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Differentiation-Specific Transcriptional Regulation of the *ESE*-2 Gene by a Novel Keratinocyte-Restricted Factor

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Abstract Epithelium specific Ets-2 (ESE-2), an epithelium-specific ETS-domain transcription factor, is highly expressed in differentiated keratinocytes. To understand the molecular mechanisms that govern the cell-type and differentiation-specific expression of ESE-2 in keratinocytes, we have focused our studies on the identification and characterization of its *cis*-regulatory elements. We first performed DNase I hypersensitive site mapping and demonstrated that the promoter region of ESE-2 is in open chromatin conformation in differentiated keratinocytes. Next, we performed transient transfection assays with several 5′ serially deleted constructs containing segments of the ESE-2 promoter. These experiments have led to the identification of a short fragment that shows remarkable sequence conservation between several species and harbors most of the transcriptional activity. Interestingly, a high level of transcriptional activity was only observed when the transfected keratinocytes were induced to differentiate by increasing the calcium concentration in the cell-culture medium. To identify the factors that mediate the transcriptional activity, we analyzed this segment by mutational and electrophoretic mobility shift assays (EMSA) experiments. Our studies have identified a critical stretch of nucleotides that is important for both basal as well as calcium responsive reporter activity and that binds to a nuclear factor, keratinocyte restricted factor (KRF). KRF is a novel transcription factor that is restricted to nuclear extracts isolated from keratinocytes and that binds to unique DNA sequences, which do not resemble any known consensus binding motif for transcription factors. Our preliminary experiments shed light on the biochemical nature of KRF and set the stage for future studies in identification of KRF and testing its role in governing *ESE*-2 gene expression in vivo. J. Cell. Biochem. 97: 766–781, 2006.

Key words: transcription factor; ESE-2; keratinocyte; differentiation

The skin is a dynamic organ composed of a dermal and an epidermal component that are separated from each other by a basement membrane. The epidermis, which constitutes the outermost segment of the skin, can be divided morphologically into distinct layers [Fuchs and Raghavan, 2002]. The innermost basal layer contains the actively proliferating keratinocytes. During epidermal differentiation, these mitotically active basal keratinocytes cease to proliferate, detach from the basement membrane, and migrate through the spinous and granular layers to the outermost terminally differentiated cornified layer of the skin. A tightly controlled program of transcriptional regulation is largely responsible for the sequential induction and repression of differentiation-specific genes, including those that encode for structural proteins and enzymes [Fuchs and Raghavan, 2002; Dai and Segre, 2004]. Although many of the structural proteins and enzymes such as the keratins, involucrin, loricrin, fillagrin, and transglutaminase are well characterized, the transcription factors that regulate the stage-specific and differentiation-specific expression of these marker genes are still in the process of being identified. However, recent evidence from many studies

Abbreviations used: ESE-2, epithelium-specific Ets-2; cDNA, complementary DNA; EST, expressed sequence tag; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region; kb, kilobase; kDa, kilodalton; bp, base pairs; RT-PCR, reverse transcription-polymerase chain reaction; KRF, keratinocyte restricted factor.

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on transcriptional control mechanisms of epidermal keratinocytes strongly suggest that multiple families of transcription factors are involved in cell development and differentiation [Eckert et al., 1997; Fuchs and Raghavan, 2002; Dai and Segre, 2004].

One such family of transcription factors belongs to the ETS class, whose members contain a signature motif consisting of a conserved 85 amino acid ETS-DNA binding domain [Sementchenko and Watson, 2000; Sharrocks, 2001]. Structural studies have revealed that the ETS-domain constitutes a winged helix-turn-helix structure that directs them to DNA sequences in promoters and enhancers of target genes containing a central GGA motif [Graves and Petersen, 1998]. It is thought that the flanking sequences surrounding the core motif provide additional recognition specificity thereby directing each family member to unique targets. While the biological role of ETS proteins in hematopoietic and neuronal development in mammals is the subject of extensive research, relatively little is known about their function in other cell types and organs [Sharrocks, 2001]. The study of Ets transcription factors in the development of epithelial cell lineages is of particular interest because epithelial cells constitute a major cell type for numerous organs and a large number of genes that are expressed in the epithelium both simple and stratified, contain functional Ets binding sites in their promoters and enhancers.

The recent discovery of Ets family members expressed exclusively in tissues and cells of epithelial origin has sparked a renewed interest in the role of Ets factors in epithelial cells. These Ets proteins, the epithelium-specific Ets (ESE) factors, ESE-1 (Ert/Jen/Elf3/Esx), ESE-2 (Elf5), ESE-3 (EHF), and PDEF (Pse) are primarily expressed in tissues of epithelial origin and more specifically, in the epithelial compartment [Andreoli et al., 1997; Oettgen et al., 1997; Choi et al., 1998; Neve et al., 1998; Zhou et al., 1998; Oettgen et al., 1999; Kas et al., 2000; Feldman et al., 2003]. ESE-1 and ESE-3 are broadly expressed in the majority of epithelial cell types, while ESE-2 expression is restricted to differentiated keratinocytes and glandular epithelium found in organs such as salivary gland, prostate, mammary gland, and kidney. Although PDEF was discovered as a prostate-specific transcription factor, it is also expressed in a wide variety of cell and tissue-types [Oettgen et al., 2000]. The amino acid sequence homologies between the ESE family members are clustered in two regions, an N-terminal pointed domain, and a C-terminal ETS-DNA binding domain [Feldman et al., 2003]. In addition, ESE-1 has a second DNA binding domain, an AT hook domain, also found in HMG family members. Although, the biological role of ESE proteins in epithelial cell types and their downstream targets are beginning to be elucidated, relatively little is known about the mechanisms that govern their cell-type specific expression. This is of importance because abnormal expression of critical transcription factors including Ets proteins have been implicated in alteration of the epithelial cell differentiation program and often shown to be associated with cancers of epithelial origin [Sharrocks, 2001].

Hence, our aim in this study was to understand the molecular mechanisms of the cell type and differentiation-specific expression of ESE-2 in epidermal keratinocytes. Analysis of epidermal-specific promoters has implicated a number of transcription factors in orchestrating keratinocyte-specific and differentiation-specific gene expression in the epidermis. We focused on the identification and characterization of the critical *cis*- and *trans*-regulatory elements in the ESE-2 promoter. Our studies show that ESE-2 expression is upregulated during keratinocyte differentiation in a cell-culture model system and that the promoter region is in an open conformation selectively in differentiated cells. Next, we characterized a short segment of the ESE-2 promoter that is evolutionarily conserved and is critical for its keratinocyte-specific and differentiation-specific expression. Biochemical analysis of the factors that bind to this segment has led us to identification of a novel keratinocyte-restricted nuclear factor (KRF) that is important for the bulk of the activity of ESE-2 promoter. We have performed extensive biochemical and mutational analysis of KRF to define the DNA sequences to which it binds. Our data suggest that KRF may be a key player in mediating the expression of not only ESE-2, but also other keratinocyte differentiation marker including involucrin. In the future, the identification and characterization of the proteins constituting KRF will shed new light into the keratinocyte differentiation program.

MATERIALS AND METHODS

DNase I Hypersensitive Site Mapping

DNase I hypersensitive mapping was performed as previously described [Sinha et al., 2000]. Briefly, nuclei were obtained from undifferentiated keratinocytes, differentiated keratinocytes (24 h after Ca^{++} switch), and NIH3T3 fibroblasts and subjected to increasing amounts of DNase I treatment. Genomic DNA was purified and digested with appropriate restriction enzymes and Southern blot was performed with Zeta-Probe GT membrane by using manufacturer's protocol (Bio-Rad). For querying the DNA segment upstream of exon 1 of ESE-2, genomic DNA was digested with BamHI–BglII and a probe corresponding to sequences upstream of the BamHI (−500 to −1,000) was used.

