Characterization of the human activator protein-2 γ (AP-2 γ) gene: control of expression by Sp1/Sp3 in breast tumour cells

Mark D. HASLETON, J. Claire IBBITT and Helen C. HURST¹

Cancer Research UK Molecular Oncology Unit, Imperial College of Science, Technology and Medicine, Hammersmith Hospital, Du Cane Road, London W12 0HS, U.K.

The activator protein-2 (AP-2) family of DNA-binding transcription factors are developmentally regulated and also play a role in human neoplasia. In particular, the AP-2 γ protein has been shown to be overexpressed in a high percentage of breast tumours. In the present study, we report the complete sequence determination of the human *TFAP2C* gene encoding the AP-2 γ transcription factor plus the mapping of the transcription start site used in breast tumour-derived cells. The 5'-end of the gene lies within a CpG island and transcription is initiated at a single site within a classical initiator motif. We have gone on to investigate why some breast tumour-derived cell lines readily express AP-2 γ , whereas others do not, and show that the proximal promoter (+191 to –312) is differentially active in the two cell phenotypes. DNase footprinting led to the identification of three Sp1/Sp3-binding

sites within this region, two of which are absolutely required both for promoter function and cell-type-specific activity. By Western blotting a panel of expressing and non-expressing breast tumour lines we show that the latter have higher levels of Sp3. Furthermore, increasing Sp3 levels in AP-2 γ -expressing cells led to the repression of AP-2 γ promoter activity, particularly when Sp3 inhibitory function was maximized through sumoylation. We propose that differences in the level and activity of Sp3 between breast tumour lines can determine the expression level of their AP-2 γ gene.

Key words: activator protein 2 factor (AP-2 factor), breast tumour line, promoter, Sp1, Sp3, sumoylation.

INTRODUCTION

Activator protein-2 (AP-2) is the term given to a family of developmentally regulated, 50 kDa, DNA-binding transcription factors, whose members include AP-2 α , AP-2 β , AP-2 γ and AP-2 δ [1–4]. During vertebrate embryogenesis, these proteins play important roles in the development and differentiation of the neural tube, neural crest derivatives, skin, urogenital tissues and heart and show overlapping, but distinct, patterns of expression within these tissues [5–8]. Three of the family members have also been analysed by homologous gene inactivation in mice. Mice lacking AP-2 α showed multiple defects in the development of the face, limbs, body wall and neural tube [9,10]. Mice lacking AP-2 β displayed fewer gross phenotypic defects but, nevertheless, died shortly after birth due to the absence of terminal kidney differentiation [11]. In contrast, AP-2 γ is absolutely required within the extra-embryonic lineages for early postimplantation development, but does not play an autonomous role within the embryo proper [12,13]. The newly described AP-2 δ gene seems to have a more restricted expression pattern, being most abundant in embryo brain, and also has a distinct DNA-binding site preference [4]. Thus, despite the close homology between these proteins, particularly within their DNA-binding and dimerization domains, there is surprisingly little redundancy in their overall function.

Expression of AP-2 proteins has also been associated with neoplasia, particularly in breast cancer, where high levels of AP-2 α and AP-2 γ have been correlated with overexpression of the proto-oncogene *ERBB2* in tumour-derived cell lines [3] and tumour samples [14,15]. Both the promoter and an intronic enhancer within the *ERBB2* gene contain binding sites for AP-2 proteins [16,17]. Moreover, further genes such as the oestrogen receptor, universal cell-cycle inhibitor p21^{cip}, insulin-like growth factor I,

E-cadherin and *ERBB3*, which also play important roles in mammary epithelial growth and differentiation, are also regulated by AP-2 proteins [14,15,18–20].

Given these data, it is important to establish how the expression of the AP-2 genes is regulated in breast epithelia. Previous analysis using immunohistochemistry has shown that, although there is little expression of AP-2 β in breast tissue, both AP-2 α and AP-2 γ are expressed in normal ductal epithelium [14]. However, AP-2 α has been shown to become overexpressed in 17–35 % of primary tumour samples [14,15], and significant overexpression of AP-2 γ was demonstrated in 84 % of breast tumours [14]. We therefore sought to establish the determinants of expression control of the AP-2 γ gene in breast tumour cell lines, comparing those with a non-expressing and an expressing phenotype. In the present study, we report the characterization of the AP-2 γ gene, *TFAP2C*, and further demonstrate that the proximal promoter of the gene is differentially active in non-AP-2 γ -expressing and AP-2 γ -expressing breast tumour-derived cell lines and implicate Sp1 and Sp3 factors as playing a major role in this regulation.

MATERIALS AND METHODS

DNA manipulation and RNase mapping

Mapping and sequencing of genomic cosmid clones employed standard methodologies. Appropriate genomic fragments were subcloned into pBluescript and the plasmids linearized to generate a probe template for RNase mapping. Labelled probes, incorporating [α -³²P]UTP, were synthesized using the Riboprobe In Vitro Transcription Kit (Promega) and purified from a preparative sequencing gel to remove any incomplete products. Total cellular RNA (25 μ g) was hybridized with the riboprobe (5×10^5 c.p.m.

Abbreviations used: AP-2, activator protein-2; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assay; FPA etc., footprint A etc.; LUC, luciferase; SUMO, small ubiquitin-like modifier; UTR, untranslated region.

¹ To whom correspondence should be addressed (e-mail Helen.Hurst@cancer.org.uk).

