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

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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.

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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

Table 1
Oligonucleotide sequences used for cloning of various ESX reporter constructs

Construct	H/M	5′/3′	Oligonucleotide sequence
−347	M	5′	CCGCCTCGAGTCAGCCCTGGCCAGGCCCCCAGG
Luc		3′	CCGCAAGCTTTAGAGCAAAGCAGGAGCTCCT
−2900	M	5′	CCGCCTCGAGGGATCCTTCCAAGGCACTGACC
Luc		3′	CCGCAAGCTTTAGAGCAAAGCAGGAGCTCCT
−351	H	5′	CCGGCTAGCCCATCTCTGGCCTGGCCCCTGGG
Luc		3′	CCGAAGCTTAGAGGATGAGGTTGGTGAGGGGAC
*Hr*II	H	5′	CCGGCTAGCGAATATGTTGAAAAGTGCTTGG
		3′	CCGGCTAGCCACTCTGTTGGCAAGGTCTTC
*Hr*III	H	5′	CCGGCTAGCCCCCATCCCCATGCCTCCAAG
		3′	CCGGCTAGCCAGGAGAGCCTGGGGCTCAGT
−2218	H	5′	CCGGCTAGCCCCCATCCCCATGCCTCCAAG
Luc		3′	GAGTGCAGTGGCGTGATATCTG
−2002	H	5′	CCGGCTAGCGGAGCACGTGAGCAGCCCTGGGG
Luc		3′	GAGTGCAGTGGCGTGATATCTG
−1976	H	5′	CCGGCTAGCGAAGCCGGTTCTCCCACATTCC
Luc		3′	GAGTGCAGTGGCGTGATATCTG
−1946	H	5′	CCGGCTAGCGGACTGGGTGGAGGGTGTGCC
Luc		3′	GAGTGCAGTGGCGTGATATCTG
−1906	H	5′	CCGGCTAGCGAGGCCCCCTGAAGGGCTGGGG
Luc		3′	GAGTGCAGTGGCGTGATATCTG

binding buffer (20 mM HEPES (pH 7.6), 100 mM KCl, 1 mM DTT, 0.1% Triton X-100, 2 mM $MgCl_2$ and 20% glycerol), 3 μg of poly(dA-dT) was added to each reaction as nonspecific DNA. For competition assays, 100-fold excess cold oligonucleotides were added to the reaction mix before adding nuclear extracts. The protein–DNA complexes were resolved by gel electrophoresis on 4% polyacrylamide gels. After electrophoresis, the gels were dried and visualized by autoradiography.

2.6. DNA constructs and oligonucleotides

All luciferase constructs were constructed using the pGL3 vector system and were sequence verified before use. Primers used to PCR out DNA regions are in Table 1.

The mouse −2900 Luc and −347 Luc constructs were cloned by PCR amplification of the relevant regions and cloned I/*Hin*dIII into pGL3-basic. The full length human −2872 region was excised from a previous pGL2-backbone construct (described in Neve et al., 2002) using a *Nhe*I–*Hin*dIII digest and shuttled into pGL3-Basic cut *Nhe*I–*Hin*dIII to create −2872 Luc. To generate truncations of −2872 Luc the truncated upstream regions were amplified using PCR. All constructs were amplified to include a 3′ *Xba*I site present at −1792 in the original construct. The PCR fragment and an *Xba*I–*Hin*dIII fragment contain the proximal promoter and the upstream sequence to −1792 were simultaneously cloned into pGL3-Basic cut *Nhe*I–*Hin*dIII. *Hr*II and *Hr*III were generated using the

