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Isolation and Characterization of a Novel Epithelium-Specific Transcription Factor, ESE-1, a Member of the *ets* Family

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We report here the isolation of a novel, highly tissue-restricted member of the *ets* transcription factor/oncogene family, ESE-1 (for epithelium-specific Ets), which has features distinct from those of any other *ets*-related factor. ESE-1 contains two putative DNA binding domains: an ETS domain, which is unique in that the 5′ half shows relatively weak homology to known *ets* factors, and an A/T hook domain, found in HMG proteins and various other nuclear factors. In contrast to any known *ets* factors, ESE-1 is expressed exclusively in epithelial cells. ESE-1 expression is induced during terminal differentiation of the epidermis and in a primary human keratinocyte differentiation system. The keratinocyte terminal differentiation marker gene, SPRR2A, is a putative target for ESE-1, since SPRR2A expression during keratinocyte differentiation correlates with induction of ESE-1 expression, and ESE-1 binds with high affinity to and transactivates the *ets* binding site in the SPRR2A promoter. ESE-1 also binds to and transactivates the enhancer of the Endo A gene, a potential target for ESE-1 in simple epithelia. Due to the important role that other *ets* factors play in cellular differentiation, ESE-1 is expected to be a critical regulator of epithelial cell differentiation.

Although several aspects of epithelium-specific gene expression have been recently elucidated, very few distinctly epithelial cell-restricted transcription factors have been characterized. This is in striking contrast to the identification of a vast number of genes transcribed exclusively in epithelial cells. Epithelium-specific gene regulation plays a critical role during embryogenesis, and the epithelial cell lineage is the first differentiated cell type to appear after fertilization. Differentiation of epithelial cells proceeds along a tightly controled pathway towards cell cycle arrest and terminal differentiation, characterized by precisely timed regulation of specific sets of genes. The majority of epithelial cancers originate as a result of aberrant gene expression leading to defects in epithelial cell differentiation and proliferation.

In search of transcriptional regulators of cell differentiation, we have focused on members of the *ets* transcription factor/oncogene family (26, 42, 54). All members of the *ets* family share a highly conserved DNA binding domain, the ETS domain (81). Outside the DNA binding domain, very little homology is common to all members of the *ets* family (81). However, *ets*-related proteins can be grouped into subclasses based on additional homologous domains shared by particular members of the *ets* family (26). The involvement of *ets* factors in human carcinogenesis has recently been highlighted by the discovery of several distinct chromosomal translocations involving specific members of the *ets* family in different cancer types (10, 19, 20, 27, 57, 85). *ets* factors play a critical role in transcriptional control of stringently regulated genes, such as genes involved in tissue development, differentiation, angiogenesis, cell cycle control, and cell proliferation (26, 81). However, relatively little is known about the role of *ets* factors in epithelial cells.

Several epithelium-specific genes have been shown to depend on *ets* factors for epithelial cell transcription, such as the transglutaminase 3, SPRR2A, Endo A/keratin 8, and Endo B/keratin 18 genes (15, 22, 38, 58, 73). Many additional epithelium-specific genes contain DNA motifs related to *ets* binding sites in their regulatory regions. We report here the cloning and characterization of ESE-1, the first member of the *ets* family that is exclusively expressed and inducible in epithelial cells and shows structural homology to both the *ets* family and HMG proteins.

MATERIALS AND METHODS

Cell culture. T84 (human colon carcinoma), A431 (human vulvar carcinoma), C-33A (human cervical carcinoma), U-937 (human monocytic), NIH 3T3 (mouse fibroblast), HUVEC (human endothelial), A-20 (murine mature B), EL-4 (murine T), and HeLa cells were grown as described previously (40). Primary renal tubular epithelial cells (RECs) were isolated from the kidneys of DBA/2J mice by gradient sieving as described by Singer et al. (77). RECs were grown on collagen-coated plates in Dulbecco's modified Eagle's medium-F12 supplemented with 10% fetal calf serum and a mixture of growth factors containing epidermal growth factor, insulin, transferrin, sodium selenite, prostaglandin E_1, T3, and hydrocortisone as described previously (77). RECs grow as monolayers and proliferate for extended, but limited, periods of time without undergoing any transformation. Primary human bronchial epithelial cells, obtained from Clonetics Corp. and grown according to the manufacturer's recommendations, were kindly provided by Nadeem Moghal.

Isolation and analysis of cDNA clones encoding a novel *ets*-related protein. To search for novel members of the *ets* family, a human expressed sequence tag (EST) cDNA database was searched for sequences homologous to known *ets* members as described previously (54). Several ESTs bearing nucleotide sequence identity were predicted to encode a novel *ets*-like protein. These ESTs originated from cDNA clones prepared from human pancreatic carcinoma, testis tumor, uterine cancer, colon, prostate, ovary, salivary gland, and kidney medulla cDNA libraries. One 1.2-kb cDNA clone from a pancreatic carcinoma cDNA library was sequenced to completion and chosen for further study.

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5′-RACE primer extension. The 1.2-kb cDNA clone contained the poly(A) tail and an open reading frame up to the 5′ end of this clone, suggesting that part of the 5′ end was missing. To determine the 5′ end of ESE-1, we performed 5′ rapid amplification of cDNA ends (5′-RACE) with human adult liver cDNA ready for 5′-RACE (Clontech) and nested primers specific for the 5′ end of the partial ESE-1 cDNA (N1 [5′-CGTTTCCGCTTCCCGTGCTTGGGATC-3′] and N2 [5′-GAGCTTGCCATCAGTGGGATCCAGGT-3′]) as described previously (54). Amplified DNA fragments were subcloned and sequenced as described previously (54). The 5′-end sequences of the ESE-1 cDNAs were confirmed by repeating 5′-RACE PCR amplification with primers specific for the 5′ ends of the longest 5′-RACE products obtained in the first two rounds of PCR amplification.

RNA isolation and Northern blot analysis. Poly(A)$^+$ mRNAs were isolated as described by Libermann et al. (39). Total cellular RNA was isolated from keratinocyte cultures, human foreskin epithelium, RECs, and bronchial epithelial cells by guanidine isothiocyanate nucleic acid extraction and cesium chloride gradient ultracentrifugation (9).

Northern blots containing poly(A)$^+$-selected mRNAs derived from different human tissues (Clontech) were hybridized with random-prime-labeled ESE-1, ELF-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs in QuickHyb solution (Stratagene) as described previously (54) and washed at 50°C with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium dodecyl sulfate (SDS).

RT-PCR analysis. cDNAs were generated from 1 μg of mRNA isolated from different cells or tissues by using oligo(dT)$_{12–18}$ priming (Gibco BRL, Grand Island, N.Y.) and Moloney murine leukemia virus reverse transcriptase (Gibco BRL) in DNase I (Gibco BRL)-treated samples. Each PCR used equivalent amounts of 0.1 ng of cDNA, 4 ng of each primer per μl, 0.25 U of *Taq* polymerase (Promega, Madison, Wis.), 150 μM each deoxynucleoside triphosphate, 3 mM $MgCl_2$, reaction buffer, and water to a final volume of 25 μl, and the mixtures were covered with mineral oil. The sequences of the ESE-1 primers were 5′-CTGAGCAAAGAGTACTGGGACTGTC-3′ (sense) and 5′-CCATAG TTGGGCCACAGCCTCGGAGC-3′ (antisense), with an expected amplification product of 188 bp. The sequences of the primers for ELF-1 were 5′-ATG GCTGCTGTTGTCCAAC-3′ (sense) and 5′-CCTGAGTGCTCTCCCCAT-3′ (antisense), with an expected amplification product of 800 bp. The sequences of the primers for Endo A were 5′-CCCTGAACAACAAGTTCGCC-3′ (sense) and 5′-CCTCATACTTAATCTGGTAC-3′ (antisense), with an expected amplification product of 600 bp. The sequences of the primers for SPRR2A were 5′-CTGGTACCTGAGCACTGATC-3′ (sense) and 5′-GGGTGGATACTTTG ACTGGC-3′ (antisense), with an expected amplification product of 242 bp. The sequences of the primers for GAPDH were 5′-CAAAGTTGTCATGGATGAC C-3′ (sense) and 5′-CCATGGAGAAGGCTGGGG-3′ (antisense), with an expected amplification product of 200 bp. Reverse transcription-PCR (RT-PCR) amplifications were carried out with a Perkin-Elmer Cetus thermal cycler 480 as follows: 20 to 30 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C followed by 15 min at 72°C. Lower numbers of cycles were used to verify linearity of the amplification signal. Ten microliters of the amplification product was analyzed on a 2% agarose gel.

In vitro transcription-translation. Full-length ESE-1 cDNA encoding the whole open reading frame was inserted downstream of the T7 promoter into the TA cloning vector. Coupled in vitro transcription-in vitro translation reactions were performed (Promega) as described previously (42).

EMSA. Electrophoretic mobility shift assays (EMSAs) were performed as described previously (40, 42) with 2 μl of in vitro translation product and 0.1 to 0.2 ng of ^{32}P-labeled double-stranded oligonucleotide probes (5,000 to 20,000 cpm) in the presence or absence of competitor oligonucleotides (1, 5, 10, 50, and 100 ng) and run on 4% polyacrylamide gels containing, as buffer, 0.5× Tris-glycine-EDTA buffer (TGE) as described previously (54).