Figure 1 Map of the 5'-end of the AP-2 γ gene

A region of approx. 2.5 kb (not to scale) around the *TFAP2C* transcription start site (arrowhead) is depicted, including key restriction sites (B, *Bam*HI; S, *Sac*I) with their position relative to +1. The sites flanking the 0.5 kb genomic *Bam*HI fragment used to generate probes for RNase mapping and DNase footprinting are shown in bold. Fragments used to generate CAT and LUC reporter constructs are delineated below the map. The approximate position of the three footprinted regions are shown by black boxes A–C.

per sample) and the RNase protection assay was performed using reagents supplied in a kit (Ambion) used according to the manufacturer's instructions. The final products were separated on a 6% (w/v) denaturing gel. Additional lanes were loaded with an aliquot of probe, an end-labelled 1 kb DNA sequencing ladder (Life Technologies) and a set of sequencing reactions carried out on the template plasmid using a primer designed to map to the extreme 3'-end of the insert. By comparing the migration of the RNase-resistant bands with the DNA marker lanes in the final autoradiograph, the position of the transcription start site could be estimated after correction for the differential gel migration of RNA and DNA species [21].

Cell lines and transfection studies

Breast tumour-derived epithelial cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, except ZR 75-1 cells which were maintained in RPMI 1640 containing 10% (v/v) fetal calf serum. The AP-2 γ -chloramphenicol acetyltransferase (CAT) and -luciferase (LUC) reporter constructs were generated by cloning the 0.5 kb genomic *Bam*HI fragment (Figure 1) into pCATbasic and pGL3 (Promega) to give p0.5CAT and p0.5LUC respectively. The p1.0CAT and p2.5CAT constructs, to -928 and -2134, extended 5' to genomic *Sac*I and *Bam*HI sites respectively, as indicated in Figure 1. Transient transfection assays were performed on cell monolayers using the Fugene 6 transfection reagent (Boehringer) according to the manufacturer's instructions. Cells were harvested after 48 h and lysed. CAT transfection efficiencies were internally controlled by including a β -galactosidase expression vector and all CAT values are presented after correction for β -galactosidase activity within the same lysate [21]. Similarly, LUC activity was assayed using the Dual Luciferase Reporter Assay system (Promega) and a Bio-Orbit 1251 luminometer (Labtech), and firefly LUC readings were normalized to thymidine kinase-*Renilla* LUC (not regulated by Sp binding). Individual footprint sites were mutated in the p0.5CAT reporter as follows: footprint A (FPA) was deleted by digestion at flanking *Stu*I and *Dra*III restriction sites and religation of repaired ends; footprint B (FPB) and footprint C (FPC) were mutated at a single base (see Figure 5 below) by using appropriate double-stranded oligonucleotides to replace wild-type sequence between flanking restriction sites. The oligonucleotides were also used as competitors and probes in electrophoretic mobility-shift assays (EMSAs) to confirm that Sp binding had been abolished and that no new binding sites were created (results not shown). Expression plasmids for Sp3 and protein inhibitor of signal transducer of transcription 1 (PIAS1) were kindly provided by Professor G. Suske, Institut für Molekularbiologie

und Tumorforschung, Marburg, Germany [22]. Small ubiquitin-like modifier (SUMO) expression plasmids have been described previously [23]. The total amount of DNA transfected was normalized using the appropriate empty vector and all experiments were performed in duplicate and repeated at least three times to allow the calculation of S.D., displayed as error bars. Student's *t* test was used to determine statistical significance.

DNase I footprinting

The 0.5 kb *Bam*HI genomic fragment was cloned into pBluescript (Stratagene), re-excised and end-labelled with [α - 32 P]dATP at either the 5'- or 3'-end. End-labelled probe (7 fmol) was incubated with crude nuclear extract [24], followed by partial DNase I treatment, proteinase K digestion, phenol/chloroform extraction and ethanol precipitation before fragments were resolved on denaturing polyacrylamide gels. Additional lanes were loaded with an A + G chemical degradation sequencing ladder generated from the same probe and a control DNase I digest of probe that had not been pre-incubated with nuclear extract. Potential factors binding to the protected sequences were predicted using MatInspector (<http://www.gsf.de/biodv/matinspector.html>).

EMSA

EMSAs were performed with end-labelled double-stranded oligonucleotide probes corresponding to each of the four protected footprint regions (see Figure 5 for sequences) as described previously [16]. 'Supershifting' experiments were performed by incubation of crude nuclear extract with the relevant antibody (Sp1, PEP2; Sp3, D20; pan Jun, D; AP-2, C-18; all from Santa Cruz Biotechnology) or preimmune serum for 1 h at 4 °C prior to addition of the probe. Competition experiments were performed with 20- and 100-fold mass excess over the labelled probe.

Western blotting

Nuclear extract (10 μ g) was separated by SDS/PAGE together with Full Range molecular-mass rainbow markers (Amersham Biosciences). Gel transfers to Hybond-ECL nitrocellulose (Amersham Biosciences) and primary and secondary antibody incubations were performed using standard techniques. The chemiluminescent detection of horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) was performed using the West Femto Maximum Sensitivity Substrate kit (Pierce) according to the manufacturer's instructions. The anti-Sp1 and -Sp3 primary antibodies (see above) were used at 1:1000

Table 1 *TFAP2C* intron–exon boundaries

First and last exons, marked by *, indicate the number of coding bases only. Splice junction sequences: exon sequence in bold; intronic sequence in normal type.

Exon number	Exon size	Following intron size (bp)	5' Splice donor	3' Splice acceptor
1	48*	1611	GAGGTGAGC	TTTCCTCCAGG
2	486	114	CAGGTGAGC	CTCTCCCCAGA
3	52	1487	AAGGTAAAT	TTCATTACAGG
4	217	581	AAGGTACTT	CTTTTCTTAGA
5	119	2343	AAGGTCAGT	GATTATTCTAGG
6	145	973	CCAGTAAGT	TGCTTCACAGG
7	283*	–		

and 1:500 dilutions respectively. AP-2 γ was detected using the 6E4 monoclonal antibody [23]. To re-probe for normalization, membranes were stripped in a solution containing 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 re-blocked prior to incubation with a goat polyclonal Ku-70 antibody (C-19; Santa Cruz Biotechnology).

