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Interdependent Transcription Control Elements Regulate the Expression of the *SPRR2A* Gene during Keratinocyte Terminal Differentiation

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Expression of the *SPRR2A* gene, a member of the small proline-rich family of cornified cell envelope precursor proteins, is strictly linked to keratinocyte terminal differentiation both in vivo and in vitro. In this study, we explored the molecular mechanisms underlying this regulation in transiently transfected primary keratinocytes induced to differentiate in vitro. Deletion mapping and site-directed mutagenesis of *SPRR2A* promoter-chloramphenicol acetyltransferase constructs indicate that four transcription control elements are essential and sufficient for promoter activity. These elements were further characterized by electrophoretic mobility shift and identified as (i) an inverted octamer doublet, bound by the POU domain factor Oct-11 (Skn-1a/i, Epoc-1), (ii) an interferon-stimulated response element recognized by interferon regulatory factors 1 and 2, (iii) an Ets binding site partially overlapping the interferon-stimulated response element, and (iv) a TG box recognized by the Sp1 family of zinc finger transcription factors. Destruction of a single terminal differentiation element is sufficient to completely abolish transcription from the *SPRR2A* promoter, indicating that these transcription control elements function in concert in an interdependent manner. Apparently, integration of signals transmitted by the above-mentioned transcription factors is necessary and sufficient to promote gene expression during keratinocyte terminal differentiation.

Keratinocyte differentiation is a multistep process which can be recognized in vivo by the presence of the different epidermal layers of the skin. Proliferating keratinocytes are attached to the basement membrane, while the cells committed to terminal differentiation migrate through the suprabasal layers to the surface of the skin. In this differentiation process, cellular hallmarks are the changes in the keratin filaments, the appearance of keratohyalin granules, and the formation of a cornified cell envelope (CE). Although the morphological aspects of keratinocyte differentiation are well described (for a recent review, see reference 31), the molecular mechanisms involved in the orchestration of this process are still poorly understood (7).

Two major experimental approaches have been developed to study keratinocyte terminal differentiation in vitro (reviewed in reference 42). Varying the concentration of calcium ions in the culture medium has been used to regulate the ability of keratinocyte cultures to stratify and engage into terminal differentiation (26). At low concentrations of extracellular calcium, desmosome formation is inhibited and stratification is impaired (64). Downregulation of integrin expression by cadherins appears to play an essential role in calcium-induced stratification (28). Inhibition of integrin function and expression is intimately linked to the initiation of keratinocyte terminal differentiation (2, 32). When stratified cultures of human keratinocytes are incubated in low-calcium medium (<0.1 mM), the suprabasal layers can be stripped off, leaving a monolayer of basal cells (35, 49). When such monolayers are refed with standard medium (1.8 mM calcium), keratinocytes will start to proliferate and will reconstitute a multilayer in which suprabasal cells express terminal differentiation markers (e.g., involucrin), whereas integrin expression is confined to the basal layer (28). We will refer to this approach as the stratification assay.

In another experimental system, terminal differentiation is induced by placing keratinocytes in single-cell suspension in medium made viscous by the addition of methylcellulose (suspension assay) (1, 18). As a result of an inhibition of cell-cell and cell-substratum contacts, cells withdraw irreversibly from the cell cycle and become committed to terminal differentiation within 5 h. By 24 h, most cells express terminal differentiation markers (9). Suspension-induced differentiation can be inhibited by addition of extracellular matrix proteins or antibodies raised against integrins (1). In contrast to the induction of stratification, suspension-induced differentiation can occur in low-calcium medium (18). In this study, we used both methods to study the regulation of the *SPRR2A* gene during keratinocyte terminal differentiation.

Originally isolated as UV-regulated genes (38), *SPRR* genes are induced during squamous differentiation both in vivo and in vitro (14, 37, 38). The gene family maps to human chromosome 1q21 and consists of two *SPRR1* genes (*SPRR1A* and *SPRR1B*), eight *SPRR2* genes (*SPRR2A* to *SPRR2H*), and one *SPRR3* gene, all clustered on a 300-kb DNA fragment (13). The *SPRR* locus is near several other keratinocyte differentiation genes such as those encoding loricrin, involucrin, profilaggrin, and trichohyalin and the S100 family of calcium-binding proteins (25, 55, 61).

The direct isolation of cross-linked SPRR peptides from purified human CE has firmly established SPRR proteins (other denominations are cornifin and pancornulin) as a new class of CE precursor proteins (57), thus confirming previous conclusions based both on theoretical (4) and experimental evi-

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dence (17, 29, 44). Recently we showed that *SPRR* genes are differentially regulated in various stratified epithelia (29), suggesting that the *SPRR* gene family has evolved to serve a highly specialized function, which might be directly related to the flexibility characteristics of cornified layers in squamous epithelia; varying the expression of the different classes of SPRR proteins might constitute an efficient mechanism of changing the physical characteristics of the CE (57). In this respect, it is interesting that *SPRR* expression is induced in response to epidermal injury (e.g., by UV light) (14, 38) and inflammatory mediators such as interleukin-1 (68) and gamma interferon (53) and in hyperproliferative (psoriasis) (8, 29) and keratinization (epidermolytic hyperkeratosis) disorders (34). *SPRR* expression is also increased during the normal aging process of keratinocytes in vivo (11) and in vitro (54).

Consequently, studying *SPRR* gene expression will allow the identification of transcription factors and regulatory processes involved in both tissue specific regulation of keratinocyte terminal differentiation and the modulation of this process by natural, accidental, and disease-related causes. In this report, we describe the identification and characterization of four terminal differentiation elements (TDEs) in the promoter region of the *SPRR2A* gene and show that these regulatory sequences function coherently as interdependent transcription control elements during in vitro-induced terminal differentiation.

MATERIALS AND METHODS

Cell culture. Primary cultures of human epidermal keratinocytes were initiated in complete medium as described by Rheinwald and Green (50), with minor modifications as specified by Ponec et al. (48). Keratinocytes were isolated from foreskin derived from circumcision and grown in the presence of a layer of lethally ^{137}Cs-irradiated mouse 3T3 fibroblasts. For experiments described here, passage 3 cultures were used. Culture in serum-free medium (KSFM; GIBCO) was initiated at the first passage of the primary keratinocytes and maintained in KSFM for two to three passages prior to experiments.

