MOLECULAR AND CELLULAR BIOLOGY, June 1997, p. 3056–3064 Vol. 17, No. 6
0270-7306/97/$04.00+0

Regulation of K3 Keratin Gene Transcription by Sp1 and AP-2 in Differentiating Rabbit Corneal Epithelial Cells

TUNG-TI CHEN,[1] REN-LONG WU,[2] FEDERICO CASTRO-MUNOZLEDO,[3] AND TUNG-TIEN SUN[1,2]*

Department of Pharmacology,[1] Epithelial Biology Unit, The Ronald O. Perelman Department of Dermatology,[2] and Kaplan Cancer Center,[2] New York University School of Medicine, New York, New York 10016, and Department of Cell Biology, Centro de Investigacion de Estudios Avanzados del IPN, Mexico City, Mexico[3]

Received 14 June 1996/Returned for modification 6 August 1996/Accepted 8 March 1997

Rabbit corneal epithelial cells cultured in the presence of 3T3 feeder cells undergo biochemical differentiation, as evidenced by their initial expression of K5 and K14 keratins characteristic of basal keratinocytes, followed by the subsequent expression of K3 and K12 keratin markers of corneal epithelial differentiation. Previous data established that mutations of an Sp1 site in a DNA element, E, that contains overlapping Sp1 and AP-2 motifs reduce K3 gene promoter activity by 70% in transfection assays. We show here that Sp1 activates while AP-2 represses the K3 promoter. Although undifferentiated corneal epithelial basal cells express equal amounts of Sp1 and AP-2 DNA-binding activities, the differentiated cells down-regulate their Sp1 activity slightly but their AP-2 activity drastically, thus resulting in a six- to sevenfold increase in the Sp1/AP-2 ratio. This change coincides with the activation and suppression of the differentiation-related K3 gene and the basal cell-related K14 keratin gene, respectively. In addition, we show that polyamines, which are present in a high concentration in proliferating basal keratinocytes, can inhibit the binding of Sp1 to its cognate binding motif but not that of AP-2. These results suggest that the relatively low Sp1/AP-2 ratio as well as the polyamine-mediated inhibition of Sp1 binding to the E motif may account, in part, for the suppression of the K3 gene in corneal epithelial basal cells, while the elevated Sp1/AP-2 ratio may be involved in activating the K3 gene in differentiated corneal epithelial cells. Coupled with the previous demonstration that AP-2 activates the K14 gene in basal cells, the switch of the Sp1/AP-2 ratio during corneal epithelial differentiation may play a role in the reciprocal expression of the K3 and K14 genes in the basal and suprabasal cell layers.

The rabbit K3 gene encodes a type II keratin which belongs to a family of intermediate filament proteins containing more than 20 members. The expression of these keratin proteins is confined to epithelial cells and, in many cases, is tissue restricted and differentiation dependent (20, 37). For example, we have shown earlier, using monoclonal antibodies against keratins, that cultured rabbit corneal epithelial cells initially express basal cell marker K5 and K14 keratins. Later, when the cells grow exponentially, they turn on the expression of hyperproliferation marker K6 and K16 keratins. Finally, as the confluent cells start to stratify, they turn off K6 and K16 expression and turn on the expression of differentiation marker K3 and K12 keratins (47). Thus, the expression of K3 and K12 keratins is a marker of corneal-type keratinocyte differentiation and is reciprocal to the expression of K5 and K14. The availability of the rabbit corneal epithelial cell culture system and keratin genes therefore offers an attractive system for studying the regulation of cellular differentiation as well as cell-type-specific gene expression.

One way to explore the basis of epithelial cell differentiation is to study how the expression of keratin genes is fine-tuned in such a way that certain keratins (e.g., K1-K10, K3-K12, K5-K14, and K6-K16) are expressed pairwise and why some of these pairs (e.g., K3-K12 and K5-K14) are expressed reciprocally (48, 49, 54). Results from intensive studies have identified a growing number of transcription factors implicated in regulating keratin gene expression. For example, transcription factor AP-2 has been shown to regulate expression of the K14 gene (32, 33). The 5′-upstream sequences of several other keratin genes, including the K3 (57), K1, K5, and K6 (33) genes, are known to harbor AP-2-binding motifs. However, in the case of the K5 and K14 genes, AP-2 alone does not seem to be sufficient for epithelial cell-specific expression (6, 33). Transcription factor Sp1 has also been shown to regulate the expression of K3 (57), and both K5 and K14 genes are known to have functional Sp1-binding motifs in their 5′-upstream sequences (6). Additionally, both ETS and the AP-1 family of transcription factors have been shown to regulate the inducible expression of certain keratin genes, including the K1 (27, 46), K5 (8), K6 (39), K18 (41), and K19 (26) genes. Recently, POU domain-containing transcription factors including Oct6 (19) and Skn-1a and Skn-1i (1) were shown to be expressed predominantly in epidermis and were suggested to play a role in epidermal development. Collectively, results from these studies have provided insights with certain aspects of keratin gene expression. However, the mechanism by which keratin genes are regulated in an epithelial cell-specific, differentiation-dependent, and reciprocal fashion remains poorly understood.

To begin elucidating how expression of the K3 keratin gene is regulated during corneal epithelial differentiation, we have previously cloned the rabbit K3 gene and identified a 300-bp 5′-upstream sequence of the gene which can function as a keratinocyte-specific promoter in cell transfection experiments (57). We found that at least two DNA elements in the 300-bp sequence are important for the expression of a reporter gene in keratinocytes. One of the elements, E, located at bp −210 to −181 of the rabbit K3 gene promoter, contains potential overlapping binding motifs for transcription factors Sp1 and AP-2 (56). We have shown that differentiated corneal epithelial cells contain an Sp1-like nuclear protein that binds to the E element and that mutations in the Sp1 site abolish over 70% of the

* Corresponding author. Mailing address: Department of Dermatology, New York University School of Medicine, 560 First Ave., New York, NY 10016. Phone: (212) 263-5685. Fax: (212) 263-8561. E-mail: sunt01@mcrcr.med.nyu.edu.

promoter activity (56). These findings suggest that Sp1 plays an important role in activating expression of the K3 gene in differentiated corneal epithelial cells. However, whether undifferentiated rabbit corneal epithelial cells express the same or additional E-element-binding activities and how these activities may regulate expression of the K3 gene are not known.

We show in this report that while undifferentiated corneal basal cells contain about equal amounts of Sp1 and AP-2 that bind to their overlapping motifs in the E element of the K3 promoter, the differentiated cells contain predominantly Sp1; such an increase in the Sp1/AP-2 ratio coincides with the stratification and differentiation of the cultured corneal keratinocytes. Mutagenesis experiments established that AP-2 suppresses the K3 promoter, while Sp1, which binds to a GC box largely overlapping the AP-2 motif, activates the promoter. The initial occupancy of the E element by the AP-2 suppressor, coupled with the polyamine-mediated inhibition of Sp1 binding to its motif, may account for the suppression of K3 expression in the basal cells, and the latter occupancy of the E element by the Sp1 activator may play a role in the differentiation-dependent activation of the K3 keratin gene.

MATERIALS AND METHODS

Cell cultures. Primary rabbit corneal epithelial cells were grown in the presence of mitomycin-treated 3T3 feeder cells in Dulbecco's modified minimal essential medium (DMEM) supplemented with 15% fetal calf serum and 0.5 μg hydrocortisone per ml as described previously (47). Rabbit corneal fibroblasts, rabbit kidney simple epithelial cells (a permanent line; ATCC CCL37), and 3T3 fibroblasts were cultured in DMEM supplemented with 10% bovine calf serum. For suspension culture (23), primary rabbit corneal epithelial cells were grown to 25% confluence, trypsinized, and resuspended in semisolid DMEM supplemented with 15% fetal bovine serum, 0.5 μg of hydrocortisone per ml, and 1.4% methylcellulose (Sigma) in a 50-ml conical tube at a concentration of 10^6 cells/ml and 5 ml/tube.

Northern blot analysis. For the isolation of total cellular RNAs, cells cultured in 100-mm-diameter dishes were washed twice with 10 ml of phosphate-buffered saline (PBS) per dish and lysed in situ by addition of 1 ml of denaturing solution (25 mM sodium citrate [pH 7.5], 4 M guanidinium thiocyanate [Fluka], 0.5% *N*-lauroylsarcosine [Sigma], 100 mM 2-mercaptoethanol) to each culture dish. The cell lysates were transferred to 1.5-ml microcentrifuge tubes containing 0.4 ml of 100% ethanol. After reduction of the viscosity of the lysates by shearing genomic DNA with a 22-gauge needle, total cellular RNAs were collected by centrifugation at room temperature for 5 min at 16,000 × *g*. The pelleted RNAs were resuspended in 300 μl of denaturing solution containing 300 mM sodium acetate. After extraction with an equal volume of phenol (pH 4.8), the RNAs were precipitated with 2 volumes of 100% ethanol and collected by centrifugation. Pelleted RNAs were dissolved in 100% formamide (Sigma) and stored at −70°C until analysis. For the determination of K3, K12, and K14 mRNA levels, 5-μg aliquots of isolated total cellular RNAs from different cells were fractionated in a 1% denaturing agarose gel. The fractionated RNAs were transferred to a piece of nitrocellulose membrane for hybridization. Specific cDNA fragments of rabbit K3 (57), rabbit K12 (58), and mouse K14 (provided by S. H. Yuspa) were ^{32}P labeled and used as probes to detect the mRNA expression levels of these keratins. For the determination of Sp1 and AP-2 mRNA levels, 20-μg aliquots of isolated total cellular RNAs from different cell types were used. In this case, ^{32}P-labeled full-length cDNA fragments of human Sp1 (29) and AP-2 (55) (provided by R. Tjian) were used as probes. The probes were detected by autoradiography.