RESULTS

Gene characterization and start site mapping for AP-2 γ and AP-2 β

The isolation of genomic cosmid clones for the human AP-2 β and AP-2 γ genes (*TFAP2B* and *TFAP2C*) has been described previously [25]. Restriction mapping and Southern blotting of both clones were performed to identify the location of the 5' exon of each gene. In the case of AP-2 γ , we used the structure of the AP-2 α gene [26] to predict exon boundaries and derived oligonucleotides that allowed us to PCR each intron and thus to map the exon–intron structure of the whole gene. The AP-2 γ gene was found to be very compact with seven short coding exons spread over 7.5 kb, as summarized in Table 1. We subsequently sequenced the complete gene, including 2.3 kb of 5'– (upstream of the AUG codon) and 1.2 kb of 3'– (downstream of the stop codon) flanking sequence (GenBank® accession number AJ315934).

The first exon of AP-2 γ contains 48 bases of coding sequence but, in order to locate accurately the promoter and potential regulatory sequences, we mapped the start site of transcription as used in breast tumour cell lines which express the gene. A genomic 0.5 kb *Bam*HI fragment (Figure 1), whose 3'-end maps within the 5'-untranslated region (UTR), was used to generate a probe for RNase protection assays and hybridized with total RNA from cell lines determined previously ([3,27]; also see Figure 8, first panel) to be either non-expressors or expressors of AP-2 γ . As shown in Figure 2, subsequent digestion of RNA hybrids led to the detection of a single protected band in AP-2 γ -expressing lines and these data were used to assign the start site of transcription (+1; Figures 1 and 2). This placed the initiation site within the motif ACA(+1)CTGT, which lies 232 bp upstream of the initiator ATG and 66 bp 5' of the sequences contained in the longest reported human cDNA clone [18]. The upstream sequence contains no obvious TATA box which, coupled with the single start site, is reminiscent of genes whose core promoter consists of an initiator over the transcription start site. Indeed the sequence around the AP-2 γ +1 is closely similar to the initiator consensus sequence YYA(+1)N(T/A)YY [28,29]. Furthermore, when we examined the AP-2 γ gene sequence for CpG frequency, we identified a CpG island over a 4 kb region of the genomic sequence that contains the presumed promoter, the transcription start site and the first

Figure 2 AP-2 γ start site mapping

An RNase protection assay using an antisense riboprobe (lane P, arrowed) corresponding to the genomic 0.5 kb *Bam*HI fragment (see Figure 1) was performed. The probe was hybridized with tRNA (lane 1, control) or total cellular RNA from non-AP-2 γ -expressing (lane 2, MDA MB 231; lane 3, MDA MB 436; and lane 4, Cal51) and AP-2 γ -expressing (lane 5, ZR 75-1; lane 6, MDA MB 435S; and lane 7, T47D,) breast tumour-derived cell lines. Labelled molecular-size markers (in bp) were run in lane M, and a sequencing reaction using a primer to the 3'-end of the riboprobe in lanes A, G, C and T. The single band defining +1 is indicated by the arrow.

three exons. These observations, plus the fact that the 5'-flanking sequences were subsequently shown to have specific promoter activity (see below), confirmed that we had located the true 5'-end of the AP-2 γ gene.

The human AP-2 β gene structure has been examined previously [11], but the transcription start sites have not been identified. A genomic 370 bp *Pst*I/*Eco*RV restriction fragment, whose 3'-end lies near the end of the first exon, was used to generate a probe for RNase protection assays. Total RNA from the two breast tumour lines (ZR 75-1 and MDA MB 453) known to express AP-2 β [3] were assayed and the results are shown in Figure 3. Two major and a number of minor initiation sites were observed, a pattern frequently found for TATA-box-dependent promoters. Indeed, a canonical TATA element is located 29 bp upstream of the more upstream major start, which was designated as +1 (Figure 3).

Identification of regulatory regions within the AP-2 γ gene

To search for regulatory sequences within the AP-2 γ gene that may be determinants of its level of activity in breast tumour-derived cells, we compared the chromatin structure around the gene in both non-expressing and expressing cells using the DNase I-hypersensitive-site assay. Genomic DNA from DNase I-

Figure 3 AP-2 β start site mapping

An RNase protection assay using an antisense riboprobe corresponding to a *TFAP2B* genomic 370 bp *Pst*I/*Eco*RV fragment (see text). The probe was hybridized with tRNA (lane 1, control) or total cellular RNA from non-AP-2 β -expressing (lane 2, MDA MB231; and lane 3, HBL100) and AP-2 β -expressing [lane 4, ZR 75-1; and lanes 5 and 6, MDA MB 453 (two separate RNA preparations)] breast tumour-derived cells. Labelled molecular-size markers (in bp) were run in lane M, and a sequencing reaction using a primer to the 3'-end of the riboprobe in lanes A, G, C and T. The migration of full-length probe is indicated by an arrow labelled P. The bands corresponding to major and minor start sites are indicated with arrows; the position of these, including the base designated as +1, is indicated on the genomic sequence below the autoradiograph, which also shows the presumed TATA box (in bold). Faint signals are apparent in lane 4 at the same positions as the bands in lanes 5 and 6 and reflect the much lower expression of AP-2 β in ZR 75-1 compared with MDA MB 453 cells [3].

treated nuclei was digested with restriction enzymes prior to Southern blotting with a number of different probes. A region from 7 kb 5' to 8 kb 3' of the gene was thus analysed, but the only hypersensitive site detected lay just 5' of the start site within the presumed proximal promoter. No difference in hypersensitivity was observed between expressing and non-expressing breast tumour lines (results not shown).