Transient transfections and CAT assay. (i) Stratification protocol. Prior to transfection, keratinocytes were cultured in complete medium until confluency. Medium was subsequently changed to Dulbecco modified Eagle medium (DMEM) lacking Ca^{2+} (DMEM −Ca) supplemented with 5% Chelex-treated calf serum (strip medium) for 2 days. At this stage, most cells already committed to terminal differentiation detach from the culture dish and can be easily removed by rinsing with the culture medium. For transfection, monolayers on 60-mm-diameter tissue culture plates were washed with DMEM −Ca, and 5 μg of chloramphenicol acetyltransferase (CAT) reporter DNA was incubated in 500 μl of DMEM −Ca with 25 μg of DOTAP (Boehringer Mannheim) for 20 min. After 1:1 dilution in DMEM −Ca, the DNA-liposome mixture was added to the cells, which were incubated for 2 h with occasional rocking of the culture dish. After addition of 4 ml of strip medium, dishes were incubated for a further 4 h. Monolayers were then washed with phosphate-buffered saline (PBS) and either maintained in strip medium or induced to stratify in complete medium lacking growth factors (1.8 mM Ca^{2+}) for 36 to 48 h.

(ii) Suspension protocol. Prior to experiments, keratinocytes were grown for at least two passages in KSFM (GIBCO) containing 0.09 mM Ca^{2+}. At 70% confluency, cells were transfected with 5 μg of CAT reporter DNA and 25 μg of DOTAP (Boehringer Mannheim) in 1 ml of DMEM −Ca and incubated as described above. Cells were then disaggregated with trypsin (0.25%) and placed as a single-cell suspension in low-calcium medium without serum (DMEM containing 0.1 mM Ca^{2+}), which was made viscous by the addition of 1.75% methylcellulose (1, 18, 64). Incubation was for 24 h (or as specified) at 37°C in a CO_2 incubator. Cells were recovered by dilution of the viscous solution 10-fold with PBS and centrifugation at 2,000 rpm for 10 min. Lysates were prepared as described by Gorman et al. (16), and CAT activity was measured by a Fluor Diffusion CAT assay (46), using Econofluor II premixed scintillation fluid (NEN Research Products) and [^{14}C]acetyl coenzyme A (Amersham) as specified by NEN Research Products. Results are expressed as counts per minute transferred to the organic solvent phase per minute of reaction time in the linear range of the reaction.

Internal controls in transfection experiments. In several experiments in which a mutant promoter construct was directly compared with the wild-type construct (pSG-2), the wild-type promoter, cloned into the luciferase assay plasmid pGL2-Basic (Promega), was used as an internal control (pSG2-*luc*). One microgram of luciferase plasmid (wild type) and 4 μg of CAT plasmid (wild type or mutant) were mixed prior to transfection and processed as described above. The same cell lysates were used for both the CAT and luciferase assays, with the only difference that the aliquot used for luciferase determination was not heat treated. The luciferase activity was determined in a Berthold Lumat luminometer by using the Promega luciferase assay system. Corrections, made by taking into account the internal control, did not significantly change the results obtained.

RNA isolation and Northern (RNA) blotting. RNA isolation and Northern blotting were done as described previously (5). Hybridization with specific probes was done as described in reference 14.

DNA manipulations. All plasmids used for transfections were maintained in *Escherichia coli* DH5α. DNA cloning procedures were essentially as described by Sambrook et al. (52). Deletions in the wild-type promoter (−1500 to +14) (14) were made either by using *Bal* 31 exonuclease digestion or by PCR. Point mutants were made in pBluescript vectors by the method of Kunkel (40), after which the insert was cloned back into the CAT reporter vector. All deletions and mutations were confirmed by DNA sequencing. Plasmid preparations for transfection were purified on Qiagen columns as specified by the manufacturer.

Nuclear extracts and EMSA. Nuclear extracts were prepared from keratinocytes grown to confluency in standard medium, using a mini-extract procedure (56). Electrophoretic mobility shift assays (EMSAs; band shifts) with octamer-binding proteins were performed in a 20-μl volume containing 3 to 5 μg of nuclear extract in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–60 mM KCl–1 mM dithiothreitol (DTT)–0.5 mM EDTA–4% Ficoll 400–1 mM phenylmethylsulfonyl fluoride with 2 μg of poly(dI-dC) and 20 fmol of end-labeled double-stranded oligonucleotide. After 30 min of incubation at room temperature, the mixture was subjected to electrophoresis on a 4% polyacrylamide–22.5 mM Tris-borate (pH 7.9)–0.625 mM EDTA–2% glycerol gel for 90 min at 10 V/cm. Interferon-stimulated response element (ISRE)-binding proteins were assayed in a 20-μl volume containing 5 μg of nuclear extract in 20 mM Tris-HCl (pH 6.8)–50 mM NaCl–1 mM DTT–20% glycerol–0.2 mM EDTA–0.25 mg of bovine serum albumin per ml with 1 μg of poly(dI-dC) and 20 fmol of end-labeled double-stranded oligonucleotide. After 30 min of incubation at room temperature, the mixture was subjected to electrophoresis on a 6% polyacrylamide–25 mM Tris–190 mM glycine–1 mM EDTA–2.5% glycerol gel for 90 min at 10 V/cm. TG-box-binding proteins were detected by incubating 5 μg of nuclear extract in 5 mM HEPES (pH 7.9)–25 mM NaCl–500 μM $ZnCl_2$–1 mM DTT–500 μM EDTA–10% glycerol with 1 μg of poly(dI-dC) and 20 fmol of end-labeled double-stranded oligonucleotide. After 30 min of incubation at room temperature, the mixture was subjected to electrophoresis on a 4% polyacrylamide–25 mM Tris–190 mM glycine–1 mM EDTA–2.5% glycerol gel for 90 min at 10 V/cm.

Oligonucleotides were labeled with T4 polynucleotide kinase, purified by denaturing gel electrophoresis and reverse-phase chromatography, and subsequently annealed to the complementary strand. Sequences of the wild-type oligonucleotides are as follows: wt1-d (octamer doublet), 5′ ATCCTGCTCATGC CAGTCATGGATAAATTTGCATCTGGCT/5′ AGCCAGATGCAAATTTAT CCATGACTGGCATGAGCAGGAT; wt1-s (octamer singlet), 5′ GGATAAA TTTGCATCTGGCT/5′ AGCCAGATGCAAATTTATCC; wt2/3 (ISRE/Ets site), 5′ GGGTAGTTTCACTTCCTGCTG/5′ CAGCAGGAAGTGAAACTAC CC; and wt4 (TG box), 5′ CCTGCTGGGTGGGGTAGCAGGCTCTA/5′ TA GAGCCTGCTACCCCACCCAGCAGG.

Oct-1-specific antibody YL15 was a generous gift of W. Herr (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); Oct-2 (C-20), Skn-1a/i (C-20), early growth response protein 1 (Egr-1) (588), and Wilms' tumor suppressor protein 1 (WT1) (C-19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif.); Oct-6 antibody was a gift of D. Meyer (Erasmus University, Rotterdam, The Netherlands); interferon response factor 1 (IRF-1) and IRF-2 antibodies were a gift of T. Taniguchi (Osaka University, Osaka, Japan); Sp1, Sp3, and Sp4 antibodies were a gift of G. Hagen and S. Philipsen (Erasmus University).

