ESE-3, a Novel Member of an Epithelium-specific Ets Transcription Factor Subfamily, Demonstrates Different Target Gene Specificity from ESE-1*

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Koen Kas‡§¶, Eduardo Finger‡, Franck Grall‡, Xuesong Gu‡, Yasmin Akbarali‡, Jay Boltax‡, Avi Weiss‡, Peter Oettgen‡, Rosana Kapeller** and Towia A. Libermann‡ ‡‡

From the ‡New England Baptist Bone and Joint Institute, Beth Israel Deaconess Medical Center, and Harvard Medical School, Boston, Massachusetts 02115, the §Laboratory for Molecular Oncology, Center for Human Genetics, University of Leuven & Flanders Interuniversity Institute for Biotechnology, Herestraat 49, B-3000 Leuven, Belgium, and **Millennium Pharmaceuticals, Cambridge, Massachusetts 02139

Most cancers originate as a result of aberrant gene expression in mainly glandular epithelial tissues leading to defects in epithelial cell differentiation. The latter is governed by distinct sets of transcriptional regulators. Here we report the characterization of epithelium-specific Ets factor, family member 3 (ESE-3), a novel member of the ESE subfamily of Ets transcription factors. ESE-3 shows highest homology to two other epithelium restricted Ets factors, ESE-1 and ESE-2. ESE-3, like ESE-1 and ESE-2, is exclusively expressed in a subset of epithelial cells with highest expression in glandular epithelium such as prostate, pancreas, salivary gland, and trachea. A potential role in branching morphogenesis is suggested, since ESE-3 transactivates the c-MET promoter via three high affinity binding sites. Additionally, ESE-3 binding to DNA sequences in the promoters of several glandular epithelium-specific genes suggests a role for ESE-3 in later stages of glandular epithelium differentiation. Although ESE-3 and ESE-1 bind with similar affinity to various Ets binding sites, ESE-3 and ESE-1 differ significantly in their ability to transactivate the promoters containing these sites. Our results support the notion that ESE-1, ESE-2, and ESE-3 represent a unique epithelium-specific subfamily of Ets factors that have critical but distinct functions in epithelial cell differentiation and proliferation.

Epithelial cell proliferation and differentiation is regulated by specific combinations of growth factors, hormones, adhesion molecules, and extracellular matrix, and epithelial-mesenchymal signaling is the critical hallmark of a developing glandular organ. How these divergent signals are integrated and governed is largely unknown, as is the nature of the transcriptional regulators involved. Ets factors constitute one important class of transcriptional regulators that play critical roles in hematopoiesis, angiogenesis, organogenesis, oncogenesis, and specification of neuronal connectivity (1–4). These proteins contain a highly conserved DNA binding domain, the Ets domain, binding to a consensus -GGA(A/T)- core sequence (3). In Drosophila and vertebrates, Ets factors have been shown to play important developmental roles (5–10). ETS1, ETS2, ERG2, and PU.1 are proto-oncogenes with mitogenic and transforming activity when overexpressed in fibroblasts (11–14). The relevance of Ets factors in human cancer has recently been highlighted by the discovery of several distinct and very specific chromosomal translocations involving various members of the Ets family in different types of human cancer. Most notably is TEL, which is found to be fused to various tyrosine kinases or transcription factor genes in different leukemias and congenital fibrosarcoma (15–20) and ERG, ERG-B/FLI-1, FEV, and E1AF/ETV4/PEA3 (21, 22), which are fused to the EWS gene in Ewing’s sarcoma and other primitive neuroectodermal tumors (23, 24). Several members of the Ets family, including Ets-1 and E1-AF, have also been directly implicated in tumor invasiveness (25).

Approximately 85% of cancers (carcinomas and adenomas) originate as a result of aberrant gene expression in mainly glandular epithelial tissues, leading to defects in epithelial cell differentiation and proliferation. Although many aspects of epithelium-specific gene expression have been determined, only a few epithelial cell-restricted transcriptional regulators are characterized to date. Because many Ets factors have been implicated in the development of cancer, epithelium-specific Ets factors may be prime candidates for being involved in certain epithelial tumors. We and others have recently discovered ESE-1 (1) (also called ESX, jen, ELF3, and ERT), the prototype of an epithelium-specific Ets transcription factor subfamily (26–28). ESE-1 is expressed in a variety of simple and stratified epithelia with particularly high expression in the epithelial lining of the gastrointestinal tract and in fetal lung. ESE-1 expression is highly inducible upon differentiation of keratinocytes correlating with induction of the terminal differentiation gene SPRR2A (26). Target genes for ESE-1 include at least five keratinocyte terminal differentiation markers, as well as the HER-2 gene, which is overexpressed in many breast cancer cell lines.

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¶ Postdoctoral fellow of the FWO.

‡ Both authors contributed equally to this work.

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cancers and other tumors (26–31). Finally, in mouse mammary gland, ESE-1 expression increases during first pregnancy in association with ductal budding, branching and the emergence of lobuloalveolar structures. Weaning also induces a dramatic increase in expression in association with glandular involution, suggesting that ESE-1 has a primary role in directing mammary gland remodeling and the early differentiation of ductal epithelium. As maximal induction of ESE-1 occurs during involution of mammary and prostate gland, this suggests an association with epithelial apoptosis (32).

Using computer-assisted EST library screening, we have identified two additional highly tissue-restricted members of the novel epithelium-specific Ets factor ESE subfamily: ESE-2 (epithelium-specific Ets factor, family member 2) (33) and ESE-3 (epithelium-specific Ets factor, family member 3), whose isolation, characterization, primary functional analysis, and comparison with ESE-1 we report here.