In order to demonstrate that the 5'-flanking sequences of the AP-2 γ gene possessed promoter activity, a series of fusion constructs were made with the CAT reporter gene (see the Materials and methods section). The 3'-end in all of these constructs was the *Bam*HI site at +191 within the 5'-UTR with sequences extending 0.5–2.5 kb 5' of this point (Figure 1). The reporter plasmids were transfected into both AP-2 γ -expressing (ZR 75-1, MDA MB 435S) and non-AP-2 γ -expressing (MDA MB 436 and MDA MB 231) cells with appropriate controls (Figure 4, and see

Figure 4 The AP-2 γ proximal promoter is only active in expressing cells

CAT reporter constructs containing AP-2 γ genomic sequences from +191 to –312 (p0.5CAT), –928 (p1.0CAT) or –2134 (p2.5CAT) were transfected into two AP-2 γ -expressing (ZR 75-1 and MDA MB 435S) and two non-AP-2 γ -expressing (MDA MB 436 and MDA MB 231) cell lines as indicated. CAT activity was normalized to pCATbasic activity (set at 1) in the same cell line to allow relative reporter activity to be compared between the lines.

Materials and methods section). In both expressing lines, we were able to demonstrate good promoter activity (approx. 10–15-fold over the promoterless control pCATbasic) with all the constructs, although sequences within the 0.5 kb construct were sufficient to confer maximal activity. In contrast, none of the constructs demonstrated significant activity above background levels in either of the non-expressing lines. Constructs containing further 5' sequences, up to 10 kb upstream of the transcription start site, did not demonstrate any increased reporter activity in either cell phenotype (results not shown). We conclude therefore that the proximal promoter sequences of the gene have differential activity between breast tumour cells that do and do not express significant levels of AP-2 γ and that sequences from +191 to –312 relative to the start site of transcription are sufficient to elicit the majority of the activity in the expressing lines. Furthermore, the differential activity of these plasmid (and consequently non-cytosine-methylated) reporter constructs in the two cell types indicates that differences in *trans*-acting factors between the two cell phenotypes are probably the main determinants of *TFAP2C* expression. Consequently, AP-2 γ expression is unlikely to be restricted in non-expressing breast tumour cells through hypermethylation of the CpG sequences at the 5'-end of the endogenous gene.

AP-2 γ promoter is bound by Sp1 and Sp3

In order to identify the transcription factors that control expression of the proximal promoter, the 0.5 kb *Bam*HI fragment (+191/–312) was end-labelled and used as a probe in DNase I footprinting experiments. Nuclear extract from both AP-2 γ -expressing and non-expressing cell lines was used in these assays and a representative autoradiograph is shown in Figure 5. Both nuclear extracts produced a pattern of three footprints designated FPA, FPB and FPC, centred at positions –200, –135 and –70 relative to the transcription start site. When the protected sequences were submitted to the MatInspector program, it was suggested that all three might provide binding sites for members of the AP-2, Sp1 or AP-1 families of transcription factors.

Double-stranded oligonucleotide probes were generated to each of the protected sequences and tested in EMSAs using nuclear extracts from AP-2 γ -expressing cells. As shown for FPA

Figure 5 Footprinting the AP-2 γ promoter reveals three factor binding sites

The 0.5 kb genomic *Bam*HI fragment was labelled at the 5'-end and incubated with crude nuclear extract from MDA MB 436 (lanes 3–5; 100, 150 and 200 μ g) or ZR 75-1 cells (lanes 6–8; 100, 150 and 200 μ g) prior to limited DNase digestion. DNase digests of probe alone are shown in lanes 1, 2 and 9 (two different incubation times). Protected sequences were identified by comparison with an A + G sequencing ladder of the probe (lane 10). FpA, FpB and FpC are indicated with the protected sequences shown; the regions similar to the Sp1 consensus binding site GGGCGGGGC are underlined (note, for FpA this sequence is the reverse complement). For FpB and FpC the base in bold was mutated to A in each case to generate the B^M and C^M mutants.

(Figure 6A, lanes 1–3), a series of protein–DNA complexes was formed that could be specifically competed by addition of excess unlabelled probe. Preincubation of extracts with antibodies to Sp1 or Sp3 caused 'supershifting' of these specific complexes, particularly when antibodies to both proteins were present (Figure 6A, lanes 4–6). Antibodies to AP-2 or Jun proteins (a component of AP-1), however, did not affect the pattern of complexes (Figure 6A, lanes 7 and 8). Essentially identical results were observed using probes to FPB and FPC and nuclear extracts from any of our panel of cell lines, including non-AP-2 γ expressors (Figure 6b and 6c, and results not shown). In each case, the upper complex represents DNA bound to either Sp1 or full-length Sp3, whereas the lower complex is actually a tight doublet representing binding by the two truncated forms of Sp3 that are synthesized from internal AUG codons (see [30] for review; and, Figure 8, third panel, below). Cross-competition assays in EMSA also showed that all three footprinted regions bound Sp1/Sp3 with

Figure 6 The AP-2 γ promoter is bound by Sp1 and Sp3 factors

(A) The FPA EMSA probe was incubated with nuclear extract from T47D cells (10 μ g/lane). Incubations included: lane 1, no additions; lanes 2 and 3, 20- and 100-fold excess of unlabelled probe; lane 4, anti-Sp1 antibody; lane 5, anti-Sp3 antibody; lane 6, anti-Sp1 and -Sp3 antibodies; lane 7, anti-AP-2 antibody; lane 8, anti-(pan Jun) antibody; and lane 9, preimmune serum (Pre). FPB (B) and FPC (C) probes were incubated with nuclear extract from MDA MB 435S cells either alone (lanes 1) or with the addition of antibody to Sp1 (lanes 2), Sp3 (lanes 3) or both Sp1 and Sp3 (lanes 4). In (C), lane 5 shows that the complex remaining after addition of Sp1/Sp3 antibodies is non-specific as it is not competed by a 200-fold excess of unlabelled probe. For all panels, protein–DNA complexes only are shown; specific complexes are marked with standard arrows, supershifted complexes with open arrows and non-specific complexes with *.

similar efficiency (results not shown). Since removal of Sp1 and Sp3 complexes using antibodies leaves no specific interactions remaining (Figure 6B, lane 4 and Figure 6C, lanes 4 and 5), we concluded that all three footprinted sites within the promoter are bound by members of the Sp-family of factors alone; no evidence for direct binding by either AP-1 or AP-2 factors was found.