Northern Blot Analysis

Total RNA was isolated from NIH3T3 fibroblasts and mouse keratinocytes (at 0, 6, 12, 24, 36, and 48 h after Ca^{++} switch), using TRIzol reagent (Invitrogen). Twenty micrograms of total RNA was separated on 1% formaldehyde-agarose gel and transferred to Zeta-Probe nylon membrane (Bio-Rad). A 450-bp fragment corresponding to the 3′-end of the mouse ESE-2 complementary DNA (cDNA) was generated by polymerase chain reaction (PCR) (nucleotides 1,550–2,000 of ESE-2 cDNA, accession number NM_010125) and used as the probe. This fragment was part of the 3′ untranslated sequence (UTR) of ESE-2 and showed less sequence similarities with any known Ets family member. Hence, this probe is likely to be specific for ESE-2 under the hybridization conditions used for Northern blot. The probe was labeled by random priming using the Prime-It random primer labeling kit (Stratagene). Hybridization was performed in ExpressHyb buffer (Clontech-BD Biosciences) using the manufacturer's protocol. The blot was stripped and reprobed with β-actin cDNA to control for RNA loading. A multiple tissue Northern blot containing poly (A) RNA from several mouse tissues was purchased from Origene and was hybridized with ESE-2 probe. The membrane was then stripped and reprobed for GAPDH to ensure equal loading.

Cell Culture

HeLa, NIH3T3, HEK293, and HaCaT cells were grown in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Wehi cells were grown in RPMI medium with 10% FBS. HepG2 cells were grown in MEM Eagle's medium with 10% FBS. A spontaneously immortalized mouse keratinocyte line (mK, also known as UG-1) was grown in a low Ca^{++} medium comprised of a 3:1 mixture of Ham's F12 and Dulbecco's modified eagle's medium supplemented with 15% chelated FBS. These mouse keratinocyte cells were induced to differentiate by increasing the Ca^{++} concentration in the medium to 1.2 mM.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSAs)

Nuclear extracts were prepared by standard methods [Sinha, 2004]. Briefly, cells were scraped from dishes into isotonic cold PBS and collected by centrifugation at 1,850*g* for 10 min. The pellet was resuspended in 5× volume of Buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, and freshly added protease inhibitors). Cells were incubated on ice for 10 min and centrifuged at 1,850*g* for 10 min. The pellet was resupended in 2× volume of buffer A and homogenized using Dounce homogenizer (10–15 strokes). Nuclei were pelleted by centrifuging for 2 min at 12,000*g*. Nuclei were resuspended in 2× volume of high-salt buffer B (20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 0.45 M NaCl, 0.2 mM EDTA, 1 mM DTT, and freshly added protease inhibitors) and rotated end-on-end on a Rota mixer for 30 min at 4°C. Nuclear extracts were collected from the supernatant by centrifugation in a microcentrifuge at 13,500*g* for 30 min, flash frozen, and stored at −80°C. Nuclear extracts from B16 and C2C12 cells were procured from Active Motif.

For EMSAs, complementary oligonucleotides spanning the regions of interest (25–35 bases) were synthesized (IDT Technologies), annealed, and 2 pmole of double-stranded oligonucleotides were used for radioactive labeling with alpha-^{32}P dCTP. A 1–3 base 5′ overhang was designed at each end to allow labeling by fill-in with Klenow polymerase. After labeling probes were purified by using G-50 Nick columns (Amersham). Binding reactions were performed at room temperature in 20 μl of DNA binding buffer (20 mM HEPES pH 7.9, 75 mM KCl, 10% glycerol, 1 mM DTT, 2.5 mM $MgCl_2$) with 4–6 μg of nuclear extracts. One microgram of poly (dA.dT) was added to each reaction as a

nonspecific DNA competitor. For competition assays, keratinocyte nuclear extracts were preincubated with competitor oligonucleotides (10- or 100-fold in excess) for 10 min prior to the addition of the labeled probe. The protein–DNA complexes were resolved by gel electrophoresis on 5% nondenaturing polyacrylamide gels at room temperature. The gels were dried and visualized by autoradiography.

Construction of Wild-Type and Mutant Promoter Constructs

The various promoter segments (EP1–EP8) were cloned into pGL3-basic vector (Promega) by using restriction enzymes Nhe I and Xho I that were introduced by PCR. A mouse BAC clone RP23-445A5 containing the ESE-2 genomic DNA was used as a template for PCR and was obtained from BACPAC Resources Center (BPRC) at Children's Hospital Oakland Research Institute in Oakland. Mutations (A–J) in the EP4 construct were introduced by using a two-step PCR-based method that has been described before [Sinha et al., 2000]. All constructs were verified by sequencing.

Transient Transfections and Reporter Assays

mK, HepG2, HeLa, and NIH3T3 cells were plated the day before transfection at 2×10^5 cells per well in 6-well plates. Transfections were performed using Fugene 6 reagent (Roche) according to the manufacturer's instructions. One microgram of each luciferase reporter construct was transfected per well along with 0.5 μg of *CMVLacZ* plasmid DNA to serve as an internal control for transfection efficiency. For inducing differentiation of mK cells, calcium levels in the medium was increased to 1.2 mM Ca^{++} (from 0.05 mM) 12 h after transfection and cells were allowed to undergo differentiation for additional 24–36 h. For reporter assays, transfected cells were scraped, and the cells were lysed in Reporter lysis reagent (Promega). The cell extracts were assayed for luciferase activity using the Luciferase Assay System (Promega) and for β-galactosidase activity using the Galacton Plus kit (Tropix) as per manufacturer's instructions. Transfection studies were performed in duplicate and minimum of three independent experiments were performed for each construct. Luciferase activity was normalized to the β-galactosidase activity and average values were determined.

Bioinformatics and In Silico Data Analysis

The sequences for the promoter region of ESE-2 from different species was obtained by performing a BLAST search of the genome database available at NCBI. The sequences were then aligned using the CLUSTALW program (http://www.ebi.ac.uk/clustalw). The Matinspector (http://www.genomatix.de) and Alibaba2.1 (http://www.alibaba2.com) programs were used for transcription factor binding site prediction.

RESULTS

ESE-2 is Induced Upon Differentiation in Mouse Skin Keratinocytes

A previous study has shown that expression of ESE-2 is induced in human keratinocytes upon their differentiation in a cell-culture model system, as detected by reverse transcription-polymerase chain reaction (RT-PCR) [Oettgen et al., 1999]. Here, we have extended this observation by performing Northern blot analysis to examine the expression patterns of ESE-2 in mouse keratinocytes grown in culture. We have also analyzed the expression of ESE-2 in multiple mouse organs, including skin. Mouse keratinocytes when cultured in medium containing a low concentration of Ca^{++} (0.05 mM) exhibit properties of basal-like cells, whereas when switched to medium containing higher concentration of Ca^{++} (1.2 mM), these cells undergo differentiation [Yuspa et al., 1988]. We isolated total RNA from keratinocytes grown in the presence of low- and high-Ca^{++} concentration at different time points. RNA isolated from NIH3T3 fibroblasts was used as a negative control since ESE-2 has been reported to be expressed only in epithelial cell types. As demonstrated in Figure 1A, Northern blot analysis showed that ESE-2 mRNA was expressed at very low levels in basal-cell like undifferentiated keratinocytes and was undetected in fibroblasts. However, upon induction of the keratinocyte differentiation program by increased Ca^{++} concentration in the media, dramatic upregulation of ESE-2 was observed as early as 6 h with maximal induction occurring 24 h post Ca^{++} switch. The differentiation effect was monitored by examining the morphological appearance of the keratinocytes and by examining known markers, such as involucrin and loricrin (data not shown). These studies establish ESE-2 as a marker for keratinocyte