MATERIALS AND METHODS

Identification of ESE-3 cDNA—A novel human expressed sequence tag (EST) cDNA fragment with significant homology to the Ets domain of human ESE-1 (26) was identified in the Millennium EST data base using the tBLASTN program (NCBI, Ref. 34). The corresponding sequence was confirmed by repeating 5′-RACE PCR amplification using primers specific for the 5′ end of ESE-3, we performed the 5′ RACE method using human adult prostate cDNA ready for RACE (Marathon ready cDNA, CLONTECH) and nested primers specific for the partial ESE-3 cDNA, N1 (5′-CCCTCGAGGTGACGGTATCGATGATA-3′) and N2 (5′-GGCCTTT-GCTGGGGTCAAGAGGAT-3′) as described (35). Amplified DNA fragments were cloned and sequenced as described (35). The 5′ end sequence of the ESE-3 cDNA was confirmed by repeating 5′-RACE PCR amplification using primers specific for the 5′ end of the longest 5′-RACE products obtained in the first two rounds of PCR amplification.

DNA Sequencing and Computer Analyses—Nucleotide sequences were determined at the Beth Israel Deaconess Medical Center DNA sequencing facility using an Applied Biosystems Automatic DNA Sequencer model 373 using the T7 DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). Sequence analysis utilized DNA Strider, Lasergene (DNASTAR), and BLAST, BEAUTY and Clustal W searches (NCBI). All oligonucleotides were purchased from Life Technologies, Inc.

Northern Blot Analysis—Expression of ESE-3 was examined by Northern blot hybridization using the 1.2-kb ESE-3b prostate cDNA clone as a probe. Human tissue RNA blots (2 μg of poly(A)+ RNA loaded/lane) and a master Northern blot containing poly(A)RNA of 27 human tissues were purchased from CLONTECH, hybridized, and analyzed by autoradiography according to standard procedures as described (35).

RT-PCR Analysis—cDNAs were generated from 1 μg of mRNA isolated from different cells or tissues using oligo(dT)12-18 priming (Life Technologies, Inc.) and M-MLV reverse transcriptase (Life Technologies, Inc.) in samples treated with deoxyribonuclease I (Life Technologies, Inc.). Each PCR reaction used equivalent amounts of 0.1 ng of cDNA, 4 ng/μl amounts of each primer, 0.25 units of Taq polymerase (Promega, Madison, WI), 150 μM amounts of each dNTP, 3 μM MgCl2 reaction buffer, and water to a final volume of 25 μl and were covered with mineral oil. The sequences of the ESE-3 primers were: sense (5′-CCCTCGAGGTGACGGTATCGATGATA-3′) and antisense (5′-CCAGGAAGAGCACCCACAG-3′), with an expected amplification product of 309 bp. The sequences of the primers for GAPDH were: sense (5′-GGATGATCGACAGCATGATAGAGGAA-3′) and antisense (5′-CCAGGAAGAGCACCCACAG-3′), with an expected amplification product of 200 bp.

RT-PCR amplifications were carried out using a Perkin-Elmer Cetus thermal cycler 480 as follows: 20–30 cycles of 1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C followed by 10 min at 72 °C. Lower numbers of cycles were used to verify linearity of the amplification signal. 10 μl of the amplification product was analyzed on a 2% agarose gel.

In vitro Transcription—Translation—In vitro transcription-translation was performed in TNT rabbit reticulocyte lysate (Promega) in the presence of [35S]methionine (NEN Life Science Products) as described (36). The ESE-3a and ESE-3b cDNAs encoding the whole open reading frame inserted downstream of the T7 promoter into TA cloning vector pCR3 (Invitrogen) were transcribed under the control of this promoter.

Electrophoretic Mobility Shift Assays (EMSAs)—EMSAs were performed as described (36, 37) using 2 μl of in vitro translation product and 0.1–0.2 ng of 32P-labeled, double-stranded oligonucleotide probes (5000–20,000 cpm) in the presence or absence of competitor oligonucleotides (1 and 10 ng) and run on 4% polyacrylamide gels, containing as buffer 0.5x TGE as described (35). Oligonucleotides for competition and/or direct binding studies are below. The numbering of the MET promoter sites indicates the position of the first nucleotide of the Ets consensus site with respect to the transcription start site.

1) Drosophila E74 wild type (WT) oligonucleotide: 5′-TCAGAAGGGCAAGGGAAGAAAGCCGACCCCG-3′

2) Drosophila E74 MUT oligonucleotide: 5′-TCAGAAGGGCAAGGGAAGAAAGCCGACCCCG-3′

3) Human MET promoter, site A (−125), WT oligonucleotide (MET-A): 5′-GCTCCGGCCCTTCCTCCGGGAGGGG-3′

4) Human MET promoter, site A (−125), MUT oligonucleotide (MET-A-MUT): 5′-GCTCCGGCCCTTCCTCCGGGAGGGG-3′

5) Human MET promoter, site B (−83), WT oligonucleotide (MET-B): 5′-TGCGAGAGGCAGAGGACCGGAAAGCCGACCCCG-3′

6) Human MET promoter, site C (−65), WT oligonucleotide (MET-C): 5′-CGCCGTGGTCCCTTCCCTCCGGG-3′

7) Human MET promoter, site C (−65), MUT oligonucleotide (MET-C-MUT): 5′-GCTCCCGCCTTCCCTCCGGG-3′

8) Human MET promoter, site D (−25), WT oligonucleotide (MET-D): 5′-TGCGAGAGGCAGAGGACCGGAAAGCCGACCCCG-3′

9) Human MET promoter, site E (+153), WT oligonucleotide (MET-E): 5′-CGCCGTGGTCCCTTCCCTCCGGG-3′