In order to establish that the footprinted regions of the AP-2 γ promoter represented interactions required for activity, we generated further reporter constructs lacking each site in turn. FPA was removed by restriction digest (see the Materials and methods section), but the resulting promoter construct was not significantly altered in activity compared with the p0.5CAT reporter in either expressing or non-expressing cells (results not shown). FPB and FPC were tested by site-directed mutagenesis whereby a single base within each core Sp1/Sp3-binding site was mutated to prevent factor binding (see the Materials and methods section and Figure 5). The activities of the individual (B^M or C^M) and the double mutant (B^M + C^M) were compared with the p0.5CAT parent construct by short-term transfection into non-AP-2 γ -expressing and AP-2 γ -expressing cells. As shown in Figure 7, there was essentially no change in reporter activity in cells that hardly express AP-2 γ , but in expressing cells loss of either FPB or

Figure 7 FPB or FPC are required for AP-2 γ promoter activity in expressing cells

The p0.5CAT reporter construct plus derivatives mutated in the FPB- (BM) or FPC- (CM) binding sites or both (BM + CM) (see the Materials and methods section) were transiently transfected into AP-2 γ -expressing (MDA MB 435S) or non-expressing (MDA MB 231) cells. CAT activity, corrected for transfection efficiency (see the Materials and methods section), is shown as the mean $\pm$ S.D. for four independent experiments. * $P = 0.007$.

FPC led to a 50 % decrease in promoter activity, whereas mutation of both led to total loss of activity ($P = 0.007$). This demonstrates that the presence of at least one of these Sp1/Sp3 sites is essential for AP-2 γ promoter activity in expressing cells.

Ratio of Sp1 and Sp3 levels modulates AP-2 γ promoter activity

Our results implied that differential AP-2 γ promoter activity could be demonstrated between expressing and non-expressing cells, although the DNA-binding factors apparently mediating this effect were the ubiquitous proteins of the Sp1 family. We therefore performed Western blotting on a panel of breast tumour lines to try to establish a link between levels of Sp1 and Sp3 and the AP-2 γ -expression status. As shown in Figure 8 (second panel), the levels of Sp1 did not vary significantly across the panel of cell lines. In contrast, levels of Sp3 appeared to be much less uniform (Figure 8, third panel) and there was a clear trend for lower levels of particularly the full-length, 105 kDa species in cell lines expressing higher levels of AP-2 γ .

The transcriptional activity of Sp3 and the precise role of each of its three isoforms has been the subject of some controversy. On promoters with multiple Sp-binding sites, however, Sp3 appears to be inactive or only to act as a very weak activator and can thus competitively antagonize the action of Sp1 [31]. An inhibitory domain has been mapped within Sp3 [32], and the core Ile-Lys-Glu-Glu sequence within this region has recently been shown to provide a conjugation site for the ubiquitin-like peptide SUMO. Furthermore, SUMO modification of Sp3 has been shown to silence Sp3 transactivation activity [22,33]. We reasoned that the higher levels of Sp3 in non-AP-2 γ -expressing cells, coupled with the multiple Sp-binding sites within the promoter, may result in repression of promoter activity by binding site competition with Sp1. To try to demonstrate that this is a probable mechanism, we sought to raise Sp3 levels in an AP-2 γ -expressing line and determine the effect on promoter activity. As shown in Figure 9(A) (compare columns 1 and 2), addition of an Sp3 expression plasmid alone had little effect on AP-2 γ reporter activity. As essentially all SUMO in mammalian cells is conjugated to target proteins, the proportion of free SUMO is consequently very small. We therefore included a SUMO expression vector in our transfection

Figure 8 Sp3 levels are elevated in non-AP-2 γ -expressing cells

Crude nuclear extract (10 μ g/lane) from a panel of breast tumour lines was separated by SDS/PAGE and blotted to a membrane. Blots were probed with primary antibodies against AP-2 γ , Sp1 and Sp3 before being reprobed for Ku-70 as a loading control, as indicated. Lanes 1–3, non-expressing lines (Cal 51, MDA MB 436 and MDA MB 231). Lanes 4–6, expressing lines (T47D, MDA MB 435S and ZR 75-1). Molecular-mass markers in kDa are shown on the right.

assays to allow modification of exogenously expressed Sp3. Initial experiments showed that SUMO2 was more active in our assays (results not shown), which may reflect the slightly more efficient conjugation of this isoform to Sp3 observed previously [22]. In addition, the conjugation of SUMO to Sp3 has been shown [22] to be stimulated by the E3 ligase-like activity of PIAS1 and thus an expression vector for this protein was also included. Using this combination of expression plasmids (Sp3, SUMO2, PIAS1) we were able to repress AP-2 γ reporter activity by up to 50 % (Figure 9A, compare columns 4–6 with column 1). To confirm that this effect was due to SUMO conjugation, we also exogenously expressed a mutant form of SUMO2 where we had deleted the two C-terminal glycine residues that are absolutely required to mediate the conjugation of SUMO to target lysine residues. This mutant form (mtSUMO) had no effect on AP-2 γ reporter activity (Figure 9B, columns 5 and 6), whereas wild-type SUMO2 again significantly repressed the promoter (Figure 9B, columns 3 and 4; $P = 0.003$).