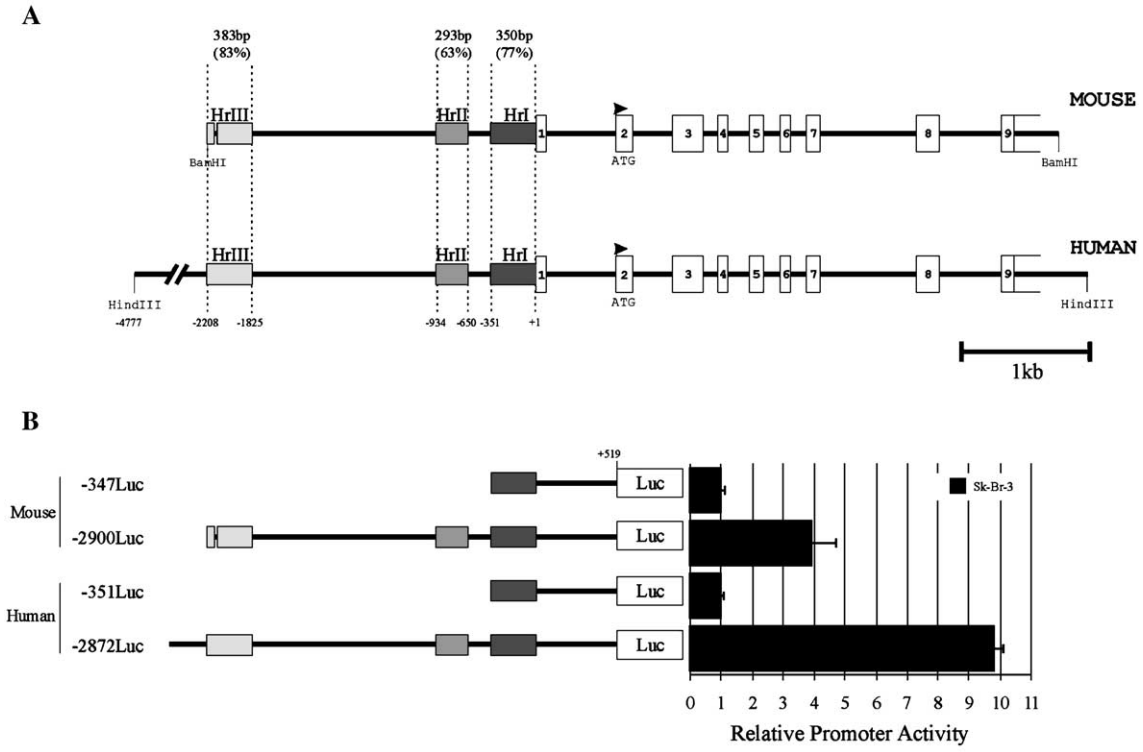

Fig. 1. (A) Genomic organization of the mouse and human ESX loci (to scale, Accession AY456682 and AF110184 respectively). Sequencing of the human and mouse genomic sequence encompassing the ESX locus shows conservation of the exons (labeled 1–9). Further comparison reveals three conserved regions of DNA upstream of the transcriptional start site, termed homology region-I, -II and -III (*Hr*I, II and III). *Hr*I encompasses the previously reported proximal promoter, *Hr*II and *Hr*III represent newly identified regions of homology upstream of *Hr*I. (B) Luciferase constructs containing either *Hr*I (Mouse: −347 Luc; Human: −351 Luc) alone or all three homology regions (Mouse: −2900 Luc; Human: −2872 Luc) for both mouse and human were transiently transfected into Sk-Br-3 cells and the basal luciferase activity measured after 24 h as described in Materials and methods. Each experiment was performed in triplicate and results are representative of at least three independent experiments.

primers in Table 1. A *Nhe*I site was generated at either end to clone into −351 Luc or pGL3-Promoter, both cut *Nhe*I. Directionality was determined by PCR analysis. All constructs containing the *Hr*I (proximal promoter) extend to +519 (see Neve et al., 2002).

Cloning of small regions of the putative enhancer elements (−2036–2002;−2002–1976;−1976–1946) was accomplished through annealing of complementary, phosphorylated oligonucleotides with *Kpn*I/*Nhe*I overhangs which were cloned into −351 Luc or pGL3-Promoter each cut *Kpn*I/*Nhe*I.