Oligonucleotides used as probes and for competition studies are as follows:

(i) murine Endo A enhancer wild-type oligonucleotide
5′-TCGACCAGACTGGACAGGAAGTAGGAACAGAC-3′
3′-GGTCTGACCTGTCCTTCATCCTTGTCTGAGCT-5′

(ii) murine Endo A enhancer M1 oligonucleotide
5′-TCGACCAGACTGGACACCAAGTAGGAACAGAC-3′
3′-GGTCTGACCTGTGGTTCATCCTTGTCTGAGCT-5′

(iii) murine Endo A enhancer M2 oligonucleotide
5′-TCGACCAGACTGGACAGGAAGTACCAACAGAC-3′
3′-GGTCTGACCTGTCCTTCATGGTTGTCTGAGCT-5′

(iv) murine Endo A enhancer M1-M2 oligonucleotide
5′-TCGACCAGACTCCACAGGAAGTACCAACAGAC-3′
3′-GGTCTGAGGTGTCCTTCATGGTTGTCTGAGCT-5′

(v) human SPRR2A promoter wild-type oligonucleotide
5′-TCGAGCAGCAGGAAGTGAAACTACCCG-3′
3′-CGTCGTCCTTCACTTTGATGGGCAGCT-5′

(vi) human SPRR2A promoter M1 oligonucleotide
5′-TCGAGCAGCATTAAGTGAAACTACCCG-3′
3′-CGTCGTAATTCACTTTGATGGGCAGCT-5′

Expression vector and luciferase reporter gene constructs. Synthetic wild-type and mutant SPRR2A promoter *ets* site and Endo A enhancer *ets* site oligonucleotides as described above containing *Sal*I and *Xho*I ends were inserted as monomers (Endo A) or dimers (SPRR2A) into the *Sal*I site of the Δ56-c-*fos*-pGL3 plasmid (54). The full-length ESE-1 cDNA was inserted into the *Eco*RI site of the pCI (Promega) eukaryotic expression vector downstream of the cytomegalovirus promoter.

DNA transfection assays. Cotransfections of 3×10^5 C-33A cells were carried out with 2 μg of reporter gene construct DNA and 3 μg of expression vector DNA with 12.5 μl of Lipofectamine (Gibco BRL) as described previously (54). The cells were harvested 16 h after transfection and assayed for luciferase activity (56). Transfections for every construct were performed independently in duplicates or triplicates and repeated three or four times with two different plasmid preparations with similar results. Cotransfection of a second plasmid for determination of transfection efficiency was omitted because potential artifacts with this technique have been reported (12) and because many commonly used viral promoters contain potential binding sites for *ets* factors.

In situ hybridization. Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4 (PBS), for 2 to 4 h at 4°C and were then transferred to 30% sucrose in PBS overnight at 4°C, frozen in OCT compound (Miles Diagnostics, Elkhart, Ind.), and stored at −70°C. In situ hybridization (ISH) was performed on 6-μm frozen sections. Details of ISH have been published previously (14). Briefly, slides were passed through xylene and graded alcohol solutions, 0.2 M HCl, Tris-EDTA with 3 μg of proteinase K per ml, 0.2% glycine, 4% paraformaldehyde in PBS, 0.1 M triethanolamine containing 1/200 (vol/vol) acetic anhydride, and 2× SSC. Slides were hybridized overnight at 50°C with ^{35}S-labeled riboprobes in a mixture containing 0.3 M NaCl, 0.01 M Tris (pH 7.6), 5 mM EDTA, 50% formamide, 10% dextran sulfate, 0.1 mg of yeast tRNA per ml, and 0.01 M dithiothreitol. Posthybridization washes included 2× SSC–50% formamide–10 mM dithiothreitol at 50°C, 4× SSC–10 mM Tris–1 mM EDTA with 20 μg of RNase per ml at 37°C, 2× SSC–50% formamide–10 mM dithiothreitol at 65°C, and 2× SSC. The slides were then dehydrated through graded alcohol solutions containing 0.3 M ammonium acetate, dried, coated with Kodak NTB 2 emulsion, and stored in the dark at 4°C for 2 weeks. The emulsion was developed with Kodak D19 developer, and the slides were counterstained with hematoxylin.

Keratinocyte culture and differentiation. Monolayer cultures of primary human foreskin keratinocytes were prepared from a pool of neonatal foreskins obtained from routine circumcisions by using a modified version of the protocol of Rheinwald and Beckett (66). Keratinocytes were isolated by dispase incubation of foreskin tissue to allow for dermal-epidermal separation. Epidermal specimens were trypsinized and plated on standard tissue culture dishes. Cells were maintained in serum-free keratinocyte growth medium (Gibco BRL). Differentiation was induced by allowing for keratinocyte growth in high-calcium medium (2.0 mM calcium) consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and L-glutamine as previously described (70). Cells were harvested for RNA isolation at different times after induction.

Nucleotide sequence accession numbers. The GenBank nucleotide sequence accession numbers for the reported sequences are U73843 and U73844.

RESULTS

Isolation and characterization of two alternative splice products of the human *ets*-related cDNA ESE-1. A human cDNA database was searched for sequences homologous to the *ets* domain, and one EST originating from a cDNA clone isolated from a human pancreatic cancer cDNA library was predicted to encode a novel member of the *ets* family of transcription factors (26). The cDNA clone contained the poly(A) tail and an open reading frame up to the 5′ end of this clone, suggesting that part of the 5′ end was missing. The 5′ end was determined by the 5′-RACE method, as described in Materials and Methods, using 5′-RACE-ready human adult liver cDNA. We have isolated and sequenced both strands of full-length cDNA clones encoding this novel member of the *ets* gene family, which we termed ESE-1 (for epithelium-specific Ets) based on its epithelium-specific expression.

Two alternative splice products of ESE-1, ESE-1a and ESE-1b, which differed by an in-frame insertion of an alternative exon of 69 nucleotides in the central part of ESE-1a, were identified (Fig. 1). The lengths of the ESE-1a (1,846 bp) and ESE-1b (1,915 bp) full-length cDNAs (Fig. 1) correlate well

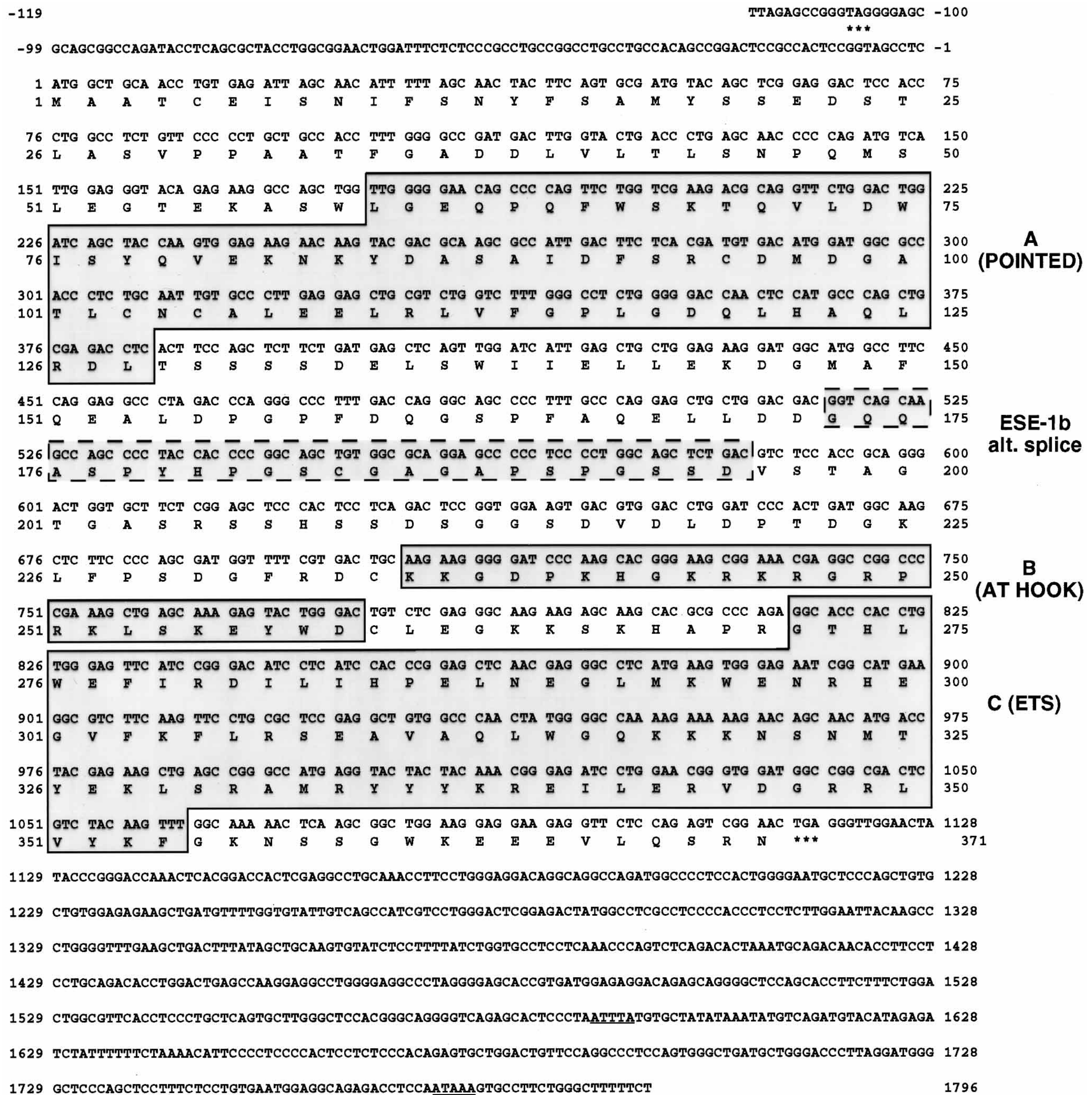


FIG. 1. Complete nucleotide sequence and predicted amino acid sequence of ESE-1. The nucleotide sequences of human ESE-1a and ESE-1b, with the deduced amino acid sequence (one-letter code) of the major open reading frame, are shown. Nucleotides are numbered at the right; amino acids are numbered at the left. The alternative (alt.) 69-bp exon of ESE-1b inserted into the central portion of ESE-1a is boxed by a dashed line and shaded. The Pointed domain, the ETS domain, and the A/T hook domain are boxed, shaded, and marked on the right. The termination codons in frame with the reading frame upstream and downstream are indicated by asterisks. The putative polyadenylation sequence, AATAAA, close to the polyadenylated 3′ end of the mRNA is double underlined. The ATTTA motif involved in mRNA turnover is underlined.

with the estimated sizes of the mRNA species detected by Northern blot analysis (see Fig. 4).

