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SCIENCE DIRECT
Gene 367 (2006) 118 – 125
GENE
SECTION
FUNCTIONAL GENOMICS
www.elsevier.com/locate/gene

Identification of an epithelial-specific enhancer regulating *ESX* expression

Richard M. Neve [b,c,*], Hema Parmar [b], Cliff Amend [a], Chira Chen [b], Angie Rizzino [d], Christopher C. Benz [a]

[a] *Buck Institute for Age Research, 8001 Redwood Boulevard, Novato, CA 94945, USA*
[b] *Cancer Research Institute, University of California San Francisco, Box 0808, San Francisco, CA 94143-0808, USA*
[c] *Lawrence Berkeley National Laboratory, 717 Potter Street, Berkeley, CA 94710-2722, USA*
[d] *Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198-6805, USA*

Received 21 June 2005; received in revised form 30 September 2005; accepted 2 October 2005
Available online 15 December 2005
Received by A.J. van Wijnen

Abstract

The Ets transcription factor, *ESX*, exhibits a unique pattern of epithelial-restricted expression and transactivates genes involved in epithelial differentiation and cancer. The aim of this study was to determine the underlying genetic basis for epithelial-specific expression of *ESX*. We have identified a 30bp *ESX* enhancer sequence (EES) approximately 3 kb upstream of the proximal promoter. This region displays enhancer activity in an epithelial-specific manner and deletion of this region abrogates *ESX* gene transcription. An EES binding protein complex (EBC) was identified through electrophoretic mobility shift assays whose degree of EES binding correlated well with endogenous *ESX* levels in epithelial cells and was regulated by epithelial differentiation. Understanding the regulation of this element will lend insight into mechanisms of epithelial differentiation and the etiology of breast cancer and may provide novel targets for cancer therapeutic intervention.

Keywords: Ets; Differentiation; Breast Cancer; ELF3; ESE1

1. Introduction

Ets transcription factors regulate gene expression during a number of biologic processes including proliferation, transformation, differentiation, morphogenesis and development. The Ets family comprises more than 40 members, many of which have been shown to be involved in the development and homeostasis of cell lineages as well as in the genesis of human cancer (Dittmer and Nordheim, 1998; Sharrocks, 2001). Null mutations of various Ets factors result in embryonic or neonatal lethality indicating a critical role in early development (Remy and Baltzinger, 2000; Ng et al., 2002) whereas genetic translocations of Ets genes are associated with early life cancers and leukemias (Dittmer and Nordheim, 1998). While there has been extensive research into the role of Ets factors in haemopoeitic lineages very little is known about Ets factors in epithelial lineages.

Recently, a number of independent reports identified a novel Ets factor, *ESX* (also termed Elf3, Jen, Ert and Ese-1), which is associated with mammary gland development, carcinogenesis and epithelial differentiation (Andreoli et al., 1997; Oettgen et al., 1997; Tymms et al., 1997; Neve et al., 1998; Chang et al., 1999; Brembeck et al., 2000; Yoshida et al., 2000). *ESX* is of particular interest as it is found exclusively expressed in terminally differentiated epithelial cells in the mammary gland, colon, trachea, kidney, prostate, uterus, stomach and skin under homeostatic conditions (Andreoli et al., 1997; Oettgen et al.,

Abbreviations: RA, retinoic acid; Act. D, actinomycin D; TGF-β, transforming growth factor beta; TGF-βRII, type II TGF-beta-receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3 kinase; EMSA, electrophoretic mobility shift assay; hFib, human fibroblasts.

* Corresponding author. Lawrence Berkeley National Laboratory, 717 Potter Street, Berkeley, CA 94710-2722, USA. Tel.: +1 510 486 6815; fax: +1 510 495 2535.
E-mail address: rmneve@lbl.gov (R.M. Neve).

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doi:10.1016/j.gene.2005.10.001

1997; Tymms et al., 1997; Neve et al., 1998). A number of genes have been identified as transcriptional targets of *ESX*, including transforming growth factor beta-type-II receptor (TGF-β RII) (Choi et al., 1998; Kim et al., 2002), several differentiation-specific markers (Andreoli et al., 1997; Oettgen et al., 1997; Brembeck et al., 2000; Yoshida et al., 2000), MIP3-α (Kwon et al., 2003), nitric oxide synthase (Rudders et al., 2001) and tumour-associated genes (Eckel et al., 2003). *ESX* is also a putative mediator of inflammatory shock and host defense (Rudders et al., 2001) and it is in response to inflammatory stimuli that *ESX* expression is induced in non-epithelial cells (Rudders et al., 2001). In breast cancer, *ESX* expression is significantly correlated with ErbB2 expression, and is a downstream transcriptional target of ErbB2 (Neve et al., 2002).

