Activator protein-2 γ (AP-2 γ) expression is specifically induced by oestrogens through binding of the oestrogen receptor to a canonical element within the 5'-untranslated region

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The activator protein 2 (AP-2) transcription factors are essential proteins for oestrogenic repression of the *ERBB2* proto-oncogene in breast cancer cells. In the present study, we have examined the possible oestrogenic regulation of AP-2 genes themselves in breast-tumour-derived lines. As early as 1 h after oestrogen treatment, AP-2 γ mRNA was markedly increased, whereas AP-2 α was down-regulated, but with slower kinetics, and AP-2 β was not affected at all. Addition of anti-oestrogens ablated these effects. Modulation of the protein levels corresponded to changes in the transcript levels, thus suggesting that in oestrogen-treated cells, an inversion of the balance between AP-2 α and AP-2 γ isoforms occurs. The 5'-untranslated region (5'-UTR) of the human AP-2 γ gene contains one consensus and one degenerate oestrogen-responsive element (ERE). Reporter constructs carrying the AP-2 γ promoter and the 5'-UTR were up-regulated by oestrogens in

transient transfection assays. Deletion of the most conserved (but not of the degenerate) ERE from reporter constructs abrogated the oestrogenic response, although both ERE-containing segments were footprinted in DNaseI protection assays. *In vitro* binding assays demonstrated the ability of oestrogen receptor α (ER α) to bind to this site, and chromatin immunoprecipitation analysis of the endogenous gene showed that ER α occupies this region in response to oestrogens. We conclude that AP-2 γ is a primary oestrogen-responsive gene and suggest that AP-2 proteins may mediate some oestrogenic responses.

Key words: activator protein-2 transcription factor, breast cancer, oestrogen receptor α , oestrogen-regulated genes, transcriptional regulation.

INTRODUCTION

Activator protein-2 (AP-2) transcription factors represent a family of closely related sequence-specific DNA-binding proteins, conserved throughout evolution [1]. In mammals, there are four AP-2 genes, AP-2 α , AP-2 β , AP-2 γ and AP-2 δ [1–3]. All family members recognize, as homo- or hetero-dimers [4], the same DNA sequence through a nearly identical DNA-binding and helix-span-helix dimerization domain. Despite these similarities, AP-2 genes have non-redundant functions in development, as testified by tissue-specific patterns of expression during embryogenesis, as well as by the different phenotypes of null-mutant animals: AP-2 α ^{−/−} mice die perinatally, showing dramatic defects in the development of craniofacial structures and thoraco-abdomino-schisis, due to incomplete neural tube closure; AP-2 β ^{−/−} mice, conversely, do not show neural defects, but die due to incomplete renal differentiation. These phenotypes reflect the embryonic expression patterns (reviewed in [1]). Finally, AP-2 γ ^{−/−} mice die at 7.5–8.5 days due to malformation of all extra-embryonic tissues, and a normal maternal-embryonic interface fails to establish [5,6]. This again mirrors the expression pattern: AP-2 γ

is expressed in pre-implanted embryos, but becomes restricted to extra-embryonal tissues at implantation.

AP-2 transcription factors regulate several genes involved in a spectrum of important biological functions, e.g. apoptosis, differentiation, migration and invasion. These include the activation of p21^{WAF1/CIP1}, transforming growth factor- α , several keratinocyte-specific genes, oestrogen receptor α (ER α), tyrosine kinase receptor c-KIT, type IV collagenase, dopamine β -hydroxylase ([1], and refs therein), *ERBB2* [4,7] and the 67 kDa laminin receptor gene [8]. AP-2 also represses transcription of several genes, for example MCAM/MUC18 (melanoma cell-adhesion molecule), C/EBP α (CCAAT/enhancer-binding product α) and c-myc [1]. In performing their transcriptional functions, AP-2 factors also display specific interactions with several proteins, among which are c-myc [1], pRB (retinoblastoma gene product) [9], YB-1 (Y-box protein-1) [10] and p53 [8,11], for which AP-2 has been suggested to act as a transcriptional co-activator. In addition, AP-2 interacts with several co-activators, i.e. PC4 [12], PARP (polyADP-ribose polymerase) [13] and some members of the CITED [CREB (cAMP-response-element-binding protein)-binding protein/p300-interacting transactivator with ED (Glu/Asp)-rich tail] family of

Abbreviations used: AP-2 γ , activator protein 2, isoform γ (*TFAP2C*); BCA, bichinchoninic acid; CAT, chloramphenicol acetyltransferase; ChIP, chromatin immunoprecipitation; CITED, CREB-binding protein/p300-interacting transactivator with ED (Glu/Asp)-rich tail; CM, complete medium; CREB, cAMP-response-element-binding protein; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; ER α , oestrogen receptor α ; rER α , recombinant ER α ; ERE, oestrogen-response element; FCS, foetal calf serum; FPA (etc.), footprint A (etc.); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Inr, initiator; LUC, luciferase; 4OH-TAM, 4-hydroxy-tamoxifen; RT, reverse transcriptase; SM, stripped medium; Sp1, specificity protein 1; TK, thymidine kinase; 5'-UTR, 5'-untranslated region; Vit, vitellogenin.

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adapter proteins, which also bind CBP (CREB-binding protein)/p300 [14,15]. Interestingly, CITED proteins can also act as specific co-activators for ER α [16].

