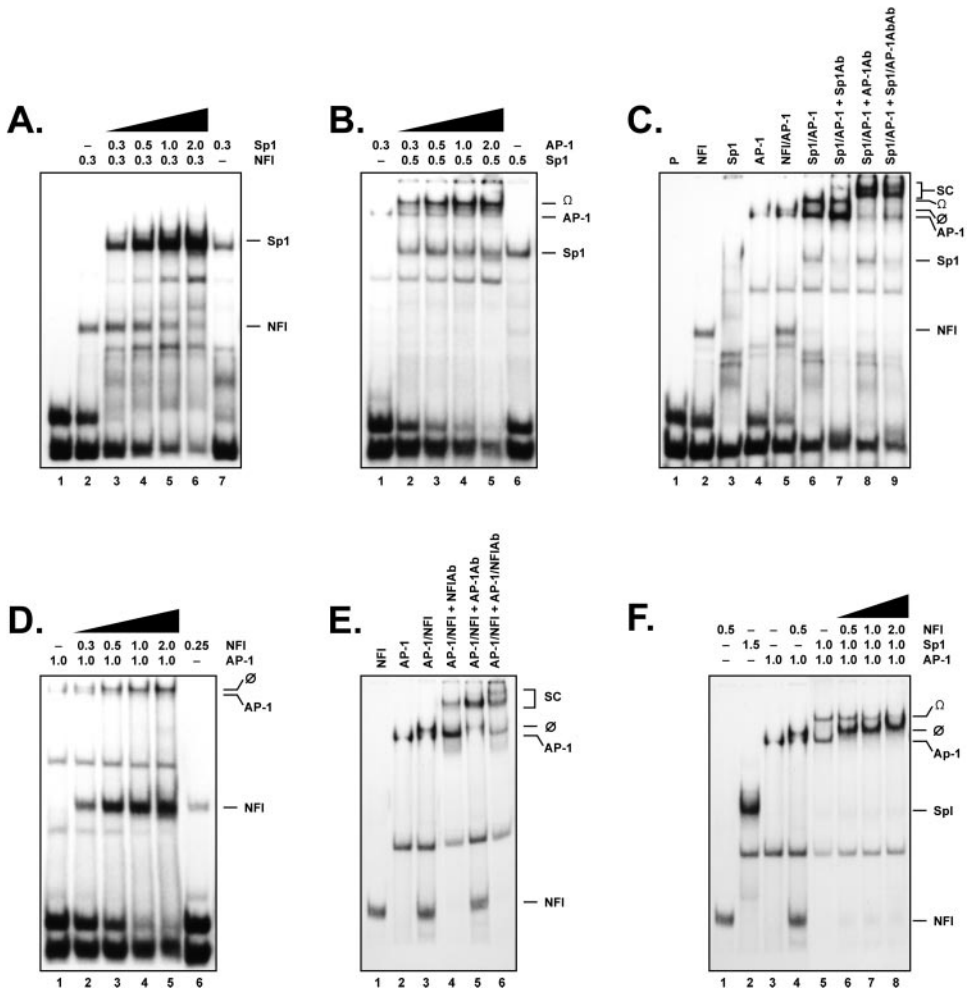


**Figure 6.** Occupancy of the α5 SPR by Sp1, NFI, and AP-1. (**A**) The α5.4 labeled probe was incubated with 0.3 μL carboxymethyl-Sepharose-enriched NFI, either alone (*lane 2*) or with increasing amounts (0.3–2.0 μL) of recombinant Sp1 (*lanes 3–6*). Formation of DNA/protein complexes was then monitored by EMSA on a 6% native gel. *Lane 1*: labeled probe alone. U, free probe. (**B**) Same conditions except that the experiment was conducted with both Sp1 (constant at 0.3 μL) and AP-1 (increasing from 0.3 to 2.0 μL). (**C**) The α5.4 probe was incubated with NFI, Sp1, or AP-1 either individually (*lanes 2–4*) or in combination (NFI and AP-1, *lane 5*; Sp1 and AP-1, *lanes 6–9*). Antibodies against Sp1 and AP-1 were then added individually (*lanes 7, 8*) or in combination (*lane 9*). The position of slow-migrating complexes resulting from the recognition of the probe by NFI and AP-1 (Ø) or Sp1 and AP-1 (Ω) is indicated, along with those resulting from the binding of any single TF. *Lane 1*: Labeled probe alone (P). (**D**) Same conditions as in (**A**) except that the experiment was conducted using NFI (increasing from 0.3 to 2.0 μL) and AP-1 (constant at 1.0 μL). (**E**) Same conditions as in (**C**) except that the supershift experiment was conducted using antibodies against NFI and AP-1 on the Ø complex. (**F**) A reaction mixture containing the α5.4 probe incubated with constant amounts of AP-1 and Sp1 (both at 1.0 μL) was added increasing concentrations (0.5–2.0 μL) of NFI. Formation of the NFI/AP-1 (Ø) or Sp1/AP-1 (Ω) multiprotein complexes was then tracked by EMSA.

negative remains in good part dictated by the type of cell examined and the gene promoter they regulate.