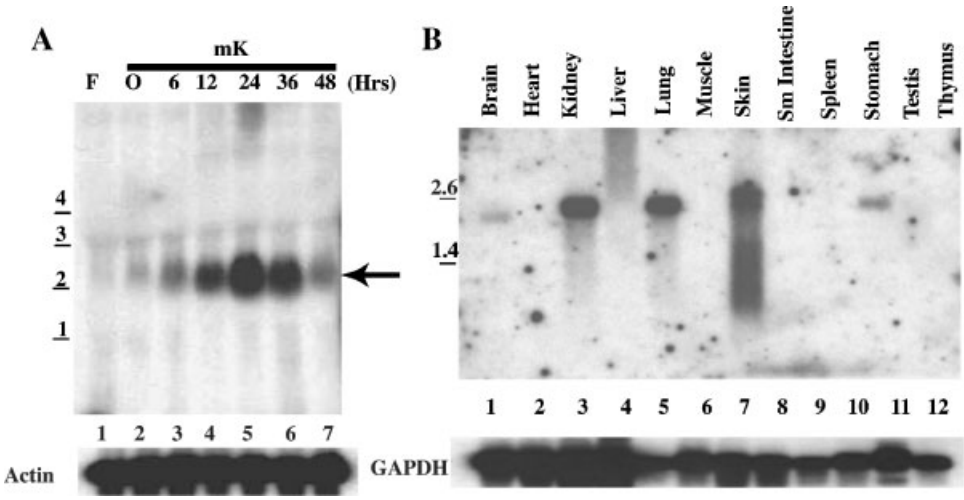

Fig. 1. Epithelium-specific Ets-2 (ESE-2) is highly expressed in skin and differentiated keratinocytes (**A**) Northern blot analysis of ESE-2 expression in mouse keratinocytes during differentiation. Twenty micrograms of total RNA isolated was from mouse keratinocytes (mK) at 0, 6, 12, 24, 36, and 48 h after Ca^{++}-induced differentiation. Total RNA from NIH3T3 fibroblasts (F) cells was used as a negative control. **Upper panel** shows the 2.4 kb ESE-2 transcript (arrow) whereas **lower panel** shows the expression of β-actin as a control for equal loading. The numbers on the left indicate markers in kilobases. **B**: Northern blot analysis of ESE-2 expression in different mouse tissues. Multiple tissue blot containing poly (A)-RNA from several mouse tissues as indicated above each lane was probed with the mouse ESE-2 probe (**upper panel**). The blot was reprobed with GAPDH as an internal control for equal loading (**lower panel**). Multiple transcripts of ESE-2 were identified in skin.

differentiation and suggest that the regulation of ESE-2 expression may occur at the transcriptional level. ESE-2 is also expressed at high levels in mouse skin tissue as well as other organs with high epithelial content such as kidney and lung (Fig. 1B). Interestingly, multiple transcripts of ESE-2 were observed in skin, which may reflect splice variants or products of alternative promoter usage.

DNase I Hypersensitive Site Mapping of the ESE-2 Gene to Determine the Location of Regulatory *Cis*-Elements

In order to identify the possible *cis*-elements that regulate ESE-2 gene expression, we analyzed its chromatin conformation in fibroblasts that do not express the *ESE-2* gene (as shown in Fig. 1) and in keratinocytes grown under both low- and high-Ca^{++} concentrations. Nuclei isolated from these cell types were treated with increasing amounts of DNase I and the genomic DNA was isolated. The DNase I treated genomic DNA was digested with various restriction endonucleases and Southern blotted with specific probes to identify potential hypersensitive sites (Hs) within the chromatin region corresponding to approximately 5 kb upstream and downstream of the putative ESE-2 promoter region. This analysis revealed that the chromatin encompassing the putative promoter segment of ESE-2 was selectively hypersensitive to DNase I in differentiated keratinocytes (high Ca^{++}) and not in undifferentiated keratinocytes (low Ca^{++}) or fibroblasts (Hs I in Fig. 2). Interestingly, an additional Hs, Hs II was observed ~1 kb downstream of the proximal promoter region in the 1st intron of the *ESE-2* gene. Hs II was observed in both differentiated keratinocytes (high Ca^{++}) and undifferentiated keratinocytes but not in fibroblasts suggesting that an element in the 1st intron may contribute to *ESE-2* gene expression in keratinocytes. These data suggested that the proximal promoter region of *ESE-2* is in an open chromatin configuration in differentiated keratinocytes and thus likely harbors the binding sites for transcription factors that regulate ESE-2.

Isolation and Characterization of the ESE-2 Proximal Promoter and Identification of a Conserved Segment Important for Transcriptional Activity

To facilitate analysis of the ESE-2 promoter we first screened a mouse BAC library to obtain a BAC clone that contained the genomic segments of the mouse *ESE-2* gene and its flanking regions. We also determined the putative site of transcriptional initiation by 5′ rapid amplification of cDNA ends (RACE)-PCR (data not shown) and by utilizing an in silico

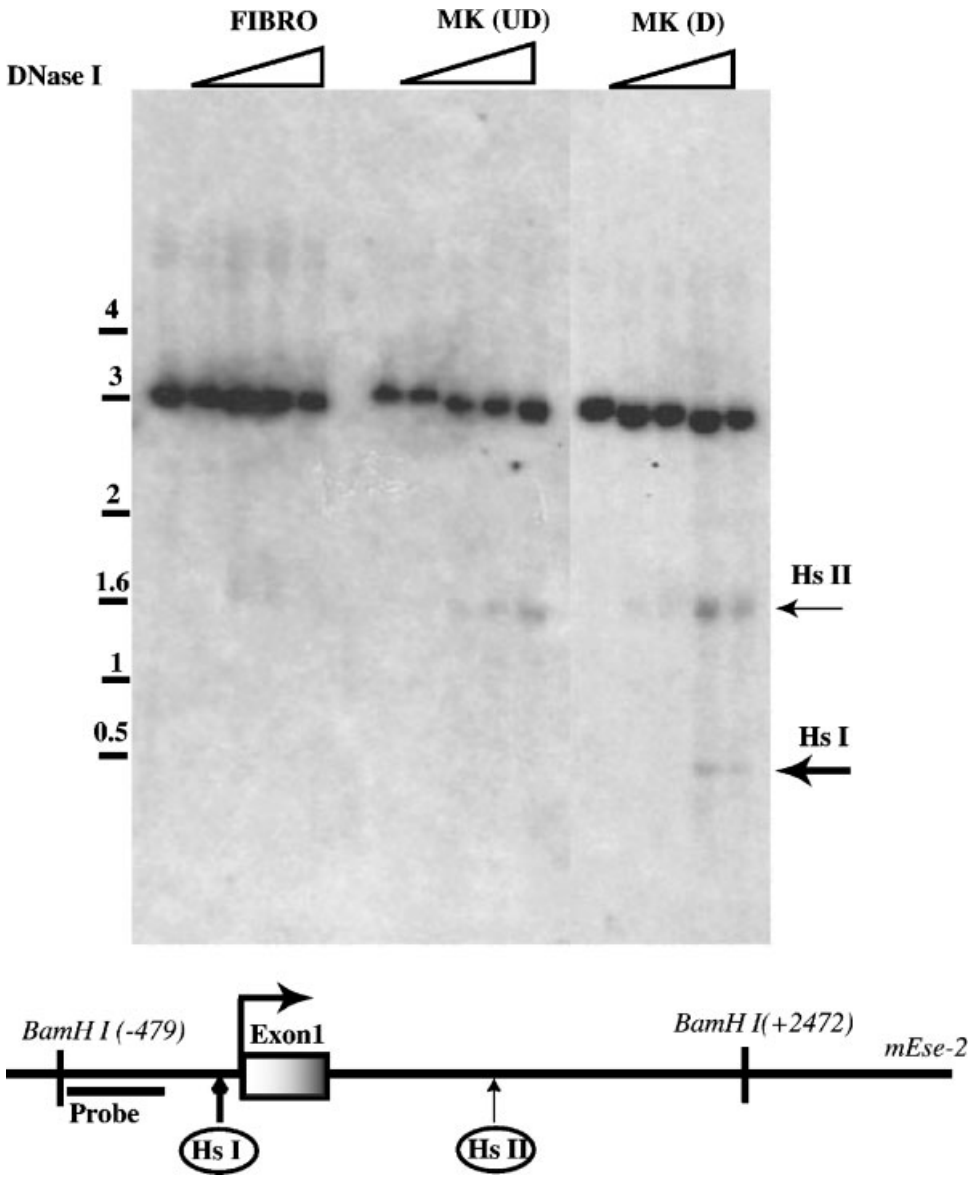

Fig. 2. ESE-2 promoter exhibits differentiation-specific chromatin structure. Genomic DNA was obtained from undifferentiated (MK-UD) and differentiated (MK-D) mouse keratinocytes and fibroblasts (Fibro) after treatment with increasing amounts of DNase I. Southern blotting was performed after digestion with BamH1 and hybridized with a probe as indicated. Two hypersensitive sites were observed (Hs I and Hs II, shown by arrows) with HS II being present in both undifferentiated and differentiated keratinocytes, whereas Hs I was restricted to differentiated keratinocytes. The **lower panel** shows a schematic diagram of the mouse *ESE-2* gene locus and the positions of the Hs sites, BamH1 site, and the probe are indicated.