Preparation of nuclear extracts. Cells were cultured in 100-mm-diameter dishes, washed twice with 10 ml of ice-cold PBS per dish, dissociated by trypsinization, and collected by centrifugation in a 50-ml tube. Pelleted cells were washed twice with 10 ml of ice-cold PBS/10^7 cells. One milliliter of ice-cold hypotonic buffer (20 mM HEPES-HCl [pH 7.6], 1 mM EDTA, 10 mM NaCl, 14 mM 2-mercaptoethanol, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg each of leupeptin and aprotinin [Sigma] per ml) was added to resuspend the cells. The cell suspension was then transferred to a 1.5-ml microcentrifuge tube and incubated on ice for 15 min. The tube was vortexed briefly after incubation and centrifuged for 30 s at 16,000 × *g* at room temperature. After being washed twice with 1 ml of ice-cold hypotonic buffer, the pelleted nuclei were extracted with 100 μl of extraction buffer (20 mM HEPES-HCl [pH 7.6], 1 mM EDTA, 430 mM NaCl, 14 mM 2-mercaptoethanol, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg each of leupeptin and aprotinin per ml) for 20 min on ice with occasional shaking. Nuclei were pelleted by centrifugation for 10 min at 16,000 × *g* at 4°C after extraction. The supernatant was transferred to a new tube without further dialysis. After determination of protein concentration, aliquots of the isolated nuclear extracts were snap frozen with liquid nitrogen and stored at −70°C until use.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts of various cell types were incubated in a final volume of 20 μl with ^{32}P-labeled double-stranded synthetic oligonucleotides for 30 min at room temperature. A typical reaction mixture contained 20 μg of nuclear proteins, 1 ng (10^5 cpm) of ^{32}P-labeled probe, 20 mM HEPES-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 12.5% glycerol, 14 mM 2-mercaptoethanol, 150 μg of poly(dI-dC) (Pharmacia) per ml, and 0.01% bromophenol blue. In selected experiments, 100-ng aliquots of unlabeled double-stranded synthetic oligonucleotides were included as specific competitors. The ^{32}P-labeled probes used included the E element (5′-CAGCGCCCGCCCCCTGCAGGGTAGGCC-3′), an Sp1-binding element (5′-ATTCGATCGGGGCGGGGCGAGC-3′) (30), an AP-2-binding element (5′-GATCGAACTGACCGCCCGCGGCCCGT-3′) (33), an AP-1-binding element (5′-CGCTTGATGAGTCAGCCGGAA-3′) (42), a c-Myc-binding element (5′-TCAGACCACGTGGTCGGGTGTTCCTGA-3′) (43), and an NF1-binding element (5′-CCTTTGGCATGCTGCCAATATG-3′) (45). In experiments involving specific antibodies against Sp1 and AP-2, the antibodies were added at the end of the first incubation as described above, and the incubation was extended for an additional 30 min. Both Sp1 and AP-2 antibodies (Santa Cruz Biotechnology) used in this study are affinity-purified rabbit polyclonal antibodies. The Sp1 antibody recognizes an epitope corresponding to amino acid residues 520 to 538 of human Sp1 (29); it reacts with the p95 and p105 Sp1 proteins. The AP-2 antibody recognizes an epitope corresponding to amino acid residues 420 to 437 of human AP-2 (55). In experiments involving recombinant human Sp1 and AP-2 (rSp1 and rAP-2; Promega), 20 μg of bovine serum albumin and 5 μg of poly(dI-dC) per ml were used. After incubation, the reaction mixtures were loaded onto a 6% native polyacrylamide gel. Electrophoresis was carried out as described previously (56), and probes were detected by autoradiography.

UV cross-linking. A probe containing the E element of the 300-bp K3 gene promoter was prepared as described below for UV cross-linking experiments. Briefly, 33 ng of a 10-mer synthetic oligonucleotide (5′-GCCAGCGCCC-3′) was annealed to 100 ng of another complementary 30-mer synthetic oligonucleotide (5′-AGGCTTACCCTGCAGGGGCGGGGGCCTGGC-3′). The annealed oligonucleotides were then filled in by the action of Klenow enzyme in the presence of 100 μCi of [^{32}P]dCTP and 0.2 mM each dATP, dGTP, and bromodeoxyuridine (Sigma). One nanogram (3×10^5) cpm of the ^{32}P-labeled E element was used in an EMSA. After electrophoresis, the gel was irradiated with UV light (254 nm) for 30 min and subsequently exposed to X-ray film for 4 h at 4°C. Selected DNA-protein complexes were detected by autoradiography, excised from the gel, and separated in a sodium dodecyl sulfate–polyacrylamide gel, using ^{14}C-labeled proteins (Gibco BRL) as molecular weight markers.

Construction of a mutant K3 promoter. Construction of the 300-bp K3 promoter with mutations in both B and E elements has been described elsewhere (57). For construction of the 300-bp K3 promoter with mutations that specifically destroy functional Sp1- or AP-2-binding motifs, we adapted the method described by Deng and Nickoloff (16). In short, two synthetic oligonucleotides, 5′-CGCCCAGCGCAATCCCCCTGCAG-3′ and 5′-GCCCCCCTGCATTTTAAGCCTGCC-3′, containing mutations in the sequences critical for the binding of Sp1 and AP-2, respectively (the mutant nucleotides are underlined), were individually annealed to denatured plasmids containing the wild-type 300-bp K3 promoter, in the presence of another synthetic oligonucleotide which serves as a selection primer. T4 DNA polymerase and ligase (New England BioLabs) were added to replicate the plasmids. The replicated double-stranded plasmids were subsequently digested by a specific restriction enzyme which recognizes only a cutting site in the selection primer of wild-type plasmids, not in plasmids containing mutations at the same position. Thus, after restriction enzyme digestion, the mutant plasmids remain intact and have a much higher efficiency for bacterial transformation. By taking advantage of this fact, K3 promoter-driven chloramphenicol acetyltransferase (CAT) reporter gene constructs containing the desired mutations were selected, and the mutated sequences were confirmed by DNA sequencing.

Transient transfection and CAT assay. Primary rabbit corneal epithelial cells were cultured to 50% confluence and fed with fresh medium before transfection. Typically, 5 μg of K3 promoter-driven CAT reporter plasmid DNA and 1 μg of RSV/lacZ plasmid DNA were mixed with 0.5 ml of solution A (250 mM $CaCl_2$ in distilled H_2O), 0.5 ml of solution B [50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4 (pH 6.95)] (10) was then added, and the mixture was incubated at room temperature for 20 min. The calcium phosphate-DNA solution was added dropwise to the entire medium, and the medium was swirled gently before the dish was returned to the incubator. Twenty-four hours later, the culture medium was removed and the cells were refed with fresh medium. Another 24 h later, the cells were harvested and CAT assays were performed as described previously (57), using β-galactosidase activity as an internal control.

Stable transfection and CAT assay. To obtain corneal epithelial cells permanently transfected with CAT reporter genes, cells from the recently established rabbit corneal epithelial line RCE1 (9) were transfected with different K3 promoter-driven CAT reporter genes and an RSV/neo plasmid (CAT/*neo* = 20/1). Sixteen hours after incubation with the DNA-calcium phosphate precipitation, the cells were washed with PBS, dissociated by trypsinization, and replated at 1/15 of the original density (one transfected 100-mm-diameter dish plated into 15

100-mm-diameter dishes) with mitomycin-treated 3T3 cells. Twenty-four hours later, the cells were fed with fresh culture medium containing 400 μg of G418 (Sigma) per ml. The culture medium was replaced with fresh selection medium every 4 days, and the selection continued for 4 weeks until G418-resistant colonies (>100 cells/colony) were established. G418-resistant colonies were pooled and amplified once for each reporter construct (~100 colonies/construct). Aliquots of the amplified cells were then plated in 60-mm-diameter dishes at 10^4 cells/dish with 2×10^5 cells of mitomycin-treated 3T3 cells per dish and cultured in medium containing 200 μg of G418 per ml. After selectively washing off the 3T3 cells with 0.02% EDTA in PBS, we harvested corneal epithelial cells at different time points during their growth and differentiation. The harvested cells were divided into two equal portions for the isolation of genomic DNA and cell extract. The average copy number of the reporter gene carried by the pooled cells was determined by Southern blot analysis using a ^{32}P-labeled CAT cDNA fragment as a probe. CAT assays were performed with the isolated cell extract, and CAT activities were normalized by the copy numbers of the reporter gene.