AP-2 proteins also play a role in neoplasia. A role for AP-2 as tumour suppressor was suggested in melanoma [17], while altered expression of both AP-2 α and AP-2 γ was found in breast tumours [18,19]. Overexpression of the *ERBB2* proto-oncogene in breast cancer is driven by AP-2 transcription factors [7,20,21]. Conversely, *ERBB2* expression is repressed by the oestrogen-activated ER [22,23]. We have shown previously that the AP-2-binding sites within the *ERBB2* promoter and the intronic enhancer are required for this oestrogenic repression [20,24]. During this work, we observed that the AP-2 α protein level was regulated by oestrogens in breast cancer cells and the AP-2 α expression level closely correlated with *ERBB2* expression. However, binding site assays failed to reveal changes in the overall AP-2-binding activity in the nuclei of treated versus untreated cells [24]. Since at least three of the AP-2 isoforms have been detected in breast-tumour-derived cells and have been shown to bind equally to a number of sites, including those within the *ERBB2* gene, we reasoned that oestrogens may be able to differentially modulate expression of the various AP-2 genes. This was investigated in the present study and our results show that oestrogens directly induce transcriptional activation of AP-2 γ , but not of other AP-2 isoforms, through binding of ER α to a canonical oestrogen-response element (ERE) within the 5'-untranslated region (5'-UTR) of the *TFAP2C* gene.

EXPERIMENTAL

Cell culture

ZR75.1, MCF7, T47D, SKBR3 and MDAMB231 human breast cancer cells were grown in complete medium (CM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) heat-inactivated foetal calf serum (FCS). MCF10A cells were maintained in DMEM/Hams F12 (1:1), supplemented with equine serum, insulin, hydrocortisone, epidermal growth factor, cholera toxin and antibiotics. For oestrogen treatment, cells were first transferred into 'stripped' medium (SM), devoid of oestrogenic activity [Phenol-Red-free DMEM supplemented with 10 % (v/v) dextran-coated charcoal-stripped FCS] for 72 h. Treatments were performed with 10^{-8} M 17 β -oestradiol (Sigma) from a 10^{-2} M stock solution in 100 % ethanol, or 10^{-6} M 4-hydroxy-tamoxifen (4OH-TAM) (Sigma) or 10^{-7} M ICI 182780 when necessary, using corresponding volumes of ethanol in control plates.

Transient transfection and chloramphenicol acetyltransferase (CAT) assays

ZR75.1 cells were plated at a density of 16 000 cells/cm² in CM and transferred to SM the following day. Transfection was performed 24 h later using the standard calcium phosphate precipitate technique. For each reporter construct tested, nine plates were transfected using a common precipitate, as previously described [25]. After 16 h, the precipitate was removed by washing three times with sterile PBS, then three plates were treated with 10^{-8} M 17 β -oestradiol, three plates with 10^{-8} M 17 β -oestradiol plus 10^{-6} M 4OH-TAM and three plates were mock-treated. In T47D transfection experiments, cells were grown in complete DMEM, transfected using FuGENETM (Roche) according to the manufacturer's instructions then treated with either 10^{-8} M 17 β -oestradiol or 10^{-7} M ICI 182780. CAT assays

were performed on cell lysates as previously described [25], and protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Sigma). CAT activity was expressed as (% c.p.m. acetylated) $\cdot$ mg⁻¹ $\cdot$ h⁻¹. CAT activity was then normalized to the mean β -gal value of the plates transfected with the same DNA mix, expressed as $A_{420} \cdot$ mg⁻¹ $\cdot$ h⁻¹. Luciferase assays were performed using commercial kits (Promega) according to the manufacturer's instructions and protein concentration was determined by the BCA protein assay (Sigma). Final luciferase readings were corrected for transfection efficiency using β -gal (ZR75.1) or *Renilla* luciferase (T47D) and finally expressed as the ratio above the activity shown by the pGL3 basic vector alone in parallel transfections under the same hormonal conditions.

Plasmids and plasmid construction

AP-2 γ reporter constructs were produced by cloning a 500 bp genomic *Bam*HI fragment (from +193 to -312) of the *TFAP2C* gene [26] into pBluescript, then subcloning a *Hind*III/*Xba*I restriction fragment into pBLCAT3 (pEREAB.CAT). A construct deleting ERE-A, p Δ EREAB.CAT, was obtained by digestion of pEREAB.CAT with *Sac*II and *Bss*HII, blunt-ending and recircularization. Similarly, digestion of pEREAB.CAT with *Bss*HII and *Spe*I gave p Δ EREAB.CAT, deleting ERE-B. Digestion of pEREAB.CAT with *Sac*II and *Spe*I gave p Δ EREAB.CAT, deleting both EREs (see Figure 3a). The oestrogen-regulated pVitA2-CAT, used as a positive-response control, contains three copies of the vitellogenin (Vit) A2 ERE upstream of the thymidine kinase (TK) basal promoter [27]. Wild-type (pEREAB.LUC) and 5 bp mutated (p Δ 5bpEREAB.LUC) AP-2 γ promoter-luciferase constructs were produced by inserting the same 500 bp genomic *Bam*HI fragment into the pGL3 basic vector (Promega). The 5 bp deletion was obtained by digesting pEREAB.LUC with *Bst*EII, blunting with S1 nuclease and recircularization. All constructs were confirmed by sequencing.

Immunoblotting

Total protein extracts were obtained by direct on-plate lysis of the cells, previously washed three times with cold PBS, with a boiling buffer containing 0.125 M Tris/HCl, pH 6.8, and 2.5 % SDS; the lysates were then homogenized by ten passages through an insulin needle and cleared at 9000 *g* for 10 min. Proteins (50 μ g) were separated by SDS/(12 %) PAGE and electroblotted on to PVDF membranes (Bio-Rad). Membranes were blocked with 150 mM NaCl, 2 mM EDTA, 50 mM Tris/HCl, pH 8.0, and 10 % BSA for 12 h, then incubated with appropriate antibodies for 2 h at room temperature (25 °C), and visualized by enhanced chemiluminescence (ECL[®]; Amersham Biosciences). Monoclonal antibodies 3B5 and 6E4 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were used to detect AP-2 α and AP-2 γ respectively. Specificity of these antibodies was confirmed by immunoblotting *in vitro*-translated AP-2 α , AP-2 β and AP-2 γ proteins (results not shown). Membranes were then stripped in 2 % (w/v) SDS, 62.5 mM Tris/HCl, pH 6.7, and 100 mM 2-mercaptoethanol for 30 min at 55 °C, and then reblocked before incubation with a goat polyclonal Ku-70 antibody (Santa Cruz Biotechnology) as a loading control.