primer extension approach that has been recently reported to be as useful in defining the transcription start site as the conventional methods [Schmid et al., 2004]. For this purpose, we performed an expressed sequence tag (EST) database search to identify a cluster within 5′ end of the ESE-2 full-length cDNAs that are likely to represent the transcription start site. Our analysis showed that ESE-2 promoter region lacked any TATA-box or CCAAT-box elements and most likely was regulated by an initiator element, since the sequences surrounding the transcriptional start site (TTATATC) showed a perfect match with the consensus (Py-Py-A + 1-NT/A-Py-Py) for a mammalian-initiator element [Butler and Kadonaga, 2002]. Based on this data, we generated a series of constructs from the BAC DNA containing sequentially truncated promoter fragments of ESE-2 upstream of the

luciferase reporter gene in the promoter-less pGL3-basic vector. These constructs containing −1,400 to +61 (EP1), −612 to +61 (EP2), −192 to +61 (EP3), and −131 to +61 (EP4) segments of the ESE-2 promoter were transfected into a mouse keratinocyte cell line, mK along with a CMV*LacZ* plasmid to serve as an internal control for transfection efficiency. All constructs showed a low level of luciferase activity in keratinocytes grown in low Ca^{++}. However, when cells were allowed to differentiate by increasing the Ca^{++} concentration, a strong upregulation (~25–40-fold) of the promoter activity was observed for all four constructs (Fig. 3A). This is in agreement with the fact that endogenous ESE-2 expression occurs primarily in differentiated keratinocytes. This upregulation was specific to the ESE-2 promoter since a 240 bp Keratin 14 (a marker for the undifferentiated basal cells) promoter fragment did not show any significant difference in luciferase activity between low- or high-Ca^{++} media when tested in similar transfection assays (data not shown). Our data also suggested that the EP4 construct corresponding to the shortest fragment of the promoter (−131 to +61) contained the sequences that were necessary for both basal and Ca^{++}-inducible activity of the ESE-2 promoter.

To determine the cell-type specificity of the ESE-2 promoter constructs, we transfected two representative constructs, EP3 and EP4 into several cell lines that do not express detectable levels of ESE-2. As in previous experiments, we co-transfected a beta-galactosidase reporter construct to correct for transfection efficiency between cell lines. The activity of the ESE-2 promoter constructs in NIH3T3, HepG2, and HeLa cells was significantly lower compared to that in differentiated keratinocytes (Fig. 3B). The transfection data corroborates with our observation that there is no hypersensitive site in the ESE-2 promoter in nonexpressing cell lines such as NIH3T3 fibroblasts. Taken together our results indicate that the ESE-2 promoter is preferentially active in differentiated keratinocytes and a short 192-bp segment harbors the necessary *cis*-elements that confer this Ca^{++}-induced activity.

Cross-species comparison of promoters provides a quick and powerful strategy to identify critical stretches of nucleotides within a regulatory region [Wasserman and Sandelin, 2004]. This in silico approach has become increasingly feasible, as large amount of genome sequence data is available for a variety of organisms. We compared the mouse ESE-2 promoter sequence with the corresponding regions from human, rat, dog, and chicken genome sequences (Fig. 4). The proximal promoter fragment of ESE-2 showed remarkable sequence similarity among the five species (almost ~98% identity between mouse and rat and ~91% between mouse and human). In contrast, sequences 5′ of this region showed significantly less sequence conservation between these species (data not shown). The high level of sequence conservation allowed us to focus on the proximal ESE-2 promoter segment and to search for potential transcription factor binding sites.

Biochemical Analysis of the *Trans*-Factors That Bind to the Critical Regulatory Region: Identification of a Novel DNA–Protein Complex

We first used the Matinspector and Alibaba algorithms to search the TRANSFAC database and identified putative sequence motifs for known classes of DNA-binding proteins, including some that are expressed in skin epithelium. To explore which of these sites bind nuclear proteins and potentially govern the activity of the ESE-2 promoter, we conducted EMSA using multiple radiolabeled oligonucleotides and nuclear extracts from both human and mouse keratinocytes and other cell-types. EMSA with one of the oligonucleotides (−47 to −19) revealed an interesting DNA–protein complex (Fig. 5). This slow-moving DNA–protein complex was detected only with nuclear extracts from keratinocytes (lanes 2–4) and not from any other cell types (lanes 5–11), including fibroblasts (NIH3T3); hepatocytes (HepG2); kidney epithelial (HEK293); B-lymphocytes (Wehi and B16); and cervical epithelial (HeLa cells). Two additional fast moving complexes were also detected in some of the cell lines, however these were either nonspecific because they could be competed away by random oligonucleotides or did not show any cell-type specificity. This DNA–protein complex (which we have named keratinocyte restricted factor, KRF) was detected in nuclear extracts from both undifferentiated and differentiated mouse keratinocytes as well as from HaCaT cells, a human keratinocyte cell line. Upon shorter exposure and competition experiments, it was clear that the band corresponding to KRF consisted of 2–

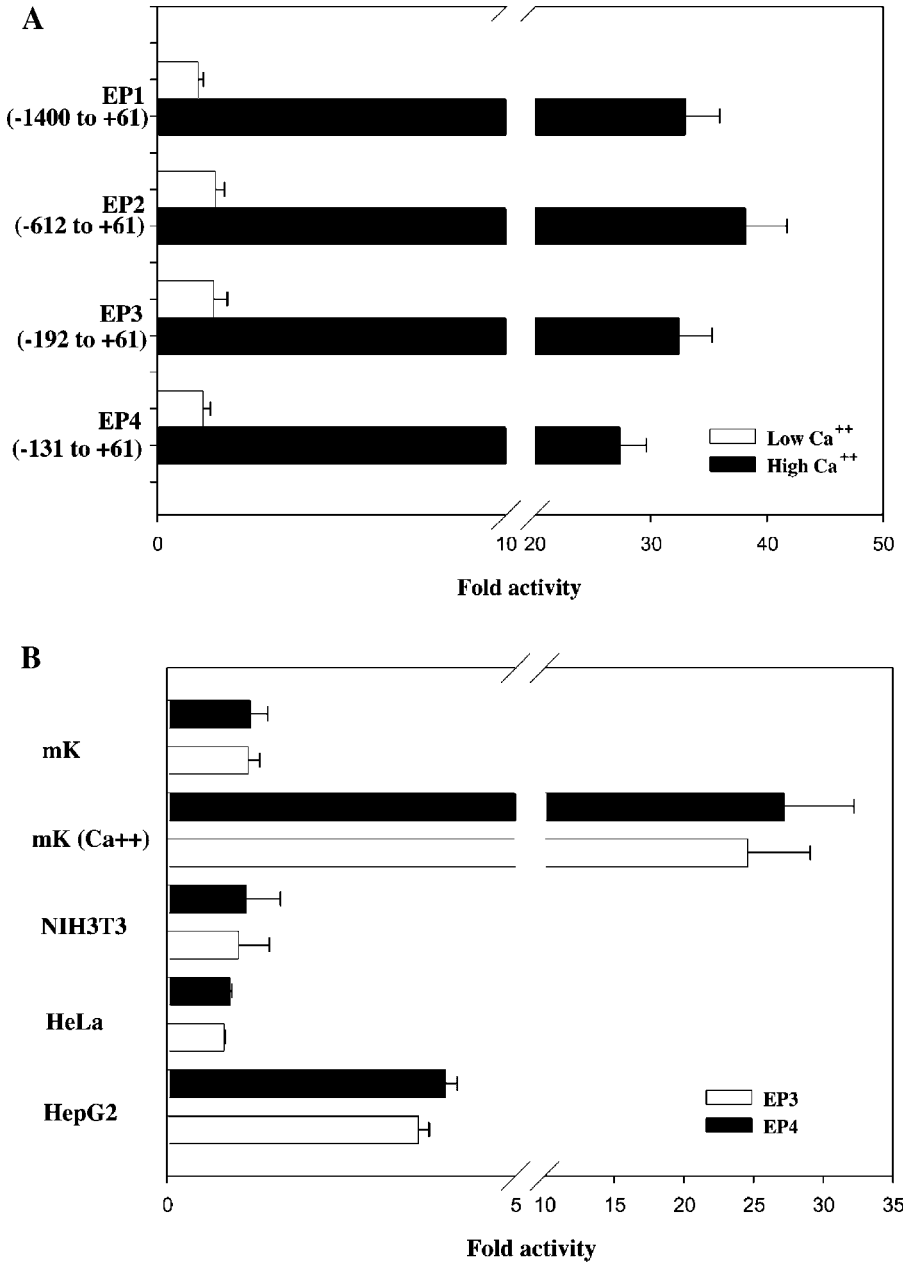

Fig. 3. A small segment of the ESE-2 promoter shows cell-type and differentiation-specific activity in transient transfection assays (**A**) deletion analysis to define the minimal promoter region of ESE-2. A series of luciferase reporter constructs containing promoter fragments with various 5′ deletions were co-transfected with pCMV*LacZ* into mK cells. After 12 h, cells were induced to differentiate by the addition of calcium. After 24 h of differentiation, cells were lysed and assayed for luciferase and β-galactosidase activities. The results are means ± SD of three independent experiments. The corrected luciferase activity of EP1 in low Ca^{++} was set to 1 and the relative fold activity was calculated. **B**: ESE-2 promoter is preferentially active in differentiated keratinocytes. Two representative promoter constructs, EP3 (−192 to +61) and EP4 (−132 to +61) were transfected into different cell types as indicated and the luciferase and β-galactosidase activity was measured. The results are means ± SD of at least three independent experiments. The corrected luciferase activity of EP3 in undifferentiated keratinocytes was set to 1 and the relative fold activity was calculated.