Predicted amino acid sequence of ESE-1. Sequence analysis revealed an open reading frame encoding a 348-amino-acid protein with a predicted molecular mass of 39.3 kDa for ESE-1a (Fig. 1) and an open reading frame encoding a 371-amino-acid protein with a predicted molecular mass of 41.4 kDa for ESE-1b (Fig. 1). The ATG initiation codon partially conforms to the consensus eukaryotic translation initiation sequence (34), with a G at position +4 and a C at positions −1 and −4. This ATG is the sole ATG present in frame, and an in-frame termination codon is found 105 bp upstream of the ATG. That this ATG is the translation initiation site is further supported by the fact that deletion of the 5′ untranslated region does not change the size of the in vitro-translated protein (53). The ESE-1 cDNA contains a long poly(A) tract which is

preceded by a classical polyadenylation site (Fig. 1) at an appropriate distance. One ATTTA motif (Fig. 1), associated with rapid mRNA turnover (68), is found in the 3′ untranslated region.

A hydropathicity plot of the predicted amino acid sequences of ESE-1a and ESE-1b reveals primarily hydrophilic proteins through the entire sequence. The deduced amino acid sequences of ESE-1a and ESE-1b predict proteins rich in glycine (8%), glutamic acid (7%), aspartic acid (8%), serine (11%), leucine (9%), and lysine (7%). The amino-terminal half of ESE-1 is characterized by a high abundance of acidic residues (Fig. 1), whereas the carboxy-terminal half contains many basic residues. The central portion of the protein contains a leucine- and serine-rich domain. Relatively few potential phosphorylation sites are present in ESE-1, including two potential cyclic AMP-dependent kinase phosphorylation sites and three potential JNK/p38/ERK kinase phosphorylation sites (S/TP) clustered in the central region of ESE-1 (4, 35, 45, 48, 78). Two of these sites are present in the alternative exon. One of these sites is followed by a tyrosine residue (SPY), making it a possible candidate for dual-specificity kinases which phosphorylate S/TPY sequences on both serine/threonine and tyrosine (41).

Sequence comparison of ESE-1 with other members of the *ets* family. Comparison of the deduced amino acid sequence of ESE-1 with those of other members of the *ets* family revealed that ESE-1 shows no striking homology to any particular subclass of *ets* factors. Alignment of the carboxy-terminal ETS domain of ESE-1 with those of other members of the *ets* family reveals highest homology to E74 (51%), ELF-1 (45%), and NERF (46%) (Fig. 2a) (54). This degree of homology, however, is far below the homologies that are characteristic among known members of the *ets* family (54). Sequence identity to other members of the *ets* family is in the range of 31 to 43%. ESE-1 is least related to Pu.1/Spi-1 (31%) (31). The carboxy-terminal half of the ESE-1 ETS domain is quite similar to those of other members of the *ets* family. In contrast, the amino-terminal half shows relatively little homology to those of any subgroup of the *ets* family, although the most highly conserved and structurally critical amino acids are conserved in ESE-1 as well (32). Thus, ESE-1 represents the prototype of a new class of *ets* factors.

The amino terminus of ESE-1 contains a region with significant homology to the Pointed domain present in several other members of the *ets* family, including tel, yan, pointed, ets-1, ets-2, fli-1, erg-2, and GABP-α (Fig. 2b), and with weak homology to the helix-loop-helix (HLH) dimerization domain of the HLH transcription factor family (20, 30, 47). This domain can be subdivided into three conserved regions, with predicted α-helical structure for the carboxy-terminal two conserved domains separated by a less conserved nonhelical potential loop region. The amino-terminal conserved region is predicted to form a flexible turn or coil.

ESE-1 contains a putative A/T hook domain. In addition to having a unique ETS domain, ESE-1 has an additional unusual feature for a member of the *ets* family. Unlike any other *ets* factor, ESE-1 contains a second putative DNA binding domain containing two closely spaced so-called A/T hook DNA binding domains located upstream of the ETS domain (Fig. 1) (65). A/T hook domains have been detected in a variety of transcription factors, in particular in HMG proteins, the *Drosophila* tramtrack factor, and the HRX/ALL-1/MLL-1 transcription factor (3, 6, 23, 44, 80). The A/T hook has been shown to bind to the minor groove of AT-rich tracts of double-stranded DNA. The A/T hook domain of ESE-1 shows highest homology to the tramtrack protein, with a stretch of 11 identical amino acids (Fig. 3) (23). Other putative A/T hook domains homologous to ESE-1 are present in, among others, HMG-I, HRX/ALL-1/MLL-1, TAF II, RFX-5, and retinoblastoma binding proteins RBP-1/2, as well as in the *Caenorhabditis elegans* LIN-15B protein and the yeast SWI-5 protein (Fig. 3).

Expression pattern of ESE-1 in human tissues. To determine the expression pattern and size of the ESE-1 transcript, poly(A)$^+$ mRNAs derived from various human tissues were analyzed by Northern blot hybridization with ESE-1 cDNA as a probe (Fig. 4). To control for RNA quality and quantity, we rehybridized the Northern blots with a GAPDH probe. The results indicate the presence of one predominant ESE-1 transcript of approximately 2.2 kb (Fig. 4). The ESE-1 gene is expressed exclusively in tissues with a high content of epithelial cells, an expression pattern strikingly different from that of any member of the *ets* family. In human fetal tissues, the largest amounts of ESE-1 were expressed in the lung (Fig. 4), with lower levels in the kidney and liver. No ESE-1 mRNA was detected in fetal heart and brain. In adult tissues, the small intestine expressed the highest levels of ESE-1 (Fig. 4). High levels of ESE-1 transcripts were also found in the prostate, ovary, colon, placenta, kidney, liver, and pancreas, whereas no expression was detected in the heart, brain, spleen, thymus, testis, and peripheral blood lymphocytes. Surprisingly, even though fetal lung expressed by far the highest levels of ESE-1 mRNA, only low levels of ESE-1 were detected in adult lung, suggesting a role for ESE-1 during early lung development. In skeletal muscle, although the 2.2-kb transcript was not expressed, two additional, thus far uncharacterized, transcripts of 1.9 kb (ESE-2a) and 1.1 kb (ESE-2b) were exclusively and highly expressed, suggesting skeletal muscle-specific alternative splice forms of ESE-1 or a highly related gene. These results suggest that ESE-1 is expressed in a very restricted set of tissues and might therefore have a very specialized function.

To compare expression of ESE-1 to expression of another *ets* factor, the Northern blots were rehybridized with a cDNA probe for ELF-1 (Fig. 4). In fetal tissues ELF-1 expression was high in the heart, lung, liver, and kidney but low in the brain. In adult tissues the highest levels of ELF-1 were found in the pancreas, spleen, thymus, and peripheral blood leukocytes. Moderate amounts of ELF-1 were expressed in the heart, placenta, lung, liver, skeletal muscle, kidney, prostate, ovary, small intestine, and colon, whereas the brain and testis expressed very little ELF-1. Thus, the expression pattern of ELF-1 is strikingly different from that of ESE-1, with ELF-1 expression especially high in the immune system and in the pancreas.

ESE-1 is expressed exclusively in epithelial cells. Since ESE-1 expression is restricted to tissues with high epithelial cell contents, we were interested to know which types of cells express ESE-1. To analyze in more detail the expression of ESE-1 in different cell types, we performed RT-PCR with mRNAs derived from different cell types by using both primary cells and cancer-derived cell lines. Only cells derived from epithelial origins, such as human foreskin epithelial cells, primary murine RECs, primary human bronchial epithelial cells, T84 colon carcinoma cells, HeLa cervical carcinoma cells, or A431 vulvar carcinoma cells, expressed ESE-1 mRNA, whereas peripheral blood leukocytes, EL4 T cells, A-20 B cells, U-937 monocytes, NIH 3T3 fibroblasts, HUVEC endothelial cells, and fetal brain cells were completely devoid of ESE-1 mRNA (Fig. 5). Interestingly, no ESE-1 mRNA was expressed in a cervical carcinoma cell line, C-33A, as well (55). Similar results were also obtained with RNase protection experiments (2). Thus, ESE-1 reveals a distinct and unique expression pattern, being restricted to epithelial cells.