RESULTS

Sp1- and AP-2-like DNA-binding activities are differentially expressed during rabbit corneal epithelial differentiation. It has been reported that the presence of an AP-2-binding motif is important for expression of the K14 gene (33) and that AP-2 mRNA is detected mainly in the undifferentiated basal cells of developing epidermis (7). These results suggest that AP-2 may play a role in determining the basal cell-specific expression of the K14 gene. Furthermore, these findings raise the question as to whether the AP-2 motif of the E element, which partially overlaps the functionally important Sp1 motif, plays a role in modulating K3 gene expression.

To determine whether cultured rabbit corneal epithelial cells express AP-2 activity during differentiation, we performed EMSAs to detect the E-element-binding activities in nuclear extracts prepared from cultured rabbit corneal epithelial cells at different stages of growth and differentiation in vitro (Fig. 1). The result showed that nuclear extracts of subconfluent rabbit corneal epithelial cells contain a high level of E-element-binding activities (Fig. 1a, lanes 5 and 6). Inclusion of a 100-fold molar excess of an Sp1- or an AP-2-binding element in the reactions affected mainly the upper and the lower parts, respectively, of the major DNA-protein complex (Fig. 1a; compare lane 1 with lanes 3 and 4). These findings suggest that undifferentiated corneal epithelial cells contain both Sp1- and AP-2-like activities that can bind to the E element.

The use of an Sp1-binding element, instead of the E element, as a probe detected a high level of Sp1 DNA-binding activity in nuclear extracts of subconfluent rabbit corneal epithelial cells; this activity dropped only slightly in the postconfluent culture (Fig. 1b, lanes 5 to 10; Fig. 2a). In contrast, AP-2 DNA-binding activity, detected with an AP-2-binding element as a probe, was abundant in nuclear extracts of subconfluent cells but dropped precipitously when the cells reached confluence and underwent terminal differentiation (Fig. 1c, lanes 5 to 10; Fig. 2a). Total AP-1 DNA-binding activity, which is present in all cells tested, was found to remain relatively constant during corneal epithelial differentiation (Fig. 1d and 2a).

To determine the mRNA levels of Sp1 and AP-2 in relation to those of the K3, K12, and K14 keratins during the differentiation of cultured rabbit corneal epithelial cells, we performed Northern blot analysis (Fig. 3). In contrast to the DNA-binding activities, which dropped during the course of corneal epithelial differentiation (Fig. 1b, 1c, and 2a), the steady-state levels of Sp1 and AP-2 mRNAs remained relatively constant during the course of corneal epithelial differentiation (Fig. 3a). As shown in Fig. 3b, the expression patterns of both K3 and K12 mRNAs are clearly differentiation dependent and are reciprocal to the expression of K14 mRNA. The differentiation-dependent and reciprocal expression of K3 and K14 mRNAs was further established by using a suspension culture system which is capable of inducing the terminal differentiation of keratinocytes (Fig. 3c) (18, 23). Collectively, these results suggest that although the mRNA levels of Sp1 and AP-2 remain relatively constant during corneal epithelial differentiation, only undifferentiated rabbit corneal epithelial cells contain high levels of both Sp1- and AP-2-like DNA-binding activities (Fig. 2a) and that the ratio of Sp1 to AP-2 DNA-binding activities increases significantly as cells differentiate (Fig. 2b).

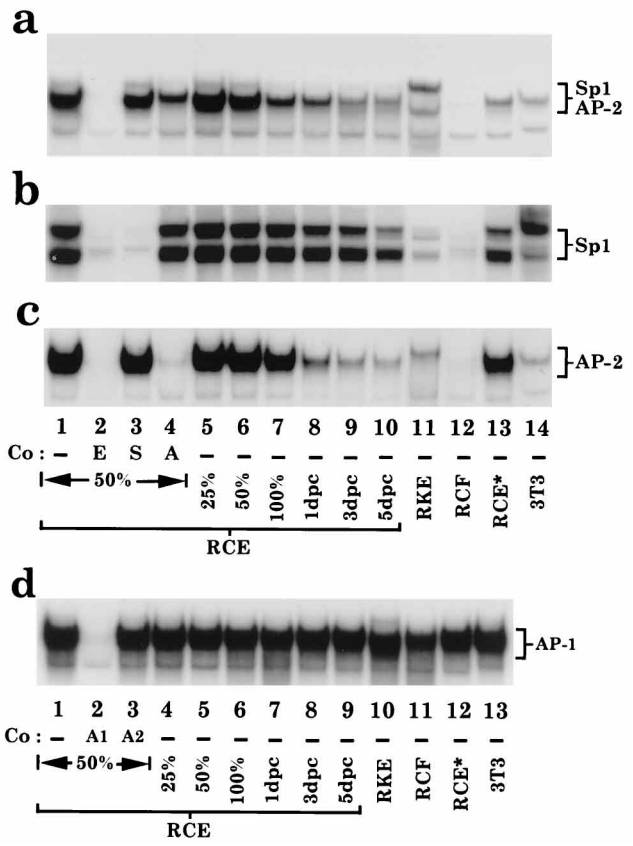

FIG. 1. Sp1 and AP-2 DNA-binding activities are differentially expressed in cultured rabbit corneal epithelial cells. (a) Nuclear extracts of rabbit corneal epithelial cells cultured in the presence of mitomycin-treated 3T3 feeder cells (RCE; lanes 1 to 10), rabbit kidney epithelial cells (RKE; lane 11), rabbit corneal fibroblasts (RCF; lane 12), rabbit corneal epithelial cells cultured in the absence of 3T3 cells (RCE*; lane 13), and 3T3 cells (3T3; lane 14) were incubated with ^{32}P-labeled E element. During incubation, either no competitor (Co) was included (lanes 1 and 5 to 14) or 100-fold molar excesses of the following competitors were included: the E element (lane 2), an Sp1-binding element (S; lane 3), and an AP-2-binding element (A; lane 4). After incubation, the reactions were subjected to EMSA and the probe was detected by autoradiography. (b to d) The same experiment was repeated except that an Sp1-binding element (b), an AP-2-binding element (c), or an AP-1-binding element (d) was used as the probe. In the AP-1 experiment, 100-fold molar excesses of the AP-1-binding element (A1, lane 2) and the AP-2-binding element (A2, lane 3) were used as specific competitors. In this and other following figures involving EMSA, the experiments were performed in the linear range and the unbound probes were not shown unless otherwise indicated. Note the formation of two specific Sp1-DNA complexes in panel b, which is consistent with other reports showing that even the binding of rSp1 with certain binding motifs can generate multiple Sp1-DNA complexes (17, 24). Also note the drastic decrease in AP-2 DNA-binding activity in confluent rabbit corneal epithelial cells. The AP-2-like activity of rabbit kidney simple epithelial cells has a mobility slower than that of corneal epithelial cells. dpc, days postconfluence.

Sp1 and AP-2 are the E-element-binding factors in undifferentiated cells, while Sp1 is the main factor in differentiated cells. To map the nucleotide sequences required for binding of the Sp1- and AP-2-like activities to the E element, we synthe-

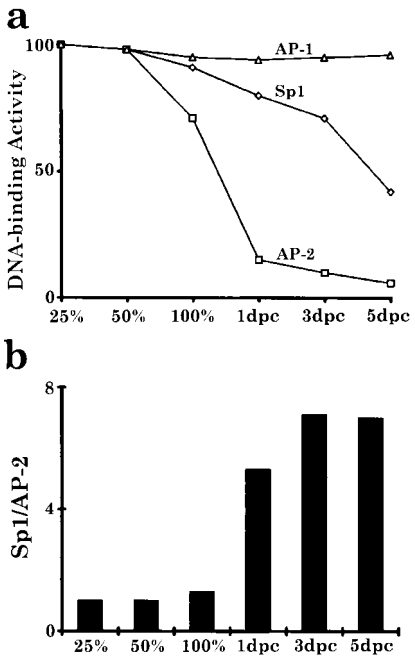

FIG. 2. The ratio of Sp1 to AP-2 DNA-binding activity increases in a differentiation-dependent manner in cultured rabbit corneal epithelial cells. Nuclear extracts of rabbit corneal epithelial cells grown to various degrees of confluence (from 25% confluence to 5 days postconfluence [5dpc]) were incubated with ^{32}P-labeled Sp1, AP-2- or AP-1-binding elements in the absence of specific competitors. After incubation, the reactions were subjected to EMSA and the probes were detected by autoradiography. Relative Sp1, AP-2, and AP-1 DNA-binding activities were quantified by scintillation counting of the excised bands. (a) Counted values; (b) ratios of Sp1 to AP-2 DNA-binding activities. Note that differentiated cells (1 day postconfluence or older) contain at least five times more Sp1 than AP-2 DNA-binding activity.

sized a panel of mutated E elements containing mutations covering the overlapping Sp1 and AP-2 motifs (Fig. 4a) and used them as competitors in EMSAs using the E element as a probe. The result indicated that the binding of nuclear factors of subconfluent corneal epithelial cells to the E element was greatly affected by mutations within a 15-bp sequence (5′-**CC GCCCCCTG**CAGGG-3′) which coincides precisely with the partially overlapping Sp1-binding (boldface) and AP-2-binding (underlined) motifs (Fig. 4b). By using an Sp1- or an AP-2-binding element, instead of the E element, as a probe, we were able to locate the sequence required for the binding of the Sp1-like activity to 5′-CCGCCCCCTG-3′ (Fig. 4c), and the AP-2-like activity to 5′-CCCTGCAGGG-3′ (Fig. 4d), of the E element. These results clearly show that the Sp1-like and AP-2-like nuclear factors of corneal epithelial cells bind to overlapping motifs in the E element of the 300-bp K3 promoter.