3 species that migrated differently. These may represent different isoforms of the same protein, proteolytic products or perhaps a family of proteins of different molecular weight but of similar DNA-binding nature. The sequence of the oligonucleotide did not reveal matches with any known transcription factor consensus binding sites suggesting that KRF may belong to a

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human          TTTCTGCATATGAGAACCATTTCCCCCCCTCCAAGGAGCCGTGTCACACTGTATGT---C
dog            TTTCTGCATGTGAGAACCATT-CCCCCCCTCCGAGGAGCCGTGTCACACTGTATGT---C
mouse     -121 TTTCTGCATGTGAAAACCACC-CCCCACCCCCGAGGAGCCGTGTCACACCGTATGT---C
rat            TTTTTGCATGTGAAAACCACC-CCCCACCCCCGAGGAGCCGTGTCACACCGTATGT---C
chicken        TTTCTGCACTTGAGAATGTTTCTCCCCCGTTCCCTCAGCTGTGTTATACTATATATTATC
               *** ****  *** **       *** *   *    *** **** * **  *** *   *

human          ACCGTCATCAAAGGGGCTGTGCGTAA--ACCTGAAAAACCAAACGGACCTGTCTGTA-GG
dog            ACCGTCATCAAAGGGGCTGTGCATAA--ACCTGAAAAACCAAACGGACCTGTCTGTA-GG
mouse          ACCGTCATCAAAGGGGCTGTGCATAA--ACCTGAAAAACCAAACGGACCTGTCTGTA-GG
rat            ACCGTCATCAAAGGGGCTGTGCATAA--ACCTGAAAAACCAAACGGACCTGTCTGTA-GG
chicken        ACTGTCATCAAAAGGGGTGTGTGTAAGAACTTGAAAAACCAAACGAACCTGTCTGCAAGG
               ** ********* *** ****  ***  ** ************** ********* * **

human          TGTCACTTATATCACAAGGCTACAGGTGTCTTTATTTCCA----CTG---CACGCTGGTG
dog            TGTTACTTATATCACAAGGCTACAGGTGCCTTTACTTCCA----CCG---TACACTGGTG
mouse          TGTCACTTATATCACACGGCTACAGGTGCCTTTATTTCTA----CGG---TCCGCTGGTG
rat            TGTCACTTATATCACAAGGCTACAGGTGCCTTTATTTCTA----CCG---TCCGCTGGTG
chicken        TGTTCCGTATATCACAGTCCCACAGGTGGCTTTATTTCCAAGCACCGGGCTGCGCTGCTG
               ***  * *********   * ******* ***** *** *    * *     * *** **

human          CTGGGAGCGCCTGCCTTC
dog            -TGGGAGCGCCTGCCTTC
mouse          CTGGGAGCGC--GCTTGC +61
rat            CTGGGAGCGC--GCCTGC
chicken        CTGACGCCTCCTGCTCCC
                **    * *  **   *
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Fig. 4. The sequences corresponding to the ESE-2 promoter are highly conserved. The sequence of the ESE-2 promoter segments from the various species as shown were obtained from the respective genome database and aligned using the ClustalW program. The proximal promoter fragment (−121 to +61) displays remarkable sequence similarities among the five species as indicated by the * sign. The arrow indicates the putative start site of transcription.

novel class of transcription factors. To characterize this complex further and to confirm that KRF binds to DNA in a sequence-specific manner, we generated several mutant oligonucleotides and performed additional EMSAs (Fig. 6 and Table I). One such mutant oligonucleotide (MT1) containing a 3-bp substitution (MT1:AAC<u>CTG</u>AAA > AAC**TGT**AAA) completely failed to bind the keratinocyte-restricted complex, whereas a second mutant oligonucleotide (MT2 AACCTG<u>AAA</u> > AACCTG**GGG**) with an adjoining 3-bp substitution showed significantly weaker binding (Fig. 6, lanes 3 and 4). To more precisely identify the critical residues in this region that were essential for DNA-binding of KRF, we then generated several single base pair mutations. While two of the single base pair mutations (MT5: AA<u>C</u>CTGAAA > AA**T**CTGAA) and (MT8: AACCT<u>G</u>AAA > AACCT**T**AA) failed to bind to KRF completely when assayed by EMSAs, other mutations (MT3: A<u>A</u>CCTGAAA > A**G**CCT-

TABLE I. Sequences of Oligonucleotides (Top Strand) Used for Electrophoretic Mobility Shift Assay (EMSA) and Competition Experiments to Determine the Critical Residues that Are Needed for KRF Binding

Oligonucleotides used for EMSA and competition
WT: 5′-GTGCATA**<u>AACCTGAAA</u>**AACCAAACGG-3′
MT-1: 5′-GTGCATAAAC**<u>TGT</u>**AAAAACCAAACGG-3′
MT-2: 5′-GTGCATAAACCTG**<u>GGG</u>**AACCAAACGG-3′
MT-3: 5′-GTGCATAA**<u>G</u>**CCTGAAAAACCAAACGG-3′
MT-4: 5′-GTGCATA**<u>G</u>**ACCTGAAAAACCAAACGG-3′
MT-5: 5′-GTGCATAAA**<u>T</u>**CTGAAAAACCAAACGG-3′
MT-6: 5′-GTGCATAAAC**<u>T</u>**TGAAAAACCAAACGG-3′
MT-7: 5′-GTGCATAAACC**<u>G</u>**GAAAAACCAAACGG-3′
MT-8: 5′-GTGCATAAACCT**<u>T</u>**AAAAACCAAACGG-3′
MT-9: 5′-GTGCATAAACCTG**<u>C</u>**AAAACCAAACGG-3′
MT-10: 5′-GTGCATAAACCTGA**<u>C</u>**AAACCAAACGG-3′
Consensus oligonucleotides used for competition
AP-1: 5′-CGCTTGATGACTCAGCCGGAA-3′
AP-2: 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′
NF-ATc: 5′-CGCCCAAAGAGGAAAATTTGTTTCATA-3′
NFκB: 5′-AGTTGAGGGGACTTTCCCAGGC-3′
SP-1: 5′-ATTCGATCGGGGCGGGGCGAGC-3′
INV: 5′-GAGAGTGGTGAAACCTGTCCATC-3′

The mutations that were introduced are in bold and underlined. The oligonucleotides corresponding to the transcription factor consensus binding sites and the promoter sequence of involucrin (INV) are shown in the lower column.