10) Human MET promoter, site F (+378), WT oligonucleotide (MET-F): 5′-TGCGAGAGGCAGAGGACCGGAAAGCCGACCCCG-3′

11) Human MET promoter, site G (+378), WT oligonucleotide (MET-G): 5′-TGCGAGAGGCAGAGGACCGGAAAGCCGACCCCG-3′

12) Human MET promoter, site H (+378), WT oligonucleotide (MET-H): 5′-TGCGAGAGGCAGAGGACCGGAAAGCCGACCCCG-3′

Sequences 1–12

Cell Culture—Human bronchial epithelial cells, obtained from Clonetics Corp. and grown according to the manufacturer’s recommendations, were kindly provided by Dr. N. Moghal. Human foreskin keratinocytes, LNCaP (human prostate), HEK293 (human fetal epithelial kidney), C-33A (human cervical carcinoma), HaCaT (human keratinocytes), A431 (human vulvar carcinoma), HeLa (human cervical carci-
motif involved in mRNA turnover is underlined and boxed.

Sato via Dr. B. Baum were grown in Dulbecco’s modified Eagle’s medium (human glioma cells), U-937 (human monocytes), human synovial sarcoma, H157 (human large cell lung carcinoma), H249 (human small cell lung carcinoma) sets were used: MET Mut A, A Mut UP (5’-CTTTCCGGTGACACTCGCCTCCCAA-3’ and 5’-GGCGGGGGTCGCGC-3’). The alternative (alt. splice) 69-bp exon of ESE-3b inserted in the central portion of ESE-3a is boxed and shaded. The Pointed domain and the Ets domain are shaded and boxed and marked on the right. Both upstream termination codons in frame with the reading frame are also indicated by asterisks. The ATTAA motif involved in mRNA turnover is underlined (40).

Fig. 1. Nucleotide sequence of the 5’-UTR, coding region, and the first 235 bp of the 3’-UTR of human ESE-3. The deduced amino acid sequence of ESE-3 is indicated below the first nucleotide of each codon, and the termination codon is marked with three asterisks. The alternative (alt. splice) 69-bp exon of ESE-3b inserted in the central portion of ESE-3a is boxed and shaded. The Pointed domain and the Ets domain are shaded and boxed and marked on the right. Both upstream termination codons in frame with the reading frame are also indicated by asterisks. The ATTAA motif involved in mRNA turnover is underlined (40).

Identification and Molecular Cloning of ESE-3 cDNAs—Homology searches of the Millennium EST data base revealed one human 575-bp EST with noticeable sequence similarity to the Ets domain of human ESE-1 (26). This cDNA clone, isolated from a human prostate cDNA library, contained a open reading frame up to the 5’ end and a termination codon at the 3’ end of this clone and a termi-

RESULTS

Construction and Characterization of ESE-3 Promoters—To investigate the regulation of ESE-3, constructs were prepared using a point mutation strategy (39). Two promoter constructs were kindly provided by Dr. Gary Quinn. The SPRR2A basic pGL2 luciferase vector (Promega) eukaryotic expression vector downstream of the cytomegalovirus promoter. The MET promoter (GenBank accession no. Z26936), was amplified from human chromosome 7 DNA using the Advan-

Recombinant DNA Manipulation—Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene), converting the different ETS binding sites from GGAA to TTAA. The following primers were used: MET Mut A, A Mut UP (5’-CTTTCCGGTGACACTCGCCTCCCAA-3’ and 5’-GGCGGGGGTCGCGC-3’). The alternative (alt. splice) 69-bp exon of ESE-3b inserted in the central portion of ESE-3a is boxed and shaded. The Pointed domain and the Ets domain are shaded and boxed and marked on the right. Both upstream termination codons in frame with the reading frame are also indicated by asterisks. The ATTAA motif involved in mRNA turnover is underlined (40).
assisted EST library screening, we discovered one human colon
cDNA clone (clone 566368), the 5' end of which (AA149006)
overlapped with the partial 3'-UTR sequence of ESE-3. The
insert of this cDNA clone was sequenced and extended our
3'-UTR sequence by 1140 bp. RT-PCR was used to confirm the
colinearity of both sequences. Since the major transcript seen
on Northern blots is 5.9 kb, we still lack a part of the 5'-UTR
and/or the 3'-UTR of full-length ESE-3.

Predicted Amino Acid Sequence of ESE-3—Sequence analy-
sis revealed an open reading frame of 277 amino acids and a
predicted molecular mass of 32.3 kDa for ESE-3a and an open
reading frame encoding a 300-amino acid protein with a pre-
predicted molecular mass of 34.9 kDa for ESE-3b (Fig. 1). ESE-3a
lacks amino acids 159–181 (DLLDSKTFCRAQISMTTTSH-
LPV), a region without homology to any known sequence in the
public data bases. The ATG initiation codon only partially
conforms to the consensus eukaryotic translation initiation
sequence (GCC(A/G)CCATGG). An in-frame termination codon
is present 12 bp upstream of the ATG. Three ATTTA motifs,
associated with rapid mRNA turnover (40), are found in the
3'-UTR. A hydropathicity plot of the predicted amino acid se-
quences of ESE-3a and ESE-3b reveals primarily hydrophilic
proteins through the entire sequence. The deduced amino
acid sequences of ESE-3a and ESE-3b predict proteins rich in as-
paragine (8%), serine (7%), and leucine (11%). The amin-
terminal half of ESE-3 is characterized by a high abundance of
acidic residues (Fig. 1), whereas the carboxyl-terminal half
contains many basic residues. The central portion of the pro-
tein contains a leucine- and serine-rich domain. Several poten-
tial phosphorylation sites are present in ESE-3b including two
potential protein kinase C phosphorylation sites, three casein
kinase II phosphorylation sites, and one potential JNK/p38/
ERK kinase phosphorylation sites ((S/T)P) in the central region
of ESE-3, just behind the alternatively spliced exon (41, 42).
One remarkable feature is the presence of two stretches of
 glutamine residues between amino acids 42 and 123, inter-
spersed with 5 or 6 amino acids. The significance of this signa-
ture (if any) is presently unclear.