To characterize the Sp1-like and the AP-2-like DNA-binding activities present in the undifferentiated rabbit corneal epithelial cells, we performed immuno-supershift assays and in vitro reconstitution. We showed in EMSAs that antibodies to Sp1 and AP-2 were able to induce supershifted bands, with the concomitant disappearance of the upper and lower parts, respectively, of the major E element-protein complex (Fig. 5a; compare lanes 1 and 6). A mixture of these two antibodies abolished the entire E element-protein complex (Fig. 5a, lane 10). Coupled with the observation that the entire E element-protein complex is abolished in the presence of a mixture of unlabeled Sp1 and AP-2 probes, this finding shows that the protein-E element complex is a duplex of Sp1-E and AP-2–E. This is confirmed by in vitro reconstitution, as the incubation of rSp1 and rAP-2 with the E element resulted in the formation of protein-DNA complexes identical in mobility to the upper and lower parts, respectively, of the major E element-protein complex (Fig. 5a; compare lane 11 with lanes 12 and 13).

To further characterize the E-element-binding proteins, we performed UV cross-linking experiments. Two major bands with apparent molecular sizes comparable to the reported molecular sizes of Sp1 (100 to 110 kDa) (29) and AP-2 (50 to 55 kDa) (55) were detected (Fig. 5c, lane 2). Taken together, these results established that a roughly equal mixture of Sp1 and AP-2 can account for >95% of the E-element-binding

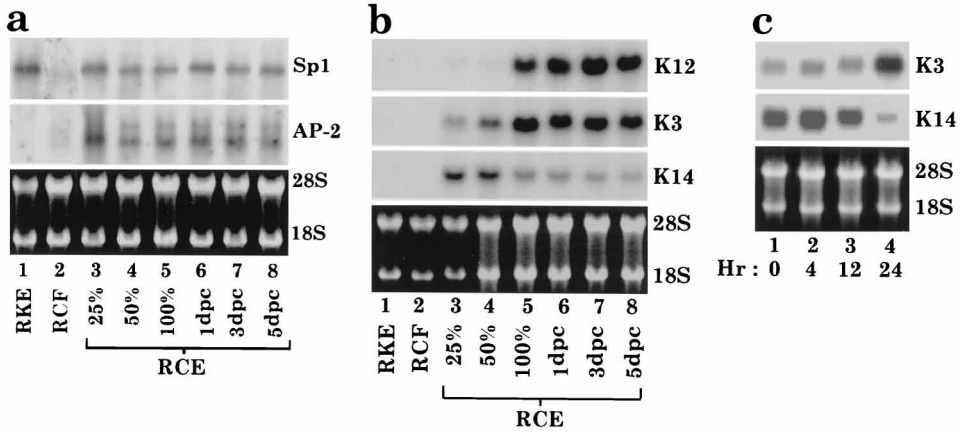

FIG. 3. Differentiating rabbit corneal epithelial cells express K3 and K14 mRNAs in a differentiation-dependent and reciprocal manner. Northern blot analyses were performed with total cellular RNAs isolated from rabbit corneal epithelial cells (RCE) grown to 25% (lane 3), 50% (lane 4), or 100% confluence (lane 5) and to 1 day postconfluence (lane 6), 3 days postconfluence (lane 7), and 5 days postconfluence (lane 8), from rabbit kidney epithelial cells (RKE; lane 1), and from rabbit corneal fibroblasts (RCF; lane 2). The probes used were full-length cDNAs of human Sp1 and AP-2 (a) and specific cDNA fragments of rabbit K3, K12, and mouse K14 (b). (c) Rabbit corneal epithelial cells were grown to 25% confluence, dissociated by trypsinization, and resuspended in culture medium containing 1.4% methycellulose. After 0 h (lane 1), 4 h (lane 2), 12 h (lane 3), and 24 h (lane 4) in suspension, total cellular RNAs were prepared and subjected to Northern blot analysis as described above. Hr, hours after cultured in medium containing methylcellulose. Note the constant level of Sp1 and AP-2, and the differentiation-dependent and reciprocal expression of K3 and K14 genes, during the course of corneal epithelial differentiation. Also note that rabbit kidney simple epithelial cells and corneal fibroblasts do not express detectable levels of AP-2 mRNA.

activities in the undifferentiated rabbit corneal epithelial cells (50% confluent [Fig. 5a]), while Sp1 is the main activity in differentiated cells (3 days postconfluent [Fig. 5b and reference 56).

Sp1 and AP-2 bind to the E element of the K3 promoter, forming distinct protein-DNA complexes. Our EMSAs in which we incubated the E probe with nuclear extracts containing both Sp1 and AP-2 DNA-binding activities showed the formation of two partially separated DNA-protein complexes (Fig. 5a). In vitro reconstitution using highly purified rSp1 and rAP-2 confirmed the formation of two distinct protein-DNA complexes (Fig. 5a, lanes 12 and 13). The binding of rSp1 and rAP-2 to the E element was quite rapid, reaching a plateau in less than 5 min (Fig. 6a, lanes 1 and 7); similar DNA-binding kinetics have been reported for the phosphorylated Max homodimer and Myc-Max heterodimer (4). Although it seemed highly unlikely that Sp1 and AP-2 would be able to bind simultaneously to their largely overlapping motifs (5 of 10 bp for both Sp1 and AP-2), we incubated a limiting amount of labeled E element with a mixture of equal amounts of rSp1 and rAP-2 and resolved the complexes under prolonged electrophoretic conditions. This clearly resolved two bands corresponding to the rSp1-E and rAP-2–E complexes, with no evidence of ternary complex formation (Fig. 6b, lane 1).

AP-2 suppresses expression of the 300-bp K3 promoter in cultured rabbit corneal epithelial cells. To determine the functional role of AP-2 in regulating expression of the K3 gene, we constructed several expression vectors in which a CAT reporter gene was driven by a 300-bp K3 promoter containing various mutations in its E element and tested them in transient transfection experiments (Fig. 7a). Consistent with our previous results (56), a mutation of the Sp1 site abolished more than 50% of the K3 promoter activity (Fig. 7b; compare constructs Wt and −Sp1). Also consistent with earlier data, simultaneous mutations (56) of both Sp1- and AP-2-binding motifs of the E element as well as the NF-κB-binding motif of the B element abolished more than 80% of the promoter activity (Fig. 7b; compare constructs Wt and −B/E). In contrast, a mutation affecting only the AP-2 motif without affecting the Sp1-binding motif actually enhanced the K3 promoter activity more than threefold (Fig. 7b; compare constructs Wt and −AP-2).

To further explore the role of the Sp1- and AP-2-binding motifs in regulating the 300-bp K3 promoter activity during in vitro growth and differentiation of rabbit corneal epithelial cells, we used a recently established permanent cell line of rabbit corneal epithelial cells, RCE1, which can recapitulate the in vitro growth and differentiation process as defined by the sequential expression of various keratin pairs (9), as well as the differential regulation of Sp1- and AP-2-like DNA-binding activities (data not shown). We stably transfected such cells with a CAT reporter gene driven by the 300-bp K3 promoter. Figure 7c shows that this promoter (Wt) indeed drove the reporter gene to express in a differentiation-dependent manner, mimicking the expression of the endogenous K3 gene. Mutation of the Sp1-binding motif of the K3 promoter had no effect on its activity in the undifferentiated cells but reduced threefold its activity in differentiated cells (Fig. 7c, −Sp1). In contrast, AP-2 motif mutation resulted in a significant enhancement of the K3 promoter activity in undifferentiated cells (Fig. 7c, −AP-2). Taken together, these results established that AP-2 can function as a repressor, particularly in the undifferentiated basal cells, of the K3 promoter. Our results also indicate that Sp1 functions primarily as an activator of the K3 promoter in the differentiated rabbit corneal epithelial cells.