RNA isolation and Northern blot analysis

Total RNA was isolated from ZR75.1 and MCF-7 cells by the acidic phenol method. RNA (15 μ g/lane) was separated on 1.2 % (w/v) formaldehyde-agarose gels, transferred on to positively charged nylon membrane HybondTM-N⁺ (Amersham

Biosciences), and fixed by UV cross-linking. For hybridization, cDNA fragments from AP-2 α (nt 363–787) and AP-2 γ (nt 1–538) were labelled with [³²P]dCTP by random priming and used as probes. Probe specificity was verified by Southern blot analysis of cloned cDNAs. The hybridization was performed in 7 % (w/v) SDS, 0.5 M NaH₂PO₄ and 5 mM EDTA at 65 °C for 16–20 h. The filters were washed twice with 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1 % (w/v) SDS at 65 °C, once with 1 $\times$ SSC and 0.1 % (w/v) SDS at 65 °C, and finally twice with 0.1 $\times$ SSC and 0.1 % (w/v) SDS at 65 °C. The blots were exposed to a PhosphorImager screen (Molecular Dynamics) for 4–24 h or to a HyperfilmTM MP film (Amersham Biosciences) for 1–10 days. The signal intensity was revealed by PhosphorImager (Storm 840, Molecular Dynamics) and bands were quantified by ImageQuant software. Blots were also probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein rPL7a to provide a reference for normalization.

Reverse transcriptase (RT)-PCR and real-time PCR

Total RNA was pre-treated with DNaseI (Promega). Reverse transcription reactions (20 μ l) contained 1 μ g of total RNA, 1 mM of each dNTP (Promega), 200 units of MMLV reverse transcriptase (Promega), 2.5 μ M random primers (Promega), 5 $\times$ RT buffer (Promega) and 40 units of ribonuclease inhibitor (Promega). To account for wide variations in RT performance, three independent reactions were performed and the cDNAs were pooled. For the PCR reactions, the following primers were used: AP-2 α , 5'-TCACTTGCTCATTTGGGATCG-3' and 5'-TCTCCGCCATCCCTATTAAC-3'; AP-2 β , 5'-GCGGCATGAATCTATTGGAC-3' and 5'-CCCAGGAAGCCGTCTTTATT-3'; AP-2 γ , 5'-GAAATGAGATGGCAGCTAGG-3' and 5'-CAGGGTTCA-TGTAGGATTTG-3'; AP-2 δ , 5'-ATATTGCCGGAGTCCCAA-AG-3' and 5'-ATCCTCCAGACCCACTCCAAT-3'. Amplicon sizes were 426 bp for AP-2 α , 97 bp for AP-2 β , 269 bp for AP-2 γ and 95 bp for AP-2 δ . The annealing temperatures were 55 °C for AP-2 α primers, 57 °C for AP-2 β primers, 52.5 °C for AP-2 γ primers and 60 °C for AP-2 δ primers. The optimum cycle number was determined empirically (see Figure legends). The GAPDH control was performed as described [22]. Bands were quantified using the Quantity-One software (Bio-Rad).

Quantitative real-time PCR monitored with SYBRGreen[®] was performed using the ABI Prism[®] 7700 (PerkinElmer) detection system. Specific primer pairs were designed with Primer Express 1.0 software (Applied Biosystems). For a reference gene, 18 S rRNA was used. Forward and reverse primers of AP-2 γ and 18 S rRNA were used at 300 nM. Primer sequences were: AP-2 γ , 5'-AACAGACCCACACACTTAGCCATT-3' (forward) and 5'-GCGGACACAAAAACCAACCA-3' (reverse); 18 S 5'-CGAACGTCTGCCCTATCAACT-3' (forward) and 5'-CTGC-CTTCCTTGATGTGGT-3' (reverse).

DNaseI footprinting and electrophoretic mobility-shift analysis (EMSA)

The 0.5 kb *Bam*HI genomic fragment was cloned into pBlue-script (Stratagene), re-excised and labelled at the 3' end. End-labelled probe (7 fmol) was incubated with ZR75.1 crude nuclear extract [28], followed by partial DNaseI treatment, proteinase K digestion, phenol/chloroform extraction and ethanol precipitation before fragments were resolved on a denaturing polyacrylamide gel. Additional lanes were loaded with an A + G chemical-degradation sequencing ladder generated from the same probe and a control DNaseI digest of probe that had not been

pre-incubated with nuclear extract. Potential factors binding to the protected sequences were predicted using MatInspector (<http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl>). EMSA was performed as previously described [7] by incubating an end-labelled double-stranded oligonucleotide probe corresponding to footprint F (FPF) (5'-AGACGCCTGGTCACCGTGACCCCG-ATTTTG-3') together with recombinant ER α made in baculovirus [29]. Supershifting experiments were performed by incubation with an anti-ER α antibody (D-12; Santa Cruz Biotechnology) for 1 h at 4 °C before addition of the probe. Competition experiments were performed with 20-, 100- and 500-fold mass excess over the labelled probe, using unlabelled oligonucleotides for FPF, or mutFPF lacking the 5 bp deleted in the p Δ 5bpERE α .LUC promoter (see above) (5'-TAGACGCCTGCGTGACCCCGATTTTG-3') or the ERE from the Vit A2 gene (5'-GATCCAGAGGTCACAGTGACCTACGA-3').