3. Results

3.1. Evidence for a cis-acting enhancer element upstream of the ESX gene

Previously we published the sequence of a 350 bp *ESX* gene proximal promoter (Neve et al., 1998). Both the mouse and human promoters showed extensive homology (77% over 350 bp) and were serum and mitogen responsive. Alignment of 3 kb of genomic sequence extending upstream of the proximal promoters of both the mouse and human *ESX* gene revealed two additional regions of significant homology (termed homology regions II (*Hr*II) and III (*Hr*III)) in addition to the proximal promoter (termed homology region I (*Hr*I))(Fig. 1A). *Hr*II and *Hr*III exhibit 83% homology over 383 bp and 63% homology over 293 bp, respectively. Mouse and human luciferase reporter constructs containing upstream sequence encompassing all three *Hr* regions (−2900 Luc and −2872 Luc, respectively) were found to have significantly higher luciferase activity in comparison to the constructs encoding the *Hr*I (−347 Luc and −351 Luc, respectively) alone (Fig. 1B) in the human breast cancer cell line, Sk-Br-3. This suggests the presence of one or more enhancer elements upstream of *Hr*I in both the mouse and human genomes.

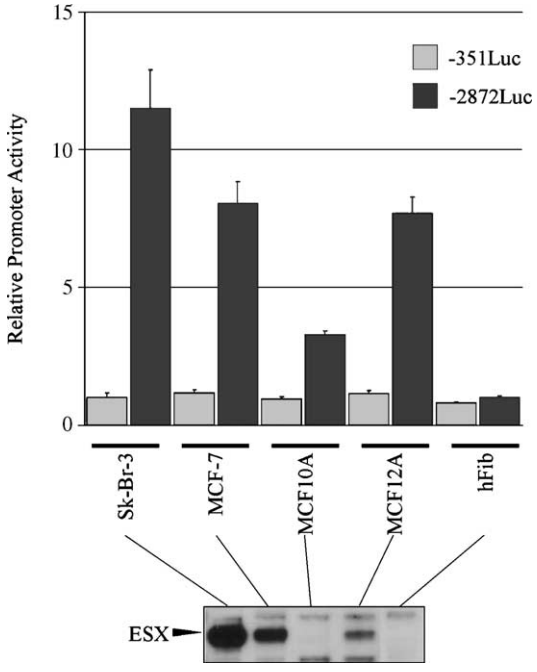

Fig. 2. The upstream enhancer shows epithelial specificity and reflects endogenous ESX protein levels. Transient luciferase assays were used to measure the transcriptional activity of both mouse and human constructs encompassing either the *Hr*1 (proximal promoter) region or all three homology regions in four human breast epithelial cell lines (Sk-Br-3, MCF-7, MCF10A and MCF12A) and one human fibroblast cell line (hFib). Luciferase assays were performed as described in Materials and methods. Each experiment was performed in triplicate and results are representative of at least three independent experiments. ESX protein levels were determined for each cell line by western analysis of nuclear extracts (lower panel) as described in Materials and methods.

The basal activity of the human −2872Luc reporter correlates well with endogenous *ESX* expression levels in a panel of cell lines (Neve et al., 2002). Comparison of the human −351 Luc with −2872 Luc activity in five untreated epithelial breast cancer cell lines with varying *ESX* expression levels revealed only the longer construct exhibited activity that varied in conjunction with the basal *ESX* level of each cell line (from barely detectable levels in MCF10a cells to highly expressed levels in Sk-Br-3 cells). This implies the upstream sequence of the *ESX* promoter likely contains a *cis*-acting enhancer region controlling cell-to-cell variation in endogenous *ESX* expression (Fig. 2). Since *ESX* expression is restricted to epithelial cells (Andreoli et al., 1997; Chang et al., 1997; Oettgen et al., 1997; Tymms et al., 1997), it is likely that this *cis*-acting enhancer region contains an epithelial-specific response element.