Polyamines differentially alter the sequence-specific DNA-binding activities of Sp1 and AP-2. Polyamines at physiological

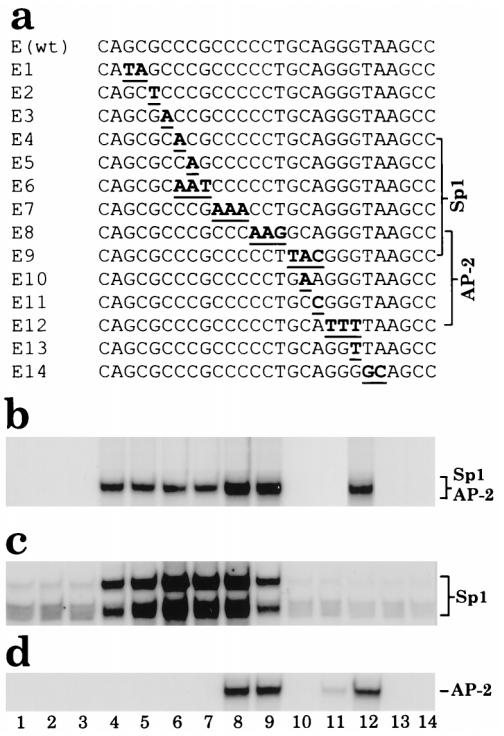

FIG. 4. Sp1 and AP-2 bind to overlapping binding sites in the E element of the 300-bp K3 promoter. (a) Nucleotide sequences of the wild-type (wt) and mutant (E1 to E14) E elements used for panels b to d. (b) EMSA was performed with nuclear extracts of cultured rabbit corneal epithelial cells (50% confluence) and ^{32}P-labeled E element as a probe in the presence of 100-fold molar excesses of competitors E1 to E14 (lanes 1 to 14). (c and d) The same experiments were repeated as described for panel b except that an Sp1-binding element (c) or an AP-2 binding element (d) was used as the probe. Note that no ternary complex is detected in panel a and that the binding motifs for Sp1 and AP-2 overlap by five nucleotides (5′-CCCTG-3′). Identical results were obtained when a longer probe that contained six additional 5′ nucleotides was used (data not shown).

concentrations are known to be able to alter the in vitro sequence-specific DNA-binding activities of several transcription factors, including the progesterone receptor (51), estrogen receptor (50), NF-κB (12), USF, NF-IL6, and YY1 (40). To determine whether polyamines can affect the DNA-binding activities of Sp1 and AP-2, we included spermine at various concentrations in EMSAs using the E element as a probe. Our results showed that spermine, at concentrations above 1 mM, inhibited the formation of the upper part of the major E element-protein complex (Fig. 8a; compare lanes 6 and 7 with other lanes). The use of an Sp1-binding element, instead of an E element, as a probe showed that spermine in this concentration range inhibits the binding of Sp1 to its binding element (Fig. 8b; compare lanes 6 and 7 with other lanes; Fig. 8g, Sp1) but has relatively little effect on the binding of AP-2 to its motif (Figs. 8c and g, AP-2). In other experiments, we found that spermine inhibits the DNA-binding activities of AP-1 and c-Myc (Fig. 8d and e) but enhanced that of NF1 (Fig. 8f). Spermidine yielded similar results (data not shown).

DISCUSSION

Mechanism of the reciprocal expression of K3 and K14 genes in differentiating corneal epithelial cells. We showed in this report that the differentiation of cultured rabbit corneal epithelial cells is accompanied by a slight decrease in Sp1 DNA-binding activity (Fig. 1b and 2a) and a severe decrease in

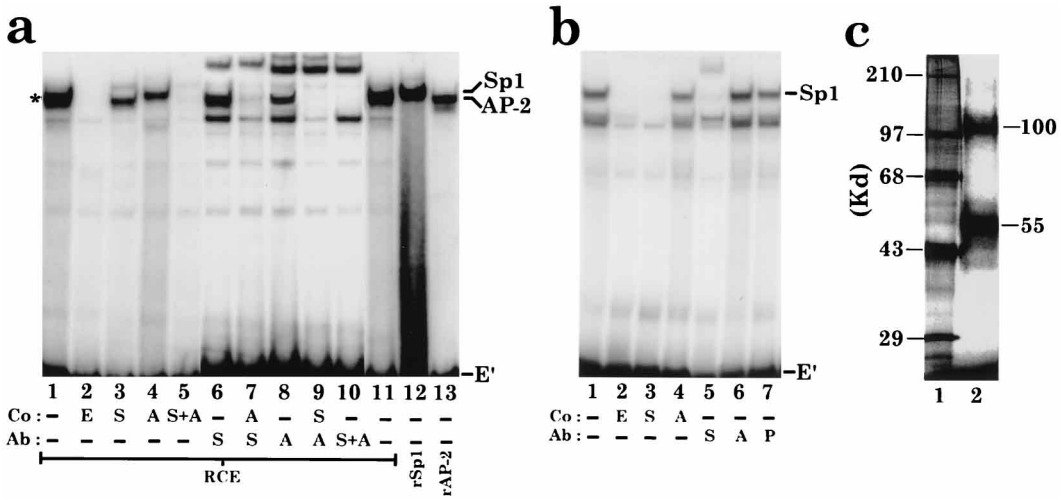

FIG. 5. The E element binds both Sp1 and AP-2 in undifferentiated corneal epithelial cells but only Sp1 in differentiated cells. (a) EMSAs were performed with ^{32}P-labeled E element as a probe and nuclear extracts of cultured rabbit corneal epithelial cells (RCE, 50% confluence; lanes 1 to 11), an rSp1 (lane 12), and rAP-2 (lane 13). Some of the reactions include 100-fold molar excesses of competitors (Co) including unlabeled E element (lane 2), an Sp1-binding element (lanes 3 and 9), an AP-2-binding element (lanes 4 and 7), and a mixture of Sp1- and AP-2-binding elements (lane 5). When indicated, antibodies (Ab) against Sp1 (S; lanes 6 and 7), AP-2 (A; lanes 8 and 9), or both (S+A, lane 10) were added. (b) The experiment was repeated as described for panel a except with nuclear extracts of corneal epithelial cells grown to 3 days postconfluence; normal rabbit serum (P; lane 7) was used as a control. (c) EMSA was performed with nuclear extracts of 50% confluent corneal epithelial cells and ^{32}P-labeled E element as a probe. After electrophoresis, the gel was UV irradiated, and the shifted band (*) shown in panel a was excised and fractionated in a sodium dodecyl sulfate–10% gel. UV-cross-linked protein-DNA complexes were detected by autoradiography. E′ represents the unbound probe. Note that the E element binds both Sp1 and AP-2 when the nuclear extracts were prepared from undifferentiated cells (50% confluence) but only Sp1 when the nuclear extracts were prepared from differentiated cells (3 days postconfluence).

AP-2 DNA-binding activity (Fig. 1c and 2a). This results in a significant increase in the ratio of Sp1 to AP-2 DNA-binding activity when the cells reach confluence and undergo stratification (Fig. 2b). Such a surge in the Sp1/AP-2 ratio coincides with activation of the K3 gene and reduced expression of the K14 gene (Fig. 3b). Three lines of evidence suggest that the balance between Sp1 and AP-2 DNA-binding activities may play a role in regulating the differentiation-dependent and reciprocal expression of the K3 and K14 genes. First, results from both transient and permanent transfection experiments showed that Sp1 is an activator (Fig. 7b and c; also see reference 56), while AP-2 is a repressor (Fig. 7b and c), of the K3 promoter. Second, under a wide range of experimental conditions, we have not been able to detect the formation of a ternary complex of the E element, Sp1, and AP-2 (Fig. 6b); such data suggest that Sp1 and AP-2 bind mutually exclusively to their overlapping motifs in the E element. Third, AP-2 is known to be an activator of the K14 promoter (33), and its expression pattern in developing epidermis coincides with that of the K14 gene (7). These considerations raise the interesting possibility that the high level of AP-2 DNA-binding activity in undifferentiated corneal epithelial cells hinders the binding of Sp1 to the E element of the K3 promoter, thus resulting in the suppression of the K3 gene. In the meantime, it is known that AP-2 can activate expression of the K14 gene in the basal cells (7). As corneal epithelial cells become differentiated, they down-regulate their AP-2 DNA-binding activity drastically but their Sp1 activity only slightly. Such a significant increase in Sp1 over AP-2 DNA-binding activity may lead to an increased occupation of the E element by Sp1 and hence the activation of the K3 gene in differentiated cells. Concurrently, the diminished AP-2 DNA-binding activity in such differentiated cells may lead to reduced expression of K14 gene. These results suggest that the differentiation-dependent and reciprocal expression of the K3 and K14 genes in differentiating rabbit corneal epithelial cells may be accomplished, in part, by a coordinated regulation of Sp1 and AP-2 DNA-binding activities.

Although AP-2 serves as an activator in the regulation of many genes, including the K14 gene, it can negatively regulate the genes encoding acetylcholinesterase (22), prothymosin, and ornithine decarboxylase (21). Whether AP-2 can also suppress the expression of any other differentiation-related keratin genes is not known. K1, like K3, is a type II basic keratin which is expressed suprabasally in the differentiated cell layers of keratinizing epidermis. Roop et al. (44) showed that a human K1 gene in a 12-kb genomic sequence is expressed in transgenic mice mainly in the differentiated suprabasal cells of the epidermis, indicating that the cloned sequence contains sufficient information for tissue-specific and differentiation-dependent expression. The 5′-proximal promoter of the cloned K1 gene contains an AP-2-binding motif capable of binding AP-2 (32) and a 3′-distal sequence contains an element capable of mediating the calcium-induced expression of the K1 gene (27, 46). Based on the earlier finding that AP-2 activates K14 gene expression, it was suggested that the AP-2 binding motif may cooperate with the 3′-distal element to activate transcription of the K1 gene (46). It would be interesting to test by mutagenesis whether the AP-2 element of the K1 gene may actually serve as a negative element analogous to that of the K3 gene reported here.