DISCUSSION

In the present study, we describe the structure of the human AP-2 γ gene, which is much more compact than the related AP-2 β and AP-2 α genes: AP-2 γ spans approx. 7.5 kb compared with approx. 20 kb in the case of AP-2 α and AP-2 β [11,26]. Nevertheless a high level of homology is retained with the seven exons being almost identical in size in each case. The human and mouse AP-2 α genes both have an IR3-like (where IR3 is third internal repeat) element within their 5'-UTR [34]. This element is not present in either the AP-2 β or AP-2 γ genes, indicating that it was inserted into AP-2 α subsequent to the duplication of the common ancestral gene.

The AP-2 γ transcription start site was mapped to a single site within the sequence ACA(+1)CTGT. The single start site and the absence of an obvious TATA box upstream indicate this to be an initiator sequence. Footprinting analysis of the AP-2 γ promoter identified three Sp1-binding sites, at positions –200, –135 and –70 (relative to the transcription start site), and mutational analysis revealed that at least the two proximal sites are essential for promoter activity (Figures 5–7). The human AP-2 α gene also has a single transcription start site within an initiator-like element but, in this case, an octamer-binding site at –49 was shown to be

Figure 9 Expression of Sp3, SUMO and PIAS1 in AP-2 γ -expressing cells leads to repression of promoter activity

(A) The p0.5LUC reporter (0.5 μ g) was transfected into AP-2 γ -expressing cells on 12-well plates alone or in combination with 0.1 μ g of an Sp3 expression plasmid, 0.2 μ g of a PIAS1 expression plasmid and 0.5, 1.0 or 2.0 μ g of a SUMO2 expression plasmid, as indicated. (B) Transfections were carried out as in (A), except that in columns 5 and 6 a mutant form of SUMO2 (mtSUMO), lacking the C-terminal glycine residues was used instead of the wild-type SUMO (wtSUMO). All transfections additionally contained 0.5 μ g of thymidine kinase-*Renilla* reporter to control for transfection efficiency. Corrected LUC values for each assay are expressed as a percentage of the activity of the p0.5LUC reporter alone, set at 100%.

critical for core promoter activity [34], whereas more distal AP-2-, basic transcription element-binding protein 1 ('BTEB-1')- and repressor of AP-2 ('AP-2rep')-binding sites also play regulatory roles [26,35]. Not surprisingly, therefore, attempts to align the AP-2 α and AP-2 γ promoter sequences did not yield any significant comparisons; however, it is interesting to note that, in an almost identical arrangement with AP-2 γ , AP-2 α has three potential Sp1-binding sites positioned approx. 1 kb upstream from the AP-2 α transcription start site. This may reflect how the duplicated genes were originally regulated in a similar fashion but have evolved separate roles and, hence, unique promoters that are regulated in different ways. In contrast with both AP-2 α and AP-2 γ , the human AP-2 β gene is initiated at two major and a number of minor start sites with a TATA box located 29 bp upstream of the first major transcription start site (Figure 3),

emphasizing the separate control mechanisms that regulate expression of this family of factors.

The Sp-binding sites within the AP-2 γ core promoter are not only necessary for core promoter function, but also apparently mediate the differential promoter activity observed between AP-2 γ -expressing and -non-expressing breast tumour-derived lines (Figure 7). Sp1 is known to be a ubiquitous activator of promoters, whereas Sp3 has been shown to be capable of both activation and repression, although it is more likely to passively inhibit Sp1-mediated activation than to actively repress transcription [31]. Despite the ubiquitous expression of these proteins, it has been proposed that cell-type-specific transcription can be regulated through a differential Sp1/Sp3 ratio. In particular, it has been suggested that a high Sp1/Sp3 ratio allows the activation of epithelial-specific cellular genes such as E-cadherin and keratin 18, whereas high Sp3 levels repress the epithelial phenotype in other cell types [36]. There are also many other promoter studies that have shown activation by Sp1 and repression by Sp3 through the same binding sites (for examples, see [37,38]). Moreover, in some cases the Sp1/Sp3 ratio has been shown to be modulated either during differentiation [39] or in response to cellular stress [40]. It seemed plausible, therefore, that Sp1 activates the AP-2 γ promoter in expressing lines, whereas Sp3 interferes with this activation in non-expressing cells by competitively binding to the same sites. Our present data showing that there were no differential hypersensitive sites or DNase footprinting patterns on the AP-2 γ promoter, coupled with the generally higher levels of Sp3 in a panel of non-AP-2 γ -expressing lines, support this as a plausible mechanism for the regulation of AP-2 γ expression in breast tumour cells.

In order to test this hypothesis, we have used transfection assays to examine AP-2 γ reporter activity in expressing cells in the presence of exogenously expressed Sp3. These experiments are notoriously difficult due to the high endogenous levels of Sp proteins in mammalian cells, and most similar studies to date have used the SL-2 insect cell line that apparently lacks Sp-like proteins. However, initiator-driven reporter constructs are not efficiently recognized in SL-2 cells [41] and we therefore tried instead to maximize the repressive action of Sp3 in human cells. This was attempted by using new information [22,33] showing that the inhibitory activity of Sp3 is increased subsequent to conjugation by the ubiquitin-like peptide SUMO. By co-transfecting SUMO2 and PIAS1 expression plasmids we were able to demonstrate significant repression of the AP-2 γ reporter in expressing cells (Figure 9). However, it is unlikely that this is the sole mechanism regulating promoter activity: it is also likely that there are other competing activities within AP-2 γ -expressing cells that may mask repression by Sp3 in this particular assay. For example, Sp3 is also acetylated on the same lysine residue used for SUMO conjugation [42]. It is possible therefore that Sp3 may be acetylated to a greater degree in AP-2 γ -expressing cells and thus act more like an activator. Alternatively, acetylation could also serve to protect Sp3 from SUMO conjugation and thus minimize its repressive action in these cells. Evaluation of these latter possibilities, however, will require the generation of specific antibodies.

