Regulation of the Integrin Subunit α5 Gene Promoter by the Transcription Factors Sp1/Sp3 Is Influenced by the Cell Density in Rabbit Corneal Epithelial Cells

Marie-Eve Gingras,[1,2] *Kathy Larouche,*[1,2] *Nicolas Larouche,*[1] *Steeve Leclerc,*[1] *Christian Salesse,*[3] *and Sylvain L. Guérin*[1]

Purpose. Expression of the α5β1 fibronectin (Fn) integrin is well recognized in the corneal epithelium and has been postulated to increase during wound healing. In the present study, the regulatory influence of the positive transcription factors Sp1/Sp3 on the activity directed by the promoter of the α5 gene was examined in rabbit corneal epithelial cells (RCECs) primary cultured at various cell densities.

Methods. Expression of the α5 subunit was assessed at the transcriptional level by semiquantitative RT-PCR analyses. The regulatory elements necessary to direct expression of the α5 gene were identified by transfecting RCECs with recombinant plasmids bearing various lengths from the α5 gene promoter fused to the CAT reporter gene. Binding of Sp1/Sp3 to the α5 promoter was assessed by both electrophoretic mobility shift assays (EMSAs) and DNaseI footprinting. Endogenous levels of Sp1/Sp3 were determined by Western blot and supershift analyses. The regulatory influence exerted by Sp1/Sp3 on the α5 promoter was evaluated both by site-directed mutagenesis and cotransfection in Sp1-deficient *Drosophila* SL-2 Schneider cells.

Results. Subconfluent RCECs expressed nearly five times more α5 transcript than 48-hour postconfluent RCECs. The activity directed by the α5 promoter was found to be affected by cell density. Strong promoter activity was observed in subconfluent RCECs, whereas a dramatic repression was measured in postconfluent cells. EMSA and DNaseI footprinting provided evidence for the binding of Sp1 to both a proximal site located within the previously reported α5 fibronectin responsive element (FRE), and a distal site located between positions −117 and −101. Cotransfection experiments in Schneider cells, as well as transfection of RCECs with recombinant constructs bearing mutations into the distal Sp1 site, confirmed the positive regulatory influence of Sp1 on both the −42/−92 and −92/−132 α5 promoter segments. Most of all, EMSA and Western blot analyses demonstrated the expression of substantial amounts of Sp1/Sp3 in subconfluent but not postconfluent RCECs.

Conclusions. These results provide support to the hypothesis that the strong reduction in the activity of the α5 promoter when RCECs reach a high cell density is the consequence of a reduced expression of Sp1/Sp3 under such cell culture conditions. (*Invest Ophthalmol Vis Sci.* 2003;44:3742–3755) DOI:10.1167/iovs.03-0191

Inside-out signaling between the extracellular matrix (ECM) and the cell is essentially mediated by a family of cell surface glycoproteins named integrins.[1,2] These transmembrane receptors are known to play major functions in many physiological and pathologic processes, including embryonic development, inflammation, metastasis, and wound healing.[3] All members of the integrin superfamily are constituted of noncovalently associated α and β subunits.[1–4] Both subunits contain a large extracellular domain, a transmembrane region, and a short cytoplasmic C-terminal tail that interacts with cytoskeletal components, such as α-actinin, vinculin, talin, tensin, and paxilin.[3,5–8] In a recent survey of the human genome, 24 α and 9 β integrin subunits were identified,[9] which implies 6 novel α-subunits and 1 novel β-subunit in addition to the previously recognized 18 α- and 8 β-subunits reported to form 24 different heterodimers.[1,3,7,10] However, the existence of these new integrin subunits remains to be firmly established.

Because of its anterior location in the eye, the cornea, and more specifically its epithelium, are continuously exposed to a large variety of injuries, including physiological and mechanical stresses. It is now well established that such types of corneal epithelial injuries in turn activate wound healing, a phenomenon that is influenced by growth factors and ECM components (for reviews, see Refs. 11–14). Many integrins have been identified in the corneal epithelium. These include α-subunit-2, -3, -4, -5, -6, -V, and -9, and β-subunit-1, -4, and -5.[15–21] Soon after a wound is inflicted on the corneal epithelium, the basal cells that border the injured area and stromal keratocytes start producing massive amounts of fibronectin (Fn).[22,23] Fn promotes corneal cell migration, both in vitro[24] and in vivo,[25] by acting as a temporary matrix to which corneal epithelial cells attach as they migrate over the wounded area.[26] Results from a few studies point toward an Fn-mediated increase in expression of the main Fn-binding integrin α5β1 during corneal wound healing.[16,27] Expression of the α5 subunit may therefore make a substantial contribution to wound healing by promoting cell adhesion and migration during this process.

Because integrins from the β1 family are known to play major functions in the regulation of cell proliferation, differentiation, and migration, their expression may therefore be closely related to the state of cell growth. Fingerman and

From the [1]Oncology and Molecular Endocrinology Research Center, and the [3]Unit of Ophthalmology, Hospital Center of the University of Québec and Laval University, Québec, Canada.

[2]Contributed equally to the work and therefore should be considered equivalent senior authors.

Supported by Medical Research Council (MRC) Grant MT-13160 (SLG, CS) and by the Vision Research Network from the Fonds de la Recherche en Santé du Québec (FRSQ). KL was supported by a studentship from the Natural Sciences and Engineering Research Council of Canada (NSERC). NL has held a studentship from the MRC of Canada. CS and SLG are FRSQ scholars.

Submitted for publication February 24, 2003; revised May 16, 2003; accepted May 29, 2003.

Disclosure: **M.-E. Gingras,** None; **K. Larouche**, None; **N. Larouche**, None; **S. Leclerc**, None; **C. Salesse**, None; **S.L. Guérin**, None

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Corresponding author: Sylvain L. Guérin, Oncology and Molecular Endocrinology Research Center, CHUL, 2705 Laurier Boulevard, Ste-Foy (Québec) G1V 4G2, Canada; sylvain.guerin@crchul.ulaval.ca.

Hemler[28] have observed that when normal human fibroblasts reach quiescence, expression of both the α2 and α3 integrin subunits, but not that of β1, is reduced. Similar results have been reported in other studies for the α5-subunit, in addition to the α2 and α3 integrin subunits.[29,30] These observations all suggest that cell-to-cell contact probably controls the level to which α integrin subunits are expressed at both the mRNA and protein levels in vitro.

In the present study, we demonstrate that expression of the gene encoding the α5 integrin subunit is dramatically altered in a manner that depends on the cell density reached in primary cultured RCECs. The increased promoter function observed when transfections were conducted in subconfluent RCECs was determined by both the previously described fibronectin responsive element (FRE) identified between α5 sequences −82 to −56,[31] and a short GC-rich segment from the α5 promoter extending between positions −92 and −132. Most of all, we provide evidence that the positive influence exerted by both of these α5 promoter segments is primarily mediated through their recognition by the positive transcription factors Sp1/Sp3, two proteins that belong to a family of GC-rich binding transcription factors, the Sp family, that includes three other proteins—Sp3, Sp4, and Sp5[32–36]—and which are expressed in primary cultured RCECs.[31,37] We demonstrate that the strong reduction in the α5 promoter function when RCECs reach postconfluence is the consequence of a dramatic reduction in the expression of both Sp1 and Sp3 when these cells are maintained at a high cell density.

Materials and Methods

Plasmids and Oligonucleotides

The plasmids −954α5CAT, −178α5CAT, −132α5CAT, −92α5CAT, and −42α5CAT, which bear the chloramphenicol acetyl transferase (*CAT*) reporter gene fused to DNA fragments from the human α5 gene upstream regulatory sequence extending up to 5′ positions −954, −178, −132, −92, and −42, respectively, all sharing a common 3′ end located at position +23, have been previously described.[37] The −132α5CAT plasmids bearing mutations into either the proximal (−132α5Mp) or the distal (−132α5Md) Sp1 sites, or both (−132α5Mp+d) were constructed through site-directed mutagenesis (QuikChange Site-Directed Mutagenesis kit; Stratagene, La Jolla, CA), using the following mutated oligonucleotides: Sp1Mp top strand, 5′-GCCGGGAGTTTGGCAAACT**AAAAAA**CGCG-TTGAGTCATTCGCCTC-TGGGAGG-3′; Sp1Mp bottom strand, 5′-CCTCCCAGAGGCGAATGAC-TCAACGCG**TTTTTT**AGTTTGCCAAACTCCCGGC-3′; Sp1Md top strand, 5′-CCCAGGAATGCCCCCC**AAA**CAGCC**AAA**TCGGCAGGCGGGGGGA-GG-3′; Sp1Md bottom strand, 5′-CCTCCCCCCGCCTGCCGA**TTT**GGCT-G**TTT**GGGGGGCATTCCTGGG-3′ (mutated residues are in bold). The Sp1 and Sp3 expression plasmids pPacSp1 and pPacSp3, respectively, which allow for a high level of expression of these proteins in *Drosophila* Schneider cells were obtained from Guntram Suske (Institute for Molecular Biology and Tumor Research, Marburg, Germany), and the mammalian Sp1 expression plasmid pSiSp1 was provided by Winnie Eskild (Department of Biochemistry, University of Oslo, Oslo, Norway). The LacZ expression plasmid pAC5/V5-His/LacZ was obtained from Invitrogen (Carlsbad, CA).