3.2. ESX expression is regulated by an epithelial-specific enhancer element

To determine whether either *Hr*II or *Hr*III of the human gene contained the putative enhancer element, each region was independently cloned upstream of the *ESX* proximal promoter driving luciferase expression (−351 Luc). *Hr*II had no effect on −351 Luc transcriptional activity, while *Hr*III enhanced the proximal promoter activity to levels equivalent to the entire upstream sequence, regardless of its orientation (Fig. 3A). *Hr*III also significantly enhanced the activity of a heterologous SV40 promoter when introduced into Sk-Br-3 cells but not upon transfection into human fibroblasts (Fig. 3B). Taken together, these results suggest that *Hr*III alone contains an epithelial-specific enhancer element. To determine the minimal element required for this full enhancer activity, we compared the luciferase activity of sequentially truncated promoter constructs (Fig. 3C). Initial experiments indicated that the enhancer lays between −2036 bp and −1819 bp within *Hr*III (not shown). Further deletions of the 5′ sequence identified a 30 bp *ESX* enhancer *s*equence (EES) (−1976 bp to −1946 bp) within *Hr*III which is required for full activity of *Hr*III (Fig. 3C, top panel). All constructs transfected into human fibroblasts exhibited promoter activity equivalent to the proximal promoter (−341 Luc), confirming the lack of significant EES enhancer activity in non-epithelial cells (Fig. 3C, lower panel).

Sequence comparison of the human and mouse *Hr*III regions revealed several clusters of conserved putative transcription factor-binding sites, several Ikaros isoforms localized to the EES are depicted (Fig. 4A) (Quandt et al., 1995). Consistent