Sequence Comparison of ESE-3 with Other Members of the
Ets Family—Protein sequence homology and conservation be-
tween various Ets family members has proven useful in iden-
tifying functional domains. Comparison of the deduced amino
acid sequence of ESE-3 with those of other members of the Ets
family revealed that ESE-3, together with ESE-1 and ESE-2,
constitute a new, separate subfamily (Fig. 2). Indeed, align-
ment of the carboxyl-terminal Ets domain of ESE-3 with that of
other members of the Ets family, besides ESE-1 and ESE-2,
reveals

![Fig. 2](image-url)
highest homology to E74 (51%), NERF (49%), MEF (48%), ELF-1 (48%), and ERP (46%) (Fig. 3A). This degree of homology, however, is far below the homologies that are characteristic among known members of the Ets family. Sequence identity to other members of the Ets family is in the range of 36–51%. ESE-3 is least related to Spi-B (36%). Besides the highly conserved Ets DNA binding domain (amino acids 206–288), the amino terminus of ESE-3 contains a region (amino acids 42–112) with significant homology to the Pointed domain present in ESE-2. The identity of the Pointed domain does not exceed 30% with several other members of the Ets family including TEL, YAN, POINTED, ETS-1, ETS-2, ERG, FLI-1/ERG-B, and GABP-α/E4TF1–60 (Fig. 4A). The conserved mitogen-activated protein kinase phosphorylation site found in the Pointed domain of ets-1, ets-2, and Drosophila Pointed, however, is lacking in the ESE family members (43). The amino acid sequences of the 26 known mammalian and 7 Drosophila Ets family members were aligned with the Clustal W program (44) and

Fig. 3A, comparison of the Ets domain of ESE-3 with all known members of the Ets gene family. Percentage of identity of the Ets domain with ESE-3 is indicated on the right side. Shaded amino acids denote amino acid identity with ESE-3. Gaps are introduced to optimize alignment. The proteins examined are indicated on the left side. Only human and Drosophila Ets factors are included for simplicity. GenBank accession numbers are as follows: ERG, M21536; ERG-B/FLI-1, Y17293; ETS-3, M88473; FEV, Y08976; ETS-6, M88475; ERF, U15655; PE-1/ETV3, L16464; GABP-α/E4TF1–60, Q06546; ELG, M88471; ETS-1, X14798; ETS-2, AF017257; POI G E N T D, S33167; ERL7/ETV2, AC002115; ERP/NET, Z36715; SAP-1, P28323; ELK1, P19419; ER51/ETV1, U17163; ERMD, X69381; PEA3/ELF4/ETV4, U18018; ETS-4, M83474; PDEF, AF071538; MEF, U32645; NERF, U43188; ELF-1, P32519; E74, A53225; TEL/ETV6, U11732; TEL-2, AF116508; YAN, Q01842; ESE-1, U73844; ESE-2, AF115402; ESE-3 (AF124439); PU.1, X52056; SpiB, X66079. B, phylogenetic analysis of the Ets domain family tree, linking members with closely homologous amino acid sequences. Phenogram representation of the inferred phylogenetic tree based on degree of amino acid sequence homology is shown. Branch lengths indicate relative similarities (short branch lengths indicate highly similar homologs). Only human and Drosophila Ets factors are included for simplicity. For GenBank accession numbers, see legend for panel a.
used to infer phylogenetic trees of the Ets domain (Fig. 3B) and the Pointed domain (Fig. 4B) by distance methodology. In the case of the Ets domain, the alignment is robust because sequence conservation within the Ets domain is quite high, with the most divergent member displaying still 36% identity to the ESE-3 sequence. The length of the horizontal lines (branches) connecting different genes indicates relative sequence similarity of their Ets domain sequences. Genes are clustered into groups of highly related homologs as indicated by short branch lengths. We identify 11 groups of Ets factors with at least two members (Fig. 3B). ESE-1, ESE-2, and ESE-3 clearly define a distinct subfamily of Ets factors. Only 12 known mammalian and 2 Drosophila Ets factors contain the Pointed domain. Remarkably, the alignment of the different Pointed domains also groups ESE-2 and ESE-3 close together, while the Pointed domain in ESE-1 is much more distinct.

Thus, ESE-1, ESE-2, and ESE-3 represent a new class of Ets factors, particularly based on structural homology in the Ets and to a lesser extent in the Pointed domain. For GenBank accession numbers, see legend to Fig. 3A. Phylogenetic analysis of the Pointed domain family tree linking members with closely homologous amino acid sequences. Phenogram representation of the inferred phylogenetic tree based on degree of amino acid sequence homology is shown. Branch lengths indicate relative similarities (short branch lengths indicate highly similar homologs). Only human and Drosophila Ets factors are included for simplicity. The sequences are compared with the SAM domain containing proteins PH (Drosophila Polyhomeotic, P39769), SCM (Drosophila Sex comb on Midleg, U47983) and EphB2 receptor tyrosine kinase (L25890). For other GenBank accession numbers, see legend for Fig. 3A.