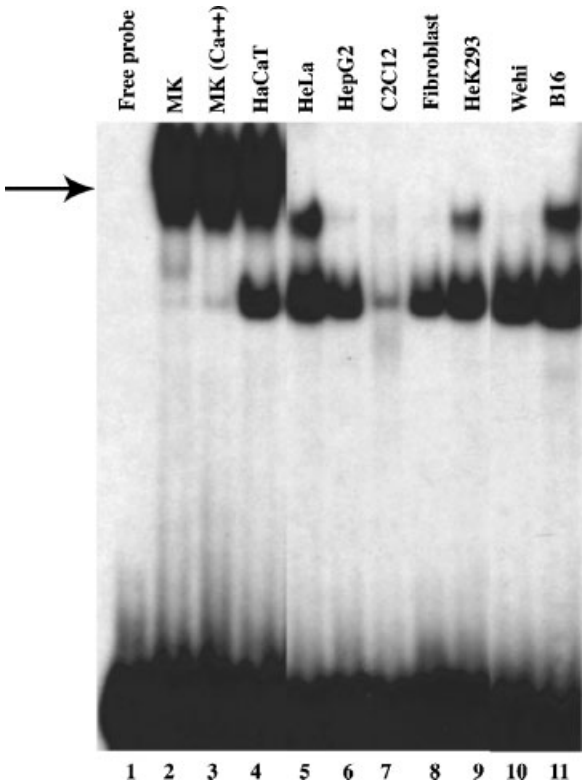

Fig. 5. Electrophoretic mobility shift assay (EMSA) experiments detect a keratinocyte-restricted factor (KRF) that binds to the ESE-2 promoter. EMSA was performed with oligonucleotides containing −47 to −19 region of the ESE-2 promoter and equal amounts of nuclear extracts from undifferentiated (**lane 2**), differentiated mouse keratinocytes (**lane 3**), HaCaT (**lane 4**), HeLa (**lane 5**), HepG2 (**lane 6**), $C2C12_2$ (**lane 7**), NIH3T3 (**lane 8**), HEK 293 (**lane 9**), Wehi (**lane 10**), and B16 (**lane 11**). An arrow indicates the KRF complex. The lower DNA–protein complexes seen in some lanes are nonspecific.

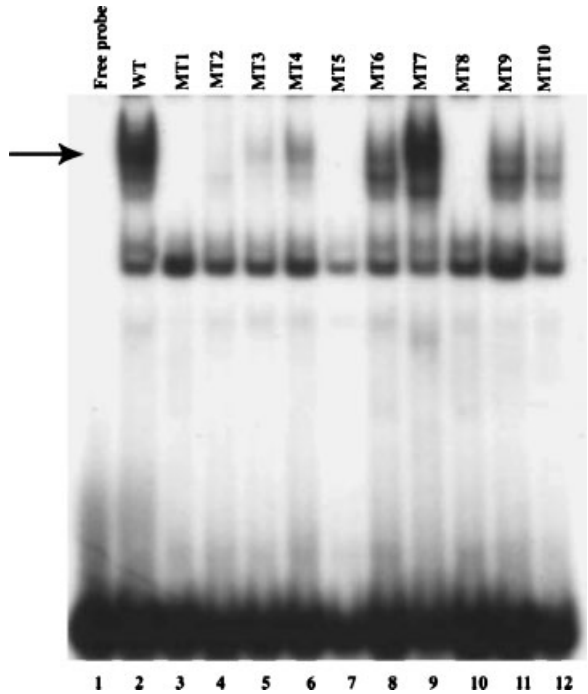

Fig. 6. Mutational analysis of the KRF-binding site identifies residues critical for DNA binding. EMSA was performed with nuclear extracts prepared from mouse keratinocytes and wild-type (WT) or various mutant oligonucleotides (MT). Three base pairs (MT-1 and MT-2) and single base pair mutations (MT-3 to MT-10) were introduced into the wild-type KRF-binding site as described in Materials and Methods and Table I. Arrow indicates the KRF complex.

GAA), (MT4: AACCTGAAA > **G**ACCTGAAA), (MT10: AACCTGAAA > AACCTGA**C**A), and (MT6: AACCTGAAA > AAC**T**TGAAA) inhibited binding to varying degrees upto 50% compared to the wild-type oligonucleotides (Fig. 6). In contrast, MT7 and MT9 oligonucleotides showed binding to KRF comparable to the wild-type oligonucleotides. These data indicated that KRF binds to a specific sequence, AACCTGAAA and that this interaction could be abrogated with 3-bp substitution as well as single base pair mutations.

To further confirm binding-specificity of KRF, we performed competition experiments with wild-type and the 10 mutant double-stranded oligonucleotides. In addition, since the binding site of KRF does not match the consensus of any known transcription factor binding sites, we also performed competition experiments with consensus AP-1, AP-2, Sp1, NFAT, and NF-κB oligonucleotides, which are commonly found in keratinocyte-specific promoters (Table I). In agreement with our previous EMSA studies, mutations MT1, MT2, MT5, and MT8 that severely disrupted binding of KRF, also failed to compete KRF from keratinocyte nuclear extracts under conditions where the wild-type (WT) oligonucleotide competed (Fig. 7A,B). Similarly, mutations that affected binding only weakly or not at all, were capable of competing KRF binding to DNA to varying degrees (MT3, MT4, MT6, MT9, and MT10). None of the oligonucleotides corresponding to AP-2, Sp1, NFAT, AP-1, and NF-κB consensus sites, could also compete with binding of KRF in keratinocyte nuclear extracts (lanes 8–17).

We next searched the literature for regulatory sequences that are involved in keratinocyte-specific gene expression and identified a sequence in the involucrin promoter (INV)

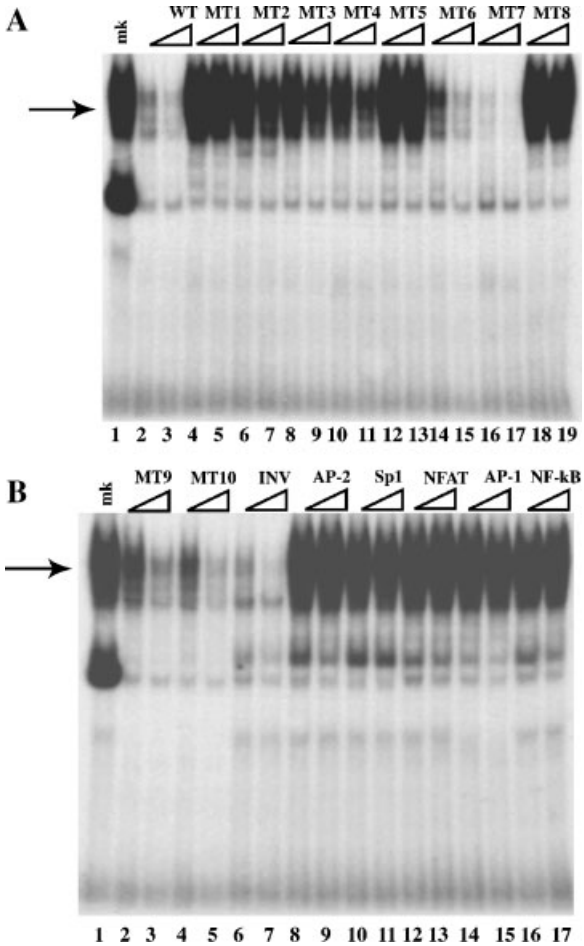

Fig. 7. KRF binds to specific sequences that does not resemble known transcription factor binding site. EMSAs were performed with nuclear extracts from mouse keratinocytes (mK). For competition experiments, 10× and 100× amounts of unlabeled oligonucleotides representing wild-type and mutant KRF binding sites (MT-1 to 10), Involucrin promoter (INV), AP-2, SP-1, NF-AT, AP-1, and NF-κB were used as described in Materials and Methods.

region (−85 GTGGTGAAACCTGT −73) with similarity to KRF-binding site [Phillips et al., 2000]. This sequence reportedly binds to a keratinocyte-specific DNA–protein complex, and is critical for the INV activity in transient transfection assays. This prompted us to test if the sequences from the INV region could compete for binding to KRF. As shown in Figure 7B, lanes 6 and 7, the oligonucleotides containing the sequences of the INV indeed competed with the formation of KRF complex when tested in EMSAs. Since involucrin is also a marker of differentiated keratinocytes, it is tempting to speculate that KRF could be involved in a broad regulation of the similarly expressed keratinocyte-specific genes.