Role of polyamines in K3 gene regulation. The fact that the ratio of AP-2 to Sp1 DNA-binding activity drops significantly during corneal epithelial differentiation (Fig. 2b) suggests that the E element is initially occupied by AP-2 in undifferentiated basal cells but is later replaced by Sp1 in differentiated cells. Since site-directed mutagenesis showed that the binding of AP-2 and Sp1 to their cognate motifs in the E element can inactivate and activate, respectively, the K3 promoter (Fig. 7), the replacement of AP-2 by Sp1 can potentially lead to the activation of the K3 gene. This hypothesis has two potential problems. The first has to do with the fact that even in the

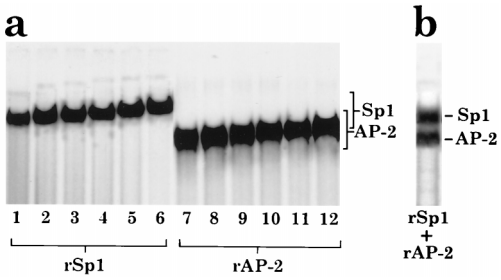

FIG. 6. Recombinant Sp1 and AP-2 bind to the E element of the K3 promoter, forming distinct protein-DNA complexes. (a) rSp1 (lanes 1 to 6) or rAP-2 (lanes 8 to 12) was incubated at room temperature with an equal molar amount of ^{32}P-labeled E element. After 5 min (lanes 1 and 7), 10 min (lanes 2 and 8), 15 min (lanes 3 and 9), 20 min (lanes 4 and 10), 25 min (lanes 5 and 11), and 30 min (lanes 6 and 12), the reaction mixtures were subjected to EMSA (electrophoresis started at 5 min). The probe was detected by autoradiography. (b) A limiting amount of ^{32}P-labeled E element was incubated with a mixture of equal amounts of Sp1 and AP-2 proteins at room temperature for 60 min before EMSA. Electrophoresis in these EMSAs was carried out for 3 (instead of 1.5) h so that the rSp1-E and rAP-2–E complexes were better separated. Note that in panel a, the binding of Sp1 and AP-2 to the E element was completed within 5 min and that in panel b, under our experimental conditions, Sp1 and AP-2 formed distinct protein-DNA complexes without any evidence of ternary complex formation.

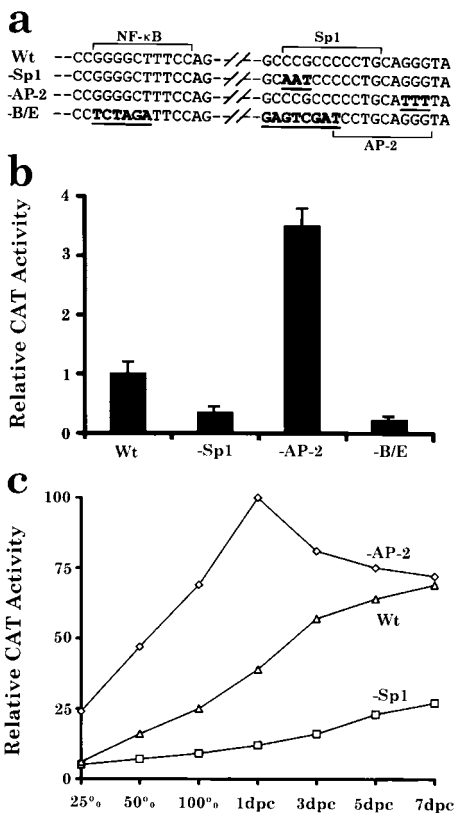

FIG. 7. AP-2 and Sp1 function as repressor and activator, respectively, of the K3 promoter. (a) Partial nucleotide sequences of the 300-bp K3 promoter showing the sequence of the B element that contains an NF-κB motif and the E element that contains overlapping Sp1 and AP-2 motifs. The mutated nucleotides are shown boldface and underlined. (b) Rabbit corneal epithelial cells grown to 70% confluence were transiently transfected with a CAT reporter gene driven by one of the four types of K3 promoter shown in panel a. The constructs are Wt (wild-type promoter), −Sp1 (promoter without a functional Sp1-binding site), −AP-2 (promoter without a functional AP-2-binding site), and −B/E (promoter without functional binding sites for NF-κB, Sp1, and AP-2). Relative CAT activities were determined 48 h after transfection. (c) A permanent cell line of rabbit corneal epithelial cells was cotransfected with a plasmid containing a *neo* gene driven by a cytomegalovirus promoter plus a the reporter construct (Wt, −Sp1, or −AP-2). G418-resistant colonies were selected and pooled for each reporter construct. Relative CAT activities were determined at different time points during the growth and differentiation of these cells. Note that in panel c, the K3 promoter without a functional AP-2-binding motif is much more active than the wild-type promoter in subconfluent cultures. dpc, days postconfluence.

5-day postconfluent culture, we can still detect some AP-2 DNA-binding activity (Fig. 1c, lane 10), which could result in the incomplete activation of the K3 promoter. This is unlikely to be the case, however, since we know that even highly stratified corneal epithelial colonies still possess an undifferentiated basal cell layer (47), which can easily account for the low level of AP-2 activity detected in these cultures (approximately 5 to 10% of the level in subconfluent cultures [Fig. 1c, lane 10]). Another potential problem has to do with the fact that even the earliest culture that we studied (at 25% confluence) contained a significant amount of Sp1 DNA-binding activity (roughly equal to the AP-2 DNA-binding activity of the same culture [Fig. 1b and c]). Although small colonies of cultured rabbit epidermal and esophageal keratinocytes are known to stratify, rabbit corneal epithelial colonies remain largely a monolayer until cells reach confluence (47). Therefore, the observed Sp1 DNA-binding activity cannot be accounted for by contaminating suprabasal cells. In this regard, our observation on the effects of polyamines on the binding of Sp1 and AP-2 to their respective motifs is of interest. Our data indicate that spermine and spermidine, at concentrations of 1 to 10 mM, which are within the physiological range (reviewed in reference 15), can drastically inhibit Sp1 binding to its motif (Fig. 8a, b, and g) but have little effect on the AP-2 binding (Fig. 8a, c, and g). It has been reported that polyamines can differentially affect the binding of transcription factors, including the progesterone receptor (51), estrogen receptor (50), NF-κB (12), USF, NF-IL6, and YY1 (40), to their cognate binding motifs. In addition, we showed here that polyamines, at physiological concentrations, can inhibit the binding of AP-1 and c-Myc, but stimulate the binding of NF1, to their binding elements (Fig. 8d, e, and f). Taken together, these data indicate that polyamines can play a potentially important role in modulating differentially the binding affinities of a large number of transcription factors to their cognate DNA motifs. The mechanism of this effect is not clear, although it is known that these polycationic molecules can induce the B-to-Z transition of DNA conformation (25, 52, 53). This differential modulation can be biologically significant because the synthesis of polyamines is known to be up-regulated in proliferating cells. Indeed, Baze et al. (3) showed that the proliferating basal cells of human epidermis contain a much higher polyamine level than the suprabasal cells. If a similar gradient of polyamines exists in corneal epithelial colonies, the high concentration of polyamines in the basal cells can potentially attenuate the binding of Sp1 to the E element, thus ensuring its occupancy by AP-2. This can result in a more complete inactivation of the K3 promoter in undifferentiated basal cells.

Regulation of Sp1 and AP-2 activities in differentiating corneal epithelial cells. Our data indicate that there is a progressive decrease in Sp1 DNA-binding activity (Fig. 1b and 2a) during the course of in vitro corneal epithelial differentiation. However, the steady-state mRNA level of Sp1 remains relatively constant during this process (Fig. 3a), raising the possibility of posttranscriptional regulation. The DNA-binding activity of Sp1 can be modulated posttranscriptionally through protein phosphorylation. Leggett et al. (34) have detected phosphorylated Sp1 in the nuclear extracts of brain, kidney,

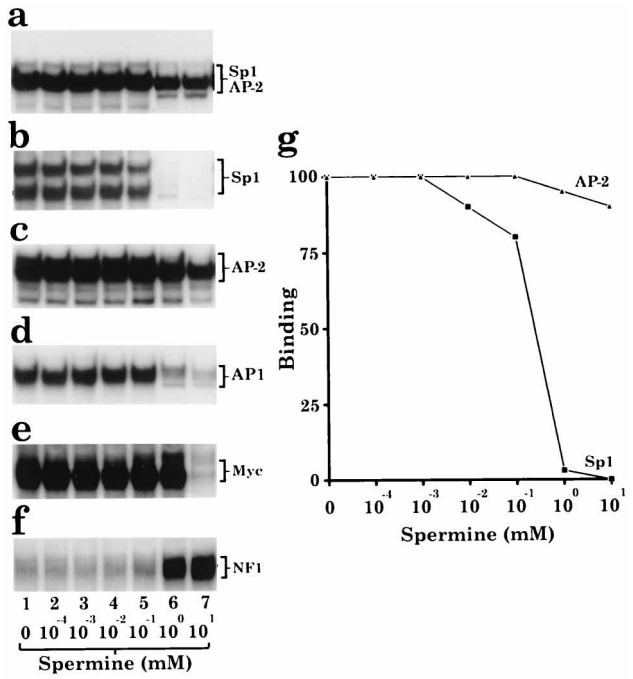

FIG. 8. Spermine differentially alters the sequence-specific DNA-binding activities of several transcription factors. (a) EMSA was performed with nuclear extracts of cultured rabbit corneal epithelial cells (50% confluence) and ^{32}P-labeled E element as a probe in the absence (lane 1) or presence of 10^{-4} mM (lane 2), 10^{-3} mM (lane 3), 10^{-2} mM (lane 4), 10^{-1} mM (lane 5), 1 mM (lane 6), and 10 mM (lane 7) spermine. (b to f) EMSA was repeated as described for panel a except that the probes used were either an Sp1-binding element (b), an AP-2-binding element (c), an AP-1-binding element (d), a c-Myc-binding element (e), or an NF1-binding element (f). (g) EMSAs were carried out as described for panels b and c. After autoradiography, specific protein-DNA complexes were excised, counted, and plotted. Note that spermine at concentrations between 0.1 and 10 mM can drastically inhibit the in vitro DNA-binding activity of Sp1 but not that of AP-2.