RESULTS

We have previously shown that a DNA fragment from the *SPRR2A* (formerly called *spr2-1*) promoter spanning positions −1500 to +14 can direct CAT expression in keratinocytes in response to inducers of terminal differentiation such as calcium ions or phorbol ester (tetradecanoyl phorbol acetate [TPA]) and UV irradiation (14).

Confluent cultures of keratinocytes were switched to medium lacking Ca^{2+} to remove the differentiated cells (stripping) and were subsequently induced to stratify by changing to medium containing 1.8 mM Ca^{2+}. The kinetics of mRNA induction were examined on a Northern blot (Fig. 1). In a parallel experiment, stripped monolayers of basal keratinocytes were transiently transfected with an *SPRR2A* promoter CAT construct (pSG-2 [14]), after which stratification was induced (see Materials and Methods). CAT activity was measured at different time intervals (Fig. 1). Comparison of the results of both experiments reveals identical kinetics of induc-

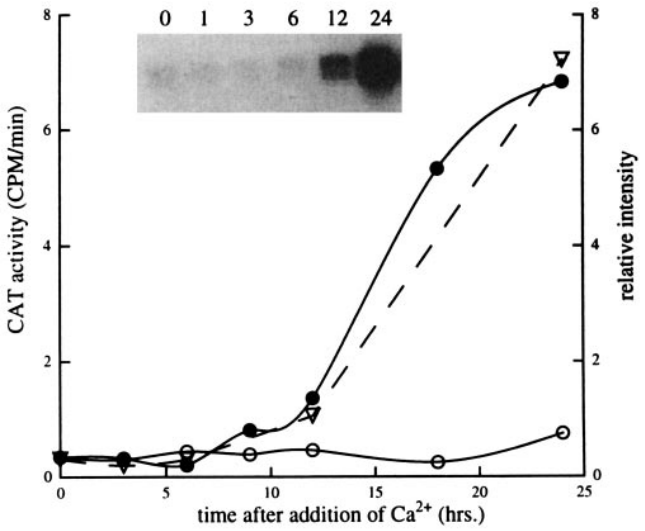

FIG. 1. For induction of *SPRR2* mRNA during keratinocyte stratification, keratinocytes were cultured in complete medium to confluency and incubated in medium without calcium to remove the differentiated cells. Monolayers were then incubated in fresh medium containing 1.8 mM calcium and lacking growth factors to regenerate a stratified culture. Total RNA was isolated at different times (see insert) after the onset of stratification. Twenty micrograms of RNA was loaded in each lane; the Northern blot was probed with an *SPRR2*-specific probe and quantified with a Betascope 603 (Betagen) (triangles). For induction of *SPRR2A* promoter-CAT construct pSG-2 during keratinocyte stratification, monolayers of keratinocytes (see above) were transfected with pSG-2 and subsequently induced to differentiate by transfer to medium with 1.8 mM calcium (closed circles) or maintained in medium lacking calcium (open circles). After 3, 6, 9, 12, 18, and 24 h, lysates were prepared and CAT activity was measured.

tion, indicating that the regulation of the *SPRR2A* gene occurs mainly at the transcriptional level. These results prompted us to determine the regulatory elements in the *SPRR2A* promoter responsible for this induction.

A 150-bp promoter fragment is necessary and sufficient for *SPRR2A* induction and contains several regulatory elements. Successive deletions from the 5′ end of the 1,500-bp promoter fragment were transfected into stripped keratinocyte cultures, and CAT activity was determined 40 h after the medium was changed to complete medium lacking growth factors. The shortest construct still fully inducible, pSG-55, contains a 150-bp promoter fragment which can drive the CAT reporter gene to the same extent as the 1,500-bp fragment (Fig. 2A). Construct pSG-56, with a promoter deletion to position −106, does not have promoter activity above that of the empty CAT vector pBA-CAT (Fig. 2A). The kinetics of induction of construct pSG-55 are the same as in Fig. 1 (data not shown).

Examination of the sequence in the region present in construct pSG-55 and absent in pSG-56 reveals a consensus octamer box (5′ ATTTGCAT) (66) between positions −110 and −103. Destruction of this sequence by insertion of 8 bp at position −107 (pSG-48) completely inactivates the promoter activity of the 1,500-bp fragment (Fig. 2B). To determine whether the octamer box is the only regulatory element needed for *SPRR2A* promoter activity, deletion mutants of the region between −97 and −34 (clone pSG-93) and the region between −51 and −34 (clone pSG-128) were tested in transient transfections (Fig. 2B). Both clones had lost their inducibility. This inactivation was not due to a change in the spacing between the octamer and the TATA box, as restitution of the wild-type distance with polylinker DNA did not result in restitution of promoter activity (clones pSG-110 and pSG-132 in Fig. 2B). A construct with a 26-bp deletion in the *SPRR2A* promoter (−96 to −71) replaced with a 25-bp polylinker (pSG-155) completely retained its inducibility compared with the wild-type pSG-2 clone. These results suggest that besides the octamer box at position −110, additional regulatory elements are present in the region between positions −71 and −34 of the *SPRR2A* promoter.

***SPRR2A* promoter activity is independent on the experimental approach used to induce keratinocyte terminal differentiation.** To confirm that the promoter activity that we measure is indeed directly related to keratinocyte terminal differentiation and not due to an irrelevant calcium response or the result of the proliferation step which precedes the onset of terminal differentiation in the stratification assay, we repeated the experiments described above but induced terminal differentiation by placing transfected cells in single-cell suspension into low-calcium (0.1 mM) medium made viscous by the addition of methylcellulose (18). In this experimental system, irreversible withdrawal from the cell cycle and commitment to terminal differentiation is achieved within 5 h as a result of inhibition of cell-cell and cell-substratum contacts (1, 9, 18, 33). The data presented in Fig. 3, 4, and 5C show that the two experimental approaches yield very similar results, indicating that the *SPRR2A* promoter activity which we measure is indeed a direct result of the induction of keratinocyte terminal differentiation in both in vitro systems.

Mutational analysis of the −71 to −34 promoter region. Examination of the promoter sequence between −65 and −50 reveals a sequence similar to an ISRE (47). Four different mutations in this region all rendered the minimal promoter unresponsive after induction of terminal differentiation (Fig. 4A). The mutation in construct pSG-212, however, should not disrupt the ISRE since this construct resembles the consensus ISRE to an even higher extent than the wild-type sequence (Fig. 4A). We will show later that the mutant phenotype of pSG-212 is due to a mutation in an Ets binding site which partially overlaps the ISRE (see Fig. 6).