Expression Pattern of ESE-3 in Human Tissues—To examine the tissue distribution of ESE-3 and the size of its transcript, we investigated the level of ESE-3 mRNA in several adult human tissues by Northern blot analysis using the ESE-3 cDNA as a probe. To control for RNA quality and quantity, Northern blots were rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. One distinct transcript of 5.9 kb was identified in the pancreas and the prostate. Lower level expression of this transcript was detected in the kidney and in colon (Fig. 5A). Absolutely no expression could be detected in heart, brain, placenta, liver, skeletal muscle, spleen, thymus, testes, and peripheral blood leukocytes. To extend our analysis to a larger number of tissues including fetal tissues, we performed mRNA dot blot analysis, showing high ESE-3 expression in salivary gland, prostate, and trachea, and a lower level of expression in colon, mammary gland, pancreas, lung, stomach, appendix, as well as fetal kidney and fetal lung (Fig. 5B). To compare expression of ESE-3 to expression of the two other ESE family members, the Northern blots were rehybridized with a cDNA probe for ESE-1 (26) and ESE-2 (33) (Fig. 5). ESE-1 expression is highest in small intestine. High levels of the same 2.2-kb ESE-1 transcript were also found in prostate, ovary, colon, placenta, kidney, liver, and pancreas, whereas no expression was detected in heart, brain, spleen, thymus, testis, and peripheral blood lym-
FIG. 5. Cell type specificity of ESE-3 as compared with ESE-1 and ESE-2. A, Northern blot analysis of poly(A)⁺ mRNA from human adult tissues including heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), pancreas (lane 8), spleen (lane 9), thymus (lane 10), prostate (lane 11), testis (lane 12), ovary (lane 13), small intestine (lane 14), colon (lane 15), and peripheral...
phocytes. In skeletal muscle, although the 2.2-kb transcript was not expressed, two additional, thus far uncharacterized, transcripts of 1.9 and 1.1 kb were exclusively and highly expressed, suggesting skeletal muscle-specific alternative splice forms of ESE-1, or a highly related gene. ESE-2 expression is mainly seen in salivary gland, trachea, kidney, prostate, mammary gland, and fetal kidney as a 2.4–2.6-kb transcript, with lower levels in fetal and adult lung, an expression pattern remarkably similar to ESE-3.

Combined, these results suggest that all three ESE family members, in contrast to other Ets family members, are expressed in a very restricted set of epithelial tissues and might, therefore, have a very specialized function.

**ESE-3 Is Exclusively Expressed in Epithelial Cells**—Since ESE-3 expression is restricted to tissues with high epithelial cell content, we were interested to know which types of cells express ESE-3. To analyze in more detail the expression of ESE-3 in different cell types, we performed RT-PCR with RNA derived from different cell types using both primary cells and cancer-derived cell lines. Only a subset of cells derived from epithelial origin such as HSG human submandibular gland cells, human foreskin epithelium cells, primary human bronchial epithelial cells, LNCaP human prostate cells, HaCaT human keratinocytes, or A431 vulvar carcinoma cells expressed ESE-3. HUVEC endothelial cells, U-87 Mg and U-138 Mg human glioma cells, U-937 (human monocytes), human synovial fibroblasts and human chondrocytes using primers specific for ESE-3 (upper panel) or GAPDH (lower panel), as described under "Materials and Methods."

Fig. 6. Expression of ESE-3 in various cell types. RT-PCR analysis of reverse transcribed total RNA from HSG (human submandibular gland epithelial duct cells) and mRNA from primary human bronchial epithelial cells, human foreskin epithelium, LNCaP (human prostate), HEK293 (human embryonic kidney), C-33A (human cervical carcinoma), HaCaT (human keratinocytes), A431 (human vulvar carcinoma), HeLa (human cervical carcinoma), H157 (human large cell lung carcinoma), H249 (human small cell lung carcinoma), HUVEC (human endothelial cells), U-87 Mg and U-138 Mg (human glioma cells), U-937 (human monocytes), human synovial fibroblasts and human chondrocytes using primers specific for ESE-3 (upper panel) or GAPDH (lower panel), as described under "Materials and Methods."

**ESE-3**

- **ESE-3** is a novel epithelium-specific Ets gene that is expressed in a very restricted set of epithelial tissues and might have a specialized function.
- ESE-3 expression is restricted to tissues with high epithelial cell content, and only a subset of cells derived from epithelial origin, such as HSG, human submandibular gland cells, express ESE-3.
- ESE-3 expression is mainly seen in salivary gland, trachea, kidney, prostate, mammary gland, and fetal kidney as a 2.4–2.6-kb transcript.
- ESE-3 expression is restricted to tissues with high epithelial cell content, and only a subset of cells derived from epithelial origin, such as HSG, human submandibular gland cells, express ESE-3.
- ESE-3 expression is mainly seen in salivary gland, trachea, kidney, prostate, mammary gland, and fetal kidney as a 2.4–2.6-kb transcript.
- ESE-3 is exclusively expressed in epithelial cells, as demonstrated by RT-PCR analysis of reverse transcribed total RNA from various cell types.