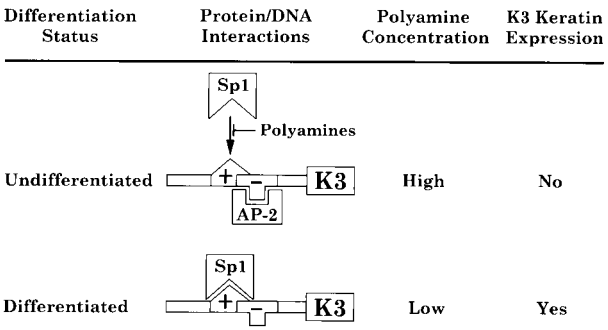

FIG. 9. Schematic diagram illustrating the hypothetical interactions between the K3 promoter and the transcription factors Sp1 and AP-2 in corneal epithelial cells. Based on the results described in this report, we hypothesize that in undifferentiated cells which do not express K3 gene, the AP-2-binding motif of K3 promoter is bound by AP-2. This AP-2 blockage, as well as the inhibitory effect of polyamine, prevents Sp1 binding to its motif, resulting in suppression of K3 gene expression. The diminished AP-2 concentration in differentiated cells allows Sp1 binding leading to the activation of the K3 gene.

and liver. Moreover, they showed that dephosphorylation resulted in a 10-fold increase in the DNA-binding affinity of Sp1 and that casein kinase II, a serine/threonine kinase, was able to phosphorylate Sp1 and to reduce its DNA-binding affinity. The DNA-binding activity of Sp1 can also be modulated posttranscriptionally through its association with the retinoblastoma-related protein p107 (14), the retinoblastoma-associated 20-kDa protein Sp1-I (11), and the ubiquitous nuclear protein p74 (38). Further investigation is required to determine whether corneal epithelial Sp1 is regulated by any of these mechanisms.

Although the DNA-binding activity of AP-2 is strikingly down-regulated in differentiated corneal epithelial cells (Fig. 1c and 2a), our data indicate that, as for Sp1, the steady-state level of AP-2 mRNA remains relatively constant (Fig. 3a). This is somewhat inconsistent with a recent report that the expression of AP-2 mRNA in developing mouse epidermis is confined to the dividing basal cells (7). Whether this is due to a difference in experimental conditions such as in vivo versus cell culture is unclear. That AP-2 may be regulated at posttranscriptional levels is in accordance with earlier results showing that AP-2 activity can be modulated by protein kinase C and cyclic AMP (28), retinoic acid (36), and tetradecanoyl phorbol acetate (2). Additionally, an AP-2 isoform, AP-2B, has recently been identified and shown to be able to inhibit the DNA-binding activity of AP-2 (5). It would be interesting to determine whether corneal epithelial cells also express AP-2B in a differentiation-dependent manner and whether this can account for the down-regulation of AP-2 DNA-binding activity.

The expression of K3 keratin is one of the hallmarks of differentiated corneal epithelium. Based on the results described in this report, we hypothesize that the transcription of K3 gene in cultured rabbit corneal epithelial cells is regulated in part by the availability of transcription factors Sp1 and AP-2, as well as by the cellular levels of polyamines, as depicted in Fig. 9. Additional studies are needed to better understand (i) the signal transduction pathways leading to the differential regulation of the Sp1 and AP-2 DNA-binding activities; (ii) how the known differences in the basement membrane composition of central and peripheral cornea lead to suprabasal and uniform K3 keratin expression, respectively (31, 35); and (iii) how such a differential pattern of the Sp1/AP-2 ratio relates to the exclusive location of corneal epithelial stem cells in peripheral cornea in the limbal zone (13, 47).

ACKNOWLEDGMENTS

We thank R. Tjian (University of California, Berkeley) for providing human Sp1 and AP-2 cDNAs and S. H. Yuspa (National Cancer Institute) for providing the mouse K14 cDNA. We also thank W. Michael O'Guin for critical reading of the manuscript.

This work was supported by National Institutes of Health grants EY04722 and AR7190-20.

REFERENCES

1. **Andersen, B., M. D. Schonemann, S. E. Flynn, R. V. D. Pearse, H. Singh, and M. G. Rosenfeld.** 1993. Skn-1a and Skn-1i: two functionally distinct Oct-2-related factors expressed in epidermis. Science **260:**78–82. (Erratum, **262:** 1499.)
2. **Andersson, G., S. Pahlman, V. Parrow, I. Johansson, and U. Hammerling.** 1994. Activation of the human NPY gene during neuroblastoma cell differentiation: induced transcriptional activities of AP-1 and AP-2. Cell Growth Differ. **5:**27–36.
3. **Baze, P. E., G. Milano, P. Verrando, N. Renee, and J. P. Ortonne.** 1985. Distribution of polyamines in human epidermis. Br. J. Dermatol. **112:**393–396.
4. **Bousset, K., M. Henriksson, J. M. Luscher-Firzlaff, D. W. Litchfield, and B. Luscher.** 1993. Identification of casein kinase II phosphorylation sites in Max: effects on DNA-binding kinetics of Max homo- and Myc/Max heterodimers. Oncogene **8:**3211–3220.
5. **Buettner, R., P. Kannan, A. Imhof, R. Bauer, S. O. Yim, R. Glockshuber, M. W. Van Dyke, and M. A. Tainsky.** 1993. An alternatively spliced mRNA from the AP-2 gene encodes a negative regulator of transcriptional activation by AP-2. Mol. Cell. Biol. **13:**4174–4185.
6. **Byrne, C., and E. Fuchs.** 1993. Probing keratinocyte and differentiation specificity of the human K5 promoter in vitro and in transgenic mice. Mol. Cell. Biol. **13:**3176–3190.