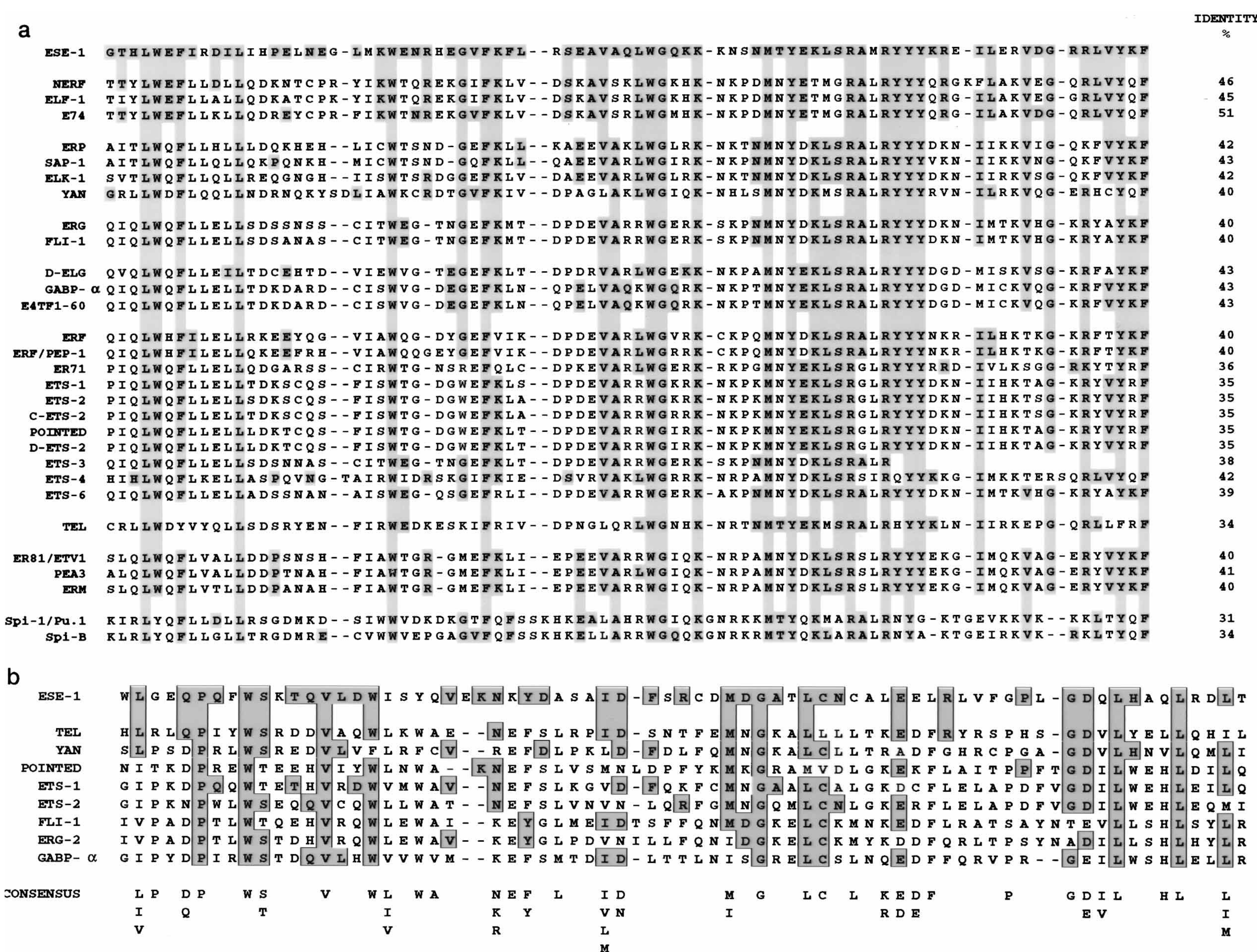


FIG. 2. (a) Comparison of the ETS domain of ESE-1 with those of all known members of the *ets* gene family. The percent identity of each ETS domain with ESE-1 is indicated on the right. Shaded amino acids denote amino acid identity with ESE-1. Gaps are introduced to optimize alignment. The proteins examined are indicated on the left and include NERF-1/2, ELF-1, E74, ERP, SAP-1, ELK-1, YAN, ERG, FLI-1, D-ELG, GABP-α, E4TF1-60, ERF/PEP-1, ER71, Ets-1, Ets-2, C-Ets-2, Pointed, D-Ets-2, Ets-3, Ets-4, Ets-6, tel, ETV-1/ER81, PEA3, ERM, Spi-1/Pu.1, and Spi-B (54). (b) Comparison of the Pointed domain of ESE-1 with those of all known members of the *ets* gene family. Shown are the homologies between the ESE-1 Pointed domain and the corresponding regions present in a subset of the *ets* family (20).

To further test the hypothesis that ESE-1 expression is restricted to epithelial cells, we performed in situ hybridization on frozen sections of neonatal foreskin, adult human lung, stomach, colon, and small intestine. In the lung, diffuse strong expression was noted in the bronchial epithelium (Fig. 6a, panels A and B). No signal was detected with the sense control probe (Fig. 6a, panels C and D). Expression in the alveolar lining epithelium was detectable only focally, and the signal was much weaker than that seen in the bronchial epithelium. No definite labeling was detected in nonepithelial cells. In both the stomach (Fig. 6a, panels E and F) and colon (Fig. 6a, panels G and H), strong labeling was seen in the crypt and surface epithelium, with a suggestion that expression was somewhat stronger at the surface than at the base of the crypts. Cross sections of normal small intestine (Fig. 6b, panels A and B) demonstrated that ESE-1 is strongly expressed in the epithelial cells lining crypts and villi. No signal was detected with the sense control probe (Fig. 6b, panels C and D). Again, no definite expression was detected in stromal cells, further supporting our Northern blot and RT-PCR data that ESE-1 is expressed exclusively in epithelial cells. In the epidermis of the neonatal foreskin, strong expression of ESE-1 mRNA was detected primarily in the granular layer and the upper spinous layer, and weaker expression was detected in the lower spinous layer, whereas only low levels were detected in the basal layer (Fig. 6c, panels A and B), indicating induction of ESE-1 expression during terminal differentiation of the epidermis. No definite expression was observed in stromal cells (Fig. 6c, panels A and B).

Induction of ESE-1 mRNA expression during in vitro differentiation of primary human keratinocytes. Since ESE-1 is expressed exclusively in epithelial cells and appears to be upregulated during epidermis differentiation, we were interested to know whether ESE-1 may be involved in epithelial cell differentiation. Very little is known about epithelial cell differentiation, and only a few models of epithelial cell differentiation using nontransformed cells have been successfully established. We have chosen a primary keratinocyte differentiation model as a tool to study expression of ESE-1 during epithelial cell differentiation in one epithelial cell type (66, 70, 74). Primary human keratinocytes were cultured under low-calcium and low-serum conditions in a specialized keratinocyte growth medium which keeps these keratinocyte cultures in an undifferentiated stage for extended periods of time (66, 70, 74). The addition of calcium and serum initiates a program of terminal differentiation in keratinocytes which progresses over the period of several days through various stages of up- and down-regulation of distinct keratinocyte marker genes, such as various members of the keratin family and various terminal differentiation markers. To evaluate ESE-1 expression during in vitro keratinocyte differentiation, we performed RT-PCR with mRNAs derived from undifferentiated keratinocytes and from keratinocytes at different times after induction with calcium and serum. To test for RNA quality and to roughly compare relative levels of expression, each RNA sample was analyzed by RT-PCR for GAPDH expression. The GAPDH gene is a housekeeping gene whose expression apparently does not change in most cells under different conditions. With equal amounts of cDNA from each time point used for PCR amplification, very similar levels of GAPDH amplification products were obtained for all samples, even when different numbers of amplification cycles were used (Fig. 7). Surprisingly, almost no expression of ESE-1 was detected in undifferentiated keratinocytes or in keratinocytes stimulated with calcium and serum for 1 or 6 h (Fig. 7). However, 12 h after stimulation, a strong amplification product for ESE-1 was visible, which remained

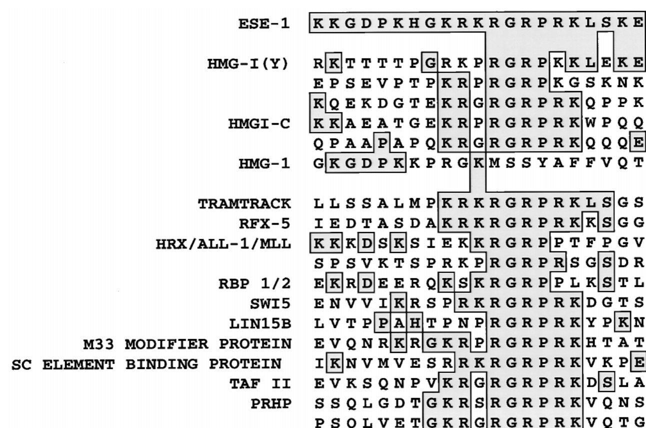


FIG. 3. Comparison of the A/T hook domains of ESE-1 and other A/T hook domain-containing proteins. Shaded amino acids denote amino acid identity with ESE-1. The proteins examined are indicated on the left.

constant up to the last time point at 72 h (Fig. 7). These results correlate with the expression pattern seen by in situ hybridization of neonatal foreskin cells (Fig. 6c) and suggest that ESE-1 expression is low in undifferentiated keratinocytes and becomes induced during in vitro differentiation of keratinocytes 12 h after stimulation. To determine whether expression of another *ets* factor, ELF-1, is inducible as well, we performed RT-PCR with ELF-1-specific primers. In contrast to that of ESE-1, expression of ELF-1 did not vary significantly during keratinocyte differentiation and was high in undifferentiated and differentiating keratinocytes (Fig. 7), indicating that ESE-1 is specifically upregulated during keratinocyte differentiation.

Induction of SPRR2A gene expression during keratinocyte differentiation correlates with ESE-1 expression. The unique expression pattern of ESE-1 in different fetal and adult tissues suggested a distinct set of epithelium-specific target genes for ESE-1, such as the transglutaminase 3, SPRR2A, Endo A/keratin 8 (K8), and Endo B/keratin 18 (K18) genes, which have been shown to contain critical *ets* binding sites in their regulatory regions (1, 15, 22, 38, 51, 58, 73). Using RT-PCR, we compared expression of the SPRR2A and Endo A genes to expression of ESE-1 during keratinocyte differentiation. SPRR2A belongs to a family of small, proline-rich, cornified envelope precursor proteins which are expressed during terminal differentiation of keratinocytes both in vivo and in vitro, whereas Endo A is a keratin expressed primarily in simple epithelia and not previously shown to be physiologically relevant for keratinocyte differentiation. As shown in Fig. 7, the SPRR2A gene is weakly expressed in undifferentiated keratinocytes, but SPRR2A expression increases 12 h after induction, at the same time when ESE-1 expression is induced. Surprisingly, the Endo A gene is weakly expressed in undifferentiated keratinocytes, and expression levels for Endo A increase 24 h after induction, about 12 h after ESE-1 expression is induced (Fig. 7). These results indicate that induction of ESE-1 expression during keratinocyte differentiation correlates with induction of SPRR2A expression and with a delayed stimulation of Endo A gene expression and that the SPRR2A and Endo A genes might be potential downstream targets for ESE-1.