The double-stranded oligonucleotides used in the present study were chemically synthesized with a commercial apparatus (Biosearch 8700; Millipore, Bedford, MA). They contained the human α5 promoter comprising positions −143 to −86 and designated α5.1 (5′-GTTTCCAGGGACCCAGGAATGCCCCCCCCGCCAGCCCCCTCGGCAG-GCGGGGGGAGGGC-3′), or shorter derivatives that extend from position −143 to −106 (α5.2: 5′-GTTTCCAGGGACCCAGGAATGC-CCCCCCGCCAGCCCCC-3′) and −105 to −86 (α5.3: 5′-TCGGCAG-GCGGGGG-GAGGGC-3′), the sequence of the α5 FRE (5′-GATCAGC-CGGGAGTTTGGCAAACTCCTCCCC-3′),[31] the DNA binding site for human HeLa CTF/NF-I in adenovirus type 2 (Ad2; 5′-GATCTTATTTT-GGATTGAAGCCAATATGAG-3′),[38] and the high-affinity binding site for Sp1 (5′-GATCATATCTGCGGGGCGGGGCAGACACAG-3′).[39]

Cell Culture

RCECs were obtained from freshly dissected rabbit corneas and cultured in 60-mm plates as described,[40] except for the following modifications: corneas were removed within 90 minutes of death from pathogen-free albino rabbits weighing 2 to 3 kg and obtained from a local abattoir. They were rinsed twice for 6-minutes each in Hanks' balanced salt solution (HBSS) containing 50 μg/mL gentamicin sulfate and a 1:100 dilution of an antibiotic mixture (5 mg/mL penicillin G in salt solution, 5 mg/mL streptomycin sulfate, and 10 mg/mL neomycin sulfate) before treatment with dispase II (2 mg/mL in calcium- and magnesium-free HBSS) for 20 minutes at 37°C. The corneal epithelia were then transferred into 25-cm^2 tissue-culture flasks (each flask contained the epithelia of three rabbit corneas) and grown in supplemental hormonal epithelial medium (SHEM) for 8 days.[40] Trypsin (0.05% trypsin, 0.5 mM EDTA) 2 mL/25 cm^2 was then added for 5 minutes and the RCECs harvested, counted, and seeded into six-well tissue culture plates. Cells were then grown either to subconfluence (5×10^4 cells/cm^2 cultured for 48 hours; this condition yielded near 70% coverage) or to postconfluence (1.5×10^6 cells/cm^2 cultured for 48 hours, which yielded 100% coverage). Cells where then maintained for an additional 48 hours in culture in SHEM, as described previously.[41] Gentamicin was added to all media at a final concentration of 15 μg/mL. *Drosophila* Schneider cells (ATCC CRL-1963; American Type Culture Collection, Manassas, VA) were cultured at 28°C without CO_2 in Schneider medium (Sigma-Aldrich, Oakville, Ontario, Canada) supplemented with 10% FBS (Invitrogen-Gibco, Burlington, Ontario, Canada) and 20 μg/mL gentamicin. Rat pituitary GH4C1 cells were grown in Ham's F10 medium (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen-Gibco) and 20 μg/mL gentamicin, in 5% CO_2 at 37°C.

Transient Transfection and CAT Assay

RCECs were grown either to subconfluence (near 70% coverage) or postconfluence (100% coverage for 48 hours in culture) and transiently transfected by using a polycationic detergent (Lipofectamine; Invitrogen-Gibco), as recommended by the manufacturer. Each transfected plate received 1.0 μg of the test plasmid and 0.5 μg of the human growth hormone (hGH)-encoding plasmid pXGH5.[42] Under those conditions, subconfluent RCECs were transfected with an efficiency of 48% ± 4%, as revealed by cotransfection of the green fluorescent protein (GFP) expression plasmid pEGFP-C3 (Clontech, Palo Alto, CA) and normalization to Hoechst-stained nuclei, whereas postconfluent cells yielded an efficiency of transfection of 39% ± 7% (data not shown). In the Sp1 overexpression experiments conducted in both postconfluent and subconfluent RCECs, 0.5 μg of the Sp1 expression plasmid pSiSp1 was added. *Drosophila* Schneider cells were transfected according to the calcium phosphate precipitation procedure[43,44] at a density of 1×10^6 cells per 60-mm culture plate. CAT activities from transfected Schneider cells were normalized to the amount of β-gal encoded by the plasmid pAC5/V5-His/LacZ and cotransfected along with the CAT recombinant constructs. Each cell-containing plate therefore received 16 μg of the test plasmid, 4 μg of pAC5/V5-His/LacZ, and 1 μg of pPacSp1, pPacSp3, or both. Levels of CAT activity for all transfected cells were determined as described,[43] normalized to the amount of hGH secreted into the culture media, and assayed with a kit for quantitative measurement of hGH (Immunocorp, Montréal, Québec, Canada). Measurement of β-gal activity was performed according to standard procedures.[45] The result presented for each test plasmid transfected corresponds to the mean ± SD of at least three separate transfections performed in triplicate. To be considered significant, each activity level had to be at least three times over the

background level caused by the reaction buffer used (usually corresponding to 0.15% chloramphenicol conversion).

Nuclear Extracts and Electrophoretic Mobility Shift Assay

Crude nuclear extracts were prepared from subconfluent (1×10^4 cells/cm^2 cultured for 72 hours usually yielded a near 70% coverage of the culture flasks) or 48-hour postconfluent RCECs (3×10^4 cells/cm^2; under such seeding conditions, cells reach 100% coverage of the culture flasks in 4 days, after which they are maintained for an additional 48 hours before they are removed from the flasks) and dialyzed against DNaseI buffer (50 mM KCl, 4 mM $MgCl_2$, 20 mM K_3PO_4 [pH 7.4], 1 mM β-mercaptoethanol, 20% glycerol) as described.[46] Extracts were kept frozen in small aliquots at −80°C until use. EMSAs were conducted by using either the 58-bp α5.1 double-stranded oligonucleotide, which bears the α5 promoter sequence from positions −143 to −86, or the Sp1 oligomer as 5′ end-labeled probes. Approximately 3×10^4 cpm labeled DNA was incubated with crude nuclear proteins (15 μg) obtained from either postconfluent or subconfluent cultures of RCECs in the presence of 25 ng poly(dI-dC). poly(dI-dC) (Pharmacia-LKB, Gaithersburg, MD) in buffer D (5 mM HEPES (pH 7.9), 10% glycerol (vol/vol), 25 mM KCl, 0.05 mM EDTA, 0.5 mM dithiothreitol [DTT], 0.125 mM phenylmethylsulfonyl fluoride [PMSF]). When indicated, molar excesses (as specified in the figure legends) of unlabeled double-stranded oligonucleotides, bearing various DNA segments from the α5 promoter (α5.1, α5.2, α5.3, an α5-FRE) or target sequences for known transcription factors (Sp1 and NF1) were added as unlabeled competitors during the assay. Incubation proceeded at room temperature for 15 minutes, at which time DNA–protein complexes were separated by gel electrophoresis through either 5% or 8% native polyacrylamide gels run against Tris-glycine buffer, as described.[31,47] Gels were dried and autoradiographed at −80°C to reveal the position of the shifted DNA–protein complexes generated.

DNase I Footprinting

A 165-bp *Bam*HI/*Xbo*I DNA fragment spanning the α5 promoter sequence from position −132 to +23 was 5′ end–labeled and used as a probe in DNaseI footprinting. DNase I digestion was performed in DNase I buffer[44] by incubating a 3×10^4-cpm–labeled probe with 10 μL of recombinant Sp1 protein (GST-Sp1-8xHis protein; kindly provided by Claude Labrie, Oncology and Molecular Endocrinology Research Center, Hospital Center Laval University (CHUL) Research Center, Québec, Canada) obtained by overexpressing the pGEX-Sp1-8xHis plasmid in *Escherichia coli* BL21 CodonPlus R1L cells before being purified as described.[48] Further analyses of the digested products on polyacrylamide sequencing gels were performed as described previously.[44]

RT-PCR and Northern Blot mRNA Analyses

Total RNA was isolated from subconfluent and 48-hour postconfluent RCECs, using extraction reagent (TRI; Molecular Research Center, Inc., Cincinnati, OH), and reverse transcribed with a commercial kit (Superscript II Transcriptase; Invitrogen-Gibco). Briefly, 10 μg total RNA was incubated in the presence of 10 mM dNTPs, 3 μg oligo dT primer, 15 mM DTT, and 1 μL RNAse inhibitor (RNAguard; Pharmacia-LKB), in first-strand buffer. Reverse transcriptase was added (2 μL of 200 U/μL), and incubation proceeded at 37°C for 90 minutes, at which time newly synthesized first-strand cDNAs were column purified (QIAquick nucleotide removal kit; Qiagen, Santa Clarita, CA) and used for PCR amplification of both the human α4 and α5 integrin subunit transcripts as well as the 18S ribosomal RNA. The DNA sequence of both the 5′ and 3′ template primers for both human α4 (5′ primer: nucleotides [nt] 2154-2174; 3′ primer: nt 2926-2907; 772-bp PCR product) and α5 (5′ primer: nt 3000-3023; 3′ primer: nt 3170-3147; 171-bp PCR product) were derived from their corresponding human genes (GenBank accession numbers XM039011 and X06256, respectively; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). The oligonucleotide primers used for the amplification of the 18S ribosomal RNA were provided in a kit (Quantum RNA 18S Internal Standards; Ambion Inc., Austin, TX). *Taq* polymerase (Pharmacia-LKB) was selected for PCR amplification. Cycle parameters were the same for all primers used (denaturation 94°C, 30 seconds; annealing 60°C, 30 seconds; extension 72°C, 30 seconds) with an identical number of cycles (26, 28, 30, 32, 34, and 36 cycles) for both sets of primers. The PCR-amplified DNAs were fractionated on a 10% polyacrylamide gel and their position revealed by ethidium bromide staining. The gel photograph was scanned with an image analyzer (Visage 110S Bioimage; Millipore), to quantify the alterations in the amounts of both the α5 and 18S PCR-amplified fragments at the various cell densities selected.

Northern blot analyses were conducted on total RNA isolated from both subconfluent and 48-hour postconfluent RCECs as detailed earlier. Total RNA was size-fractionated on a 1.2% formaldehyde-agarose gel and blotted onto a membrane (Hybond-N+; Amersham Canada, Oakville, Ontario, Canada), as described elsewhere. The membrane was then hybridized at 42°C in 50% (vol/vol) formamide 5× SSPE (1× SSPE is 0.15 M NaCl; 10 mM NaH_2PO_4; 1 mM EDTA [pH 7.4]), 5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone, 0.02% single-density gradient [Ficoll] and 0.02% bovine serum albumin), 0.1% SDS, and sheared denatured salmon sperm DNA at 0.3 mg/mL. Blots were washed once at 25°C in 2× SSC-0.1% SDS (1× SSC is 150 mM NaCl and 15 mM sodium citrate), and twice at 42°C in 0.2× SSC-0.1% SDS. The labeled probe used for hybridization consisted of an 800-bp *Eco*RI-*Eco*RI restriction fragment derived from the α4 cDNA.[49] Membranes were autoradiographed at −70°C for the indicated period.