Deletion or substitution of the segment between positions −51 and −34 by foreign DNA inactivates the promoter (Fig. 2B, clones pSG-128 and pSG-132). Constructs pSG-149 and pSG-150 each contain specific point mutations in the region from −51 to −34 while keeping the rest of the minimal promoter intact. Construct pSG-149 has completely lost its inducibility, while pSG-150 has retained wild-type expression (Fig. 4B). The sequence mutated in pSG-149 consists of a direct repeat of the sequence 5′ TGGG, which resembles the sequences bound by the zinc finger proteins Sp1, Egr-1, and WT1 (12, 19, 21, 36).

The octamer box is part of an imperfect palindrome recognized by Oct-11. We have shown above that a sequence matching the consensus of an octamer box is essential for *SPRR2A* expression. Examination of the region flanking this element revealed that this sequence is part of an imperfect palindrome of two octamer boxes arranged in a head-to-head fashion (octamer doublet) (Fig. 5C). In an EMSA with the double-stranded probe wt1-d, which contains the octamer doublet, several specific complexes were observed after incubation with keratinocyte nuclear extracts (Fig. 5A). Antibodies raised against different octamer-binding proteins (the POU family of transcription factors) were used to elucidate the nature of these complexes. The uppermost complex reacted with a monoclonal antibody against Oct-1, while Oct-2 and Oct-6 antibodies did not seem to react with proteins present in the bandshift. The Oct-11/Skn-1 (3, 15) antibody reacted with a complex just below the Oct-1 complex. The mobility of the Oct-11 complex is slower than expected for a monomeric Oct-11–DNA complex but would be consistent with that of a

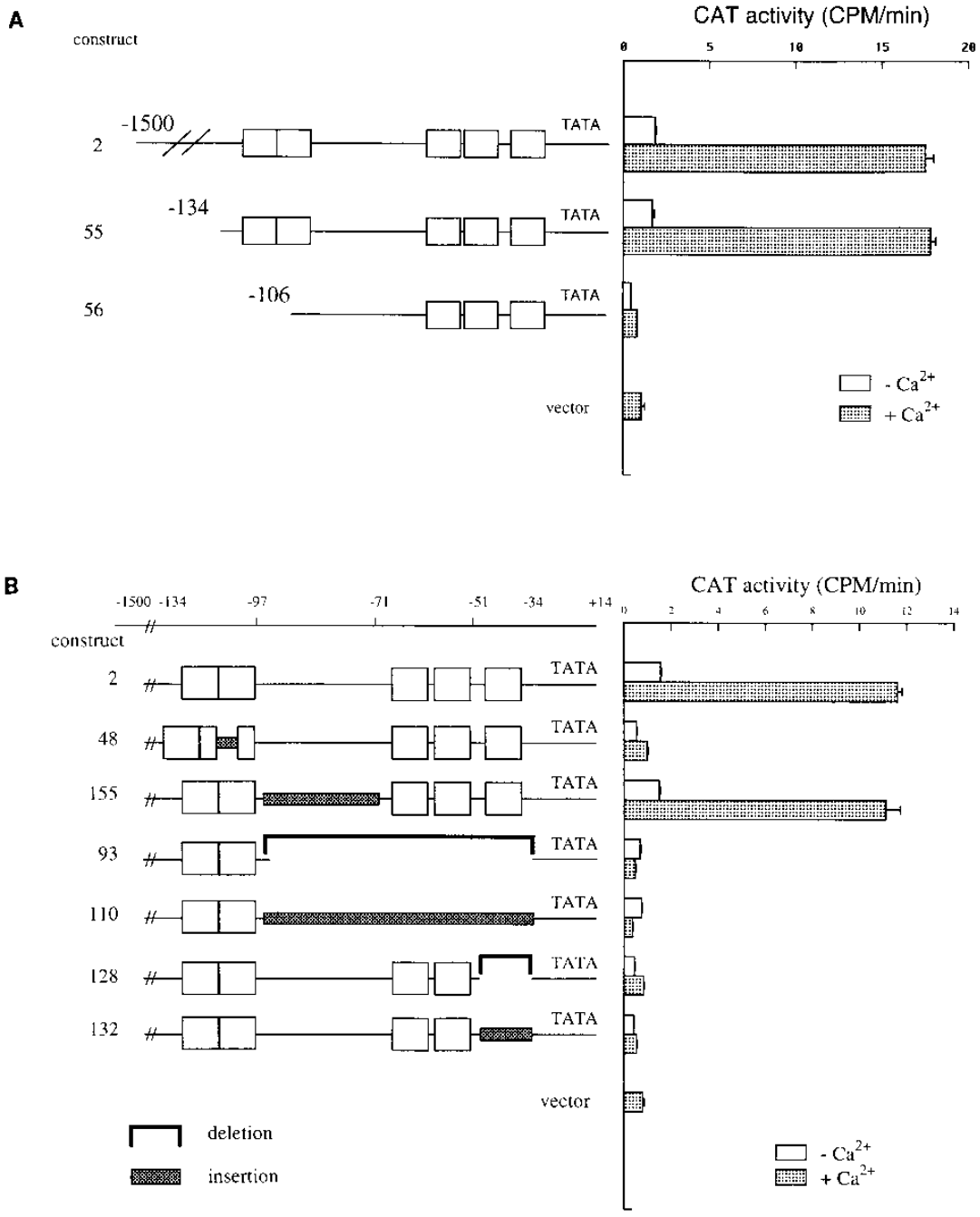

FIG. 2. Deletion analysis of the *SPRR2A* promoter. Constructs with 5′ deletions (A) or clones with internal promoter deletions (B) were transfected into keratinocytes, and CAT activity was assayed 40 h after induction of stratification. Results are representative for five to seven individual experiments. Each error bar represents the standard deviation of the reaction rate for a single measurement. CAT activity is represented as counts per minute of acetylated chloramphenicol transferred to the organic phase per minute in the linear phase of the reaction.

dimeric complex. Competition with excess unlabeled wt1-d reflects the specificity of both the Oct-1 and the Oct-11 complexes (Fig. 5A). Interestingly, for complete competition of the Oct-11 complex, a much higher concentration of competitor DNA is required (Fig. 5B, left panel). When oligonucleotide wt1-s, which contains an octamer singlet, is used as a competitor, only the Oct-1 complex, not the Oct-11 complex, can be competed for (Fig. 5B, right panel). Furthermore, the Oct-11 complex was never observed with wt1-s as a probe, whereas binding of Oct-1 is as efficient to the singlet as to the doublet oligonucleotide (Fig. 5B). Apparently, Oct-1 requires only a single octamer box for DNA binding, whereas Oct-11 requires the doublet site. As this finding provides a way to verify which of these POU factors is involved in *SPRR2A* expression, point mutations were created in both octamer boxes. Since POU proteins are bipartite DNA-binding proteins recognizing two half-sites in the octamer sequence (the A/T-rich half-site is recognized by the POU homeodomain, and the other half-site