ESE-1 binds to the SPRR2A promoter and Endo A enhancer *ets* binding sites. The SPRR2A gene contains a keratinocyte-specific promoter which consists of four critical regulatory elements, including an *ets* binding site essential for promoter

FIG. 4. Expression of ESE-1 in different human fetal and adult tissues. Northern blot analysis of poly(A)$^+$ mRNAs from the indicated human fetal and adult tissues is shown. PBL, peripheral blood leukocytes. The blot was sequentially probed with ESE-1, ELF-1, and GAPDH cDNA probes under stringent conditions as described in Materials and Methods. Numbers on the right indicate sizes of major mRNA bands. The sizes of molecular size markers are indicated on the left.

activity during keratinocyte terminal differentiation (15). To determine whether the correlation of SPRR2A gene expression with ESE-1 expression during keratinocyte differentiation might be reflected in the ability of ESE-1 to bind to the SPRR2A promoter *ets* site and whether ESE-1, despite its distinct ETS domain, can specifically interact with canonical *ets*-related binding sites, ESE-1 was in vitro transcribed and translated into protein in a reticulocyte lysate, revealing as the major product a protein of the expected molecular weight (Fig. 8a). Minor amounts of additional, faster-migrating proteins were present as well, due to either partial proteolysis, internal translation initiation, or premature translational termination.

We tested the ability of in vitro-translated full-length ESE-1 protein to bind specifically to an oligonucleotide containing the SPRR2A promoter *ets* binding site, which has been demonstrated to bind *ets*-related factors. We compared the abilities of reticulocyte lysate containing ESE-1 protein and of unprimed reticulocyte lysate to form complexes with the SPRR2A promoter *ets* site. EMSA analysis revealed that the SPRR2A oligonucleotide formed one major complex with proteins present in the ESE-1 extract (Fig. 8b), which was not formed by the control extract (Fig. 8b). An SPRR2A oligonucleotide with mutations in the core of the *ets* binding site was tested in competition assays for the specificity of ESE-1 binding. SPRR2A mutant M1 did not compete with the ESE-1-specific complex in EMSAs, whereas wild-type SPRR2A competed with it, indicating that mutation of the *ets* binding site abolishes binding

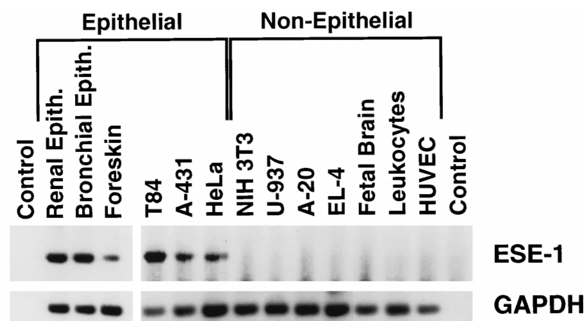
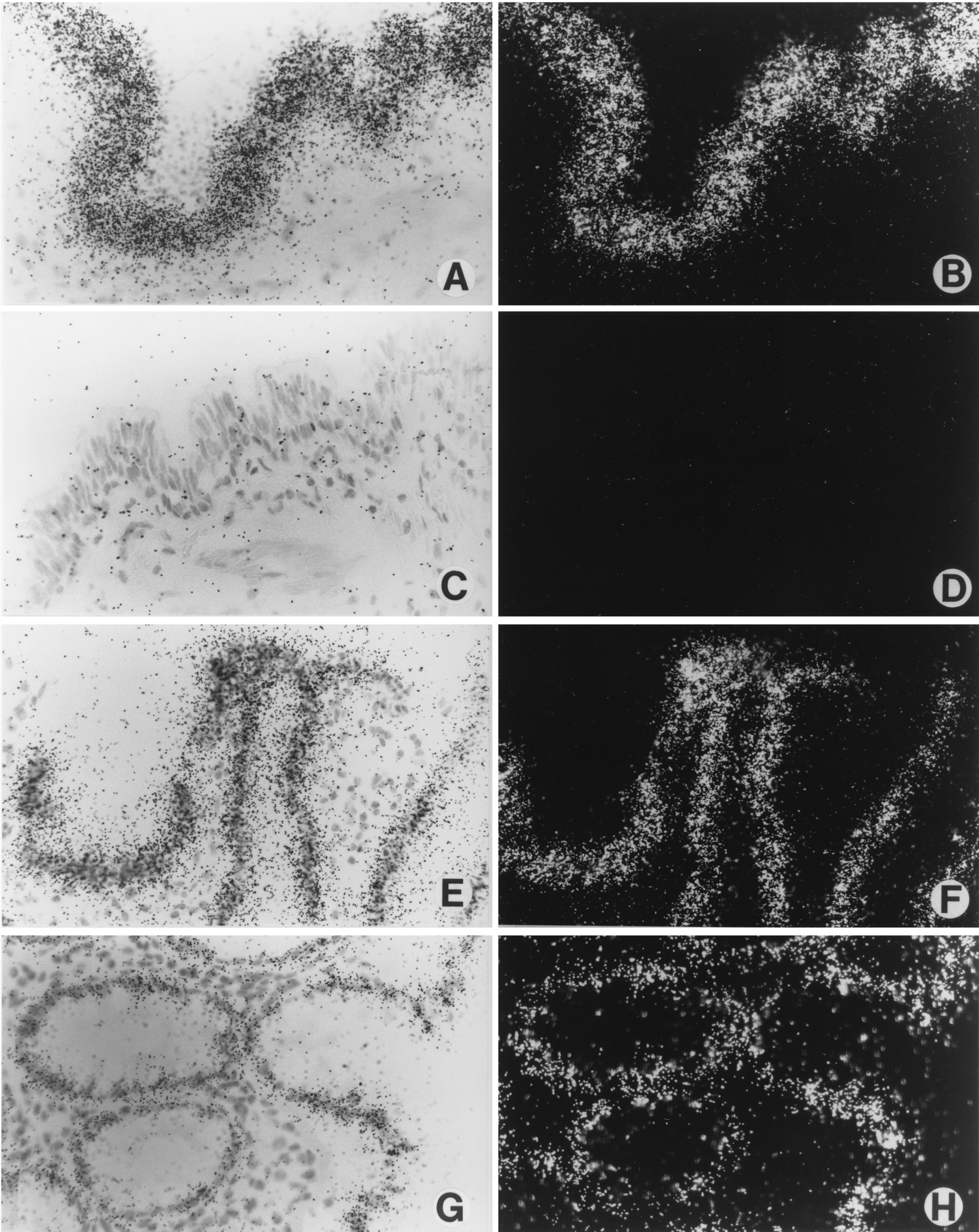


FIG. 5. Expression of ESE-1 in various cell types, showing RT-PCR analysis of poly(A)$^+$ mRNAs from human foreskin epithelium, primary human bronchial epithelial cells (bronchial epith.), primary murine RECs (renal epith.), and T84, A-431, HeLa, NIH 3T3, U-937, A-20, EL-4, and HUVEC cells with ESE-1 or GAPDH specific primers as described in Materials and Methods.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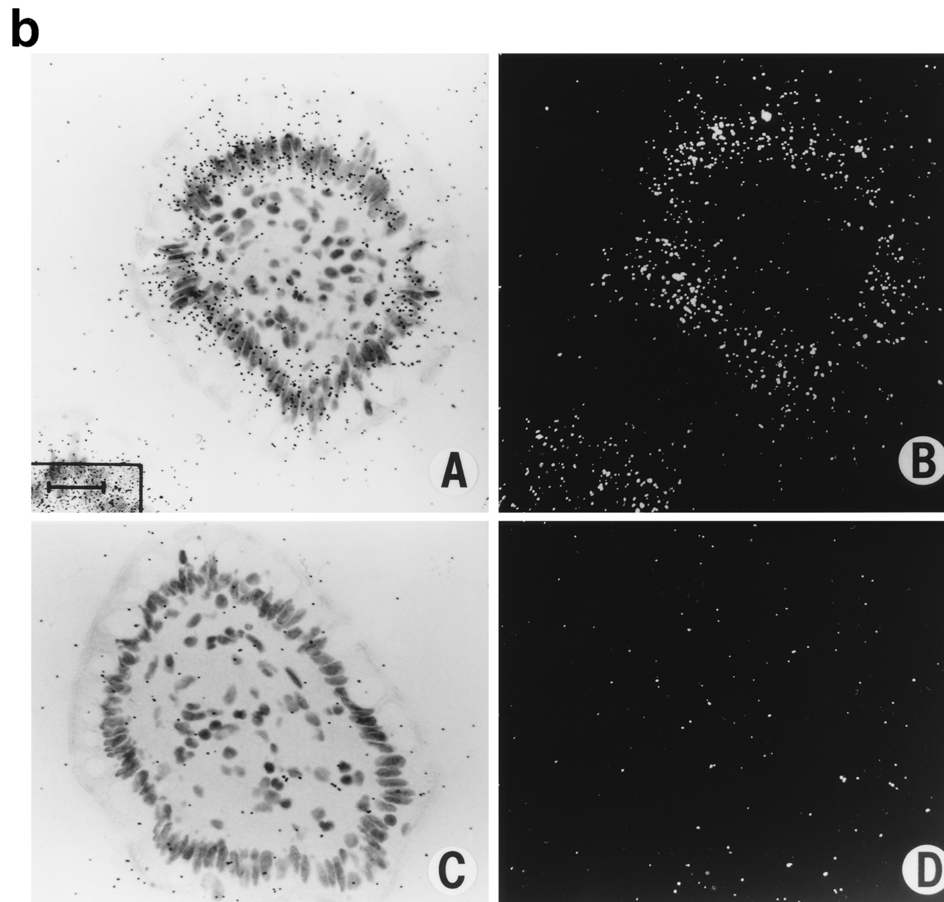

FIG. 6—*Continued.*

of ESE-1 (Fig. 8b). An additional, slower-migrating weak complex formed by both the control and the ESE-1 extract competed with both the wild-type and mutant SPRR2A oligonucleotides, demonstrating that this complex is formed by binding of a reticulocyte factor not related to *ets* to another region of the SPRR2A oligonucleotide.