SDS-PAGE and Western Blot

The protein concentration from postconfluent and subconfluent RCEC crude nuclear extracts was evaluated by the Bradford procedure and further validated after Coomassie blue staining of SDS-polyacrylamide fractionated nuclear proteins. Approximately 20 μg of proteins was added to 1 volume of sample buffer (6 M urea, 63 mM Tris [pH 6.8], 10% [vol/vol] glycerol, 1% SDS, 0.00125% [wt/vol] bromophenol blue, 300 mM β-mercaptoethanol) and then size fractionated on a 10% SDS-polyacrylamide minigel before being transferred onto a nitrocellulose filter. A full set of protein molecular mass markers (Invitrogen-Gibco) was also loaded as a control to evaluate protein sizes. The blot was then washed once in TS buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.4]) and four times (5 minutes each at 22°C) in TSM buffer (TS buffer plus 5% [wt/vol] fat-free dried milk and 0.1% Tween 20). Then, a 1:500 dilution of a rabbit polyclonal antibody raised against the transcription factors Sp1 or Sp3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or a 1:10,000 dilution of a mouse monoclonal antibody (C-2-10[50]) raised against the C-terminal end of the DNA binding domain of bovine poly(ADP-ribose) polymerase (PARP; kindly provided by Guy G. Poirier, Unit of Health and Environment, CHUL Research Center, Québec, Canada), was added to the membrane-containing TSM buffer and incubation proceeded for 2 hours at 22°C. The blot was then washed in TSM buffer and incubated an additional 1 hour at 22°C in a 1:1000 dilution of a peroxidase-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratory, West Grove, PA). The membrane was successively washed in TSM (four times, 5 minutes each) and TS (two times, 5 minutes each) buffers before immunoreactive complexes were revealed with Western blot chemiluminescence reagents (Renaissance; NEN Dupont, Boston, MA) and autoradiographed.

Results

Effect of Cell Density on the α5 Promoter Function

Although expression of the α5 integrin subunit has been reported in the human corneal epithelium,[16,51–53] such primary

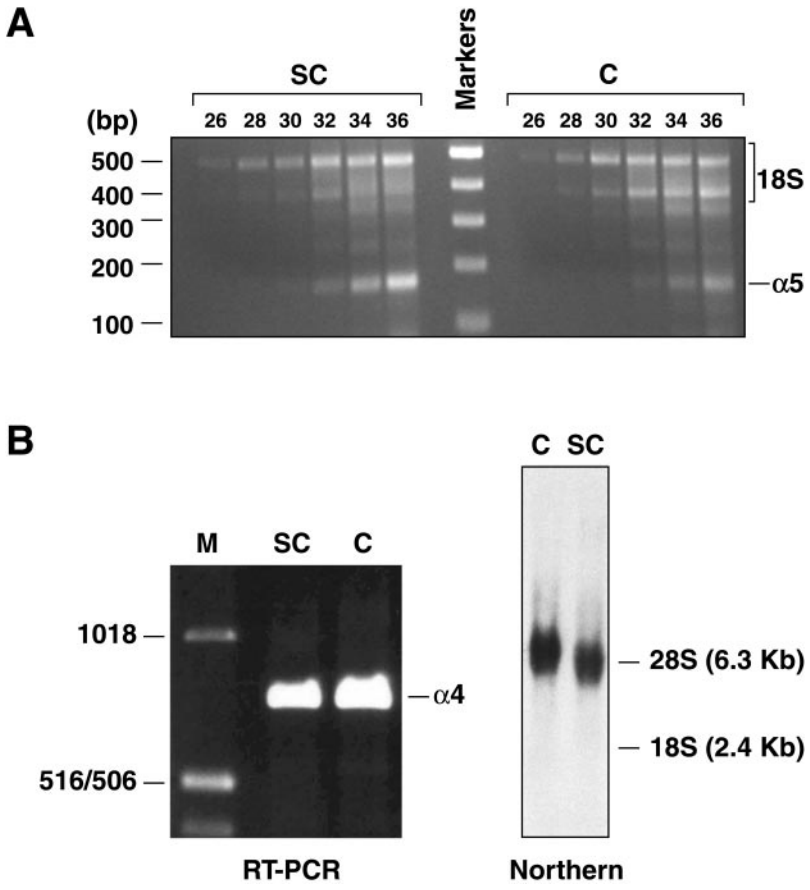

FIGURE 1. RT-PCR analysis of α5 mRNAs in postconfluent and subconfluent RCECs. (**A**) Total RNA from both postconfluent (C) and subconfluent (SC) RCECs were reverse transcribed and PCR coamplified, with α5 and 18S ribosomal RNA–specific primers. The positions of both the amplified 171-bp α5 and 489-bp 18S fragments are indicated, along with that of the most relevant markers (*left*). Results are shown for PCR amplification cycles 26, 28, 30, 32, 34, and 36. (**B**) Both RT-PCR and Northern blot analyses were conducted on the total RNA extracted in (**A**) to detect transcripts encoding integrin subunit-α4. The position of both the α4 772-bp RT-PCR amplification product and the α4 6.5-kb mRNA identified by Northern blot is indicated, along with that of appropriate markers (1018- and 516/506-bp fragments) and both the 18S and 28S ribosomal RNA (for the Northern blot).

cultured cells are inappropriate as a model for conducting detailed gene promoter studies, primarily because of the difficulties encountered in both culturing and transfecting these cells in vitro.[52,54] In contrast, RCECs are easy to maintain in culture without the need for feeder cells. Most of all, they can be transfected with high efficiency by a polycationic detergent used as a transfection reagent (Lipofectamine; Invitrogen-Gibco)–mediated gene transfer[31,55,56] which makes them an ideal cellular model to conduct gene promoter studies. As the proliferative state of a particular cell type changes with cell density, probably as a result of altered gene expression, we exploited semiquantitative RT-PCR analyses to investigate whether transcription of the α5 integrin subunit gene is under the influence of the cell density reached by primary cultured RCECs (Fig. 1). PCR amplifications were performed on reverse-transcribed total RNA obtained from both subconfluent and postconfluent RCECs. Coamplification of the 18S ribosomal RNA was also performed for normalization purposes. The specific α5 PCR product, which appeared as a single DNA fragment of the expected size (171 bp) under all culture conditions (subconfluent and postconfluent RCECs) was detectable on the gel after 30 cycles of amplification and remained linear up to 34 cycles (Fig. 1A). However, a significant reduction was observed for the corresponding PCR amplification cycles in postconfluent cells. Normalization of the α5 signal to that of the 18S rRNA, which appear as a single band of the appropriate size (489 bp) in subconfluent RCECs but as a doublet in postconfluent cells (probably as a result of alternative splicing events occurring at high cell density), provided evidence that the amount of α5 transcript is on average 4.6 ± 0.3 times lower in postconfluent RCECs than in subconfluent cells (as revealed through densitometric analyses of the amplified PCR products). As a control, we also examined whether the expression of a class II gene, the transcription of which is not regulated by Sp1, remains unaltered by the state of cell density. For this purpose, we selected the gene encoding the human α4 integrin subunit, the expression of which has been found only in a restricted number of tissues, including the cornea,[16] both lymphoid and myeloid cells,[57] and differentiating skeletal muscle.[58] We have reported the binding of transcription factors to the α4 basal promoter,[56] of which none belong to the Sp1 family. Besides, no GC-rich Sp1 target sites are found in the sequence of the α4 promoter.[49] The selection of this gene also relies on the fact that cotransfection of the α4 promoter along with either the Sp1 or Sp3 expression plasmids (pPACSp1 and pPACSp3) in Sp1-deficient *Drosophila* Schneider cells had no influence at all on the transcriptional activity directed by the α4 promoter (data not shown). As revealed on Figure 1B (left), PCR amplification yielded the expected 772-bp α4 fragment. However, no alteration was observed in the amount of α4 amplification product between reverse-transcribed mRNAs prepared from postconfluent and subconfluent cells on normalization to the 28S ribosomal RNA. We next isolated total RNA from either postconfluent or subconfluent RCECs and examined the α4 transcript by Northern blot analysis. After only a few hours of exposure, a single mRNA species of approximately 6.5 kb corresponding to the previously reported major α4 transcript[49] was observed just above the 28S rRNA (Fig. 1B, right). As expected, expression of the α4 transcript was not altered when RNA was isolated from postconfluent RCECs. We therefore conclude that the relative concentration of the α5 mRNA, but not that of the α4 transcript, is considerably reduced when RCECs reach confluence.