ER α chromatin immunoprecipitation (ChIP)

Protein–DNA cross-linking was performed by adding 1 % (w/v) formaldehyde to ZR75.1 culture medium for 10 min at 37 °C. Cells were washed with ice-cold PBS containing protease inhibitors (1 mM PMSF, 1 μ g/ml aprotinin and 1 μ g/ml pepstatin A) and lysed with lysis buffer [1 % (w/v) SDS, 10 mM EDTA and 50 mM Tris/HCl, pH 8.1] for 10 min on ice. The lysate was sonicated to reduce DNA length to 200–1000 bp and debris was removed by centrifugation for 10 min at 9750 *g* at 4 °C. The supernatant was diluted 10-fold in dilution buffer [0.01 % (w/v) SDS, 1.1 % (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris/HCl, pH 8.1, and 167 mM NaCl] containing protease inhibitors (as above). Soluble chromatin was pre-cleared by incubation with 45 μ l of Protein A–agarose (or Protein G–Sephadex), 2 μ g of salmon sperm DNA and 24 μ l of preimmune rabbit (or mouse) IgG, for 2 h at 4 °C with agitation. Immunoprecipitation of 1 ml of soluble chromatin was performed overnight at 4 °C using 10 μ l of the anti-ER α monoclonal antibody Ab10 (NeoMarkers) or 5 μ l of an anti-(acetylated-H4 histone) polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.) as a positive control or no antibody as a negative control. A further control was obtained using an irrelevant antibody (anti-Mcl-1 monoclonal antibody; Transduction Laboratories, Lexington, KY, U.S.A.). Immunocomplexes were collected with 45 μ l of Protein A–agarose (or Protein G–Sephadex) for 1 h at 4 °C with rotation. Beads were pelleted and were washed sequentially with the buffers: 0.1 % (w/v) SDS/1 % (v/v) Triton X-100/2 mM EDTA/20 mM Tris/HCl (pH 8.1)/150 mM NaCl; 0.1 % (w/v) SDS/1 % (v/v) Triton X-100/2 mM EDTA/20 mM Tris/HCl (pH 8.1)/500 mM NaCl; 0.25 M LiCl/1 % (v/v)/Nonidet P-40/1 % (w/v) sodium deoxycholate/1 mM EDTA/10 mM Tris/HCl (pH 8.1) and twice with Tris-EDTA (pH 8.0). Immunocomplexes were eluted by adding 250 μ l of 1 % (w/v) SDS in 0.1 M NaHCO₃ to pelleted beads, before incubation at room temperature for 15 min with rotation. Elution was repeated and eluates were combined. Cross-linking was reversed at 65 °C overnight and DNA was purified on Qiaquick spin columns (Qiagen) and eluted in 50 μ l of water. Specific sequences from immunoprecipitated and input DNA were detected by PCR using specific primers for the ERE-B sequence of the AP-2 γ promoter region and control primers from an upstream and a downstream region of the AP-2 γ gene: ERE-B, 5'-GTCCAGTGACCCGGACAG-3' (forward) and 5'-ACCTCG-CAGTCCTCTTCGTA-3' (reverse); control upstream, 5'-GCGT-TTGTGTGGAAGGAAGT-3' (forward) and 5'-CTCTACTCGC-CCTGGGAAA-3'; control downstream, 5'-GCACGAAAACA-ACCGAAGTT-3' (forward) and 5'-CTGCGTCTTCTTCCTC-TTCC-3'. The annealing temperature was 60 °C for ERE-B

primers and 55 °C for the upstream and downstream control primers. Amplicon sizes were 196 bp for ERE-B and 180 bp for the upstream and 187 bp for the downstream controls.

RESULTS

Expression levels of AP-2 genes were firstly assayed at the mRNA level by RT-PCR using a panel of five human breast cancer and one non-tumorigenic mammary cell line, together displaying a range of growth properties. MCF-7, ZR75.1 and T47D cells show epithelial morphology, are ER α -positive and have different degrees of dependency on oestrogens for growth; SKBR3 have epithelial-like morphology, are ER-negative and possess a high degree of amplification of the *ERBB2* oncogene, while MDAMB231 are fibroblast-like cells with no ER and very low levels of *ERBB2* expression. Finally, MCF10A is a non-tumorigenic cell line, with epithelial morphology and no ER. As shown in Figure 1(a), mRNA for the different AP-2 isoforms, with the exception of AP-2 δ , was present to a variable degree in all of these cell lines. Notably, all tumour-derived lines express AP-2 α and AP-2 γ , whereas non-tumorigenic MCF10A showed very little expression of AP-2 γ . Higher levels of AP-2 γ were seen in ER-positive cells and in SKBR3 cells, where amplification of the AP-2 γ gene has been reported [4]. In contrast, AP-2 β was detected only in ZR75.1 cells. For this reason, we chose this line for further investigations.