The Endo A gene contains an epithelium-specific enhancer 3′ of the gene which consists of a repeat of six direct repeats of dual *ets* binding sites essential for enhancer activity (17, 73). We tested the ability of in vitro-translated full-length ESE-1 protein to bind specifically to an oligonucleotide containing one repeat of the dual Endo A enhancer *ets* binding sites, which have been demonstrated to bind *ets*-related factors. The Endo A oligonucleotide formed one major complex in EMSA with proteins present in the ESE-1 extract (Fig. 8c), which was not formed by the control extract (Fig. 8c). An additional complex was formed by both the control and the ESE-1 extract. The oligonucleotide of the Endo A enhancer contains two potential adjacent *ets* binding sites. Endo A oligonucleotides with mutations in the core of each of the *ets* binding sites or in both sites together were tested in competition assays for the specificity of ESE-1 binding. Endo A mutant M1, which contains a mutation in the 5′ *ets* binding site, did not compete with the ESE-1-specific complex in EMSAs but competed with the reticulocyte lysate background complex, whereas wild-type Endo A and Endo A mutant M2, containing a mutation in the 3′ *ets* binding site, competed with both complexes (Fig. 8b). The complex formed in the control lysate appears not to be related to *ets* factors, since both wild-type and mutant M1 and M2 oligonucleotides competed with this complex. These results demonstrate that (i) even though the homology of the ETS domain of ESE-1 to the ETS domains of other *ets* factors is less than 50%, ESE-1 is able to bind specifically to binding sites which are recognized by other *ets* factors, such as ets-1, ets-2, and Fli-1 (17, 73); (ii) ESE-1 interacts with only one of the two *ets* binding sites in the Endo A enhancer repeats; and (iii) ESE-1 can bind to the promoter and enhancer of at least two epithelium-specific genes, and thus these may represent potential targets for ESE-1 function. These results indicate that most of the amino acids essential in generating the three-dimensional protein structure for contacting DNA are conserved in ESE-1. Indeed, we have evidence that ESE-1 can bind a variety of different canonical *ets* binding sites (2), suggesting that specificity and selectivity might be determined by other criteria, such as protein-protein interactions with other transcription factors binding to other regulatory elements in a particular gene. The presence of a putative second DNA binding domain in ESE-1, the A/T hook domain, would suggest the involvement of AT-rich sequences in the binding site for ESE-1. However, so far we have been unable to detect any binding of ESE-1 to canonical A/T hook DNA binding sites.

ESE-1 acts as a transactivator of the SPRR2A promoter and Endo A enhancer *ets* sites. To determine whether ESE-1 acts as a repressor or enhancer of transcription and to further evaluate the possibility that the SPRR2A and Endo A genes are targets for ESE-1, full-length ESE-1 inserted into a eukary-

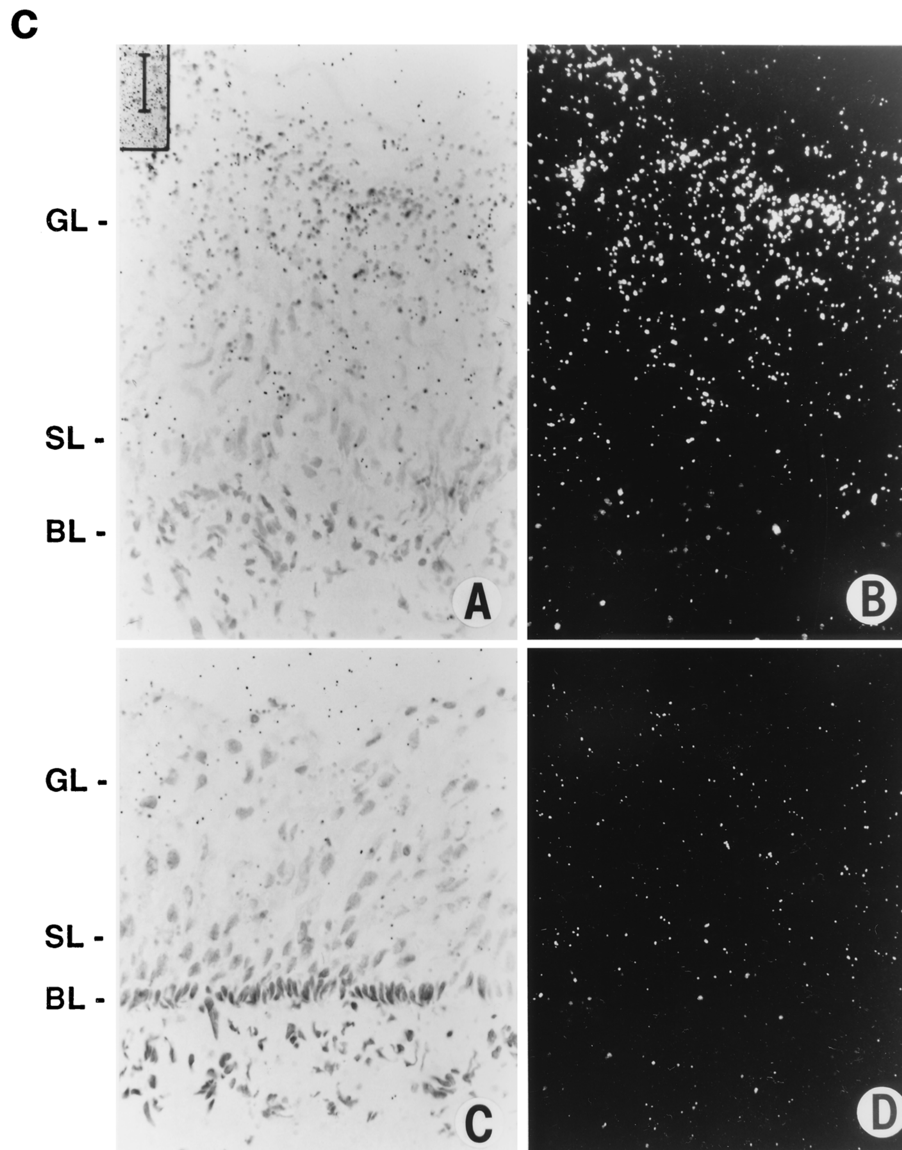

FIG. 6. In situ hybridization studies. (a) Paired bright-field (A, C, E, and G) and corresponding polarized fluorescence (B, D, F, and H) photomicrographs. Intense labeling of the bronchial epithelium in the normal lung is seen with an antisense probe to ESE-1 mRNA (A and B). No labeling of the bronchial epithelium is seen with a control sense probe (C and D). Normal gastric (E and F), and normal colonic (G and H) epithelia are also labeled strongly for ESE-1 mRNA. No significant labeling is noted in stromal tissues surrounding the epithelium. Magnification, ×239. (b) Paired bright-field (A and C) and corresponding polarized fluorescence (B and D) photomicrographs. Strong labeling of the normal small intestinal epithelium is seen with an antisense probe to ESE-1 mRNA (A and B). No specific labeling is seen with a control sense probe (C and D). No significant labeling is noted in stromal tissues surrounding the epithelium. Bar, 25 μm. (c) Paired bright-field (A and C) and corresponding polarized fluorescence (B and D) photomicrographs. Strong labeling of the upper granular layer (GL) and weaker labeling of the spinous layer (SL) of the epidermis in the neonatal foreskin is seen with an antisense probe to ESE-1 mRNA (A and B). No significant labeling is noted in the basal layer (BL) of the epidermis or in stromal tissues surrounding the epithelium. No specific labeling is seen with a control sense probe (C and D). Bar, 25 μm.

otic expression vector (pCI/ESE-1) was cotransfected into ESE-1-negative C-33A cells together with a pGL3 reporter gene construct containing the luciferase gene in which two copies of the SPRR2A promoter *ets* site or one copy of the dual Endo A enhancer *ets* binding site was inserted upstream of the minimal c-*fos* promoter (18). Cotransfection with pCI/ESE-1 resulted in a ca. fivefold transcriptional stimulation of the wild-type SPRR2A promoter *ets* site compared to that with the parental pCI vector (Fig. 8d). Mutation of the *ets* binding site M1 slightly reduced the basal level and drastically reduced inducibility by ESE-1 (Fig. 8d). Thus, the SPRR2A gene contains a high-affinity binding site for ESE-1 which can be transactivated by ESE-1, demonstrating that ESE-1 is a positive regulator of transcription and that SPRR2A might indeed be a relevant epithelium-specific target for ESE-1. Similarly, cotransfection with pCI/ESE-1 resulted in a fivefold transcriptional stimulation of the wild-type Endo A enhancer *ets* site compared to that with the parental pCI vector (Fig. 8e). Consistent with the EMSA results, mutation of the first *ets* binding site, M1, drastically reduced the basal level and inducibility by ESE-1, whereas mutation of the second *ets* binding site, M2, had a less pronounced effect (Fig. 8e). Combined mutation of both sites abolished inducibility. Thus, the Endo A gene can also be transactivated by ESE-1 and might be a second epithe-

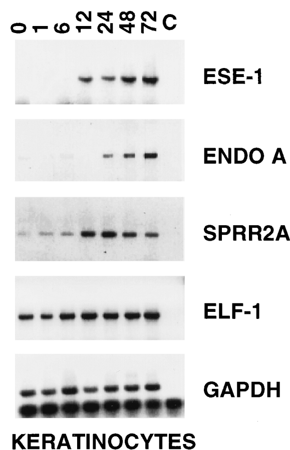

FIG. 7. Expression of ESE-1, SPRR2A, and Endo A during keratinocyte differentiation, showing RT-PCR analysis of total RNA isolated from primary human keratinocytes induced to differentiate with calcium and serum from 0 to 72 h, as indicated at the top, with ESE-1-, Endo A-, SPRR2A-, ELF-1-, and GAPDH-specific primers as indicated on the right (see Materials and Methods). C, control.

lium-specific target for ESE-1. Mutations of the Endo A enhancer *ets* sites reduced the basal level of the luciferase constructs, suggesting that C-33A cells might contain endogenous *ets*-related factors which can transactivate the Endo A enhancer *ets* sites.