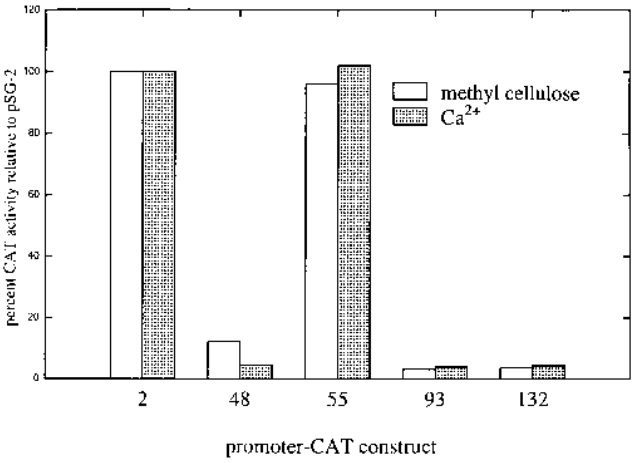

FIG. 3. Direct comparison of *SPRR2A* promoter activity as measured by the stratification or the suspension assay. Transient transfections were performed as described in Materials and Methods. In both assays, CAT activity was measured 40 h after the induction of differentiation. Activities of several promoter constructs relative to that of the original 1,500-bp promoter construct pSG-2 are depicted.

is recognized by the POU-specific domain [27]), we created four mutants, one in each half-site. All four mutants resulted in 50 to 70% loss of activity (Fig. 5C). Apparently both octamer elements are equally important for *SPRR2A* expression, indicating that Oct-11 (but not Oct-1) is involved in the up-regulation of this gene.

The ISRE overlaps an Ets binding site. An EMSA of keratinocyte nuclear extracts with oligonucleotide wt2/3 containing the putative ISRE (−68 to −48) shows three complexes (Fig. 6A). All three complexes were competed for by the oligonucleotide with the wild-type sequence, which demonstrates their specificity, but different results were obtained when competition was performed with the four mutant oligonucleotides (see Fig. 4A for sequences). The two upper complexes were competed for by oligonucleotides m211 (partial) and m212 but not by oligonucleotides m148 and m210. The lower complex was competed for by m148 and m210 but not by the two other mutant sequences. The two upper and the lower complexes thus seem to have overlapping yet nonidentical binding sites. The identities of the different complexes were further examined with antisera raised against IRF-1 and IRF-2, two transcription factors known to bind ISRE sequences (22). The results presented in Fig. 6B show that the two upper complexes contain IRF-1 (band 1) and IRF-2 (band 2). In addition, especially the IRF-1 complex is induced in keratinocytes treated with gamma interferon (Fig. 6C), and both the IRF-1 and IRF-2 complexes are competed for by an oligonucleotide containing the HLA-A2 ISRE (39) (Fig. 6A).

Examination of the 3′ part of the ISRE reveals the tetranucleotide 5′ TTCC, which is the core sequence recognized by the Ets transcription factor family (reviewed in reference 63). Because this sequence is mutated in m211 and m212 (Fig. 4A), we performed a competition experiment with a polyomavirus enhancer PEA3 site (43), which is known to bind several Ets family members. The PEA3 oligonucleotide specifically inhibits the formation of the lower complex (Fig. 6A). Furthermore, when PEA3 is used as a probe, a band shift similar to that for the wt2/3 lower complex is observed with keratinocyte nuclear extracts (Fig. 6B, lane 5). Thus, the sequence from −68 to −48 in the *SPRR2A* promoter contains both an ISRE to which IRF-1 and IRF-2 can bind and an Ets binding site to which an as yet unidentified protein binds.

The TGGG repeat is recognized by a Zn^{2+}-containing transcription factor. An EMSA of keratinocyte extracts with oligonucleotide wt4 spanning region −54 to −30 shows five Zn^{2+}-dependent complexes that can be competed for by an excess of unlabeled wt4 oligonucleotide and by the m150 but not the m149 oligonucleotide (Fig. 7). Since the sequence mutated in m149 resembles the TG-rich sequences bound by the transcription factors Sp1, WT1, and Egr-1, competition was performed with an oligonucleotide containing the Sp1 binding sites from simian virus 40. This oligonucleotide completely abolished binding of the keratinocyte nuclear proteins to wt4 (Fig. 7A). Antibodies raised against Sp1, Sp3, Sp4, Egr-1, and WT1 were used to identify the complexes in the EMSA. Sp1 and Sp3 antibodies reacted with the uppermost complexes, while Sp4, Egr-1, and WT1 antibodies did not affect the EMSA (Fig. 7B and data not shown). From this analysis, it appears that the two upper complexes correspond to Sp1 and Sp3, respectively, whereas the three lower (major) complexes are due to the binding of three unidentified proteins.

DISCUSSION

We have previously shown that *SPRR* genes are differentially expressed in various squamous epithelia and that in each case expression is strictly confined to cells committed to terminal differentiation (29, 37). In this study, we have identified the regulatory elements in the *SPRR2A* gene responsible for this regulation. Two complementary in vitro approaches were used to induce keratinocyte terminal differentiation. We believe that such a dual approach is necessary to ensure that the regulatory elements which are identified are genuine TDEs and not involved in responses inherent to the use of one particular in vitro assay. The minimal region of the *SPRR2A* promoter required for induction is confined to a 134-bp fragment directly upstream of the transcription start site. In this region we identified four TDEs, all of which were unconditionally required for promoter activity.

TDE-1 contains an octamer doublet, arranged as an imperfect palindrome, in which the central 8 bp (A or T) constitute the two POU homeodomain recognition parts and the outer 4 bp on each side correspond to the POU-specific recognition sequences. Our analysis shows that this duplicated octamer site is specifically recognized by the POU domain transcription factor Oct-11; furthermore, expression studies with promoter constructs with individual mutations in the four different half-sites identify Oct-11 as the POU factor responsible for *SPRR2A* induction. Involvement of Oct-11 in regulating gene expression during keratinocyte terminal differentiation has previously been postulated, as Skn1a/i and Epoc-1 (the homologous factors in rats and mice) are selectively expressed in terminally differentiating epidermis and hair follicles (3, 70). *SPRR2A* constitutes the first example of a human gene for which a direct involvement of Oct-11 in regulation during keratinocyte differentiation has been identified. An interesting observation is that point mutations in a single octamer half-site still have some residual activity (30 to 50%), in contrast to two mutants in which the octamer doublet has been completely destroyed either by linker insertion (pSG48) or by complete deletion (pSG56). This finding suggests that in the octamer doublet, some sequence variability might be allowed, though at the expense of a loss in binding affinity. In this respect, the results of Yukawa et al. (70) are interesting, as these authors identified in the E6/E7 promoter of human papillomavirus a palindromic sequence which is recognized by Epoc-1. This