Functional Assay of the Mutants in Transient Transfection Experiments: Effect of KRF Mutation on Basal and Differentiation-Specific Expression

Identification of KRF led us to question whether the factor(s) that bind to this site have a functional role in activation of the ESE-2 promoter. We also wanted to test if additional factors were capable of binding to the 192-bp fragment that were potentially important and were missed during our gel shift analysis. To test this we generated 10 3-bp mutations (A–J) spanning the promoter segment in the EP4 luciferase reporter construct (−131 to +61). We chose elements that were evolutionarily conserved and were sites for putative transcription factor binding as predicted by MatInspector algorithm. Two of the mutations (B and C) were the same 3-bp mutations that abolished the binding of KRF in our previous EMSA studies (Fig. 6, lanes 3 and 4). These mutant constructs were then analyzed in transient transfection experiments in keratinocytes. Mutation of the KRF site reduced the basal-promoter activity whereas the rest of the mutations had little effect (Fig. 8). Interestingly, when cells were switched to high Ca^{++}, unlike the wild-type EP4 construct which showed 25-fold higher activity, mutants B and C that do not bind to KRF showed only 2–3-fold activity. In addition, mutant B, which failed completely to bind to KRF in EMSA showed more severe effect than mutant C, which showed weak but detectable binding of KRF. However, both the mutant promoters still exhibited partial Ca^{++} responsive behavior and exhibited higher activity of luciferase in differentiated keratinocytes compared to those that were undifferentiated. This suggested that although, KRF is a critical regulator of the basal ESE-2 promoter activity, part of the Ca^{++}-induced upregulation is probably mediated by additional, as yet unknown *cis*- and *trans*-elements. Alternatively, it is possible that the mutations though incapable of binding to KRF in EMSA experiments may behave differently and allow some degree of binding in vivo. None of the eight other mutations (A, D, E–J) showed any significant effect on ESE-2 promoter activity under both basal and differentiation-specific conditions.

We also performed deletion studies to narrow down the boundaries of the minimal promoter

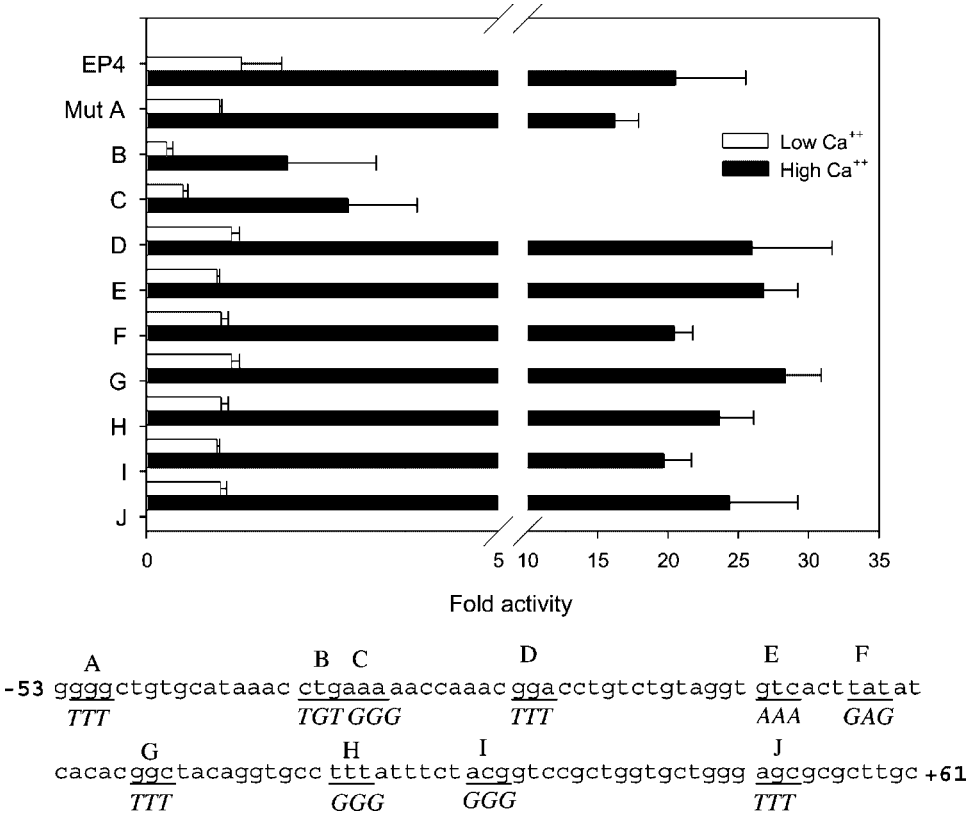

Fig. 8. Mutational analysis of the ESE-2 promoter to identify the regulatory elements luciferase reporter constructs containing 10 3-bp mutations (A–J) spanning the promoter region were generated in the EP4 construct and transfected into mouse keratinocytes. Luciferase and β-galactosidase activities were measured under both undifferentiated (low Ca^{++}) and differentiated (high Ca^{++}) conditions. The results are means ± SD of at least three independent experiments. The luciferase activity of EP4 in low Ca^{++} was set to 1 and the relative-fold activity was calculated. The sequence of the ESE-2 promoter is shown on the bottom, with the mutated residues for each mutant underlined and the changes are indicated underneath.

even further. For the 5′-deletion studies, we generated two additional constructs, EP5 (−62 to −61) and EP6 (−11 to +61), the latter lacking the KRF binding site. Whereas EP5, which still contained the putative KRF binding site, behaved similarly to EP4 and showed a high level of expression upon Ca^{++} switch, EP6, lacking the KRF-binding site had a dramatic effect on both the basal and Ca^{++}-induced promoter activity (Fig. 9A). Compared to EP4, the basal activity of EP6 was sixfold lower (1.2 vs. 0.2) whereas the Ca^{++}-induced activity was affected more dramatically to 47-fold (33 vs. 0.7). These data further validates our results from previous mutational analysis and underscores the importance of KRF in mediating the Ca^{++}-induced promoter activity of ESE-2. We also shortened the 3′ downstream boundary from +61 by generating two additional deletions containing ESE-2 promoter segments from −62 to +42 (EP7) and −62 to +18 (EP8) and tested these constructs similarly. These constructs in particular EP8, had higher basal activity in undifferentiated keratinocytes (e.g., ∼2–5-fold higher than EP4) and still displayed Ca^{++}-responsive behavior albeit to a lesser extent because of higher basal activity. These data suggested that there might be a repressor element in the ESE-2 promoter between +18 and +61.

To further test if the element corresponding to the KRF site can confer keratinocyte-specific expression on its own, we created a construct containing two copies of the KRF site upstream of the heterologous minimal thymidine kinase (TK) promoter. This construct was then tested in transient transfection assays in both undifferentiated and differentiated keratinocytes. As shown in Figure 9B, the multimerized KRF site showed fourfold higher activity than the basal TK promoter in undifferentiated keratinocytes, however the activity was ninefold higher in keratinocytes induced to differentiate. This data taken together with our previous