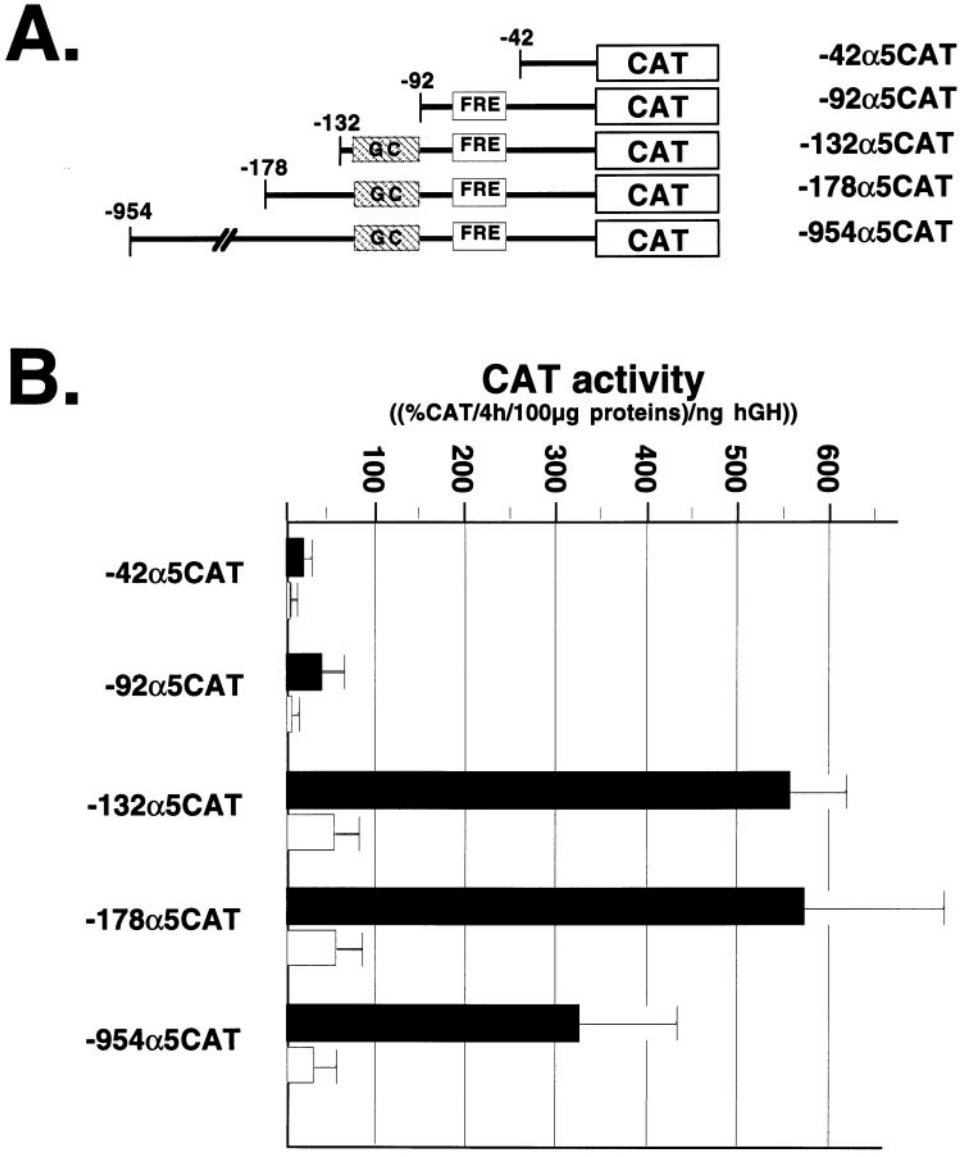

FIGURE 2. Transient transfection analysis in RCECs. (**A**) Schematic representation of the plasmids used. Both the FRE and the GC-rich area from the α5 promoter are shown. (**B**) The plasmids shown in (**A**) were transiently transfected into primary cultured RCECs plated at either subconfluence (■) or confluence (□). Cells were harvested and CAT activities determined and normalized to secreted hGH. Data are expressed as %CAT activity per 4 hours per 100 μg protein per nanogram hGH. Error bars, SD.

To determine whether the cell-density–dependent alterations observed in the amount of α5 mRNA also translate into similar alterations in the activity directed by the α5 gene promoter, transfection analyses were conducted in RCECs. Because the upstream regulatory sequences from the rabbit α5 integrin subunit gene have not been cloned yet, we selected the human α5 gene to conduct the following experiments, because recombinant constructs bearing the CAT reporter gene under the control of various lengths from the human α5 gene promoter were already available.[37] These constructs were therefore transiently transfected into either postconfluent or subconfluent RCECs (Fig. 2). Consistent with a previously published study,[37] most of the basal α5 promoter activity was found to be determined by the α5 sequences extending up to position −132 (in plasmid −132α5CAT), the shorter α5 promoter-bearing construct (in this case, −42α5CAT) being 22 times less active than the former when RCECs were cultured to subconfluence (Fig. 2B). Extending further up to α5 position −178 or −954 did not increase further but rather resulted in a weak reduction (with the −954α5CAT plasmid) in α5 promoter activity. Consistent with the near fivefold reduction in the amount of α5 transcript observed when RCECs reach confluence, transfection of postconfluent RCECs with the α5/CAT plasmids also resulted in a 9- to 13-fold reduction in the α5 promoter activity when compared with the activity measured in subconfluent cells (Fig. 2B). We therefore conclude that the transcriptional activity directed by the promoter of the α5 gene is dramatically affected by the extent of cell density reached by RCECs in vitro.

Binding of Nuclear Proteins to the α5 Promoter

To determine whether the changes in the activity directed by the α5 promoter result from altered binding of nuclear regulatory proteins to the α5 regulatory elements in postconfluent versus subconfluent RCECs, differential EMSA experiments were conducted. For this purpose, we focused our attention on the −92 to −132 DNA segment from the α5 promoter, because it was shown to be required to reach maximum α5 promoter activity after transfection into RCECs (Fig. 2B). In the present study, deletion of this region from the α5 promoter yielded a drastic 18- and 13-fold reduction of CAT gene activity after transfection of either postconfluent or subconfluent RCECs, respectively (Fig. 2B). We therefore synthesized a dou-

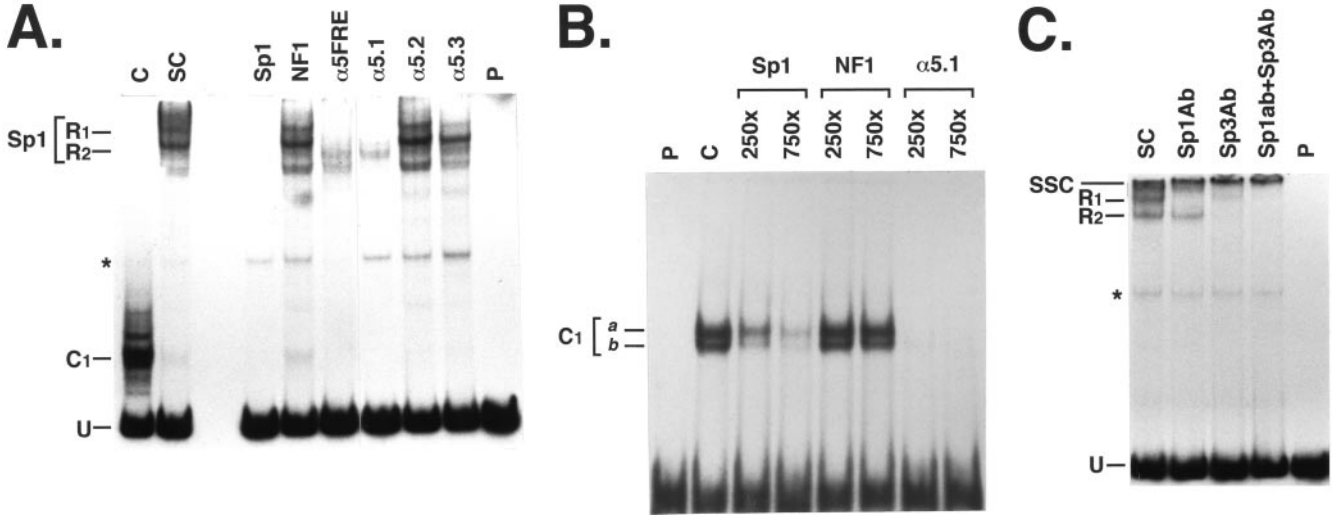

FIGURE 3. EMSA analysis of the nuclear proteins from RCECs binding to the α5 basal promoter. (**A**) The α5.1 oligonucleotide was 5′ end labeled and incubated with nuclear proteins (15 μg) obtained from either postconfluent (C) or subconfluent (SC) RCECs. Formation of DNA–protein complexes was then monitored by EMSA on a 8% native polyacrylamide gel. The position of two DNA–protein complexes (denoted R1 and R2) is shown along with that of a fast-migrating complex (C1) that appeared only with the extract from postconfluent RCECs. The α5.1 labeled probe was also incubated with 15 μg proteins from subconfluent RCECs in the presence of a 150-fold molar excess of various double-stranded oligonucleotides (Sp1, NF1, α5FRE, α5.1, α5.2, and α5.3) used as unlabeled competitors. Formation of both the R1 and R2 complexes was then monitored. A nonspecific complex often observed in the extract from subconfluent RCECs is also indicated (✶). U, unbound fraction of the labeled probe; P, labeled probe alone. (**B**) The α5.1-labeled probe was incubated with 15 μg nuclear proteins from postconfluent RCECs in the presence of increasing concentrations (250- and 750-fold molar excesses) of various unlabeled oligonucleotides (Sp1, NF1, and α5.1) as competitors. Formation of C1, which is made up of two closely migrating complexes (C1a and C1b) was then monitored by EMSA on a native 5% polyacrylamide gel. C, labeled probe incubated with proteins in the absence of unlabeled competitors. (**C**) Approximately 15 μg protein from subconfluent RCECs was incubated with the α5.1 labeled probe in the presence of either no (SC) or 1 μL of a polyclonal antibody directed against either Sp1 (Sp1Ab) or Sp3 (Sp3Ab), or both (Sp1Ab+Sp3Ab). Formation of both the R1 and R2 Sp1/Sp3 complexes, as well as that of supershifted complexes (SSC) was then monitored by EMSA.

ble-stranded oligonucleotide bearing the GC-rich −132 to −92 region from the α5 promoter (designated α5.1) and used it as a labeled probe in EMSAs. Incubation of the α5.1-labeled probe with nuclear proteins from RCECs revealed the formation of two, low electrophoretic mobility DNA–protein complexes (denoted R1 and R2) when nuclear proteins from subconfluent, but not from postconfluent, cells were used in the assay (Fig. 3A, left). In contrast, a fast-migrating complex (designated C1) was yielded by the extract from postconfluent, but not by that from subconfluent, RCECs. Considering the high content of GC residues in the −132/−92 α5 promoter and the fact that this sequence bears multiple putative binding sites for the transcription factors that belong to the Sp1 family, competition experiments in EMSAs were then performed. As shown on Figure 3A (right), both unlabeled α5.1 and an oligonucleotide bearing the high-affinity target site for Sp1 could compete for the formation of the R1 and R2 complexes. As expected, the high-affinity target site for the unrelated NF1 protein did not compete for the formation of both of these complexes.