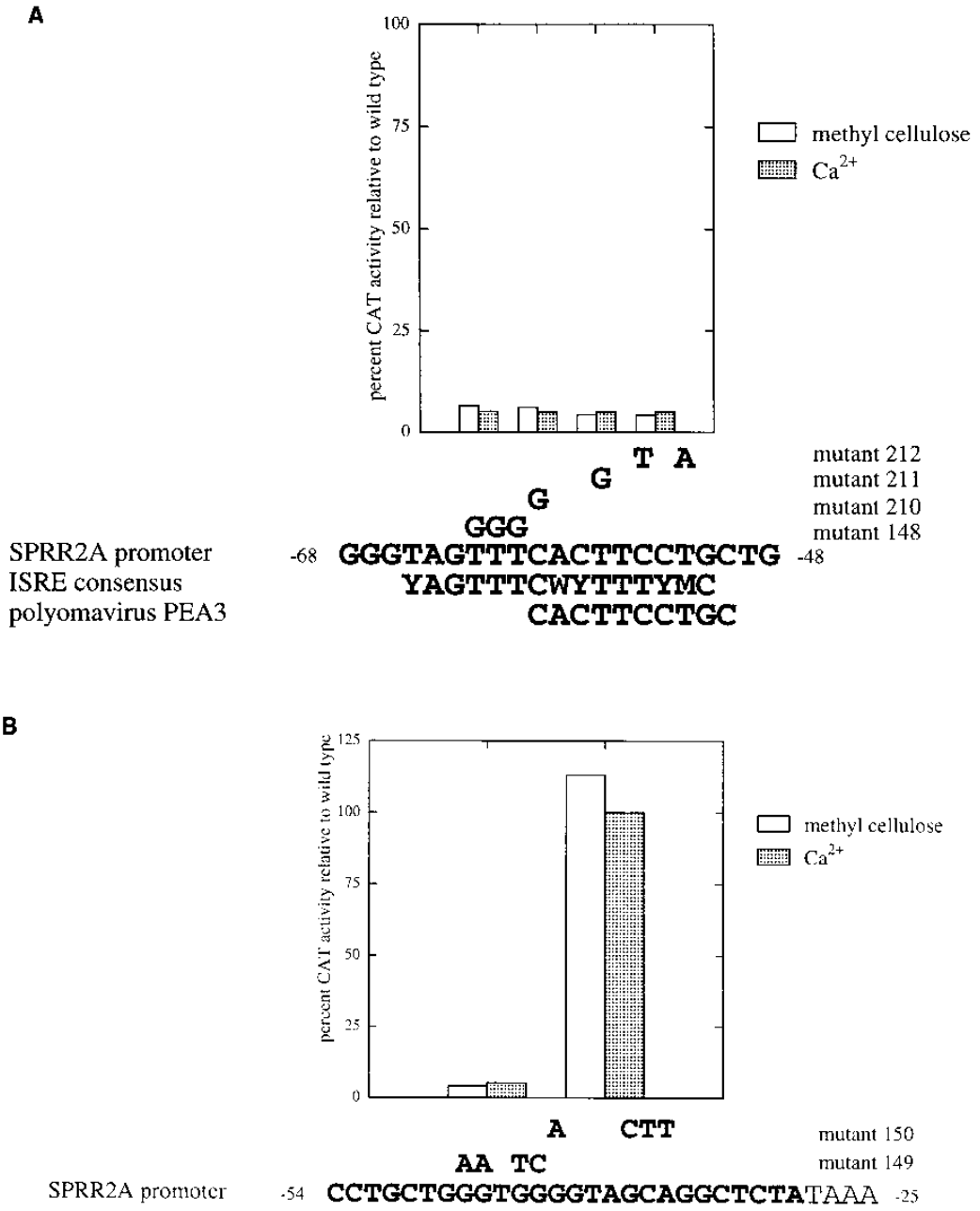

FIG. 4. Mutational analysis of regions from −68 to −48 (A) and −51 to −34 (B) of the *SPRR2A* promoter. The different mutant constructs were tested in both the stratification and the suspension assay. CAT activity is related to that of the wild-type construct pSG-2 which was set at 100%. Code for bases: Y, pyrimidine; W, A or T; M, A or C.

sequence resembles the octamer doublet described here to some extent but has not been recognized as such by the authors. Apparently, because of dimerization and the recognition of a duplicated sequence, the recognition sequence requirements are less stringent for Oct-11 than for the related Oct-1 and Oct-2 factors, which recognize a single octamer sequence (27). In rat skin, Andersen and coworkers (3) have identified two splice variants, Skn-1a and Skn-1i. Skn-1i contains an N-terminal dimerization domain which functions as an inhibitory domain and prevents Skn-1i from binding to a single octamer sequence (3). Skn-1a does not have this domain and can bind to a single octamer site. Taken together with the results presented here, these findings suggest a model whereby a dimer of Skn-1i is able to recognize specifically an octamer doublet, whereas Skn-1a binds as a monomer to the canonical octamer site. It remains to be determined whether the same splice variants are also expressed in human keratinocytes.

TDE-2 conforms to the consensus sequence of an ISRE (47) and is recognized specifically by the transcription factors IRF-1 and IRF-2. Both factors were originally identified as regulators of the alpha/beta interferon system (60). IRF-1 is a transcriptional activator of interferon-regulated genes, whereas IRF-2 functions as an antagonistic repressor of IRF-1 (22, 24). Besides their function in interferon signaling, these transcrip-