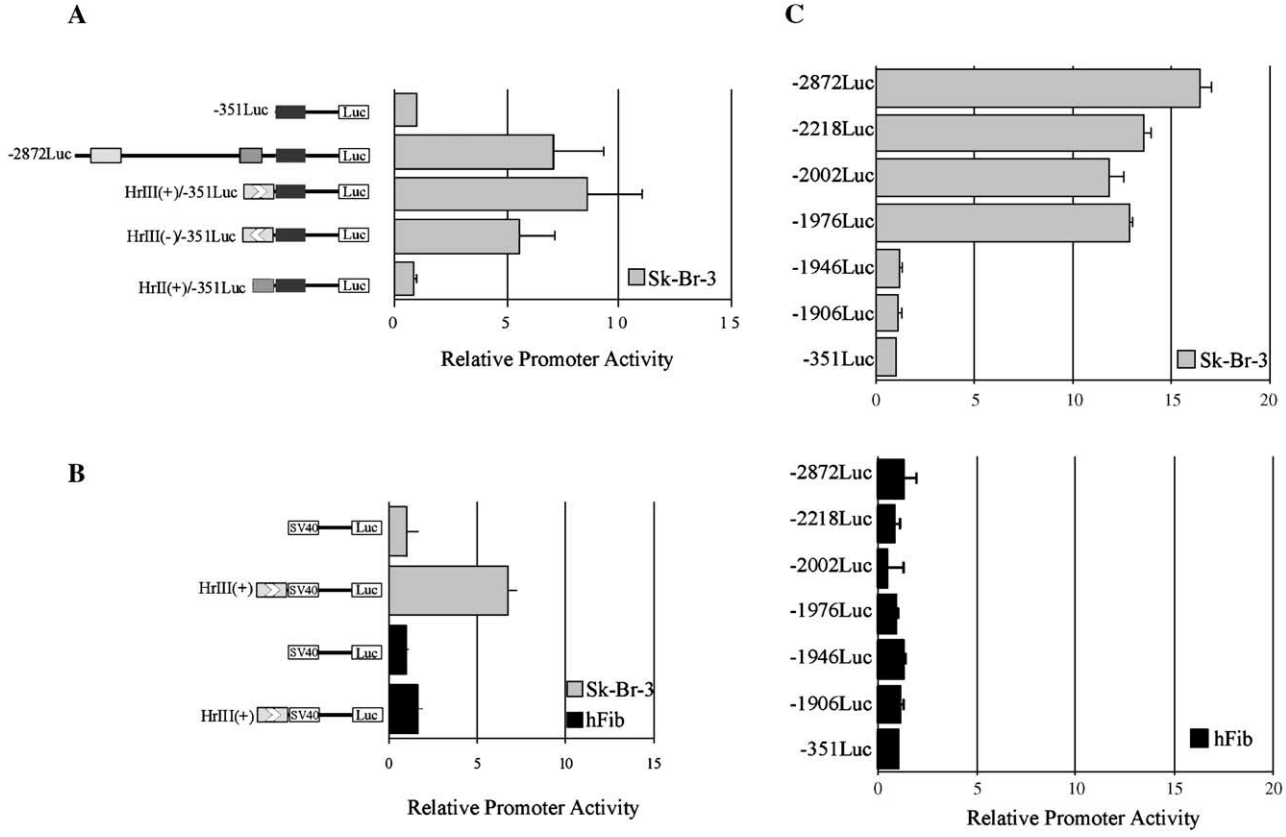

Fig. 3. *Hr*III encodes a putative epithelial-enhancer element. Transient transfection of (A) luciferase constructs containing either *Hr*II or *Hr*III (in positive (+) or negative (−) orientation) cloned upstream of *Hr*I (proximal promoter) into Sk-BR-3 cells, (B) *Hr*III (in positive (+) or negative (−) orientation) cloned upstream of the SV40 promoter (pGL3-promoter vector) into Sk-Br-3 cells or human fibroblasts, (C) serial deletions of the upstream sequence of the −2872 Luc promoter into Sk-Br-3 cells (upper panel) or human fibroblasts (lower panel) were performed as in Materials and methods. Each experiment was performed in triplicate and results are representative of at least three independent experiments.

with the deletion constructs, the EES (−1976bp to −1946bp) is sufficient for full enhancer activity when placed upstream of the −341 Luc promoter construct, independent of orientation (Fig. 4B). Similarly, when a single copy of the EES was placed upstream of the heterologous SV40 promoter a ten-fold enhancement of basal promoter activity is observed (Fig. 4C). Three tandem copies of the EES sequentially arranged upstream of the SV40 promoter increases basal promoter activity more than 60-fold (Fig. 4C). In keeping with the putative epithelial specificity of the EES, none of the *Hr*III constructs affect promoter activity when introduced into human fibroblasts (Fig. 4C).

3.3. An EES binding complex (EBC) controls ESX enhancer activity

EMSA assays, using radiolabelled oligonucleotides corresponding to the EES sequence, revealed two EES-binding protein complexes. While both protein–DNA complexes were specifically competed by excess, unlabelled EES oligonucleotide (100 × EES), the slower-migrating complex was completely competed away in contrast to minimal competition of the faster migrating (non-specific) complex (Fig 4D, right panel, lane 2). This indicates a direct relationship between EES-binding EBC activity, the magnitude of EES *trans*-activation potential, and endogenous ESX protein expression among various cell lines. The EBC EMSA data are consistent with the promoter–reporter studies and argue that EES constitutes the core enhancer element within the *Hr*III and likely determines the relative level of *ESX* transcription in any given epithelial cell.

3.4. EBC formation is dependent on epithelial differentiation signals

Since *ESX* expression is restricted to epithelial tissues and is involved in epithelial differentiation (Oettgen et al., 1997; Brembeck et al., 2000; Yoshida et al., 2000; Kim et al., 2002; Ng et al., 2002) we tested whether epithelial differentiation is associated with EBC binding to the EES, as detected by EMSA. We have shown *ESX* is up-regulated in HC11 cells during lactogenic competency in the presence of growth factors (Neve et al., 1998). Using this cell system, we observe that the EBC–EES complex was induced upon reaching lactogenic competency (Fig. 5, lanes 2–4). Since our *ESX* c-terminal-specific antisera does not cross-react with murine *ESX*, we were unable to determine ESX protein levels in these mouse cells; however, RT-PCR analysis showed an increase in *ESX* mRNA levels, consistent with the EMSA result (Fig. 5A). To test how the EBC activity is affected by differentiation signals, the breast cancer epithelial cell line, MCF-7, was used a model