Differential regulation of AP-2 genes by oestrogen

The effect of treatment with 10^{-8} M 17 β -oestradiol for 24 or 72 h was investigated on ZR75.1 cells. As shown in Figure 1(b), addition of oestrogen to cells maintained in SM significantly increased levels of AP-2 γ mRNA, whereas AP-2 α mRNA levels decreased slightly and AP-2 β did not change appreciably. Densitometry data showed a reduction in the levels of AP-2 α mRNA (70% of the level of untreated cells, at days 1 and 3) and a marked increase in the levels of AP-2 γ mRNA (300%). This inverse effect of oestrogens on AP-2 α and AP-2 γ was investigated further, using both ZR75.1 and MCF-7 cells, which display a much lower AP-2 α to AP-2 γ expression ratio and no AP-2 β (Figure 1a), using probes derived from the less-conserved 5'-end of the appropriate cDNA clones (see Experimental). Figure 2 presents results of treatment for 6, 24 and 72 h in MCF-7 (Figure 2a) and ZR75.1 (Figure 2b) cells. AP-2 γ mRNA was clearly visible as a single sharp band in both cell lines and showed a clear increase under oestrogen treatment, which is more marked in ZR75.1 cells, at all time points examined. Densitometric evaluation of the bands and normalization to the GAPDH signal, showed a 2- (24 h) to 5- (72 h) fold increase in levels of AP-2 γ mRNA in oestrogen-treated ZR75.1 cells (Figure 2b) and a 2- (24 h) to 4- (72 h) fold increase in MCF-7 cells (Figure 2a). Similar results were obtained when normalizing against another housekeeping gene, rpl7a (results not shown). AP-2 α mRNA was detected as at least two bands. This agrees with results from other groups and reflects the presence of differentially sized, reactive RNA species, perhaps representing alternative splicing forms (see [1] and references therein). In MCF-7 cells, the AP-2 α mRNA bands were faintly detectable, confirming that in this cell line, AP-2 γ is the most represented isoform, as also observed by RT-PCR. In both cell lines, AP-2 α mRNA was reduced by oestrogenic treatments. Reduction was variable and densitometric quantification (that is complicated by the presence of multiple bands) allowed estimation of a 0.5-fold mean reduction.

Figure 1 RT-PCR analysis of AP-2 isoform expression

(a) RT-PCR analysis of AP-2 α , AP-2 β , AP-2 γ and AP-2 δ expression in breast epithelial lines. Total RNA (1 μ g) was reverse-transcribed and aliquots of cDNA were subjected to 34 (AP-2 γ), 30 (AP-2 α and AP-2 β), 35 (AP-2 δ) and 27 (GAPDH) cycles of amplification. The two right-hand lanes represent the no-template (N) and cDNA positive (P) controls. (b) RT-PCR analysis of AP-2 expression in oestrogen-treated ZR75.1 cells. Cells were maintained in SM for 72 h before the addition of 10^{-8} M 17 β -oestradiol. Total RNA was extracted 24 or 72 h after oestrogen addition and was analysed by RT-PCR as above.

Interestingly, in both cell lines, the effect of oestrogens on AP-2 α mRNA was much more evident with treatment at 24 h than at 72 h, perhaps reflecting a transient modulatory regulation. In order to verify whether the effects observed could be due to the classical ER activation pathway, the anti-oestrogen 4OH-TAM was also used in combination with oestrogen in all the experiments. Results presented in Figures 2(a) and 2(b) clearly demonstrate that 4OH-TAM reversed the oestrogen effects on both AP-2 γ and AP-2 α mRNA expression. Since it is known that 4OH-TAM can act as a partial agonist, the experiment was repeated using ICI 182780, a pure antagonist. Real-time PCR evaluation of mRNA showed a 2.5-fold increase in AP-2 γ expression with 17 β -oestradiol, which

Figure 2 Northern and Western blot analysis of AP-2 α and AP-2 γ in hormone-manipulated breast-tumour-derived cells

Northern blots of total RNA from MCF-7 (**a**) and ZR75.1 cells (**b**) treated with 10^{-8} M 17β -oestradiol as above or 10^{-8} M 17β -oestradiol plus 10^{-6} M 4OH-TAM (TAM) were probed for AP-2 α , AP-2 γ or GAPDH, as indicated. (**c**) Northern blot analysis of total RNA from MCF-7 cells. Cells were starved of oestrogens for 72 h then treated with 10^{-8} M 17β -oestradiol for 1, 3 or 6 h. Blots were probed for AP-2 γ and GAPDH. (**d**) Immunoblotting analysis of AP-2 α and AP-2 γ proteins in whole-cell lysates of ZR75.1 cells, treated as above. Blots were re-probed for Ku-70 antigen to control for equal loading.

is completely blocked in the presence of either 4OH-TAM or ICI 182780 (results not shown).

A shorter time-course experiment was then performed using MCF-7 cells to obtain evidence that AP-2 γ may represent a primary oestrogen-responsive gene. As shown in Figure 2(c), AP-2 γ mRNA levels were clearly enhanced after 1 h of treatment with 17β -oestradiol (2-fold), strongly suggesting that oestrogenic induction is a direct effect. Regulation of mRNA was accompanied by a corresponding regulation at the protein level. Figure 2(d) shows results of immunoblotting analysis of AP-2 α and AP-2 γ proteins in ZR75.1 cells, using isoform-specific antibodies (verified using recombinant proteins; results not shown). AP-2 γ protein was up-regulated in a time-dependent fashion, reaching a 4-fold induction after 72 h of oestrogen treatment. Immunoblotting confirmed the decrease in AP-2 α as a result of oestrogen treatment and, again, 4OH-TAM reversed the oestrogenic effects on both AP-2 γ and AP-2 α protein levels. Taken together, these results demonstrate that the induction of AP-2 γ is a specific and primary response to oestrogenic treatment of ER α^+ breast cancer cell lines, whereas AP-2 α displays limited down-regulation, perhaps as a secondary response.