Recently, we reported the binding of Sp1 to a GA-rich target site that is part of a regulatory element from the α5 promoter located between positions −82 and −56 (and designated FRE) that confer responsiveness to Fn.[31] As with both the Sp1 and α5.1 oligonucleotides, an oligomer bearing the sequence from the α5 FRE competed for the formation of both R1 and R2 (Fig. 3A, right). The use of shorter derivatives of the α5.1 oligonucleotide that extend from position −143 to −106 (α5.2) and −105 to −86 (α5.3) did not alter significantly the formation of both the R1 and R2 complexes, therefore suggesting that the proteins yielding R1 and R2 probably recognize a target site that overlaps the −106/−105 junction of these oligonucleotides. A similar competition experiment was conducted on the C1 complex detected when crude nuclear proteins from postconfluent RCECs were used in the EMSA. To improve separation of the complexes that constitute the C1 complex, the concentration of the native polyacrylamide gel was reduced to 5%. As shown on Figure 3B, C1 is made up of two closely migrating DNA–protein complexes that we designated C1a and C1b. Formation of both complexes was abolished by the unlabeled Sp1 and α5.1 oligonucleotides but not by the unrelated NF1 oligomer, suggesting that the proteins yielding C1a and C1b must be closely related to Sp1.

As further evidence that members of the Sp1 family bind the −132/−92 sequence of the α5 promoter, supershift experiments in EMSA were conducted. As shown in Figure 3C, formation of the R1 complex, but not that of R2, was totally prevented by the addition of an antibody directed against Sp1. A supershifted complex with very low electrophoretic mobility (SSC) also appeared on the gel on incubation with the Sp1 Ab. That the supershifted complex is located at the top of the gel is not uncommon[31] and results from the shorter time of migration that is necessary to visualize properly the position of the free probe. Similarly, formation of R2 (and to some extent that of R1) was efficiently prevented by the addition of an antibody directed against Sp3. Taken together, these results suggest that complex R1 contains both Sp1 and Sp3 and that complex R2 results solely from the recognition of the α5.1-labeled probe by Sp3. Indeed, the addition of both the Sp1 and Sp3 Abs to the reaction mix totally prevented formation of both complexes R1 and R2, as expected (Fig. 3C). That both Sp1 and Sp3 yield DNA–protein complexes that comigrate in EMSA is not unique and has been reported in many studies.[59–61]

The results presented in Figure 3A suggest that dramatic alterations in either the concentration or the DNA binding affinity of both Sp1 and Sp3 for their −132/−92 target site

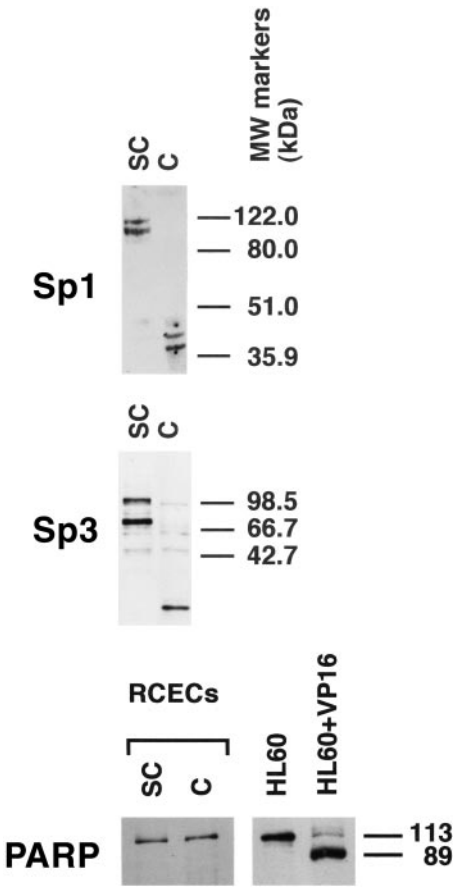

FIGURE 4. Western blot analysis of Sp1/Sp3 in nuclear extracts from postconfluent and subconfluent RCECs. Approximately 30 μg crude nuclear extracts from both postconfluent (C) and subconfluent (SC) RCECs were examined in Western blot analyses by using the Sp1 and Sp3 antisera. The positions of the nearest molecular mass markers (β-gal; molecular mass 122 kDa); phosphorylase B (98.5 kDa); bovine serum albumin (80.0 kDa, Invitrogen-Gibco; 66.7 kDa, BioRad); ovalbumin (51 kDa, Invitrogen-Gibco; 42.7 kDa, BioRad); and carbonic anhydrase (35.9 kDa) are provided. The nuclear extracts (15 μg each) from both postconfluent (C) and subconfluent (SC) RCECs were also blotted with the anti-PARP C-2-10 mAb. Crude nuclear proteins prepared from either untreated or VP16-treated HL60 cells were included as the control for the expression of the mature 113-kDa PARP protein or its 89-kDa apoptotic cleavage derivative, respectively.

occur in RCECs grown to different cell densities. To determine which of these two hypotheses account for the alterations in the formation of both Sp1/Sp3 complexes R1 and R2, Western blot analyses were conducted on the nuclear extracts used in Figure 3A. As revealed in Figure 4, the pattern of Sp1/Sp3 protein expressed by both subconfluent and postconfluent RCECs perfectly matched that observed in the EMSA, the maximum Sp1/Sp3 expression being observed at subconfluence, whereas both proteins totally disappeared when cells were maintained at postconfluence for 48 hours. Much shorter protein derivatives that were recognized by both the Sp1 and Sp3 mAbs were observed in the same extracts that also support formation of the C1 complex (estimated molecular mass of 40 to 45 kDa with the Sp1 mAb and under 40 kDa with the Sp3 mAb). It is worth pointing out that the Sp1 mAb recognized a doublet of closely migrating proteins in the crude extract from 48-hour postconfluent RCECs that closely resemble that yielded by the C1a and C1b complexes in EMSA (Fig. 3B). To eliminate the possibility that extinction of Sp1/Sp3 may have resulted from their proteolytic cleavage by apoptotic caspases as a consequence of the postconfluent RCECs' progressing into apoptosis, blotted proteins were probed with a monoclonal antibody (C-2-10) raised against the bovine PARP protein[50] whose cleavage by caspase 3 is well recognized as an early marker of apoptosis.[62] The epitope recognized by the C-2-10 antibody is localized near the C-terminal end of the PARP DNA binding domain, a region shown to be totally conserved between murine, bovine, and human PARP.[50] Cleavage of the 113-kDa mature PARP by caspase-3 yields two degradation products with molecular masses of 89 and 24 kDa, of which the larger, which bear the intact epitope, can be recognized efficiently by the C-2-10 mAb.[63] As shown on Figure 4, a single clear signal corresponding to the 113-kDa mature PARP protein was observed with the protein preparation from both subconfluent and 48-hour postconfluent RCECs. Crude nuclear proteins from HL60 cells exposed or not to the cell death inducer VP16 (and kindly provided by Guy G. Poirier) were also included as the control. The 89-kDa PARP caspase-3 cleavage product, which was easily detectable in the extract from apoptotic VP16-treated HL60 cells, was not detectable in postconfluent RCECs (as well as in subconfluent cells), clear evidence that postconfluent cells are not committed to apoptosis.

As a next step, we wanted to determine whether disappearance of both Sp1 and Sp3 at postconfluence indeed results from their proteolytic degradation through the action of proteases expressed in postconfluent RCECs but missing in subconfluent cells. Crude nuclear proteins prepared from pituitary GH4C1 cells, which express high levels of Sp1/Sp3, were used as a source of exogenous Sp1/Sp3 proteins in these experiments. A constant amount from the GH4C1 extract (5 μg) was then incubated with increasing concentrations (1–10 μg) of nuclear proteins obtained either from postconfluent (C) or subconfluent (SC) RCECs and incubated for 90 minutes at 37°C before the addition of the Sp1-labeled probe and further analysis through EMSA. As shown in Figure 5A, incubation in the presence of the extract from subconfluent RCECs had no influence on the formation of the Sp1/Sp3 complexes normally yielded by the GH4C1 extract. In contrast, the addition of increasing amounts of extracts from confluent RCECs resulted in a complete disappearance of both transcription factors. The formation of intermediate, fast-migrating complexes that are thought to result from the proteolytic degradation of these proteins by unknown proteases (as their formation is inhibited by the unlabeled Sp1 oligonucleotide; data not shown) was also observed when the extract from postconfluent RCECs was added (Fig. 5A). A similar experiment was repeated by varying the time of incubation, rather than the protein concentration, during the assay. The Sp1/Sp3-containing extract from GH4C1 cells (5 μg) was therefore incubated with a constant amount of nuclear proteins (5 μg) from both subconfluent and postconfluent RCECs and incubated for various periods of time (0–120 minutes) at 37°C before the addition of the Sp1-labeled probe and further analysis through EMSA. As shown on Figure 5B, an incubation of 120 minutes at 37°C was necessary to obtain a substantial reduction (but no total disappearance) in the formation of the Sp1/Sp3 complexes in EMSA. Only 30 minutes were required, however, when the extract from confluent cells was used, with no detectable Sp1/Sp3 signals present after 60 minutes of incubation. We therefore conclude that the lack of formation for both complexes R1 and R2 when nuclear extracts from postconfluent RCECs are used in EMSA can be accounted for by the corresponding lack of expression of both Sp1 and Sp3 in these cells and not by alterations in the affinity of these proteins for their target sequences. Disappearance of Sp1 and Sp3 at a high cell density appears to be the consequence of their proteolytic degradation by an unknown pro-