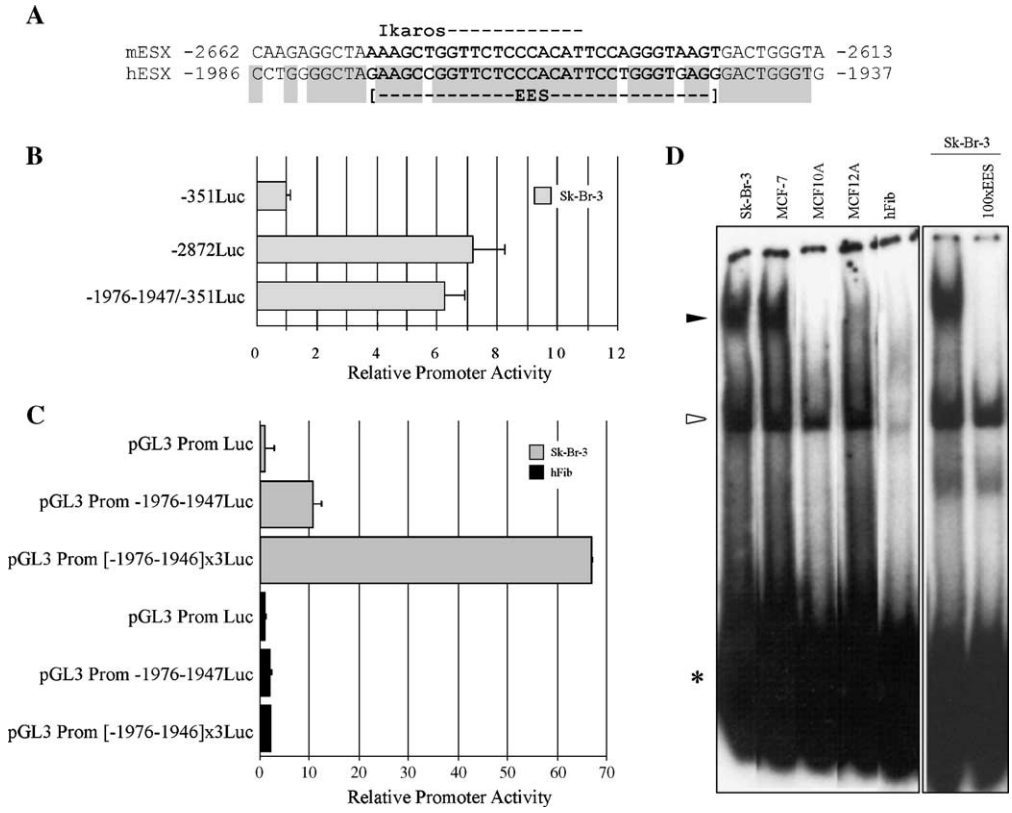

Fig. 4. A conserved 30 bp epithelial enhancer element controlling ESX expression. (A) Sequence comparison of *Hr*III between mouse and human. Alignment of mouse and human *Hr*III regions, overall homology with the EES enhancer sequence indicated in bold. Shaded regions indicate perfect homology between the two sequences. Putative transcription factor binding sites are indicated above the sequence alignment. (B) Transient transfections of the EES cloned upstream of the *Hr*I (proximal promoter) (−1976–1947/−351 Luc) in Sk-Br-3 cells compared to −351 Luc (*Hr*I, the proximal promoter) and −2872 Luc (containing *Hr*I, *Hr*II and *Hr*III). (C) Transient transfection of the EES and three tandem copies of the EES placed upstream of the SV40 promoter (pGL3 Promoter) in Sk-Br-3 cells and human fibroblasts. Transient transfections are described in the Materials and methods and each experiment was performed in triplicate and results are representative of at least three independent experiments. (D) Electromobility shift assays (EMSA) showing nuclear factors binding the EES. Nuclear extracts from four epithelial cell lines and one human fibroblast cell line, with differing levels of ESX protein were analysed for DNA–protein complex formation. Results are reflective of several independent experiments. * = unbound probe; filled arrow = specific band shift; open arrow = non-specific band.

system, since it undergoes differentiation in response to retinoic acid (RA) (Wang et al., 2000). We found that RA treatment induced endogenous *ESX* mRNA and protein levels and increased EES–protein complex within 1 h (Fig 5A, lanes 5–6). The transcriptional up-regulation of *ESX* was dependent on de-novo RNA synthesis, since treatment of MCF-7 cells with RA in the presence of actinomycin D prevented an increase in *ESX* mRNA levels and ESX protein levels. These results confirm that binding of EBC to EES appears responsive to epithelial differentiation signals and may mediate the observed up-regulation of *ESX* expression that occurs during mammary gland development and tumorigenesis.