DISCUSSION

We report here the isolation and characterization of the prototype of a new subgroup of the *ets* family, ESE-1. Structurally, ESE-1 expresses some unusual features. ESE-1 contains two putative DNA binding domains, an ETS domain and an apparent A/T hook domain repeat. The ETS domain does not fit into any of the known subgroups of the *ets* family, since in ESE-1 several highly conserved amino acids are distinct from any other member of the *ets* family and the overall homology of this domain to any *ets* factor is far below the commonly observed homologies within a specific subgroup. Nevertheless, ESE-1 binds with high affinity to binding sites recognized by the highly divergent ets-1 and ets-2 ETS domains. The carboxy-terminal half of the ESE-1 ETS domain, encoding two alpha helices predominantly involved in contacting the DNA, is much more conserved than the amino-terminal half, and most of the amino acids important for contacting DNA appear to be conserved in ESE-1 (32, 59, 60). Interestingly, the two *ets* binding sites in the SPRR2A and Endo A genes identified by us to interact with ESE-1 show a striking similarity with each other (CAGGAAGT), extending beyond the core recognition site for *ets* factors. Determination of the consensus DNA binding motif for ESE-1 will clarify whether ESE-1 has a high-affinity binding site very distinct from those of other *ets* factors.

In addition to its DNA binding function, the ETS domain is also involved in protein-protein interactions with a variety of transcription factors, and it has recently been demonstrated that subtle amino acid differences among highly related ETS domains determine specificity and can distinguish the ability to interact with a particular transcription factor (16, 28, 75). Thus, the high degree of nonconserved amino acids in the ESE-1 ETS domain may suggest the interaction of a unique set of transcription factors with ESE-1.

A/T hook domains have been detected in a variety of transcription factors, in particular in HMG proteins, the *Drosophila* tramtrack factor, and the HRX/ALL-1/MLL-1 transcription factor (3, 6, 23, 44, 80). The A/T hook has been shown to bind to the minor groove of AT-rich tracts of double-stranded DNA. Each A/T hook is apparently able to mediate contact with an AT tract of 4 to 6 bp or more (44). Speculation about the function of A/T hook domains has also focused on the binding to cruciform DNA and bending of DNA (84). The typical *ets* binding site A/GGAA/T is strikingly different from the A/T hook binding sequences, suggesting that the combination of the ETS domain with A/T hook domains in ESE-1 may create a novel and potentially extended recognition sequence for ESE-1 which diverges from typical *ets* binding sites or that ESE-1 can bind to two different sites cooperatively, similar to the interaction of ELF-1 with HMG-I (28). However, we are so far unable to detect any binding or contribution to binding of the putative ESE-1 A/T hook domains to DNA.

The amino terminus of ESE-1 shows homology to the Pointed domain found in several members of the *ets* family (20, 30, 47). The function of this domain is not clear, but due to its weak homology to the HLH domain, it has been suggested that it plays a role in dimerization (20, 30, 47, 72). Other reports have shown an involvement in transactivation (63, 76). Recently it has been demonstrated that the *ets* factor tel can indeed dimerize via the Pointed domain (29, 47). Furthermore, the involvement of the tel Pointed domain in various chromosomal translocations in different types of leukemia (19, 20, 47) suggests a role of the Pointed domain in transformation. Due to the high conservation of this domain in several members of the *ets* family, an important function may be predicted.

Most members of the *ets* family of transcription factors are widely distributed in different tissues and cell types, with only a few of them demonstrating a cell-type-specific expression pattern (26). These selected members of the *ets* family, such as Pu.1 and SpiB, are usually restricted to the immune system (31, 64). The critical involvement of Pu.1 in early immune system development has been directly demonstrated by homologous recombination studies (46, 71). Various other members of the *ets* family have been directly linked to cell differentiation, highlighting the importance of the *ets* family in developmental processes (5, 49, 52). We now report the isolation of ESE-1, the first member of the *ets* family which is expressed exclusively in epithelial cells. Expression of ESE-1 appears, furthermore, to be highly regulated during epithelial cell differentiation, as exemplified by the upregulation of ESE-1 expression during terminal differentiation of the epidermis and upon in vitro keratinocyte differentiation, suggesting a crucial role for ESE-1 in epithelial cell differentiation. Although epithelial cells contribute to the biological function of the majority of organs and many epithelium-specific genes have been characterized, epithelium-specific gene regulation and epithelial cell differentiation are poorly understood.

Several transcription factors which are involved in epithelium-specific gene expression have been characterized, such as TTF-1, AP-2, LFB3, and skn-1a (13, 21, 24, 25, 36, 37, 43, 79, 83); however, very few of these transcription factors are restricted to epithelial cells. ESE-1 thus represents not only the first epithelium-specific member of the *ets* family but one of the first truly epithelium-specific transcription factors isolated so far. Strong evidence for epithelial cell specificity is provided by both RT-PCR analysis of individual cell types and in situ hybridization of several different tissues. High levels of ESE-1 transcripts in adult human tissues are found throughout the gastrointestinal tract in surface and crypt epithelia as well as in bronchial epithelial cells of the lung. Surprisingly, lung alveolar epithelial cells express very low levels of ESE-1 and do so only