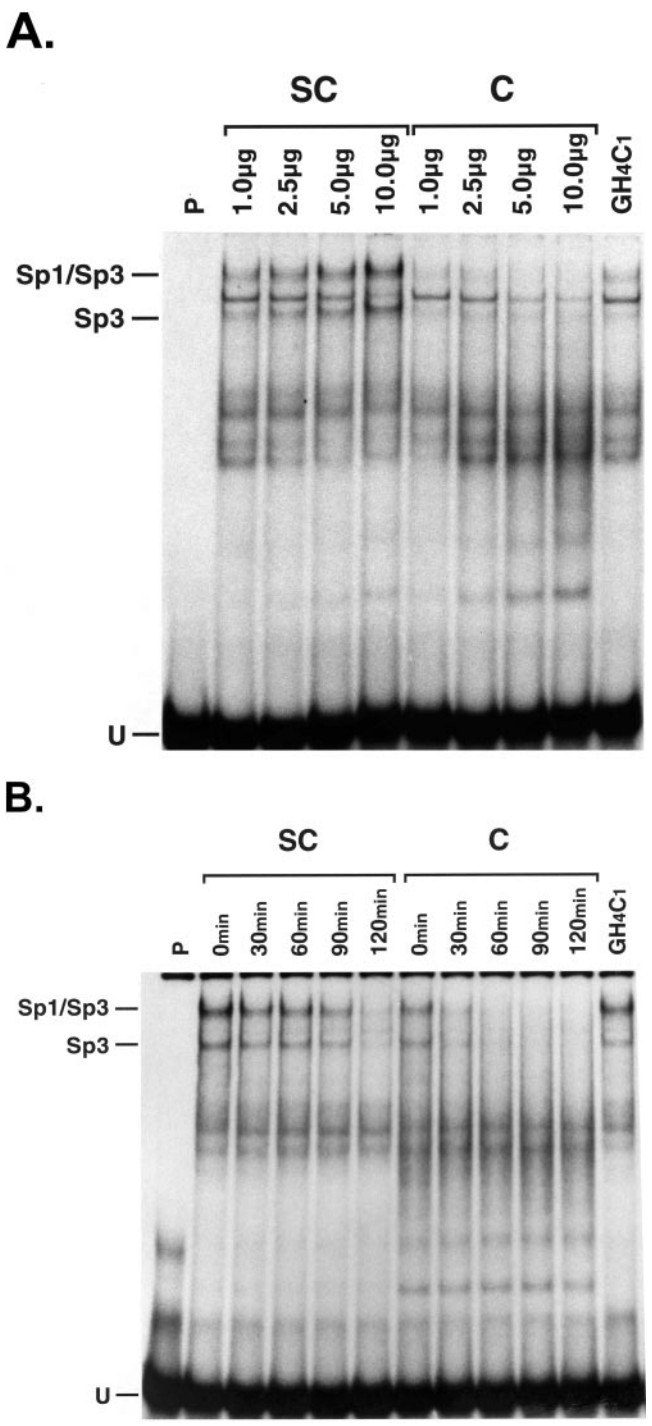

FIGURE 5. Degradation of Sp1/Sp3 by a protease activity present in postconfluent RCECs. (**A**) Crude nuclear proteins (5 μg) from pituitary GH4C1 cells were added increasing amounts (1–10 μg) of nuclear proteins obtained either from postconfluent (C) or subconfluent (SC) RCECs and incubated in buffer D for 90 minutes at 37°C before the addition of a 5′ end–labeled oligonucleotide bearing the high-affinity binding site for Sp1. Formation of the Sp1 and Sp3 DNA–protein complexes was then examined by EMSA, as in Figure 3. (**B**) Approximately 5 μg from the GH4C1 Sp1/Sp3-containing extract was incubated with 5 μg nuclear proteins from both subconfluent and postconfluent RCECs and incubated for various times (0–120 minutes) at 37°C before the addition of the Sp1-labeled probe. Formation of the Sp1 and Sp3 DNA–protein complexes was then examined by EMSA, as in (**A**). U, unbound fraction of the labeled probe; P, labeled probe alone.

tease that is highly active (or expressed) only in postconfluent RCECs. Such degradation of Sp1/Sp3 is a progressive event, because cleavage intermediates are clearly detectable when crude nuclear protein preparations obtained from RCECs cultured at 100% confluence for 0 and 1 days are used as the source of protein for the Western blot analysis (data not shown).

Regulatory Influence of Sp1/Sp3 on the α5 Promoter

To determine the precise position of the Sp1 target sites within the −82/−56 α5 FRE and the −92/−132 α5 promoter segment, DNaseI footprinting experiments were conducted with a labeled probe that covers the entire basal promoter area. As shown in Figure 6A (and also summarized in Fig. 6B), purified Sp1 protected a region from the α5 FRE located between positions −50 and −71, which we designated the proximal Sp1 site (Sp1p). In addition, a weaker, partial protection was observed between positions −101 and −117. This other site, which we designated the distal Sp1 site (Sp1d), overlaps the junction between the α5.2 and α5.3 oligonucleotides (Fig. 6B) that have been used as unlabeled competitors in the EMSA experiment shown in Figure 3A, which also explains why neither could compete efficiently for the formation of the R1 and R2 Sp1/Sp3 DNA–protein complexes. Both the proximal and distal Sp1 sites from the recombinant plasmid −132α5CAT were mutated either individually or in combination, using site-directed mutagenesis to assess precisely the regulatory influence of each site on the transcriptional activity directed by the α5 promoter. The wild-type −132α5CAT plasmid as well as its mutated derivatives were transfected into either subconfluent or 48-hour postconfluent RCECs. At subconfluence, mutation of the proximal Sp1 site (in plasmid −132α5Mp) resulted in a fivefold reduction in the activity normally directed by the α5 promoter, whereas the distal Sp1 site had little influence on the activity directed by the −132α5CAT when mutated alone (in plasmid −132α5Md; Fig. 6C). However, mutating both the proximal and the distal sites together (in plasmid −132α5Md+p) resulted in a reduction in α5 promoter function nearly twice that seen with the −132α5Mp construct (eightfold reduction) therefore providing evidence that the distal Sp1 site indeed contributes, although to a lesser level than the proximal site, to basal α5 promoter function. When transfected in 48-hour postconfluent RCECs, mutation of the proximal Sp1 site yielded only a moderate (2.6-fold) reduction in the α5 promoter activity and unlike in subconfluent cells, no further reduction occurred when the distal Sp1 site was also mutated (2.5-fold reduction at postconfluence versus eightfold reduction in subconfluent RCECs). We therefore conclude that two distinct Sp1 target sites from the α5 promoter, a proximal and a distal one, are bound in vitro by Sp1 and that the positive regulatory influence they mediate is under the influence of the cell density reached by primary cultured RCECs.

To evaluate the respective contribution of both Sp1 and Sp3 on the activity directed by the α5 promoter, cotransfection experiments were conducted into *Drosophila* SL2 Schneider cells. These cells are known to be deficient in producing these transcription factors, which make them an ideal system for studying gene expression or transcription factors function (for a review, see Ref. 64). The plasmids −132α5CAT, −92α5CAT, and −42α5CAT were cotransfected into Schneider cells, either alone or with recombinant plasmids (pPacSp1 and pPacSp3) containing either the Sp1 or Sp3 cDNA under the control of the *Drosophila* actin gene promoter and therefore ensuring high levels of both these proteins in Schneider cells. As shown in Figure 7, none of these three recombinant plasmids could