The 5'-UTR of *TFAP2C* contains a functional ERE

The structure of the human AP-2 γ gene, *TFAP2C*, has been examined and the transcriptional start site (+1) assigned to a single base within a presumed initiator (Inr) core promoter whose activity is modulated by the presence of three upstream Sp1/3 (specificity protein 1/3) sites, centred at -70, -135 and -200 [26]. Inspection of the AP-2 γ gene sequence revealed one perfect (ERE-B) and one less conserved (ERE-A) ERE (consensus palindromic sequence GGTCAN₃TGACC) which both lie within the 232 bp 5'-UTR (Figure 3a). The functional significance of these EREs was examined by transient transfection of a CAT reporter construct, carrying the genomic *Bam*HI fragment (-312 to +193) containing the whole promoter and the majority of the 5'-UTR sequence of the human AP-2 γ gene, into ER-positive cells. Transfection into ZR75.1 cells showed increased CAT activity in response to a 16 h 17β -oestradiol treatment (up to 2-fold for the wild-type construct, pEREABCAT). This was similar to the response shown by the widely used oestrogen-responsive reporter plasmid pVitA2-CAT, containing three copies of the chicken Vit A2 ERE upstream of the TK promoter, in the same assay

Figure 3 Analysis of the promoter and the 5'-UTR region of the *TFAP2C* gene

(a) Comparison of the 5'-UTR of murine and human AP-2γ genes, showing the conservation of ERE-B. ERE-A has three substitutions compared with a consensus ERE and no longer resembles an ERE in the mouse. (b) Schematic map of the human AP-2γ 5'-flanking sequence (+193 to -312) showing the positions of the transcriptional start site (TSS +1) and the putative EREs. The structure of the wild-type pEREAB.CAT construct and the derived mutants plus the relative position of key restriction enzyme sites mentioned in the text are also shown. (c) ZR75.1 cells, maintained for 24 h in the absence of oestrogen, were transfected with control (VitA2.CAT) or wild-type or mutated AP-2γ CAT reporter constructs as indicated and then incubated for 48 h in the absence (white bars; control) or presence (black bars) of 10^{-8} M 17β-oestradiol. Normalized relative CAT activity data are shown from a single representative experiment, performed in triplicate. This experiment was repeated four times with comparable results. (d) ZR75.1 cells, maintained as above, were transfected with either wild-type pEREAB.LUC or pΔ5bpERE.B.LUC, which carries a 5 bp deletion within ERE-B and then incubated for 48 h in the absence of ligand (white bars; control) or in the presence of 10^{-8} M 17β-oestradiol (black bars) or 10^{-6} M 4OH-TAM (grey bars). Normalized relative luciferase activity from two different experiments, each performed in triplicate is shown. (e) The same reporter vectors were transfected into T47D cells, maintained in CM, which were subsequently either maintained in 10^{-8} M 17β-oestradiol (black bars) or supplemented with 10^{-7} M ICI 182,780 (hatched bars) for 48 h. Normalized relative luciferase activity from two different experiments, each performed in triplicate, is shown.

(see Figure 3c) and confirmed that AP-2γ responsiveness to oestrogens could be mediated by these 5'-flanking sequences. Comparable responses to oestrogens were verified on longer constructs, containing 5'-flanking sequences up to -300 and -598 (results not shown). In order to evaluate the possible contribution of the ERE-A and ERE-B sequences present in the 5'-UTR, mutants of the wild-type construct pEREAB.CAT, were produced using restriction digests to delete either ERE-A, ERE-B or both (see the Experimental section). As shown in Fig-

ure 3c, deletion of ERE-A (pΔERE.A.CAT) resulted in lower basal activity than the wild-type construct, but identical inducibility by oestrogens, whereas the ERE-B deletion (pΔERE.B.CAT) was no longer induced by oestrogens, but with basal activity comparable with that of the wild-type construct. Deletion of both EREs (pΔEREAB.CAT) showed low basal activity and no induction by oestrogens. From these studies, we could conclude that the more highly conserved ERE-B is capable of mediating AP-2γ responsiveness to oestrogens in reporter assays. Thus, to directly

Figure 4 DNaseI footprinting of the AP-2 γ 5'-UTR

A 0.5 kb genomic *Bam*HI fragment (–312 to +191) was labelled at the 3' end and incubated with either 100 μ g (lanes 2 and 3) or 200 μ g (lanes 4 and 5) of nuclear extract from ZR75.1 cells, or buffer alone (lane 1), before limited digestion with DNaseI. Samples were resolved on a 6% denaturing gel and auto-radiographed. Footprints A–F are marked and were mapped to the gene sequence with the aid of an A + G chemical-sequencing ladder (L) generated from an aliquot of the probe (lane 6). Footprints E and F coincide with sequences ERE-A and ERE-B respectively.

demonstrate the involvement of ERE-B in oestrogen response, we produced a specific smaller mutation affecting precisely the ERE-B sequence, by deleting 5 bp, corresponding to one half site of the ERE sequence and thus ablating ER α binding (see Figure 5), in a luciferase reporter vector (p Δ 5bpERE-B.LUC). This vector was tested for oestrogen and anti-oestrogen-responsiveness using ZR75.1 and T47D cells. As shown in Figures 3(d) and 3(e), the 5 bp deletion completely abrogated responsiveness to

Figure 5 ER α binds specifically to ERE-B

EMSA was performed using end-labelled double-stranded oligonucleotides to the sequences protected by FPF, which contains ERE-B, as the probe. The probe and recombinant ER α were incubated together either alone (lane 2) or with the addition of an anti-ER α antibody (lane 1) or 20-, 100- or 500-fold of unlabelled competitor oligonucleotides as follows: Vit (lanes 3–5), FPF (lanes 6–8) or mutant FPF, lacking one half-site (mFPF, lanes 9–10). DNA–protein complexes and free probe were separated by non-denaturing gel electrophoresis and revealed by autoradiography; only the DNA–protein complexes are shown.

oestrogens, in both cell lines. In ZR75.1 cells, the mutant reporter showed higher basal activity than the wild-type construct (pEREAB.LUC), whereas in T47D cells, they had the same activity in anti-oestrogenic conditions. Anti-oestrogen treatment clearly reversed the oestrogen effect in both cell lines. These transfection experiments were set up in slightly different ways and used different anti-oestrogens (namely the partial agonist, 4OH-TAM and the total antagonist, ICI 182 780) to demonstrate further the robust and 'classic' nature of the oestrogen response by the AP-2 γ 5'-flanking sequences.