Here we sought to identify *cis*-acting elements within the *ESX* locus that control the epithelial-specific expression of *ESX*. We describe a 30-bp enhancer element approximately 3 kb upstream of the transcriptional start point that directly controls *ESX* expression. This *ESX* enhancer sequence (EES) is regulated in response to differentiation and appears to be positively controlled by an EES binding protein complex (EBC).

2. Materials and methods

2.1. Cell culture and transient transfection assays

Sk-Br-3 cells were grown in McCoys5A medium supplemented with 10% fetal calf serum. MCF7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) 1 g/L glucose supplemented with 10% fetal calf serum and 0.01mg/ml bovine insulin. MCF10A and MCF12A cells were grown in a 50:50 mix of DMEM/Hams F12 supplemented with 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 50 ng/ml hydrocortisone and 5% horse serum. HC11 cells were grown in RPMI medium supplemented with 10% fetal calf serum, 5 μg/ml insulin and 10 ng/ml EGF. Human fibroblasts were maintained in a 50:50 mix of DMEM/Hams F12 supplemented with 5 μg/ml Insulin and 10% fetal calf serum. All cells were grown at 37 °C and 5% CO_2.

Transient transfections were carried out using Effectene (Qiagen) transfection reagent according to manufacturer's instructions. Briefly, cells were grown in 6-well plates and transfected at approximately 50% confluency. For each well 1 μg of the luciferase reporter plasmid and 0.5 μg of *LacZ* plasmid (internal control) were co-transfected to control for transfection efficiency. After 24 h cells were washed in phosphate-buffered saline (PBS) and extracted using passive lysis buffer (Promega) and luciferase assays performed with a luciferase assay system (Promega) and luminometer. Beta-galactosidase values were obtained using a chemiluminescent reporter assay system (Tropix Galactolight Assay) and used to normalize luciferase values.

2.2. Immunochemical techniques

For immunoblot analysis, cleared cell lysates were electrophoretically resolved on denaturing sodium doedecyl sulphate (SDS)-polyacrylamide gels (4–12%), transferred to polyvinylidene difluoride (PVDF, Millipore), and probed with specific antisera. The primary antibody to *ESX* was prepared and purified as in Chang et al. (1997).

2.3. Northern analysis

For Northern blotting, total cellular RNA was purified from cells using RNeasy (Qiagen). RNA (20 ug/sample lane) was electrophoresed through 1% formaldehyde-agarose gels and transferred to nylon membranes. Blots were probed with a c-terminal single-stranded PCR-amplified DNA fragment corresponding to the anti-sense strand of the last 400 bp of the 3′ *ESX* cDNA sequence. After annealing and a final wash at 65 °C in 0.2× SSC the membrane was exposed to film and the autoradiograph developed.

2.4. Reverse-transcriptase PCR

RNA was isolated from cells using the RNeasy kit (Quiagen), and semi-quantitative RT-PCR was performed using the Superscript One Step RT-PCR system (Invitrogen). Briefly, the reactions were prepared with 0.5 μg total RNA and primers for *ESX* (forward: 5′-GATCATTGAGCTGCTGGAGAAGGA-3′, reverse: 5′-GTCCCAGTACTCTTTGCTCAGCTT-3′) that yield a 317 bp product. The cDNA was generated at 50 °C for 30 min. Subsequent denaturation for 2min at 94 °C was followed by 40 cycles of 94 °C, 30 s; 56 °C, 30 s; 72 °C, 45 s. The RT-PCR products were separated on a 0.8% agarose gel and visualised under UV light.

2.5. Cell lysates, nuclear extracts and EMSAs

For preparation of protein lysates, cells were washed in ice cold PBS containing 1mM phenylmethylsulfonyl fluoride (PMSF) and then with a buffer containing 50mM HEPES (pH7.5), 150 mM NaCl, 25 mM β-glycerophsphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 15 mM pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium molybdate, leupeptin (10 μg/ml), aprotinin (10 μg/ml) and 1 mM PMSF. Cells were extracted in the same buffer containing 1% Nonidet-P40. Lysates were then clarified by centrifugation and frozen at −80 °C. Protein concentrations were determined using the Bio-Rad protein assay kit.

Nuclear extracts were prepared using the method of Dignam et al. (1983). Electrophoretic mobility shift assays (EMSAs) were performed using 5 μg of nuclear extract and 3×10^4 cpm of end-labelled double stranded oligonucleotides (EEC sense; 5′-GAAGCCGGTTCTCCCACATTCCTGG-GTGAG-3′, antisense; 5′-CTCACCCAGGAATGTGGGAGAACCGGCTTC-3′). Binding reactions were performed at room temperature for 15 min. Reactions were carried out in 20 μl of DNA