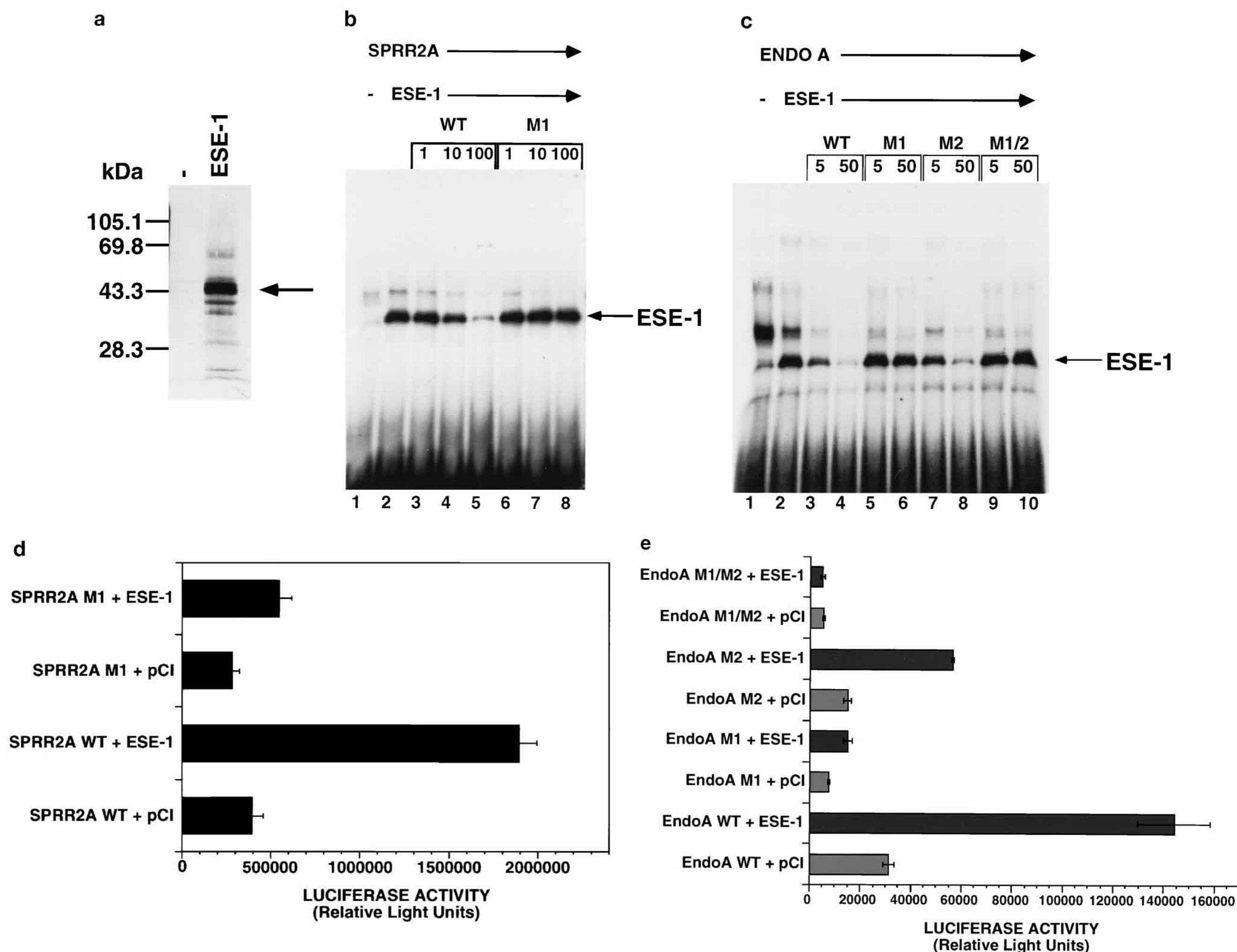

FIG. 8. The SPRR2A promoter *ets* site and the Endo A enhancer *ets* site are targets for ESE-1. (a) In vitro translation products of unprogrammed reticulocyte lysate (−) and full-length ESE-1b separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. (b) DNA binding of ESE-1 to the SPRR2A promoter *ets* site. DNA binding of full-length ESE-1 in an EMSA with synthetic oligonucleotides coding for the SPRR2A promoter *ets* site is shown. The full-length ESE-1 construct described in Materials and Methods was transcribed and translated in vitro in a reticulocyte lysate extract. Equal amounts of the translation products as assayed by SDS-polyacrylamide gel electrophoresis were incubated with the labeled SPRR2A promoter oligonucleotide. Lane 1, unprogrammed reticulocyte lysate; lanes 2 to 8, reticulocyte lysate programmed with the ESE-1 RNA as indicated at the top. EMSA of in vitro-translated ESE-1 and unprogrammed reticulocyte lysate incubated with the labeled SPRR2A promoter *ets* site oligonucleotide probe was carried out with either no competitor (lanes 1 and 2); 1, 10, or 100 ng of unlabeled wild-type (WT) SPRR2A promoter oligonucleotide (lanes 3 to 5, respectively); or 1, 10, or 100 ng of mutant M1 SPRR2A promoter oligonucleotide (lanes 6 to 8, respectively). (c) DNA binding of ESE-1 to the Endo A enhancer *ets* sites. DNA binding of full-length ESE-1 in an EMSA with synthetic oligonucleotides coding for the Endo A enhancer *ets* sites is shown. The full-length ESE-1 construct described in Materials and Methods was transcribed and translated in vitro in a reticulocyte lysate extract. Equal amounts of the translation products were incubated with the labeled Endo A enhancer oligonucleotide. Lane 1, unprogrammed reticulocyte lysate; lanes 2 to 10, reticulocyte lysate programmed with the ESE-1 RNA as indicated at the top. EMSA of in vitro-translated ESE-1 and unprogrammed reticulocyte lysate incubated with the labeled Endo A enhancer *ets* site oligonucleotide probe was carried out with either no competitor (lanes 1 and 2) or 5 or 50 ng of unlabeled wild-type Endo A enhancer oligonucleotide (lanes 3 and 4, respectively), mutant M1 Endo A enhancer oligonucleotide (lanes 5 and 6, respectively), M2 Endo A enhancer oligonucleotide (lanes 7 and 8, respectively), or M1-M2 Endo A enhancer oligonucleotide (lanes 9 and 10, respectively). (d) Transcriptional activation of the SPRR2A promoter *ets* site by ESE-1. C-33A cells were cotransfected with the indicated ESE-1 expression vector construct or the parental pCI expression vector and luciferase constructs containing two copies of the wild-type or mutant M1 SPRR2A promoter *ets* site. Luciferase activity in the lysates was determined 16 h later as described elsewhere (56). Data shown are means and standard deviations for triplicate measurements from one representative transfection. The experiment was repeated three times with different plasmid preparations with comparable results. (e) Transcriptional activation of the Endo A enhancer *ets* sites by ESE-1. C-33A cells were cotransfected with the indicated ESE-1 expression vector construct or the parental pCI expression vector and luciferase constructs containing one copy of the wild-type or mutant Endo A enhancer *ets* sites. Luciferase activity in the lysates was determined 16 h later as described elsewhere (56). Data shown are means and standard deviations for duplicate measurements from one representative transfection. The experiment was repeated four times with different plasmid preparations with comparable results.

focally, suggesting that ESE-1 expression and function vary within the epithelial cell lineage. Northern blot analysis demonstrates that ESE-1 is highly expressed in the human fetal lung, whereas ESE-1 expression in the adult lung is very weak. ESE-1 expression during lung development thus may correlate with the high percentage of bronchial epithelial cells in the fetal lung and the expansion of apparently low-expressing alveolar epithelial cells after birth.

Interestingly, two ESE-1-related transcripts, tentatively named ESE-2a and ESE-2b, are abundantly and exclusively expressed in skeletal muscle. However, these transcripts are smaller than the epithelium-specific transcript, and the epithelium-specific

transcript is not expressed in skeletal muscle, suggesting that these transcripts either represent alternative skeletal muscle-specific splice products of ESE-1 or are transcribed from an ESE-1-related gene. We cannot exclude the possibility that these skeletal muscle-specific transcripts encode the same protein as the epithelium-specific transcript. However, since these transcripts are restricted to skeletal muscle and many other *ets* factors express tissue-restricted alternative splice products encoding protein isoforms, it is likely that alternative ESE isoforms are encoded by the different transcripts (33, 54, 61, 67). A unique function of these ESE-1-related mRNAs in skeletal muscle differentiation can be envisioned, and we are now in the process of determining the nature of these mRNAs.

Based on our Northern blot analysis, in situ hybridization, and RT-PCR analysis, ESE-1 is expressed in a variety of epithelial cells, including stratified and nonstratified epithelia. In the epidermis, ESE-1 expression is restricted to the suprabasal layers, with increasing expression towards more-differentiated keratinocytes in the upper spinous and granular layers. Very few in vitro differentiation systems for primary epithelial cells have been established which allow one to study the regulation of epithelium-specific genes during differentiation. We have used, as a first attempt to explore the role of ESE-1 in one type of epithelial cells, one of the better-characterized in vitro differentiation systems, involving primary human keratinocytes, which has been successfully used to determine several aspects of keratinocyte terminal differentiation (11, 15, 50, 62, 69, 82). In correlation with the in situ hybridization results for the epidermis, ESE-1 expression is low in undifferentiated keratinocytes and becomes induced during terminal differentiation 12 h after induction by calcium. The expression pattern of ESE-1 suggests that ESE-1 regulates a distinct group of epithelium-specific genes. Only a few epithelium-specific genes have been previously shown to be regulated by *ets* factors, and in our first approach to identify targets for ESE-1, we have focused on two of these genes, SPRR2A and Endo A. SPRR2A is a small proline-rich protein whose expression is induced in stratified epithelia during squamous differentiation both in vivo and in vitro as well as in response to epidermal injury, inflammation, hyperproliferation, keratinization, and aging (15). In the epidermis, SPRR2A is expressed in the upper spinous and granular layers, similar to the expression pattern of ESE-1. Recently, an *ets* binding site in the SPRR2A promoter was identified as one of four interdependent regulatory elements unconditionally required and sufficient for regulation of SPRR2A expression during in vitro keratinocyte terminal differentiation (15). The particular keratinocyte *ets* factor was not identified. We now provide strong evidence that ESE-1 binds with high affinity to the SPRR2A promoter *ets* binding site and can transactivate the SPRR2A promoter *ets* site. Furthermore, we show that ESE-1 expression is induced during terminal differentiation of the epidermis and during keratinocyte in vitro differentiation concommitant with induction of SPRR2A expression, indicating that ESE-1 might be the *ets* factor in keratinocytes which regulates SPRR2A gene expression.

Endo A, a second potential target for ESE-1, is expressed primarily in simple epithelial cells. Binding sites for *ets* factors in the 3′ Endo A enhancer have been shown to be critical for epithelium-specific Endo A gene expression (17, 73). Previously, Fli-1, ets-1, and ets-2 have been shown to transactivate the Endo A enhancer; however, ets-1 is highly expressed primarily in the immune system, which does not express the Endo A gene, and Fli-1 and ets-2 are expressed in epithelial and nonepithelial cells (17, 73). Furthermore, antibodies against ets-1 and ets-2 have failed to recognize the epithelial nuclear factor interacting with the Endo A enhancer (17, 73), suggesting that neither ets-1 nor ets-2 is the epithelial nuclear factor regulating the Endo A gene. We now provide evidence that ESE-1 may be a likely candidate for the epithelium-specific *ets* factor regulating Endo A gene expression, since ESE-1 binds to the Endo A enhancer and transactivates the Endo A enhancer *ets* site. In addition, we have unexpectedly observed induction of Endo A expression during in vitro keratinocyte differentiation 12 h after induction of ESE-1 mRNA expression. Endo A expression is usually associated with simple epithelium, and no function for Endo A in keratinocyte differentiation has been demonstrated, although Endo A gene expression can be induced in keratinocytes upon activation of the ras pathway or by transforming growth factor α (7, 8). We thus have identified two putative epithelium-specific target genes for ESE-1, even though these two genes show different expression patterns within the epithelial cell lineage. Our observation that ESE-1 expression is induced during keratinocyte differentiation and might be a regulator of the SPRR2A gene, which is induced during terminal differentiation, suggests that ESE-1 might play a role during keratinocyte differentiation. However, the expression pattern of ESE-1 clearly demonstrates that the function of ESE-1 is not restricted to stratified epithelia but might be more generally related to epithelial cell gene regulation and possibly differentiation or proliferation.

In summary, ESE-1 is the first *ets* factor expressed exclusively in epithelial cells. Since other members of the *ets* family, such as Pu.1, are essential regulators of cell differentiation, ESE-1 may play a similar critical role in epithelial cell differentiation. Indeed, our results demonstrate that ESE-1 expression is induced during terminal differentiation of keratinocytes, and ESE-1 may regulate expression of keratinocyte terminal differentiation genes. Isolation of this structurally and functionally unique *ets* factor provides exciting opportunities to test the hypothesis that aberrant ESE-1 expression plays a role in epithelial carcinogenesis, since several members of the *ets* family have been directly implicated in human malignancies.

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