**ESE-3**

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- ESE-3 expression is restricted to tissues with high epithelial cell content, and only a subset of cells derived from epithelial origin, such as HSG, human submandibular gland cells, express ESE-3.
- ESE-3 expression is mainly seen in salivary gland, trachea, kidney, prostate, mammary gland, and fetal kidney as a 2.4–2.6-kb transcript.
- ESE-3 is exclusively expressed in epithelial cells, as demonstrated by RT-PCR analysis of reverse transcribed total RNA from various cell types.
Fig. 7. Interaction of ESE-3 with Ets binding sites in the MET promoter and 5'-UTR and binding of ESE-1 and ESE-3 to SPRR2A, PSMA and E74 Ets sites. A, EMSAs of in vitro translated ESE-3b and ESE3a incubated with the labeled E74 oligonucleotide probe were carried out with no competitor (left panel), 1 and 10 ng of unlabeled wild type E74 oligonucleotide, or 1 and 10 ng of mutant E74 oligonucleotide (right panel). The arrow indicates the specific DNA-protein complex. B, DNA binding of in vitro translated ESE-3a in an EMSA using synthetic oligonucleotides coding for the 6 c-MET promoter Ets sites (MET-A (lane 2) to MET-F (lane 12)), always compared with binding of unprogrammed reticulocyte lysate (−) (lanes 1, 3, 5, 7, 9, and 11). The arrow indicates the specific DNA-protein complex. C, Direct DNA binding of ESE-3a to the MET promoter Ets site A. EMSAs of in vitro translated ESE-3a incubated with the labeled MET site A oligonucleotide probe were carried out with either no competitor (lane 2), 1 and 10 ng of unlabeled wild type site A oligonucleotide (lanes 3 and 4), or 1 and 10 ng of mutant site A oligonucleotide (lanes 5 and 6). The arrow indicates the specific DNA-protein complex. D, DNA binding of in vitro translated ESE-1 and ESE-3b in an EMSA using synthetic oligonucleotides coding for the SPRR2A (lane 1 and 2), PSMA (lanes 5 and 6), and E74 Ets sites (lane 8 and 9), always compared with binding of unprogrammed reticulocyte lysate (−) (lanes 3, 4, and 7). The arrow indicates the specific DNA-protein complex.
contains 6 putative Ets binding sites and has previously been shown to be responsive to the Ets factor ETS-1 (47). However, the expression pattern of ETS-1 does not correlate very well with the expression and function of c-MET, since ETS-1 is highly expressed in B and T lymphocytes, but not in epithelial cells. Furthermore, knock-out mice for ETS-1 have primarily shown defects in the immune system, suggesting that other Ets factors are responsible for epithelial cell expression of c-MET. To analyze whether ESE-3 may be the epithelial Ets factor that interacts with the human c-MET promoter and 5'-UTR Ets sites, we performed EMSAs with in vitro-translated full-length ESE-3a protein and the unprogrammed reticulocyte lysate and oligonucleotides encoding the six putative c-MET promoter Ets binding sites as probes. A nonspecific complex was formed with both the unprogrammed reticulocyte lysate and ESE-3a. The oligonucleotides encoding site A and site C of the c-MET promoter formed strong, specific protein-DNA complexes, which were only seen with the ESE-3a protein. The oligonucleotides encoding site B and site F showed weaker binding to ESE-3a and the ones encoding site D and site E showed no significant ESE-3 binding (Fig. 7B). The ability of ESE-3 to bind to MET promoter Ets sites A and C specifically was further confirmed by competition experiments. Binding of ESE-3 to site A (Fig. 7C, lane 2) was competed efficiently by 1 or 10 ng of wild type site A itself (lanes 3 and 4) but not by the mutant site A oligonucleotide (lanes 5 and 6). The same holds true for binding to site C (data not shown). These results demonstrate that ESE-3 binds with high affinity to several binding sites in the c-MET promoter. Like ESE-3, ESE-1 binds with similar affinities to the c-MET promoter Ets sites (data not shown).

To further extend our analysis of potential target genes for ESE-3, we chose a spectrum of different glandular epithelium-specific genes containing putative binding sites for Ets factors, including the CRISP-1, CRISP-3, MP6, PSA, PSMA, PSP94, and PSP genes. We also included the SPRR2A gene, which contains a keratinocyte-specific promoter consisting of four critical regulatory elements including an Ets binding site essential for promoter activity during keratinocyte terminal differentiation (29), as well as the Endo A gene, containing 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 (48, 49). Both have been shown previously to specifically bind ESE-1, the closest homolog to ESE-3 (26). EMSA analysis showed strong binding of ESE-3a and ESE-1 to a subset of the promoter Ets sites of the genes encoding parotid secretory protein PSP, cysteine-rich secretory protein CRISP-1, proline-rich protein MP6, PSA, and the prostate-specific membrane antigen PSMA. ESE-3 can also bind to the promoter and the enhancer SPRR2A and EndoA sites respectively, as does ESE-1. Binding of in vitro translated full-length ESE-1 and ESE-3b to the SPRR2A, PSMA, and E74 sites is shown in Fig. 7D. These results demonstrate that ESE-3 can bind in addition to the c-MET promoter to Ets binding sites in the regulatory regions of several glandular epithelium-specific genes. The relevance of this data is currently under investigation. These data further illustrate that ESE-1 and ESE-3 have similar binding specificities for a number of Ets sites, which is also reflected in the high conservation of the Ets DNA binding domain.

**ESE-3b Acts as a Transactivator of the c-MET Promoter and Demonstrates Distinct Transactivation Specificities from ESE-1—**To determine whether ESE-3 acts as a repressor or enhancer of transcription and to further evaluate the possibility that the c-MET promoter is a biologically relevant target for ESE-3, both ESE-3 isoforms were inserted into a eukaryotic expression vector (pCI/ESE-3a, pCI/ESE-3b) and co-transfected into HEK293 cells together with a pGL2 reporter gene construct containing the 600-bp c-MET promoter and 5'-UTR in front of the luciferase gene. Transfection of the reporter by itself (the promoterless pGL2 vector) or pGL2 plus the parental expression vector pCI (the expression cloning vector) alone failed to induce luciferase activity (Fig. 8). Co-transfection with the two ESE-3 isoforms resulted in a 5-fold transcriptional stimulation of the c-MET promoter only by ESE-3b compared with that with the parental pCI vector, whereas ESE-3a was only marginally active (Fig. 8a). This difference in transactivation capacity between ESE-3a and ESE-3b does not appear to be due to different levels of protein expression (data not shown). Thus, the c-MET gene contains two high affinity binding sites for ESE-3, which, as part of the regulatory region controlling c-MET gene expression, can be specifically transactivated by ESE-3b. These data show that ESE-3b is a positive regulator of transcription and that the c-MET gene might indeed be a relevant epithelium-specific target for ESE-3b.