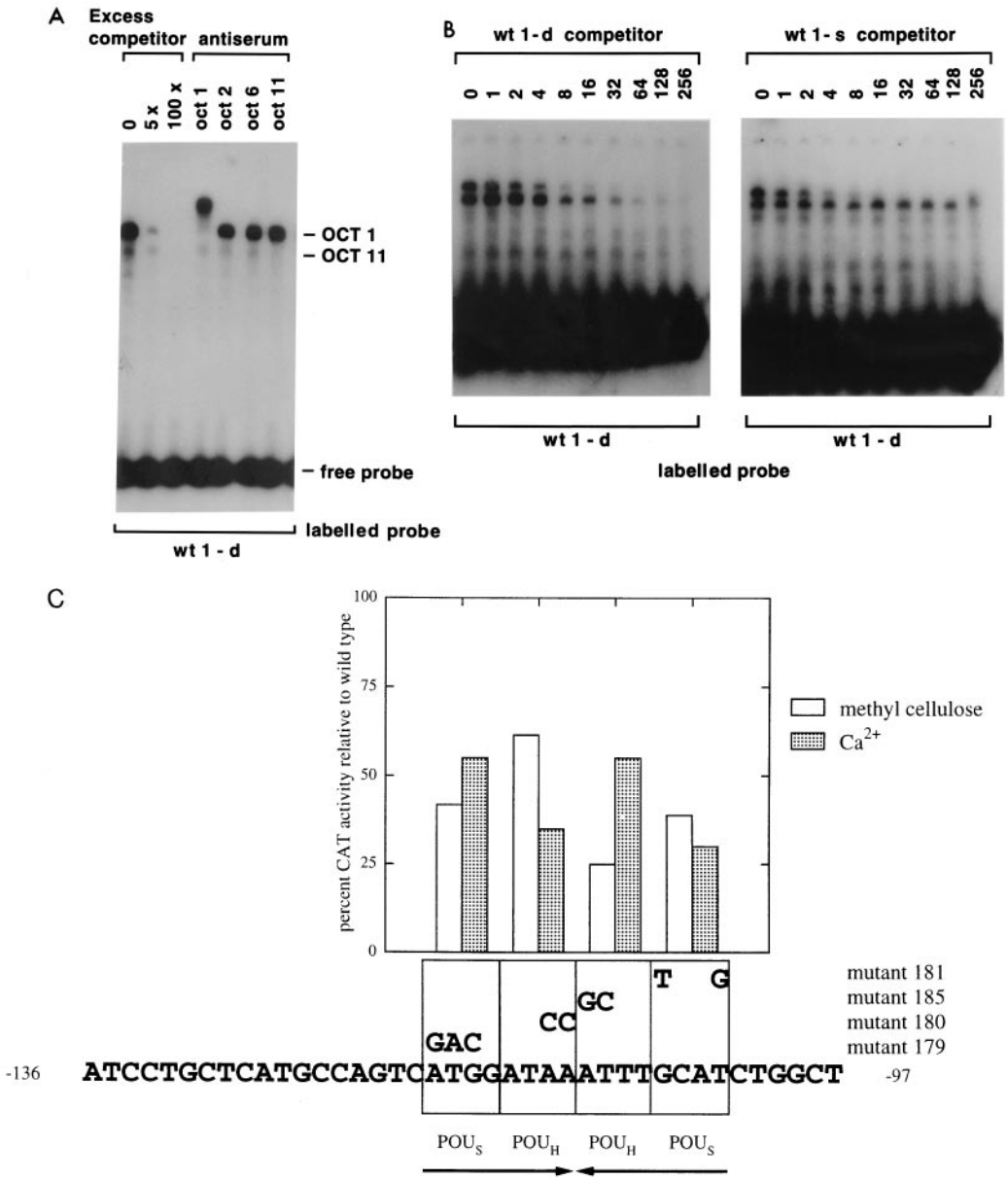

FIG. 5. (A) EMSA of keratinocyte nuclear extracts with probe wt1-d (octamer doublet). Lane 1, 5 μg of nuclear extract; lane 2, as in lane 1 but competed for with a 5-fold excess of unlabeled wt1-d oligonucleotide; lane 3, as in lane 1 but competed for with a 100-fold excess of competitor oligonucleotide; lanes 4 to 7, 5 μg of nuclear extract pretreated with antibodies raised against Oct-1, Oct-2, Oct-6, and Oct-11/Skn-1, respectively. (B) Oct-11/Skn-1 binds preferentially to a duplicated octamer site. Three micrograms of nuclear extract was probed with 20 fmol of labeled wt1-d oligonucleotide and competed for with increasing amounts of either unlabeled wt1-d (octamer doublet) (left) or unlabeled wt1-s (single octamer site) (right). The excess of competitor oligonucleotide is indicated above each lane. (C) Both octamer boxes are equally important for *SPRR2A* expression. Mutants 179, 180, 181, and 185 were tested after induction of keratinocyte terminal differentiation by either the stratification or the suspension protocol. Expression levels relative to the wild-type construct are shown. Results of one of two representative experiments are presented.

tional regulators were shown to be developmentally regulated (24) and involved in the control of cell growth: the IRF-1 gene has characteristics of a tumor suppressor gene, whereas the IRF-2 gene behaves as an oncogene (23). Recently it was shown that IRF-1 is involved in apoptotic cell death (58, 59). In view of the experiments described here, it is interesting that nullizygous IRF-2 mice have been reported to show, besides the predominant alterations in hematopoiesis, a greater physical vulnerability, resulting in skin erosions and ulcers around the neck, back, and abdomen, leading to scarring and hair loss in older animals (45). Whether this phenotype is directly linked to a possible function of IRF-1 and IRF-2 in keratinocyte terminal differentiation as suggested by the present work is not yet known. In fact, interferons are well-known modulators of keratinocyte differentiation (69), and the expression of a number of growth-regulatory and differentiation-specific genes (including the *SPRR* genes) has been shown to be affected by treatment of human keratinocytes with gamma interferon (reference 53 and our unpublished observation). The results presented here constitute a first indication that IRF-1 and IRF-2 might be involved in these regulations.

TDE-3 is recognized by the Ets family of proteins. These related transcriptional regulators are unique in that their activity appears to rely more on their interaction with other

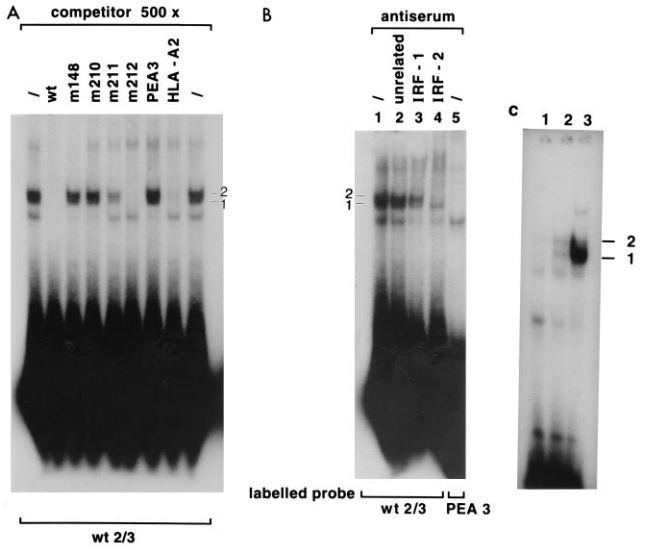

FIG. 6. Identification of ISRE- and Ets-binding factors. (A) Five micrograms of keratinocyte nuclear extract was assayed with 20 fmol of the wt2/3 oligonucleotide. Competition was performed with no competitor (/) or with 10 pmol of the following unlabeled oligonucleotides: wt (wild-type sequence wt2/3); m148, m210, m211, and m212 (mutant oligonucleotides as in clones pSG148, pSG210, pSG211, and pSG212, respectively [sequences are presented in Fig. 4A]); PEA3 (oligonucleotide containing the polyomavirus enhancer PEA3 consensus sequence); and HLA-A2 (ISRE site from the human HLA-A2 gene). (B) Identification of proteins binding to the wt2/3 oligonucleotide. Nuclear extracts from keratinocytes were tested in an EMSA with the wt2/3 oligonucleotide (lanes 1 to 4) or with the polyomavirus enhancer PEA3 site (lane 5). In lane 2, an antibody directed against an unrelated transcription factor was added to the incubation mixture; in lane 3 an IRF-1-specific antibody and in lane 4 an IRF-2-specific antibody were preincubated with the nuclear extracts. (C) Binding of IRF-1 and IRF-2 to wt2/3 is enhanced by pretreatment of the cells with gamma interferon. Nuclear extracts from untreated keratinocytes (lane 1) or from keratinocytes treated with 50 (lane 2) or 200 (lane 3) IU of gamma interferon for 24 h were assayed with the wt2/3 oligonucleotide as described in Materials and Methods.