Equally we cannot completely exclude the possibility that variations in Sp1 activity between the different breast lines also contribute to determining AP-2 γ -expression levels. The DNA-binding activity of Sp1 has been reported to vary in response to post-translational modification such as phosphorylation [30]. Consequently, although we did not observe any major discrepancy between Sp1 levels and binding potential in our nuclear extracts, it is possible that Sp1 activity may be relatively attenuated in lines with low AP-2 γ levels. As a number of different signalling pathways have been proposed to modulate Sp1 and breast cancer is

a notoriously heterogeneous disease, there may not therefore be a single mechanism to alter the ratio between Sp1 and Sp3 level and/or activity. However, the data in the present study demonstrate that differences in this ratio between breast tumour-derived cells are likely to be a major determinant of their AP-2 γ expression level.

We would like to thank our colleagues Jyrki Eloranta and Nick Lemoine for comments on the manuscript, and Professor Guntram Suske for generously providing reagents and communicating results prior to publication. We also gratefully acknowledge the Association for International Cancer Research for supporting M. D. H.

REFERENCES

- Williams, T., Admon, A., Luscher, B. and Tjian, R. (1988) Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. *Genes Dev.* **2**, 1557–1569
- Moser, M., Imhof, I., Pscherer, A., Bauer, R., Arnselgruber, W., Sinowatz, F., Hofstädter, F., Schüle, R. and Buettner, R. (1995) Cloning and characterisation of a second AP-2 transcription factor: AP-2 β . *Development* **121**, 2779–2788
- Bosher, J. M., Totty, N. F., Hsuan, J. J., Williams, T. and Hurst, H. C. (1996) A family of AP-2 proteins regulates c-erbB-2 expression in mammary carcinoma. *Oncogene* **13**, 1701–1707
- Zhao, F., Satoda, M., Licht, J. D., Hayashizaki, Y. and Gelb, B. D. (2001) Cloning and characterisation of a novel mouse AP-2 transcription factor, AP-2 δ with unique DNA binding and transactivation properties. *J. Biol. Chem.* **276**, 40755–40760
- Chazaud, C., Ouladabdelghani, M., Bouillet, P., Decimo, D., Chambon, P. and Dolle, P. (1996) AP-2.2, a novel gene related to AP-2, is expressed in the forebrain, limbs and face during mouse embryogenesis. *Mech. Dev.* **54**, 83–94
- Moser, M., Ruschoff, J. and Buettner, R. (1997) Comparative analysis of AP-2 α and AP-2 β gene expression during mouse embryogenesis. *Dev. Dyn.* **208**, 115–124
- Satoda, M., Zhao, F., Diaz, G. A., Burn, J., Goodship, J., Davidson, H. R., Pierpont, M. E. and Gelb, B. D. (2000) Mutations in *TFAP2B* cause Char syndrome, a familial form of patent ductus arteriosus. *Nat. Genet.* **25**, 42–46
- Brewer, S., Jiang, X., Donaldson, S., Williams, T. and Sucov, H. M. (2002) Requirement for AP-2 α in cardiac outflow tract morphogenesis. *Mech. Dev.* **110**, 139–149
- Zhang, J. A., Hagopianaldson, S., Serbedzija, G., Elsemore, J., Plehndujowich, D., McMahon, A. P., Flavell, R. A. and Williams, T. (1996) Neural-tube, skeletal and body-wall defects in mice lacking transcription factor AP-2. *Nature (London)* **381**, 238–241
- Schorle, H., Meier, P., Buchter, M., Jaenisch, R. and Mitchell, P. J. (1996) Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature (London)* **381**, 235–238
- Moser, M., Pscherer, A., Roth, C., Becker, J., Mucher, G., Zerres, K., Dixkens, C., Weis, J., Guaywoodford, L., Buettner, R. and Fassler, R. (1997) Enhanced apoptotic cell-death of renal epithelial cells in mice lacking transcription factor AP-2 β . *Genes Dev.* **11**, 1938–1948
- Auman, H. J., Nottoli, T., Lakiza, O., Winger, Q., Donaldson, S. and Williams, T. (2002) Transcription factor AP-2 γ is essential in the extraembryonic lineages for early post-implantation development. *Development* **129**, 2733–2747
- Werling, U. and Schorle, H. (2002) Transcription factor AP-2 γ is essential for early murine development. *Mol. Cell. Biol.* **22**, 3149–3156
- Turner, B., Zhang, J., Gumbs, A. A., Maher, M. G., Kaplan, L., Carter, D., Glazer, P. M., Hurst, H. C., Haffty, B. C. and Williams, T. (1998) Expression of AP-2 transcription factors in human breast cancer correlates with the regulation of multiple growth factor signalling pathways. *Cancer Res.* **58**, 5466–5472
- Gee, J. M. W., Robertson, J. F. R., Ellis, I. O., Nicholson, R. I. and Hurst, H. C. (1999) Immunohistochemical analysis reveals a tumour suppressor-like role for the transcription factor AP-2 in breast cancer. *J. Pathol.* **189**, 514–520
- Bosher, J. M., Williams, T. and Hurst, H. C. (1995) The developmentally-regulated transcription factor AP-2 is involved in c-erbB-2 overexpression in human mammary carcinoma. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 744–747