In order to determine whether the highly related ESE-1 is also able to transactivate the c-MET promoter, we compared the transactivation capacity of ESE-3 with ESE-1. In contrast to ESE-3, ESE-1 did not transactivate the c-MET promoter, demonstrating that ESE-3 and ESE-1 have distinct specificities. These differences in transactivation capacities between ESE-3 and ESE-1 were further confirmed in co-transfection experiments with the PSA and PSMA promoter as well as SPRR2A promoter and EndoA enhancer oligo luciferase reporter constructs. Whereas ESE-3b activated the PSA promoter 2-fold, ESE-1 strongly repressed the PSA promoter (Fig. 8b). In contrast, ESE-3b has no effect on the PSMA promoter, while ESE-1 activates the PSMA promoter 3-fold (Fig. 8c). Two copies of the SPRR2A promoter Ets site oligo were induced 4-fold by ESE-1, but only 2-fold by ESE-3b (Fig. 8d). Finally ESE-3b and ESE-1 activate the EndoA enhancer Ets site oligo equally well (Fig. 8c). These results most vividly demonstrate that despite their significant structural similarities ESE-3 and ESE-1 express strikingly distinct functional activities. This distinct function cannot be explained by differences in their binding specificities, since ESE-3 and ESE-1 express practically undistinguishable binding affinities toward different Ets sites and bind indeed with similar affinity to the c-Met promoter, PSA, PSMA, EndoA, and SPRR2A Ets sites. These data further support the notion that the specificity of a particular Ets factor for a promoter is determined by distinct protein-protein interactions with other transcription factors or co-activators/co-repressors binding to regulatory elements in one, but not the other promoter.

**Multiple Ets Sites Are Involved in the Transactivation of the c-MET Promoter by ESE-3—**In order to determine which of the four binding sites (A, B, C, and F) within the c-MET promoter that interact with ESE-3 are involved in transcription by ESE-3, we introduced point mutations in each of these binding sites in the context of the c-MET promoter/luciferase construct. The wild type and mutant c-MET promoter Ets sites were compared with the parental pCI vector, whereas ESE-3a was only marginally active (Fig. 8a). This difference in transactivation capacity between ESE-3a and ESE-3b does not appear to be due to different levels of protein expression (data not shown). Thus, the c-MET gene contains two high affinity binding sites for ESE-3, which, as part of the regulatory region controlling c-MET gene expression, can be specifically transactivated by ESE-3b. These data show that ESE-3b is a positive regulator of transcription and that the c-MET gene might indeed be a relevant epithelium-specific target for ESE-3b.
mutated together. Transactivation of this triple mutant by ESE-3b was drastically reduced by 80–90% (Fig. 9). These data demonstrate that ESE-3b transactivates the c-MET promoter via at least three high affinity binding sites.

DISCUSSION

We have isolated a novel member of the Ets transcription factor/oncogene family, ESE-3, with two alternative splice products ESE-3a and ESE-3b. Comparison of the deduced amino acid sequence of ESE-3 with those of other members of the Ets family reveals the highest level of homology to the Ets domain of the epithelium-specific Ets factors ESE-1 (ESX, jen, ELF3, ERT) (84%) (26) and ESE-2 (ELF5) (65%) (33) (Fig. 3). In the Pointed domain at the amino terminus, ESE-3 shows higher homology to ESE-2 than to ESE-1 (Fig. 4). The fact that this domain is not found in the Ets factors ELF1, NERF, and MEF, the next closest Ets factors to ESE-3 besides ESE-1 and ESE-2, is further support for the classification of the three ESE proteins in one subfamily (Figs. 3 and 4). The Pointed domain belongs to the class of SAM domains. This domain plays a functional role in mediating homo- and heterodimerization, as was shown for the Ets transcription factor TEL (50, 51), polycomb proteins (52), and Eph receptors (53). Despite the high conservation of the Pointed domain among a set of Ets factors, only Tel has been shown up to now to be able to homodimerize. Our phylogenetic analysis clusters all 26 known mammalian Ets factors (as of February, 1999), including two newly discovered, unpublished sequences of our laboratory (PDEF and TEL-2) as well as seven Drosophila genes (Figs. 3 and 4). Based on the Ets domain, we distinguish 11 Ets families with at least two members (Fig. 3). Ets genes containing a Pointed domain cluster basically to the same group, regardless of whether the Ets domain or the Pointed domain are used to make the alignments. ESE-1 is a clear exception to this rule, as its Pointed domain is more divergent than all the other Pointed domains thus far described (Fig. 4).

With a few exceptions, Ets proteins are broadly expressed in different tissues and cells. The only other known Ets factors with a very restricted pattern of expression are the Ets factor
subfamily Spi-1/Pu.1, Spi-B, and Spi-C, which are solely expressed in the immune system (Fig. 3). ESE-3, described here, together with ESE-1 (26) and ESE-2 (33), constitute the second subfamily of Ets proteins, which not only share considerable sequence homology (Fig. 2), but which are all expressed in a similar subset of epithelial tissues (Fig. 5). Noteworthy, the Ets factor subfamily members ERG, FLI-1/ERG-B, and FEV, while less confined with regard to expression (Fig. 3), all have been detected as translocation partners of the EWS gene in Ewing sarcoma (21, 23). This again might point to a functional homology, surpassing their structural homology. Of note is the presence in the dbEST library of a murine eight-cell embryo expressed sequence tag (EST, accession no. AU019064) homologous to the 3′-UTR of murine ESE-3, pointing to an important function of ESE-3 already at the earliest stages of development at a time when the first differentiation processes toward the epithelial cell lineage are in progress.