5'-UTR of *TFAP2C* interacts with oestrogen-activated ER α

To determine if transcription factors can interact with either ERE-A or ERE-B, this region of the AP-2 γ gene was used as a probe for *in vitro* DNaseI footprinting (see the Experimental section) using nuclear extracts derived from ZR75.1 cells cultured in CM (i.e. containing oestrogenic activity). As shown in Figure 4, three previously identified Sp1 sites (FPA, FPB and FPC) were observed at the top of the gel, whereas three new footprints were observed over the 5'-UTR. One of these, FPD, maps over the presumed Inr containing the transcription start site [26], whereas FPE and FPF lie over the putative ER binding sites, ERE-A and ERE-B, respectively. Thus one or both of these sites might bind the ER directly. This was examined further using EMSAs. Initial experiments using nuclear extracts identified an ER-like complex over ERE-B, with no specific complex identified using ERE-A sequences as a probe (results not shown). Further experiments therefore compared the ability of oligonucleotide probes containing sequences to ERE-B (i.e. FPF) with a well-characterized ERE from the chicken Vit A2 gene to bind recombinant ER α (rER α). As shown in Figure 5, the labelled FPF probe formed a complex with rER α (lane 2) which was supershifted by the addition of an anti-ER α antibody (lane 1). Competition experiments were performed by the addition of excess unlabelled oligonucleotide for Vit (lanes 3–5) or FPF (lanes 6–8) or mutant FPF (lanes 9–11). The pattern of competition was similar for both FPF and Vit, showing them to be equally avid ER α -binding sites, at least *in vitro*, whereas an oligonucleotide carrying a 5 bp mutation in FPF, corresponding to the mutated site in the p Δ 5bpERE-B.LUC reporter construct (see above) (lanes 9–11), can no longer compete, demonstrating that deletion of an ERE half-site completely abrogated ER α binding.

Figure 6 ER α occupancy of the *TFAP2C* 5'-UTR

ZR75.1 cells were incubated with or without 10^{-8} M 17β -oestradiol for 1 h. Chromatin was isolated and immunoprecipitated with either an anti-ER α antibody or a 'mock' control, as indicated. DNA purified from both the immunoprecipitated (IPT) fraction and from the unbound fraction was subjected to PCR amplification for the AP-2 γ 5'-UTR (+86 to +282). The two right-hand lanes represent the no-template (N) and cDNA positive controls for PCR (P).

To test whether ER α binds to the 5'-UTR of AP-2 γ *in vivo* in response to activation by oestrogens, we analysed the AP-2 γ gene in ZR75.1 cells by ChIP assay. Specific AP-2 γ PCR amplicons from +86 to +282, but not from upstream (−818 to −998) or downstream (+722 to +909) regions (results not shown), were obtained from chromatin immunoprecipitated using an anti-ER α antibody, but not from mock-immunoprecipitated controls. As shown in Figure 6, 1 h after the addition of 10^{-8} M 17β -oestradiol to the culture, there was a significant increase in the amount of ER α bound to the region containing the EREs. Taken together, our results demonstrate that the AP-2 γ gene, *TFAP2C*, is directly regulated by oestrogens through the binding of ER α to a conserved site within the 5'-UTR of the gene.

DISCUSSION

The data presented here demonstrate that AP-2 γ is induced by oestrogens in ER-positive breast cancer cells as a primary response. In addition, our results show that AP-2 α expression is reduced in the presence of oestrogens, possibly as a secondary response. Data from RT-PCR, and Northern and Western blotting using two different ER α -positive cell lines combine to demonstrate that the ratio between the different AP-2 protein isoforms is consistently changed as a result of oestrogen exposure. Reporter assays, using wild-type and mutant constructs, indicate that the oestrogenic regulation of AP-2 γ depends on a ERE element (ERE-B) that lies within the 5'-UTR of the gene. The combined results obtained by DNaseI footprinting, EMSA and ChIP assays clearly demonstrate that ER α binds to ERE-B *in vivo* and *in vitro*. Total or partial deletion of this element, while eliminating ER α binding, abrogated the response to oestrogen by the reporter constructs in two different ER-positive cell lines. This element is both highly similar to a consensus ER-binding site and is conserved in sequence and position within the mouse AP-2 γ gene (Figure 3a). The presence of a functional ERE within the 5'-UTR of a gene is not frequent, yet several examples have been reported, including the vascular endothelial growth factor (VEGF) gene [30], the *CYP19A* genes in zebrafish [31] and the recently described Ras-related and oestrogen regulated growth inhibitor gene (RERG) whose expression has been correlated with ER expression in breast tumour samples [32].