Culturing RCECs in the presence of FN considerably improved the nuclear stability of Sp1, NFI, and AP-1, but distinctions were observed as the cell density was changing. At low confluence, a condition in which cell adhesion and migration are predominantly increased, FN caused a dramatic increase in the DNA-binding properties of AP-1 that clearly predominated over Sp1 and NFI. As the cell density increased toward postconfluence, a dramatic shift toward predominance by NFI rather than AP-1 occurred. We have already provided evidence that Sp1 and NFI responded positively to the presence of FN in primary cultured cells.[24,54] In support of our results, FN was recently reported to stimulate the expression of MMP-9 through increased AP-1/DNA binding and c-Fos protein expression through ERK and PI3K signaling pathways in proliferative H1838 human cells.[55] The density-dependent extinction of TFs such as those discussed in this study, combined with the FN-mediated increase in the expression of transcriptional suppressors such as NFI, represent extremely efficient and powerful devices for the recently reepithelialized wound to moderate the proliferative rate of its constituting epithelial cells. In addition to the cell-density effect, other factors, such as the variation in the composition of the ECM that occurs during the wounding process, may contribute to the change in the properties of corneal epithelial cells. Indeed, FN, whose staining is absent in the BM of normal corneas, becomes massively secreted within hours of damage to the corneal epithelium,[13,56]

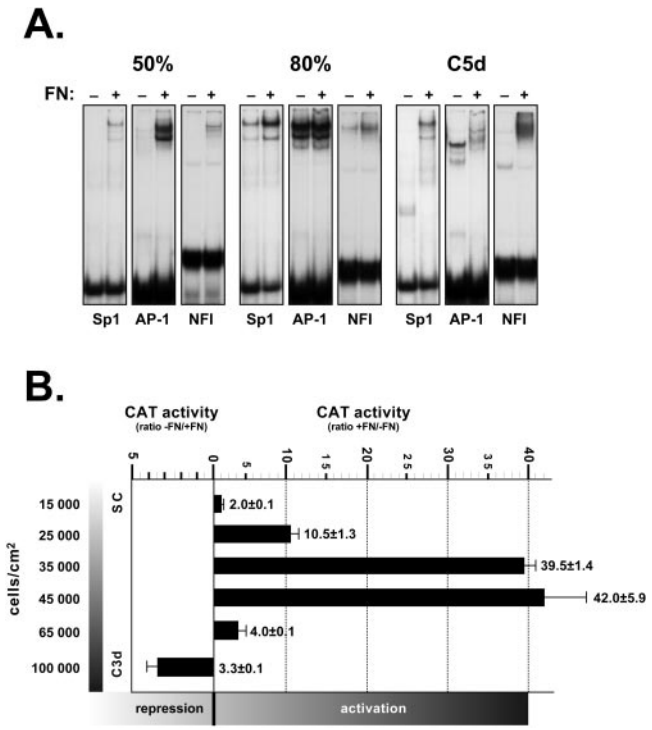


**FIGURE 7.** Cell-density dependent influence of FN on DNA binding of Sp1, NFI, and AP-1. (**A**) RCECs were grown to 50% or 80% confluence or to postconfluence for 5 days (C5d) on BSA- or FN-coated plates and then were used for preparation of nuclear extracts. Proteins (7.5 $\mu$g) from each cell culture condition were incubated with Sp1, AP-1, and NFI-labeled probes, and DNA-protein complexes were examined by EMSA. (**B**) RCECs seeded at varying cell densities on BSA- or FN-coated plates were transfected with the plasmid −92$\alpha$5-CAT and harvested 48 hours later, and CAT activities were determined. Values are expressed as the ratio of the CAT activity from cells grown in the presence of FN over that from cells grown on BSA (+FN/−FN) when activation was observed on FN or as the opposite (−FN/+FN) when repression occurred on FN.

which precisely coincides with activation of the migration and proliferation properties of basal epithelial cells. Conversely, as epithelial cells completely cover the damaged corneal surface and cell proliferation and migration are no longer required, FN expression and secretion are turned off but that of LM is turned on because the BM stains strongly for this ECM component 48 hours after corneal injury.[13] These clinical findings suggest that FN may promote cell migration and proliferation in response to tissue injury, whereas LM would signal exactly the opposite by restricting both these properties and forcing the cells to progress to growth arrest or to differentiate. Interestingly, though FN is strictly reported to increase cell migration and proliferation at low cell density, LM is often associated with growth arrest of normal cells and cancer cells.[57,58] Through interaction with its corresponding integrin receptor $\alpha6\beta4$, LM recently has been reported to cause a dramatic extinction of Sp1 in RCECs and HCECs.[36] Therefore, the secretion of LM concurrent with the degradation of FN during the wounding process may signal to epithelial cells the proper time to stop migrating by inducing the degradation of key TFs by the proteasome, such as Sp1, and thereby shutting down expression of a large number of genes required for cell adhesion and proliferation.

## *Acknowledgments*

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