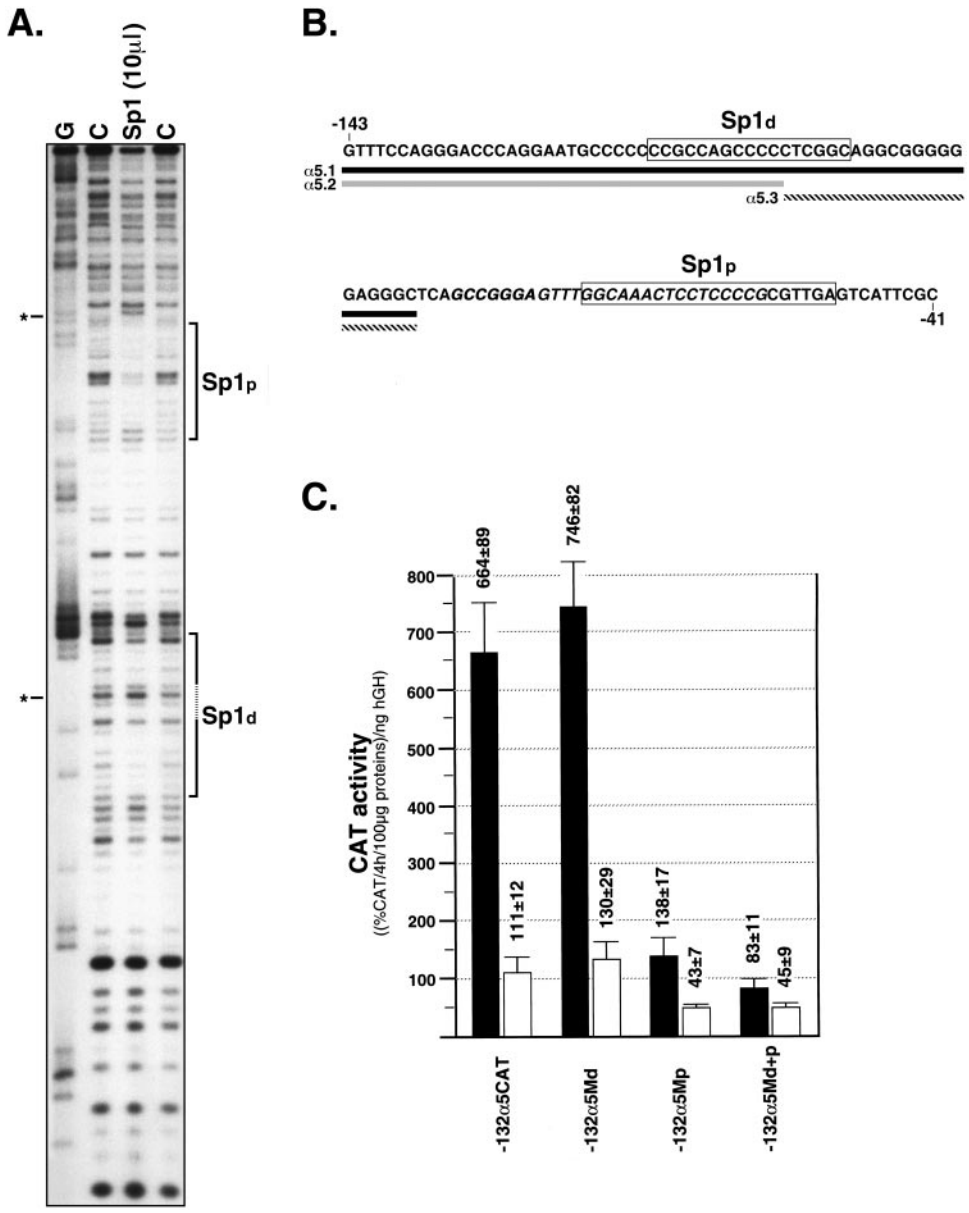

FIGURE 6. DNaseI footprinting and site-directed mutagenesis of the Sp1 sites from the α5 promoter (**A**) A 165-bp *Bam*HI/*Xbo*I DNA-labeled probe spanning the entire α5 promoter sequence was incubated with 10 μL of a purified preparation of recombinant Sp1 before being digested with DNaseI. The position of two Sp1 sites (a proximal site, Sp1p, and a distal site, Sp1d) protected from digestion by DNaseI is shown along with that of two DNaseI hypersensitive sites (✶). G, Maxam and Gilbert G sequencing ladder; C, labeled DNA digested by DNaseI in the absence of proteins. (**B**) Schematic representation of both the proximal (Sp1p) and distal (Sp1d) Sp1 target sites (*boxes*) identified by DNaseI footprinting along the sequence of the α5 gene promoter. The nucleotide sequence corresponding to the α5.1, α5.2, and α5.3 oligonucleotides used for the competition experiments in EMSA are also depicted, along with the position of the previously described FRE (nucleotides in *italic*). (**C**) The recombinant plasmid −132α5CAT, or derivatives bearing mutations into either the proximal (−132α5Mp) or the distal (−132α5Md) Sp1 sites, or both (−132α5Mp+d) were transiently transfected into both subconfluent (■) and 48-hour postconfluent (□) RCECs. Cells were harvested 48 hours after transfection and CAT activities were determined, normalized, and expressed as detailed in Figure 2.

determine high basal promoter activity when transfected alone in Schneider cells. Cotransfection of either the Sp1 or Sp3 expression plasmids (or the combination of both) did not have any influence at all on the CAT activity directed by the −42α5CAT plasmid, providing evidence that the 42 bp upstream from the α5 mRNA start site are insufficient to direct transcription in vitro (Fig. 7). Extending further the α5 promoter up to position −92 yielded 20- and 6.5-fold increases in CAT activity on cotransfection with the Sp1 and Sp3 expression plasmids, respectively. Cotransfection of pPacSp1 with the −132α5CAT recombinant plasmid increased CAT activity by fivefold over the level directed by the plasmid −92α5CAT (Fig. 7). These results provide evidence that the −132/−92 α5 promoter segment contribute to the Sp1-mediated positive regulatory influence directed by this promoter in vitro, although to a lesser level than that directed by the −42/−92 segment (which yielded a near 20-fold increase with Sp1 by comparison to the fivefold increase mediated by the −92/−132 segment). The findings are also consistent with the results obtained after transfection of both the wild-type and mutated constructs into RCECs (Figs. 2B, 6C). Cotransfection of the −132α5CAT with pPacSp3 did not yield promoter activation significantly greater than that obtained with the −92α5CAT plasmid (8- and 6.5-fold activation, respectively). Unlike results reported in other studies,[65–67] no synergistic nor antagonistic influence resulted from the cotransfection of both pPacSp1 and pPacSp3 along with any of these α5CAT recombinant plasmids (Fig. 7). We therefore conclude that Sp1 exerts its synergistic positive influence on the activity directed by the α5 promoter through the recognition of both its proximal and distal target sites. In contrast, Sp3 appears to exert its 6.5- to 8-fold positive influence through the recognition of a single regulatory element located on the 50-bp −42/−92 DNA segment (most likely through the proximal site also recognized by Sp1).

Because postconfluent RCECs do not express Sp1/Sp3 proteins with the appropriate molecular mass, we examined whether restoring expression of Sp1 into Sp1-deficient postconfluent RCECs would alter the activity normally directed by the α5 promoter. Both the −92α5CAT and −132α5CAT plas-

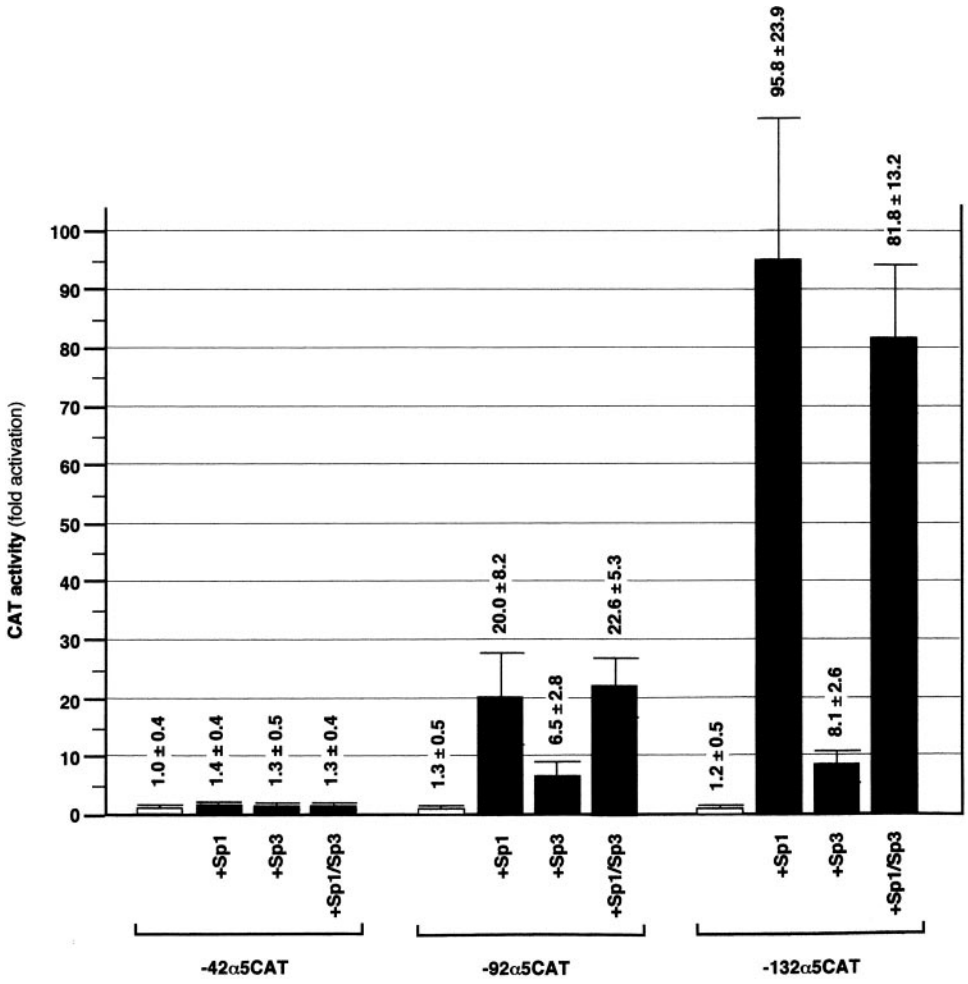

FIGURE 7. Transient transfection in Sp1/Sp3-deficient SL2 *Drosophila* Schneider cells. The recombinant plasmids −132α5CAT, −92α5CAT, and −42α5CAT were transfected either alone (□) or in combination with the Sp1 (+Sp1) or Sp3 (+Sp3) expression plasmids pPacSp1 and pPacSp3, respectively, or both (+Sp1/Sp3; ■), into SL2 *Drosophila* Schneider cells. Cells were harvested 48 hours later and CAT activity (expressed as the change in activity relative to the level directed by the −42α5CAT promoter construct alone was determined and normalized. Standard deviation is also provided.

mids were transfected in subconfluent and 48-hour postconfluent RCECs in either the absence or presence of the Sp1 expression plasmid pSiSp1 (which was selected over pPacSp1 essentially because it bears the Sp1 cDNA fused to the simian virus [SV]40 promoter, which allows expression of Sp1 in mammalian cells). As shown in Figure 8, cotransfection of pSiSp1 had no influence on the CAT activity directed by either α5CAT plasmids in subconfluent RCECs. This result was anticipated, because subconfluent RCECs already express substantial amounts of endogenous Sp1, therefore suggesting that both α5 promoter constructs are already under the positive influence of Sp1, and no further increase in activity can be reached, despite the forced expression of Sp1 encoded by pSiSp1. However, performing the same experiment in Sp1-deficient postconfluent RCECs resulted in a modest but statistically significant 2.3- and 2.6-fold increase in the promoter activity directed by the plasmids −92α5CAT and −132α5CAT, respectively.