4. Discussion

Epithelial cells play a critical role in the development and homeostasis of many organs. The same mechanisms controlling normal epithelial processes are often found perturbed in human disease such as cancer. However, there is very little information regarding the control of genes, especially transcription factors, regulating epithelial differentiation and maintenance. *ESX* is clearly involved in the development, differentiation and carcinogenesis of epithelial organs including the mammary gland, however, *ESX* gene targets and mechanisms of action remain elusive. Here we report the *cis*-acting regulatory elements that control the expression of human *ESX*.

We previously isolated and sequenced the mouse and human *ESX* loci and reported on the transcriptional activity of the *ESX* proximal promoter (Neve et al., 1998). The proximal promoter element and genomic organization of *ESX* was subsequently described (Oettgen et al., 1999). Comparison of the human and mouse upstream genomic sequences has revealed two additional regions of high homology, and detailed analysis of these regions now reveals an upstream enhancer region bearing a 30 base pair minimal enhancer element. This newly identified upstream enhancer element described here and termed EES, exhibits epithelial-specific activity when transfected into a panel of epithelial and non-epithelial cell lines. This activity correlates with endogenous levels of *ESX* in these cells. In contrast, the proximal promoter element shows similar basal activity in all cell lines tested. These observations suggest that endogenous transcription factors capable of binding to the EES during unstressed conditions are restricted to epithelial cells and positively control *ESX* expression. In all experiments this