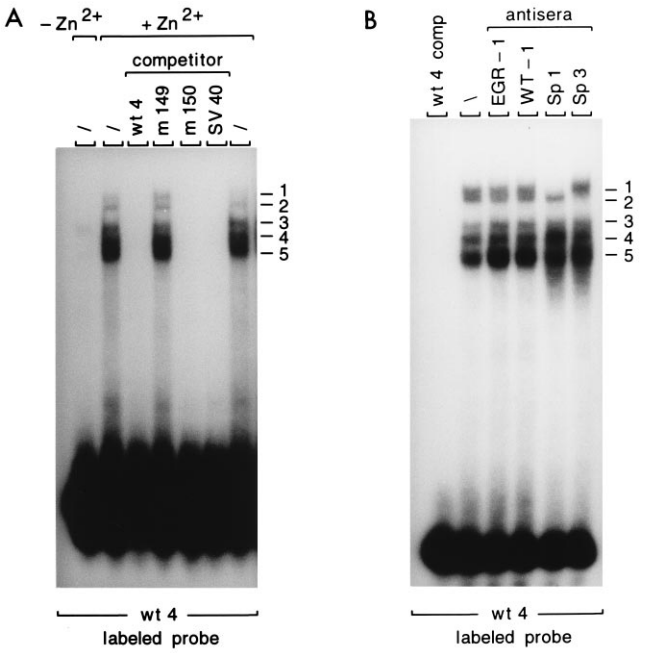

FIG. 7. The wt4 oligonucleotide (TG box) is recognized by the zinc finger family of transcription factors. Nuclear extracts were incubated with 20 fmol of labeled wt4 oligonucleotide in the presence or absence of 0.5 mM $ZnCl_2$ and assayed as described in Materials and Methods. Unlabeled competitor oligonucleotides were wt4 (wild-type sequence), m149 and m150 (mutant oligonucleotides as present in pSG149 and pSG150, respectively [Fig. 4B]), and SV40 (Sp1 binding site from the simian virus 40 early promoter region). Antisera were directed against Egr-1, WT1, and the transcription factors Sp1 and Sp3.

transcription factors than on their specific interactions with their DNA target sequences (recently reviewed in references 6 and 63). Indeed, Ets factors are weak transcriptional activators and function mainly as components of larger transcription complexes. This might explain why Ets proteins are involved in a large variety of rather diverse biological processes (6). Many of these transcription factors are also responsive to a large variety of stimuli, including mitogens, calcium, and protein kinase C activators (63). An involvement of an Ets factor in keratinocyte differentiation has recently been described for the human transglutaminase 3 gene (41).

TDE-4 is bound by several factors, including the transcription factors Sp1 and Sp3 (19, 36). Whereas Sp1 has the characteristics of a classical transcriptional activator (36), Sp3 was shown to be an inhibitor of Sp1-mediated gene activation (20). Although these proteins are ubiquitously expressed and their binding sites have been identified in many different promoters, a recent analysis of the developmental expression of Sp1 suggests that elevated expression of this factor is linked with the process of cellular differentiation (51). An involvement of Sp1 factors in the regulation of several keratinocyte-specific genes has previously been observed (41, 67). Sp1 and Sp3 are probably not the only factors involved in the induction of the *SPRR2A* gene via TDE-4. Our EMSAs indicate that this regulatory element is recognized by three additional binding proteins which have not yet been identified. As these activities bind specifically to the wild-type sequence and to the active (m150) but not to the inactive (m149) mutant sequence, each of these factors could be involved in the regulation of this gene. The finding that complex formation between TDE-4 and these nuclear proteins is strongly induced by the addition of zinc ions suggests that all TDE-4-binding factors belong to the zinc finger family of transcription factors. A requirement of zinc for DNA binding has been documented for Sp1 (65). More research will be needed to determine the identities of the three additional binding proteins.

We have previously suggested that a TPA-responsive element at position −200 of the *SPRR2A* promoter might be involved in the regulation of this gene during the normal process of keratinocyte differentiation (14). Similar suggestions have been made by others (10), all based on the finding that this sequence element (5′ TGAGTCA) is conserved in the promoter region of all major CE precursor genes (involucrin and loricrin genes and the three classes of *SPRR* genes). Although evolutionary conservation in the promoter sequence is a reasonable criterion to suggest coregulation of the different CE precursors, such a view is not in agreement with the in vivo expression patterns of the corresponding genes, which appear to be differentially regulated: involucrin gene is present in all squamous epithelia, loricrin gene expression is typical for orthokeratinizing epithelia (30), *SPRR1* expression is confined to follicular epidermis and oral mucosa, *SPRR2* is expressed coherently in the upper spinous and granular layers of epidermis, and *SPRR3* is specific for internal squamous epithelia (29). These data clearly indicate that in vivo under normal conditions, the different CE precursor genes are subjected to individual regulation. It is more likely that the TPA-responsive element, which is bound by the AP-1 transcription factor, is implicated in the modulation of gene expression by external agents which can interfere with keratinocyte terminal differ-

entiation rather than involved in the normal tissue-specific expression of these genes. Such a view is in agreement with the finding that the Jun and Fos family members (which constitute AP-1) are important targets for mitogen-activated and stress-activated protein kinases (62). However, a possible activating function of the AP-1 factors at very late stages of keratinocyte differentiation in vivo, which might not be measurable in our in vitro system, cannot be completely dismissed.

The present analysis shows that the four TDEs which we have identified function in a strictly interdependent manner. Mutations in a single element are sufficient to completely abolish *SPRR2A* promoter activity. Apparently, signals transduced by different transcription factors have to converge in a concerted manner at these regulatory elements in order to allow transcription of the *SPRR2A* gene. Additionally, as it appears from our deletion and mutation analysis that there is little room left for additional regulatory elements in the minimal promoter region, we believe that the four TDEs which we have identified are sufficient to promote *SPRR2A* expression. An interesting observation is that the different classes of transcription factors which interact with these TDEs have previously been linked with different biological determinants, including skin specificity (Oct-11), propensity for programmed cell death (IRF-1), ability to differentiate (Sp1), and general enhancement of transcription (Ets). The research presented here suggests that integration of signals originating from these different biological determinants is necessary and sufficient to promote gene expression during keratinocyte terminal differentiation.

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