DISCUSSION

Although expression of the α5 integrin subunit has been unquestionably recognized in the corneal epithelium, the way by which its gene is regulated during the process of corneal wound healing remains obscure. The present study was undertaken to shed light on the variations that may exist in the level of expression of this gene during corneal wounding by exploiting both postconfluent and subconfluent cultures of RCECs as a model to study α5 gene promoter function in vitro. The

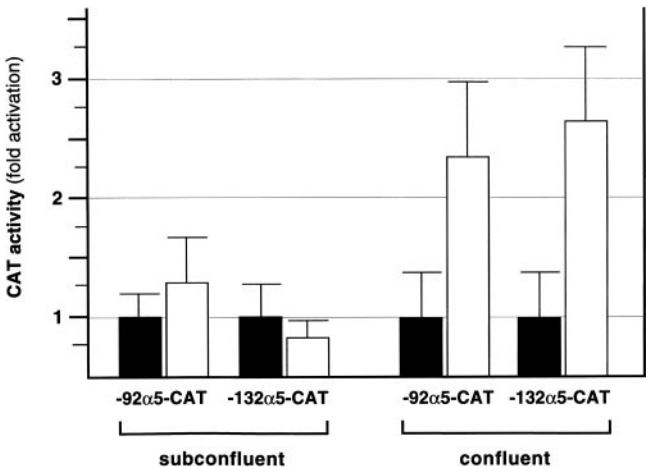

FIGURE 8. Transfection in postconfluent and subconfluent RCECs that transiently express high levels of Sp1. The recombinant plasmids −132α5CAT and −92α5CAT were transfected either alone (■) or in combination with the Sp1 expression plasmid pPacSp1 (□) into both postconfluent and subconfluent RCECs. Cells were harvested and CAT activity ± SD (expressed as the change in activity relative to the level directed by the α5CAT promoter construct alone (■) was determined and normalized.

results shown in the present study demonstrated clearly that efficient transcription of the α5 gene, but not that of the α4 gene (the expression of which is apparently not under the influence of Sp1/Sp3), is dependent on the recognition of two target sites (a proximal and a distal site) from the α5 basal promoter by transcription factors that belong to the Sp1 family. We demonstrated that the activity directed by the α5 promoter is substantially abrogated when RCECs reach confluence (as revealed by both RT-PCR analyses and transfection of the CAT/α5 promoter recombinant constructs). The strong reduction in the activity of the α5 promoter when RCECs reach a high cell density appears to be the consequence of a reduced expression of Sp1/Sp3 that apparently results from their proteolytic cleavage by yet unknown proteases.

Considering the ubiquitous nature of the expression of the α5 integrin subunit, it is no surprise that transcription of its gene would turn out to be dependent also on the positive influence of ubiquitously expressed transcription factors. The results presented herein demonstrate clearly the critical function played by Sp1, and to some extent also Sp3, in the activity directed by the α5 promoter. We directed our attention primarily toward Sp1 and Sp3, and not Sp2, Sp4, and Sp5 for the following reasons: (1) Sp4 was expressed at only very low levels in primary cultures of both human[68] and rabbit (data not shown) corneal epithelial cells, as revealed by Western blot analyses, and was not shown to bind GC-rich target sites in supershift experiments; (2) Sp2 was not reported to recognize the classic GC box that is bound by the remaining members of this family, but rather targets GT-rich elements; and (3) Sp5 is tissue-specifically expressed only during early development.[36,69] Besides, Sp5 bears only one Sp1-like zinc finger and apart from this Zn finger, it has little similarity to any other member of the Sp1 family. Results from *Drosophila* cell transfections provided evidence that Sp3 must exert its regulatory influence through the recognition of a target site located on the −42/−92 α5 promoter segment because extending further the length of the α5 promoter to position −132 did not increase further its positive influence. Sp1, however, appeared to act synergistically on the activity directed by the α5 promoter, because extending the α5 basal promoter, which already bears a strong Sp1 site, from position −92 to position −132 resulted in a further increase in the α5 promoter's strength. Synergistically acting Sp1 target sites are not uncommon, because they have been identified in the promoter of many genes[29,30,70] including the gene for PARP.[71] We recently provided evidence that Sp1 interacts with a GA-rich target site from the α5 promoter, located between positions −82 and −56 (the FRE element), which also confers responsiveness to the α5β1 ligand Fn.[31] This positive regulatory influence of Sp1 on the α5 promoter was shown to be mediated by its interaction with two target sites, a proximal site located from positions −50 to −71, and a distal site, located from positions −101 to −117. Although the proximal Sp1 site was clearly the most important, both the proximal and distal sites are necessary to reach maximum activation in *Drosophila* SL2 cells. That mutating the distal Sp1 site had little influence on the α5 promoter activity (Fig. 6) whereas deletion of the −132 to −92 α5 promoter segment resulted in a dramatic reduction in the basal promoter activity (Fig. 2) can be explained by the presence of target sites for transcription factors other than Sp1 that substantially contribute to the influence of the −92/−132 upstream sequence in primary cultured RCECs. Although target sites for nuclear transcription factors other than Sp1 have been identified in the α5 promoter downstream of position −92, including sites for both AP-1 and C/EBP,[72] no such protein has been reported to bind the −92/−132 α5 promoter segment.

Sp3 also binds GC- or GA-rich target sites and is therefore likely to bind the GA-rich Sp1 binding site (Sp1p) identified in the α5 FRE. The lack of synergy between Sp1 and Sp3 when both expression plasmids are transfected in Schneider cells support this hypothesis. Both must therefore compete with one another for binding their respective target site. The fact that the transactivation properties of Sp1 remained unaltered, even in the presence of Sp3, also suggests that binding of Sp1 predominates over that of Sp3 for the FRE GA-rich target site.

It is noteworthy that 48-hour postconfluent RCECs, unlike subconfluent cells, lose expression of the typical Sp1 and Sp3 proteins. This is somehow consistent with the results published by Chen et al.,[73] who reported a substantial reduction, but no complete disappearance, of Sp1 in 5-day postconfluent RCECs. A cell-density–mediated upregulation in the amount of Sp1 has also been reported to occur in primary cultures of human corneal stromal cells.[74] However, the immunohistochemical staining conducted by these investigators clearly indicated that the cells plated at the highest cell density selected (1.25×10^6 cells/cm^2 for 18 hours) barely reached 75% confluence, a condition we consider to be subconfluent and under which Sp1 is also highly expressed.

The disappearance of full-length Sp1 and Sp3 in 48-hour postconfluent RCECs combined with the presence of much shorter protein versions that are immunologically recognized by both the Sp1 and Sp3 mAbs and the fact that the nuclear extract from postconfluent RCECs possess the ability to degrade Sp1 from a foreign source (for instance that from GH4C1 cells) points toward the possibility that both proteins are subjected to proteolysis when RCECs reaches a high cell density. This hypothesis is supported by the fact that unlike the DNA-binding activity of Sp1, which has been reported to drop considerably during the course of corneal epithelial cell differentiation, no corresponding alteration in the steady state level of Sp1 mRNA occurs.[73] Cleavage of Sp1 by caspases has recently been shown to occur as a consequence of cells involved in the process of apoptosis.[75,76] Indeed, caspase 3, and the caspase 3-activated downstream caspases 6 and 7, have been shown to cleave Sp1 into three distinctive products of 68, 45, and 22 kDa. It is interesting to point out that the smaller Sp1 cleavage product cannot be detected immunologically through Western blot, because it lacks the epitope recognized by the Sp1 antibody, and has been postulated to function as a dominant negative Sp1 derivative, since it may still bind DNA but no longer possesses the ability to activate transcription.[75] However, we clearly demonstrated that postconfluent RCECs were not committed to apoptosis, because no cleavage of mature PARP by caspase 3, which is considered an early event of apoptosis, occurs in postconfluent RCECs. Besides, although the size of the Sp1 cleavage product detected in postconfluent RCECs (40–45 kDa) may match that of the Sp1 apoptotic fragment of 45 kDa, no fragment with a molecular mass of 68 kDa has ever been observed in RCECs under these or any other culture conditions. We therefore postulate that once RCECs reach postconfluence, they either activate or start producing one protease (or a few) for which one of the immediate downstream target proteins is Sp1. Inactivating Sp1 and Sp3 through their degradation by one or a few proteases that would either be activated or expressed once the cells reach confluence would also explain why we could not fully restore levels of CAT activity similar to that observed in subconfluent RCECs when postconfluent cells were forced to express Sp1 from the plasmid SpSp1 (Fig. 8). Under such a cell culture condition, it is likely that a large proportion of the de novo synthesized Sp1 is inactivated in the same way as endogenous Sp1.

Cleavage of Sp1 by one or more cysteine proteases as cells progress through differentiation has been reported before.[77,78]

In one such study, differentiation of embryonal carcinoma cells into parietal ectoderm-like cells was accompanied by a decrease in nuclear levels of Sp1 as a consequence of its cleavage by cysteine-like nuclear protease activity.[77] Although transcription factors other than Sp1 (ATF-1, NF-YA, NF-YB, and the octamer-binding proteins Oct-1 and Oct-3) also have been shown to be targets of this cysteine protease, they possess a very restricted activity because these nuclear proteins were the only recognized substrates of this enzyme. Such results would support the idea that antiproliferative effects or cell differentiation somehow increases nuclear protease activity and decreases the level of specific transcription factors including Sp1. One must also consider, however, that increasing stimulation of one or a few signal transduction pathways through cell–cell contacts as cells are reaching confluence may activate early cleavage of Sp1/Sp3 and therefore preclude the growth arrest that is typical of postconfluent cells. Considering the high number of genes with transcription regulated by Sp1/Sp3, this would clearly represent a remarkable means for arresting cell growth and preventing cell proliferation after wound healing, especially considering the critical role Sp1 plays during the cell cycle.[79,80] Indeed, Sp1 has been shown to interact directly with many nuclear proteins, including some, such as cyclin D1, the retinoblastoma protein (Rb), and the Rb-related proteins p107, E2F, p53, and mdm2, which play critical functions during the cell cycle.[79,81–87] Most of all, Sp1 expression has been shown to predominate during the G_1 phase of the cell cycle and is then subjected to proteasome-dependent degradation before the S phase, a process that is thought to be dependent on the level of Sp1 phosphorylation.[88] However, these studies have been conducted in transformed culture cells and their relevance to primary cultured, untransformed cells remains unknown.

Acknowledgments

The authors thank Marcelle Giasson for valuable help in culturing RCECs and Thomas M. Birkenmeier (Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO) for the generous gift of the α5 promoter-CAT constructs.

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