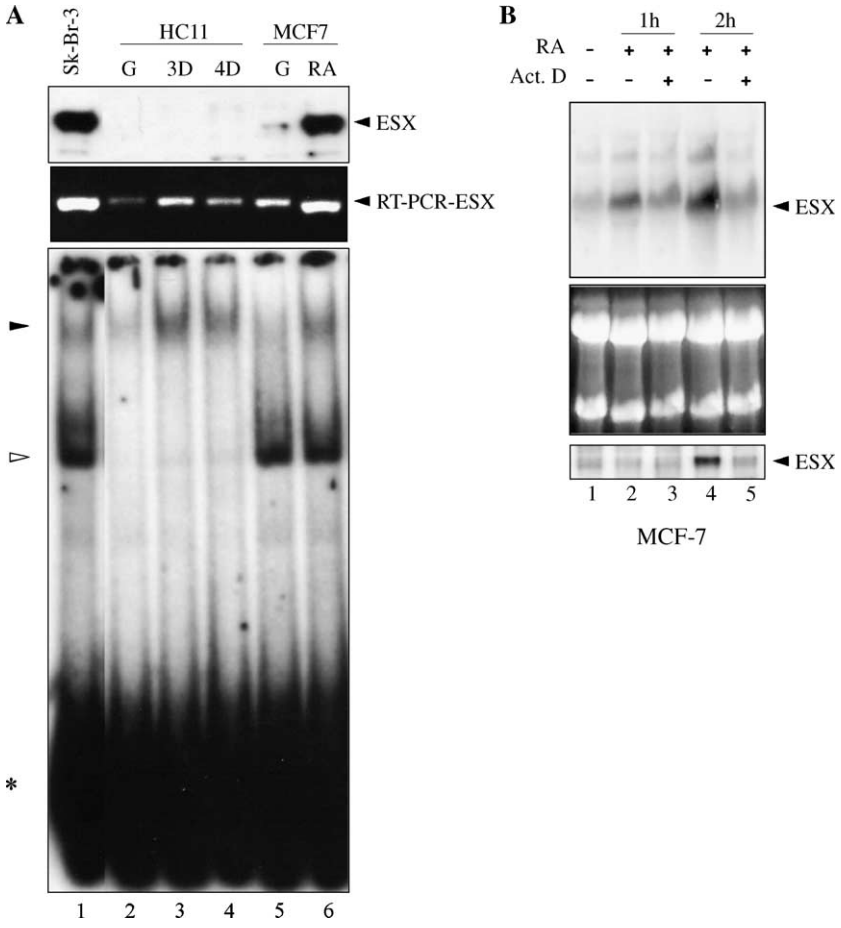

Fig. 5. *ESX* transcriptional activation and epithelial differentiation. (A) The response of *ESX* transcription to differentiation signals in two epithelial cell lines was tested. *ESX* mRNA (measured by RT-PCR), protein levels and EES–EBC binding (measured by EMSA) were observed in control and lactogenic competent HC11 cells, and MCF-7 cells treated with the differentiation-inducing factor, retinoic acid (RA). ESX protein levels can not be detected in HC11 cells as this antibody does not cross-react with mouse *ESX*. Results are reflective of several independent experiments. *=unbound probe; filled arrow=specific band shift; open arrow=non-specific band. (B) To test whether the increase of ESX mRNA and protein levels in response to RA requires de-novo RNA synthesis, MCF-7 cells were treated with actinomycin D (Act. D) prior to administration of RA. ESX mRNA was detected by Northern blotting (top panel) versus total RNA loading (middle panel) and *ESX* protein levels were determined by Western blotting (lower panel). All protocols were performed as described in Materials and methods and are illustrative of several independent experiments. G=growing; 3D=cells at 3 days confluent; 4D=cells at 4 days confluent.

complex associated with the EES and correlated well with endogenous *ESX* expression levels in control cells and in differentiating mouse and human epithelial cells.

The EES element is distinct from one identified by Park et al. in the *ESX* proximal promoter region in human gastric cancers (Park et al., 2001). We found no significant enhancer binding activity using their DNA binding motif to probe our panel of breast cancer cell lines (not shown). Recently a mouse enhancer element was identified (Hou et al., 2004) which is distinct from the EES. We have found that the mouse enhancer, which is not present in the human *ESX* gene, is not functional in Sk-Br-3 cells; whereas the human *ESX* enhancer exhibits weak, but significant, activity in mouse F9 differentiated cells (Rizzino, unpublished data). Overall this points to a complex regulation of *ESX* that is species-dependent and organ-dependent within a species.

One hallmark of cancer is a loss of cell differentiation (Tenen, 2003). *ESX* plays roles in epithelial differentiation including that of primary human mammary epithelial cells in vitro and during in vivo normal mammary gland development and breast tumorigenesis (Neve et al., 1998, 2002). Our data suggests that increased *ESX* expression in breast epithelial cells decreases cellular proliferation (Neve, unpublished data), which appears contrary to the function of a cancer-related gene, but is in keeping with its regulatory role during mammary gland development. Indeed, reports indicate potential transforming and apoptotic roles for *ESX* under specific biological conditions (Prescott et al., 2004; Schedin et al., 2004). We have previously shown that transcriptional up-regulation of *ESX* is downstream of ErbB receptor activation, primarily mediated by PI3K/PKB rather than MAPK signaling (Neve et al., 2002); and thus it is possible that oncogene-associated up-regulation of *ESX* is necessary to confer cancer-defining cell behavior such as invasiveness and metastatic potential rather than proliferation. Of note in this regard, TGF-βRII is a transcriptional target of *ESX* (Choi et al., 1998; Kim et al., 2002), and co-activation of TGF-β signaling in conjunction with ErbB2/*neu* activation has recently been shown to confer an anti-mitogenic but pro-metastatic

phenotype (Muraoka et al., 2003; Siegel et al., 2003). By identifying a critical genetic regulatory element that controls *ESX* expression in an epithelial-and tissue-specific manner we have uncovered a key mechanism potentially common to both normal epithelial development and carcinogenesis; as such, future identification of the components of EBC may yield new breast cancer therapeutic targets.

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