- Newman, S. P., Bates, N. P., Vernimmen, D., Parker, M. G. and Hurst, H. C. (2000) Cofactor competition between the ligand-bound oestrogen receptor and an intron 1 enhancer leads to oestrogen repression of *ERBB2* expression in breast cancer. *Oncogene* **19**, 490–497
- McPherson, L. A., Baichwal, V. R. and Weigel, R. J. (1997) Identification of erf-1 as a member of the AP-2 transcription factor family. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4342–4347
- Zeng, Y. X., Somasundaram, K. and Eldeiry, W. S. (1997) AP-2 inhibits cancer cell-growth and activates p21^{waf1/cip1} expression. *Nat. Genet.* **15**, 78–82
- Skinner, A. and Hurst, H. C. (1993) Transcriptional regulation of the c-erbB-3 gene in human breast-carcinoma cell-lines. *Oncogene* **8**, 3393–3401
- Ausubel, F. M., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. and Struhl, K. (1994) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York
- Sapetschnig, A., Rischitor, G., Braun, H., Doll, A., Schergaut, M., Melchior, F. and Suske, G. (2002) Transcription factor Sp3 is silenced through SUMO modification by PIAS1. *EMBO J.* **21**, 5206–5215
- Eloranta, J. J. and Hurst, H. C. (2002) Transcription factor AP-2 interacts with the SUMO-conjugating enzyme UBC9 and is sumoylated *in vivo*. *J. Biol. Chem.* **277**, 30798–30804
- Dignam, J., Lebowitz, R. and Roeder, R. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475–1489
- Williamson, J. A., Bosher, J. M., Skinner, A., Sheer, D., Williams, T. and Hurst, H. C. (1996) Chromosomal mapping of the human and mouse homologs of two new members of the AP-2 family of transcription factors. *Genomics* **35**, 262–264
- Bauer, R., Imhof, A., Pscherer, A., Kopp, H., Moser, M., Seegers, S., Kerscher, M., Tainsky, M. A., Hofstaedter, F. and Buettner, R. (1994) The genomic structure of the human AP-2 transcription factor. *Nucleic Acids Res.* **22**, 1413–1420
- Zhu, C.-H. and Domann, P. E. (2002) Dominant negative interference of transcription factor AP-2 causes inhibition of ErbB-3 expression and suppresses malignant cell growth. *Breast Cancer Res. Treat.* **71**, 47–57
- Javahery, R., Khachi, A., Lo, A., Zenzie-Gregory, B. and Smale, S. T. (1994) DNA sequence requirements for transcriptional initiator activity in mammalian cells. *Mol. Cell. Biol.* **14**, 116–127
- Lo, K. and Smale, S. T. (1996) Generality of a functional initiator consensus sequence. *Gene* **182**, 13–22
- Suske, G. (1999) The Sp-family of transcription factors. *Gene* **238**, 291–300
- Hagen, G., Muller, S., Beato, M. and Suske, G. (1994) Sp1-mediated transcriptional activation is repressed by Sp3. *EMBO J.* **13**, 3843–3851
- Dennig, J., Beato, M. and Suske, G. (1996) An inhibitor domain in Sp3 regulates its glutamine-rich activation domains. *EMBO J.* **15**, 5659–5667
- Ross, S., Best, J. L., Zon, L. I. and Gill, G. (2002) SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localisation. *Mol. Cell* **10**, 831–842
- Creaser, P. C., Dargenio, D. A. and Williams, T. (1996) Comparative and functional-analysis of the AP-2 promoter indicates that conserved octamer and initiator elements are critical for activity. *Nucleic Acids Res.* **24**, 2597–2605
- Imhof, A., Schuierer, M., Werner, O., Moser, M., Roth, C., Bauer, R. and Buettner, R. (1999) Transcriptional regulation of the promoter by BTEB-1 and AP-2rep, a novel wt-1/egr-related zinc finger repressor. *Mol. Cell. Biol.* **19**, 194–204
- Apt, D., Watts, R. M., Suske, G. and Bernard, H. U. (1996) High Sp1/Sp3 ratios in epithelial cells during epithelial differentiation and cellular transformation correlate with the activation of the HPV-16 promoter. *Virology* **224**, 281–291
- Feng, X., Teitelbaum, S. L., Quiroz, M. E., Cheng, S. L., Lai, C. F., Avioli, L. V. and Ross, F. P. (2000) Sp1/Sp3 and PU.1 differentially regulate β_5 integrin gene expression in macrophages and osteoblasts. *J. Biol. Chem.* **275**, 8331–8340
- Kwon, H. S., Kim, M. S., Edenberg, H. J. and Hur, M. W. (1999) Sp3 and Sp4 can repress transcription by competing with Sp1 for the core cis-elements on the human ADH5/FDH minimal promoter. *J. Biol. Chem.* **274**, 20–28
- Fandos, C., Sanchez-Feutrie, M., Santalucia, T., Vinals, F., Cadefau, J., Guma, A., Cusso, R., Kaliman, P., Canicio, J., Palacin, M. and Zorzano, A. (1999) GLUT1 glucose transporter gene transcription is repressed by Sp3. Evidence for a regulatory role of Sp3 during myogenesis. *J. Mol. Biol.* **294**, 103–119
- Discher, D. J., Bishopric, N. H., Wu, X., Peterson, C. A. and Webster, K. A. (1998) Hypoxia regulates β -enolase and pyruvate kinase-M promoters by modulating Sp1/Sp3 binding to a conserved GC element. *J. Biol. Chem.* **273**, 26087–26093
- Emami, K. H., Burke, T. W. and Smale, S. T. (1998) Sp1 activation of a TATA-less promoter requires a species-specific interaction involving transcription factor IID. *Nucleic Acids Res.* **26**, 839–846
- Braun, H., Koop, R., Ertmer, A., Nacht, S. and Suske, G. (2001) Transcription factor Sp3 is regulated by acetylation. *Nucleic Acids Res.* **29**, 4994–5000