Several transcription factors that are involved in epithelium-specific gene expression have been characterized, but few of these are restricted to epithelial cells. The identification of these three epithelium-specific Ets transcription factor genes will undoubtedly lead to exploration of the nature of epithelial cell differentiation. ESE-1 (26), independently isolated as ESX (28), ELF3 (54), JEN (27), and ERT (55), is expressed in many, but not all epithelial cell types and plays a role during terminal differentiation of the epidermis, mammary gland remodeling and the early differentiation of ductal epithelium (26, 27, 32).

ESE-3 is expressed mainly in epithelial cells of glandular organs. Given this restricted expression pattern, we reasoned that ESE-3 might be involved in epithelial differentiation, i.e. in the regulation of glandular epithelium-specific genes or in the process of branching morphogenesis. One of the major players involved in epithelial differentiation is the c-MET gene encoding the receptor for scatter factor or hepatocyte growth factor. This heterodimer has biological activities on epithelial sheets, including mitogenesis, cell-cell dissociation, stimulation of migration into the extracellular matrix, induction of cell polarization, and branched tubulogenesis. Oncogenically activated c-MET confers transforming, invasive, and metastatic properties to normal cells (56). Our transfection experiments demonstrate that ESE-3, but not ESE-1 can indeed transactivate the c-MET promoter, but also show a differential transactivation capacity of both ESE-3 isoforms, which might point to an important activation domain encoded by the alternative exon present in ESE-3b or differences in protein expression or stability. Alignment of the human and mouse promoter and 5′-UTR region of the c-MET gene reveals that Ets binding sites A, B, and E are conserved, and that sites A and B localize to a subregion of the promoter which exhibits enhancing effects on the MET promoter (57, 58). Interestingly, mutation of a single Ets site does not eliminate transactivation by ESE-3. Only combined mutation of sites A, B, and C, which all bind to ESE-3, leads to a drastic decrease in ESE-3-mediated transactivation. Combined, these data suggest that at least three Ets sites, A, B, and C, are critical for the activation of the c-MET promoter by ESE-3 (Fig. 9). The cooperative effect of several Ets sites within a regulatory region is a phenomenon commonly seen in a variety of genes that are regulated by Ets factors such as the immunoglobulin heavy chain enhancer (3, 36).

Tubulogenesis and branching morphogenesis are developmental processes common to the formation of many organs, most prominently lung, trachea, salivary gland, mammary gland, pancreas, prostate, and kidney (Ref. 59 and references therein), i.e. the same tissues that express ESE-2 and ESE-3. The ESE family of transcription factors might be important regulators that control the appropriate spatiotemporal pattern of gene expression during normal organogenesis. Recently, the mouse homolog of ESE-3b was described as Ehf (46). Bochert and co-workers (46) isolated Ehf using differential display analysis from mouse pituitary somatotroph tumor tissue, indicating another possible role for ESE-3, i.e in the regulation of somatotroph development or pituitary tumorigenesis. Besides a function in tubulogenesis and branching morphogenesis, ESE-3 may be involved in the regulation of genes encoding proteins secreted by glandular organs. Indeed promoters of various genes specifically expressed in glandular tissues such as CRISP-1, CRISP-3, MP6, PSA, P5P, PSMA, and PSP94 contain high affinity binding sites for ESE-3 (Fig. 7D) (60–65). To investigate whether ESE-3 has distinct properties from the closely related ESE-1, we compared the transcriptional activation capacities of both factors as well as their binding affinities toward different Ets sites. As becomes clear in Fig. 8 (a–c), different scenarios exist. For the PSA promoter, ESE-3b weakly activates while ESE-1 strongly represses (Fig. 8b); the c-MET promoter is activated by ESE-3b, but not by ESE-1, the PSMA promoter gets activated by ESE-1 and not by ESE-3 (Fig. 8c); both ESE-1 and ESE-3 transactivate the SPRR2A oligo but to a different extent (Fig. 8d); ESE-1 and ESE-3 activate the EndoA oligo in a comparable way (Fig. 8e).

These data clearly indicate that both ESE proteins behave...
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Finally, using YAC clone/computer aided chromosomal localization, ESE-3, as well as ESE-2, have been assigned by us to chromosome 11p14.1,2 near the WAGR syndrome (Wilms tumor, aniridia, genito-urinary anomalies and mental retardation) deletion region (66).

In summary, we have identified and studied the function of a novel, glandular epithelium-specific member of the Ets trans-cription factor family, ESE-3, the third member of the tissue-specific Ets factor ESE subfamily. Identification and characteri-zeration of a novel Ets factor may contribute to a better understanding of the role that Ets factors play in normal development and pathologic processes.

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difference with regard to transcriptional activation. Nevertheless, both ESE-3 and ESE-1 bind with similar affinity to the various Ets sites tested. Since the DNA binding domains of both ESE-3 and ESE-1 are very similar and their DNA binding specificity is almost identical, it is unlikely that differences in their trans-activation capacities toward different promoters is due to differ-ences in DNA binding. These differences are most likely reflections of the distinct amino-terminal regions of both pro-teins and the resulting differences in protein-protein inter-ac-tions with other transcription factors and/or co-activators/co-repressors that bind or interact with regulatory elements unique for each promoter. Additional differences could be the result of distinct post-translational modifications such as phos-phorylation by different sets of kinases.

Finally, using YAC clone/computer aided chromosomal localization, ESE-3, as well as ESE-2, have been assigned by us to chromosome 11p14.1,2 near the WAGR syndrome (Wilms tumor, aniridia, genito-urinary anomalies and mental retardation) deletion region (66).