However, the possibility that additional EREs at other locations in the AP-2 γ gene may contribute to the regulation of AP-2 γ transcription should be assessed. In particular, we have considered whether oestrogen-inducibility may be mediated in part via the Sp1 sites within the AP-2 γ proximal promoter [26], following

reports that several genes can be transcriptionally activated either by direct ER–Sp1 protein–protein interactions [33] or possibly through a common co-activator, SNURF (small nuclear RING finger protein) [34]. However, this seems unlikely in the context of AP-2 γ owing to the fact that deletion of ERE-B alone prevented oestrogen-responsiveness (Figures 3c–3e). This would also rule out a role for the second ERE-like element within the 5'-UTR, ERE-A. This region appears to contribute to overall promoter function, since its deletion resulted in weaker reporter activity (Figure 3c). However, despite showing binding to nuclear factors in a DNaseI footprinting assay, we have not been able to demonstrate ER α binding to this site, and the fact that reporter activity remains inducible when ERE-A is deleted indicates strongly that this site does not contribute to AP-2 γ responsiveness to oestrogens. The reduction in the basal activity of the p Δ 5bpEREb.LUC vector is possibly due to the squelching of co-activators by the co-transfected ER α . Finally, although the positional resolving power of ChIP is low, the fact that we never observed any amplification with upstream (−818 to −998) or downstream (+722 to +909) primer pairs from DNA immunoprecipitated with an anti-ER α antibody also supports the conclusion that oestrogen-responsiveness is centred around the ERE-containing region of the AP-2 γ gene. Indeed, the pattern of ER α occupancy over the AP-2 γ ERE, as revealed by our ChIP analysis, is strongly reminiscent of results with the well-characterized oestrogen-responsive gene, pS2. For both genes, ER α binding can be detected in the absence of ligand and this binding is dramatically increased in the presence of oestrogen ([35,36]; Figure 6). This implies that both genes are poised for transcriptional activation and underlines that AP-2 γ is also a primary oestrogen-responsive gene.

The fact that oestrogens, which represent fundamental mitogens and morphogens in the mammary gland, alter the balance between different isoforms of developmentally regulated AP-2 transcription factors, by directly inducing transcription of the AP-2 γ gene, strongly suggests that a number of AP-2 target genes may be regulated by oestrogens as a secondary response, as in the case of *ERBB2* [20,24]. From Northern and Western blotting, our data indicate that, under oestrogenic conditions, AP-2 α and AP-2 γ are present in roughly equal amounts, whereas in anti-oestrogenic milieu, AP-2 α predominates. Since heterodimerization was demonstrated for all AP-2 proteins, it is conceivable that not only homodimers, but also heterodimeric arrangements, of the different AP-2 proteins may display distinct transcriptional activities, at least in certain gene contexts. AP-2 factors are thought of as being fairly weak activators of gene expression, but have also been described as repressing expression of certain genes. Of the four family members described, three (AP-2 α , β and γ) have been shown to be expressed in overlapping patterns in developing tissues, yet have apparently non-degenerate gene targets, since mice homozygously deleted for each of these isoforms have completely distinct phenotypes [1,5–6]. Moreover, even in *in vitro* assays, differences in activity can be noted; AP-2 α and γ were three to four times more active than AP-2 β at activating an *ERBB2* reporter construct [4]. However, homo- and hetero-dimers of these same three family members have been shown to have essentially identical DNA-binding specificity, reflecting the extreme conservation of their dimerization/DNA-binding domains. Presumably, therefore, the specificity of AP-2 activity derives from specific interactions with other transcription factors and/or with different co-activators. For example, AP-2 proteins were reported to interact with p53 in at least three promoter contexts {the gelatinase A promoter, the p21^{WAF/CIP1} gene (see [1] and references therein) and the 67 kDa laminin-receptor gene intronic enhancer [8]}, the interaction resulting in

either co-activation or repression. In these studies, interaction with p53 was demonstrated for both AP-2 α and AP-2 γ , but it would be very interesting to determine whether different arrangements of homo- and hetero-dimers of the AP-2 isoforms can display differential affinities for p53. Similarly, it has been shown recently that the CITED proteins are specific co-activators of AP-2 factors [14]. In a further characterization of CITED co-activation, it was shown that CITED4 was significantly weaker than CITED2 for co-activation of AP-2 γ , but this was not apparent for other AP-2 proteins [15], suggesting that the co-activator complement in a given cell might determine the specific transcriptional activities of AP-2 isoforms.

Changes in the balance of AP-2 isoforms may affect the expression of genes such as *ERBB2*, where regulation of AP-2 transactivation by oestrogens was originally suggested [20,24]. During these studies, it was shown that transfection of AP-2 γ and AP-2 β , but not AP-2 α , into ZR75.1 cells not only reduced transcription of a reporter vector carrying the *ERBB2* promoter, but also abrogated oestrogenic repression of this promoter [24], suggesting that the presence of elevated levels of AP-2 γ or AP-2 β alters the way this promoter is regulated. In addition, whereas expression of either AP-2 α , AP-2 β or AP-2 γ singly in AP-2-negative HepG2 cells resulted in activation of an AP-2 reporter construct carrying three copies of a consensus AP-2-binding site [4], co-expression of AP-2 α and AP-2 γ did not activate transcription (M. De Bortoli, unpublished work). Thus, at least in certain gene contexts, the balance between homo- and hetero-dimers can result in different transcriptional outputs. Thus, although *in vitro* AP-2 α and AP-2 γ appear to be equally potent activators of the *ERBB2* gene [4], in the chromatin context, oestrogenic repression at the *ERBB2* promoter [24] may be linked to an increase in AP-2 γ over AP-2 α , which could result in less-efficient activation.

A further interesting observation is that AP-2 factors and ER α may form a control loop regulating each other's transcription. In fact, AP-2 γ was identified as the ERF-1 factor that binds to and maintains transcription of the ER α gene in MCF-7 cells (see [1] for a review). The fact that, in breast tumours, an inverse relationship exists between ER α and *ERBB2* expression also suggests that the interaction between AP-2 factors may be critically dependent on promoter context. In conclusion, we have demonstrated in the present study that one of the pathways of secondary gene control by oestrogens may depend on the balance between AP-2 transcription factors. We now have to understand how these different balances of AP-2 factors operate in terms of differential gene regulation on both known AP-2 target genes, as well as at the genome-wide level.

Note added in proof (received 18 November 2003)

A fifth member of the AP-2 family was recently described [37].

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