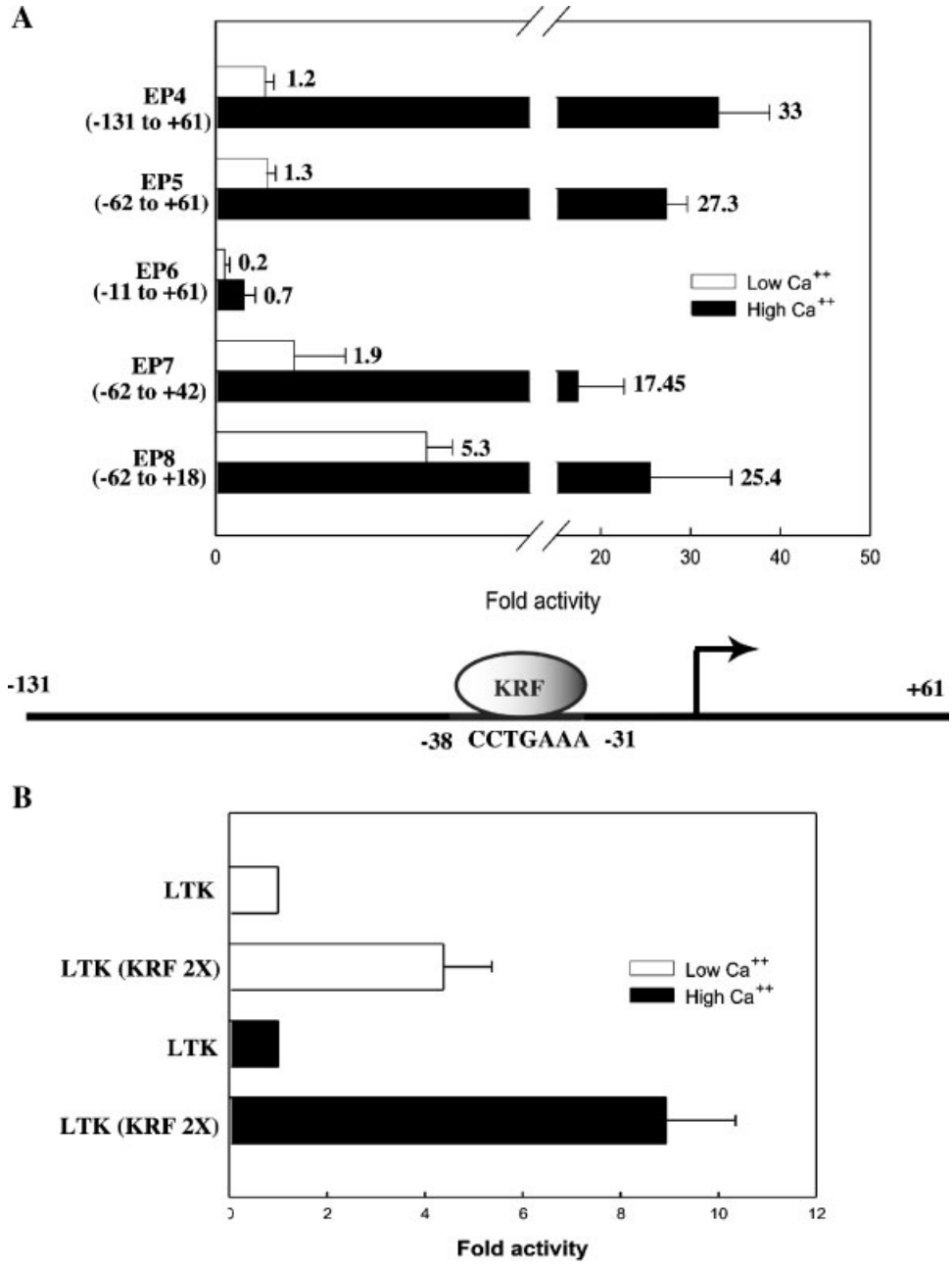

Fig. 9. KRF binding site is important for differentiation-specific activity in keratinocytes. **A**: Deletion of KRF binding site in EP5 (−62 to +61) minimal promoter construct reduces calcium-induced promoter activity. Promoter constructs with a 5′ deletion encompassing the KRF binding site (−11 to +61) and two 3′ deletions EP7 (−62 to +42), and EP8 (−62 to +18) were co-transfected with pCMV*LacZ* into mouse keratinocyte cells and assayed for luciferase and β-galactosidase activities under low- and high-Ca^{++} conditions. The results are means ± SD of three independent experiments. The luciferase activity of EP4 in low Ca^{++} was set to 1. The numbers indicate the relative fold activity. **B**: KRF binding sites shows higher activity in differentiated keratinocytes. Luciferase reporter constructs containing the minimal thymidine kinase (LTK) promoter and two copies of the KRF binding site (−48 to −25) upstream of the TK promoter LTK (KRF2X) were transfected into mouse keratinocytes and induced to differentiate as described before. The results are means ± SD of three independent experiments. The luciferase activities of LTK in low- and high-Ca^{++} were set to 1. LTK (KRF2X) shows fourfold higher activity compared to TK alone in undifferentiated keratinocytes, whereas the activity was increased ninefold in differentiated keratinocytes.

mutational analysis further demonstrate that the KRF site may be important for keratinocyte-specific expression of ESE-2 and may contribute in part to the differentiation-specific activation of its promoter.

DISCUSSION

Keratinocytes are the major cell type of the skin epidermis that undergo a pre-set program of differentiation and morphological and biochemical changes [Fuchs and Raghavan, 2002]. This process is controlled in part by combinatorial action of multiple transcription factors that function to activate or repress-specific target genes via binding to *cis*-regulatory elements [Dai and Segre, 2004]. Hence, identification of promoters and enhancers and their characterization is critical for better understanding of the processes that regulate keratinocyte-specific and differentiation-specific gene expression at the transcriptional level.

The epithelial-specific Ets family member ESE-2, is expressed in epithelial-rich tissues including the prostate, mammary gland, trachea, lung, and kidney [Feldman et al., 2003]. Here, we show that ESE-2 is also expressed in mouse skin at high levels and shows a dramatic induction during keratinocyte differentiation in a cell-culture model system. Although, much is known about the patterns of expression of structural genes in the skin epidermis during differentiation, much less is known about how these patterns are established during development and how programs of terminal differentiation are controlled at the transcriptional level. Transcription factors such as those belonging to the POU, Sp1, AP-2, Oct, KLF, and retinoic acid receptor families have been shown to be important for some of these processes, although their target genes still remain largely unknown [Dai and Segre, 2004]. It is important to note that studying the regulation of genes expressed specifically in keratinocytes has led to the identification of many of the transcription factors commonly associated with this process.

The differentiation-specific expression of ESE-2 in keratinocytes prompted us to utilize this as a model system to identify the *cis*- and *trans*-regulatory elements that control Ca^{++}-induced differentiation-specific expression. We show that the chromatin structure surrounding the ESE-2 promoter region is sensitive to DNase I digestion suggesting that it is in an open chromatin conformation predominantly when keratinocytes are differentiated. Our data suggest that upon initiation of the keratinocyte differentiation program, remodeling of the chromatin region surrounding the transcriptional start site of ESE-2 occurs and this may be a critical step in allowing specific transcription factors to bind to the promoter region.

The identification of a short segment of ESE-2 promoter that showed robust Ca^{++}-inducible differentiation-specific transcriptional activity allowed us to examine these sequences in more detail. Database searches and EMSA assays show that this element binds to a novel KRF and that KRF-binding element is critical for the keratinocyte-specific and differentiation-specific expression of ESE-2 promoter. Extensive mutational analysis has allowed us to define the residues that are critical for binding of KRF and have further substantiated that the binding site for KRF does not resemble that of any known transcription factor. However, promoter segments containing mutations and deletions that abolish KRF binding, still exhibit some degree of calcium-responsiveness suggesting that the differentiation-specific activity of the ESE-2 promoter is partly due to the KRF and is not totally dependent on it. Indeed, Ca^{++} response elements have been characterized for other differentiation-specific genes such as involucrin, loricrin, keratin1, sprr, and transcription factors such as AP-1 and Ets have been implicated [Rothnagel et al., 1993; DiSepio et al., 1995; Lee et al., 1996; Ng et al., 1996; Sark et al., 1998; Crish et al., 2002]. The ESE-2 promoter does not contain any sites that match AP-1 and Ets binding sites and it is possible that the residual Ca^{++} responsiveness of the ESE-2 promoter devoid of KRF-binding sequences may come from unknown elements. Alternatively, this activity may be mediated through transcription factors that can also bind to nonconsensus sequences or are being recruited through protein–protein interactions. One intriguing aspect of KRF is that its activity as measured by EMSA with nuclear extracts seems to show no difference between the undifferentiated and differentiated keratinocytes. At present, without knowing theidentity of KRF, we have no means to address this issue; presumably KRF may show differentiation-specific activity by interacting

with a co-activator that is restricted in differentiated keratinocytes or by undergoing a differentiation-specific post-translational modification that may confer higher transcriptional activity.

What is the identity of KRF? Although at this stage, we do not know the answer, two lines of evidence suggest that KRF may consist of more than one polypeptide. First, to identify KRF we performed initial biochemical characterization of the KRF including partial purification of KRF from keratinocytes using a heparin–agarose column. The partially purified KRF fractions were subjected to Southwestern blot analysis using the KRF-binding site in an attempt to identify the molecular weight of polypeptide encoding for KRF. However, despite multiple attempts, these experiments have failed to show KRF–DNA complex on a membrane suggesting that KRF might consist of more than one polypeptide that are needed for the DNA-binding activity. Second, UV cross-linking experiments that have been performed by Rice and co-workers on the DNA–protein complex that binds to the involucrin promoter revealed multiple proteins, consisting of two major and two minor bands [Phillips et al., 2000]. Based on our competition experiments and the fact that this DNA–protein complex was also shown to be keratinocyte-restricted, we have strong reason to believe that the complex that binds to INV is either KRF or a closely related family member. In future, it will be useful to search the database for presence of KRF-binding sites in keratinocyte-restricted promoters and enhancers, in particular those for differentiation-specific genes. We hypothesize that many such targets probably exist. Such studies are underway and along with the identification and cloning of the cDNAs for KRF should provide us with the right tools to investigate the transcriptional control mechanisms that govern not only ESE-2 but also other keratinocyte-specific genes.

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