7. **Byrne, C., M. Tainsky, and E. Fuchs.** 1994. Programming gene expression in developing epidermis. Development **120:**2369–2383.
8. **Casatorres, J., J. M. Navarro, M. Blessing, and J. L. Jorcano.** 1994. Analysis of the control of expression and tissue specificity of the keratin 5 gene, characteristic of basal keratinocytes. Fundamental role of an AP-1 element. J. Biol. Chem. **269:**20489–20496.
9. **Castro-Munozledo, F.** 1994. Development of a spontaneous permanent cell line of rabbit corneal epithelial cells that undergoes sequential stages of differentiation in cell culture. J. Cell Sci. **107:**2343–2351.
10. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
11. **Chen, L. I., T. Nishinaka, K. Kwan, I. Kitabayashi, K. Yokoyama, Y. H. Fu, S. Grunwald, and R. Chiu.** 1994. The retinoblastoma gene product RB stimulates Sp1-mediated transcription by liberating Sp1 from a negative regulator. Mol. Cell. Biol. **14:**4380–4389.
12. **Clark, L., J. R. Matthews, and R. T. Hay.** 1990. Interaction of enhancer-binding protein EBP1 (NF-kappa B) with the human immunodeficiency virus type 1 enhancer. J. Virol. **64:**1335–1344.
13. **Cotsarelis, G., S. Z. Cheng, G. Dong, T.-T. Sun, and R. M. Lavker.** 1989. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell **57:**201–209.
14. **Datta, P. K., P. Raychaudhuri, and S. Bagchi.** 1995. Association of p107 with Sp1: genetically separable regions of p107 are involved in regulation of E2F- and Sp1-dependent transcription. Mol. Cell. Biol. **15:**5444–5452.
15. **Davis, R. H., D. R. Morris, and P. Coffino.** 1992. Sequestered end products and enzyme regulation: the case of ornithine decarboxylase. Microbiol. Rev. **56:**280–290.
16. **Deng, W. P., and J. A. Nickoloff.** 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal. Biochem. **200:**81–88.
17. **Dennig, J., G. Hagen, M. Beato, and G. Suske.** 1995. Members of the Sp transcription factor family control transcription from the uteroglobin promoter. J. Biol. Chem. **270:**12737–12744.
18. **Drozdoff, V., and W. J. Pledger.** 1993. Commitment to differentiation and expression of early differentiation markers in murine keratinocytes in vitro are regulated independently of extracellular calcium concentrations. J. Cell Biol. **123:**909–919.
19. **Faus, I., H. J. Hsu, and E. Fuchs.** 1994. Oct-6: a regulator of keratinocyte gene expression in stratified squamous epithelia. Mol. Cell. Biol. **14:**3263–3275.
20. **Fuchs, E., and K. Weber.** 1994. Intermediate filaments: structure, dynamics, function, and disease. Annu. Rev. Biochem. **63:**345–382.
21. **Gaubatz, S., A. Imhof, R. Dosch, O. Werner, P. Mitchell, R. Buettner, and M. Eilers.** 1995. Transcriptional activation by Myc is under negative control by the transcription factor AP-2. EMBO J. **14:**1508–1519.
22. **Getman, D. K., A. Mutero, K. Inoue, and P. Taylor.** 1995. Transcription factor repression and activation of the human acetylcholinesterase gene. J. Biol. Chem. **270:**23511–23519.
23. **Green, H.** 1977. Terminal differentiation of cultured human epidermal cells. Cell **11:**405–416.
24. **Hagen, G., S. Muller, M. Beato, and G. Suske.** 1994. Sp1-mediated transcriptional activation is repressed by Sp3. EMBO J. **13:**3843–3851.
25. **Hasan, R., M. K. Alam, and R. Ali.** 1995. Polyamine induced Z-conformation of native calf thymus DNA. FEBS Lett. **368:**27–30.
26. **Hu, L., and L. J. Gudas.** 1994. Activation of keratin 19 gene expression by a 3′ enhancer containing an AP1 site. J. Biol. Chem. **269:**183–191.
27. **Huff, C. A., S. H. Yuspa, and D. Rosenthal.** 1993. Identification of control elements 3′ to the human keratin 1 gene that regulate cell type and differentiation-specific expression. J. Biol. Chem. **268:**377–384.
28. **Imagawa, M., R. Chiu, and M. Karin.** 1987. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell **51:**251–260.
29. **Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian.** 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell **51:**1079–1090.
30. **Kadonaga, J. T., and R. Tjian.** 1986. Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA **83:**5889–5893.
31. **Kolega, J., M. Manabe, and T.-T. Sun.** 1989. Basement membrane heterogeneity and variation in corneal epithelial differentiation. Differentiation **42:**54–63.
32. **Leask, A., C. Byrne, and E. Fuchs.** 1991. Transcription factor AP2 and its role in epidermal-specific gene expression. Proc. Natl. Acad. Sci. USA **88:**7948–7952.
33. **Leask, A., M. Rosenberg, R. Vassar, and E. Fuchs.** 1990. Regulation of a human epidermal keratin gene: sequences and nuclear factors involved in keratinocyte-specific transcription. Genes Dev. **4:**1985–1998.
34. **Leggett, R. W., S. A. Armstrong, D. Barry, and C. R. Mueller.** 1995. Sp1 is phosphorylated and its DNA binding activity down-regulated upon terminal differentiation of the liver. J. Biol. Chem. **270:**25879–25884.
35. **Ljubimov, A. V., R. E. Burgeson, R. J. Butkowski, A. F. Michael, T.-T. Sun, and M. C. Kenney.** 1995. Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms. Lab. Invest. **72:**461–473.
36. **Luscher, B., P. J. Mitchell, T. Williams, and R. Tjian.** 1989. Regulation of transcription factor AP-2 by the morphogen retinoic acid and by second messengers. Genes Dev. **3:**1507–1517.
37. **Moll, R., W. W. Franke, D. L. Schiller, B. Geiger, and R. Krepler.** 1982. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell **31:**11–24.
38. **Murata, Y., H. G. Kim, K. T. Rogers, A. J. Udvadia, and J. M. Horowitz.** 1994. Negative regulation of Sp1 trans-activation is correlated with the binding of cellular proteins to the amino terminus of the Sp1 trans-activation domain. J. Biol. Chem. **269:**20674–20681.
39. **Navarro, J. M., J. Casatorres, and J. L. Jorcano.** 1995. Elements controlling the expression and induction of the skin hyperproliferation-associated keratin K6. J. Biol. Chem. **270:**21362–21367.
40. **Panagiotidis, C. A., S. Artandi, K. Calame, and S. J. Silverstein.** 1995. Polyamines alter sequence-specific DNA-protein interactions. Nucleic Acids Res. **23:**1800–1809.
41. **Pankov, R., N. Neznanov, A. Umezawa, and R. G. Oshima.** 1994. AP-1, ETS, and transcriptional silencers regulate retinoic acid-dependent induction of keratin 18 in embryonic cells. Mol. Cell. Biol. **14:**7744–7757.
42. **Piette, J., S. Hirai, and M. Yaniv.** 1988. Constitutive synthesis of activator protein 1 transcription factor after viral transformation of mouse fibroblasts. Proc. Natl. Acad. Sci. USA **85:**3401–3405.
43. **Prendergast, G. C., D. Lawe, and E. B. Ziff.** 1991. Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. Cell **65:**395–407.
44. **Roop, D. R., S. Chung, C. Cheng, P. M. Steinert, R. Sinha, S. H. Yuspa, and D. S. Rosenthal.** 1989. Epidermal differentiation and its modulation by retinoids. Karger, Basel, Switzerland.
45. **Rossi, P., G. Karsenty, A. B. Roberts, N. S. Roche, M. B. Sporn, and B. de Crombrugghe.** 1988. A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor-beta. Cell **52:**405–414.
46. **Rothnagel, J. A., D. A. Greenhalgh, T. A. Gagne, M. A. Longley, and D. R. Roop.** 1993. Identification of a calcium-inducible, epidermal-specific regulatory element in the 3′-flanking region of the human keratin 1 gene. J. Invest. Dermatol. **101:**506–513.
47. **Schermer, A., S. Galvin, and T.-T. Sun.** 1986. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J. Cell Biol. **103:**49–62.
48. **Schermer, A., J. V. Jester, C. Hardy, D. Milano, and T.-T. Sun.** 1989. Transient synthesis of K6 and K16 keratins in regenerating rabbit corneal epithelium: keratin markers for an alternative pathway of keratinocyte differentiation. Differentiation **42:**103–110.
49. **Sun, T.-T., S. C. Tseng, A. J. Huang, D. Cooper, A. Schermer, M. H. Lynch, R. Weiss, and R. Eichner.** 1985. Monoclonal antibody studies of mammalian epithelial keratins: a review. Ann. N. Y. Acad. Sci. **455:**307–329.
50. **Thomas, T., M. A. Gallo, C. M. Klinge, and T. J. Thomas.** 1995. Polyamine-mediated conformational perturbations in DNA alter the binding of estrogen receptor to poly(dG-m5dC) · poly(dG-m5dC) and a plasmid containing the estrogen response element. J. Steroid Biochem. Mol. Biol. **54:**89–99.
51. **Thomas, T., and D. T. Kiang.** 1988. Modulation of the binding of progesterone receptor to DNA by polyamines. Cancer Res. **48:**1217–1222.
52. **Thomas, T. J., U. B. Gunnia, and T. Thomas.** 1991. Polyamine-induced B-DNA to Z-DNA conformational transition of a plasmid DNA with (dG-dC)n insert. J. Biol. Chem. **266:**6137–6141.
53. **Thomas, T. J., and T. Thomas.** 1994. Polyamine-induced Z-DNA conformation in plasmids containing (dA-dC)n · (dG-dT)n inserts and increased binding of lupus autoantibodies to the Z-DNA form of plasmids. Biochem. J. **298:**485–491.
54. **Weiss, R. A., R. Eichner, and T.-T. Sun.** 1984. Monoclonal antibody analysis of keratin expression in epidermal diseases: a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes. J. Cell Biol. **98:**1397–1406.
55. **Williams, T., A. Admon, B. Luscher, and R. Tjian.** 1988. Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. Genes Dev. **2:**1557–1569.
56. **Wu, R.-L., T.-T. Chen, and T.-T. Sun.** 1994. Functional importance of an Sp1- and an NFκB-related nuclear protein in a keratinocyte-specific promoter of rabbit K3 keratin gene. J. Biol. Chem. **269:**28450–28459.
57. **Wu, R.-L., S. Galvin, S. K. Wu, C. Xu, M. Blumenberg, and T.-T. Sun.** 1993. A 300 bp 5′-upstream sequence of a differentiation-dependent rabbit K3 keratin gene can serve as a keratinocyte-specific promoter. J. Cell Sci. **105:**303–316.
58. **Wu, R.-L., G. Zhu, S. Galvin, C. Xu, T. Haseba, C. Chaloin-Dufau, D. Dhouailly, Z. G. Wei, R. M. Lavker, W. Y. Kao, et al.** 1994. Lineage-specific and differentiation-dependent expression of K12 keratin in rabbit corneal/limbal epithelial cells: cDNA cloning and northern blot analysis. Differentiation **55:**137–144.