

The 5' flanking region of the pS2 gene contains a complex enhancer region responsive to oestrogens, epidermal growth factor, a tumour promoter (TPA), the c-Ha-ras oncoprotein and the c-jun protein

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Expression of the pS2 gene which is transcriptionally controlled by oestrogens in the breast cancer cell line MCF-7 is oestrogen independent in stomach mucosa. We show here that the level of MCF-7 cell pS2 mRNA can also be increased by the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). We further demonstrate, using transient transfection assays, that the –428 to –332 5' flanking sequence of the pS2 gene contains DNA enhancer elements responsive to oestrogens, TPA, EGF, the c-Ha-ras oncoprotein and the c-jun protein.
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Introduction

A significant fraction of human breast cancers are oestrogen dependent. These tumours are characterized by the presence of variable levels of oestrogen receptor (Jensen *et al.*, 1982). The natural history of these human breast cancers often involves two major steps. During the first, oestrogen-responsive period, the tumours undergo regression when deprived of oestrogen or when treated with anti-oestrogens (Jensen *et al.*, 1982). Then, during the second period, there is a spontaneous progression from oestrogen responsive to oestrogen independent, more malignant cancer. The mechanisms involved in growth control during this evolution are not understood. The MCF-7 cell line, which is derived from a pleural effusion of a human breast cancer (Soule *et al.*, 1973) and expresses oestrogen receptor (Brooks *et al.*, 1973), represents a good *in vitro* model system for hormone-dependent breast cancer. In culture, these cells show accelerated growth following treatment with physiological concentrations of oestradiol (Lippman *et al.*, 1976; Darbre *et al.*, 1983). *In vivo*, when implanted into ovariectomized athymic mice, they require the presence of oestrogen to exhibit tumorigenicity and invasiveness (Shafie, 1980; Siebert *et al.*, 1983). In order to understand the molecular basis of oestrogen action, several groups have characterized, identified and isolated a variety of proteins, mRNAs and secreted growth factors which are under oestrogen control in MCF-7 cells (Adams *et al.*, 1983; Rochefort, 1983; Dickson *et al.*, 1986; Huff *et al.*, 1986, 1988; Bronzert *et al.*, 1987; Knabbe *et al.*, 1987; Sheen and Katzenellenbogen, 1987; Bates *et al.*, 1988). Our laboratory has reported the isolation of a cDNA clone, pS2, whose corresponding

mRNA is increased specifically by oestradiol treatment of MCF-7 cells (Masiakowski *et al.*, 1982; Jakowlev *et al.*, 1984). We have also shown that the pS2 protein is synthesized by MCF-7 cells in the presence of oestradiol, and is secreted into the culture medium as a 6.5 kd polypeptide (Nunez *et al.*, 1987). Furthermore, it has been shown that the expression of the pS2 gene in human breast cancers is highly correlated with the presence of the oestrogen receptor (i.e. 98% of tumours producing pS2 mRNA expressed the oestrogen receptor, see Rio *et al.*, 1987). The function of the pS2 protein is unknown, although an interesting homology exists between the pS2 protein and a porcine pancreatic protein which has been shown to inhibit gastrointestinal motility and gastric acid secretion (Jorgensen *et al.*, 1982; Thim *et al.*, 1985; Thim, 1988), as well as with a protein present in *Xenopus* skin (Hoffman, 1988). Recently, our laboratory has also shown that the pS2 protein is also normally expressed and secreted in individuals of both sexes by gastric mucosa cells which do not contain the oestrogen receptor (Rio *et al.*, 1988).

The pS2 gene has been cloned (Jeltsch *et al.*, 1987) and it has been demonstrated that its induction by oestradiol in MCF-7 cells is a primary transcriptional event (Brown *et al.*, 1984). Previous studies, in which chimeric recombinants transfected into MCF-7 cells (Roberts *et al.*, 1988) or co-transfected into HeLa cells with a vector expressing the human oestrogen receptor (hER) (Kumar *et al.*, 1987) have indicated that the 5' flanking region of the pS2 gene contains an element(s) responsive to oestradiol. The presence of pS2 mRNA in gastric mucosa cells which do not contain the oestrogen receptor (Rio *et al.*, 1988), prompted us to investigate whether the pS2 gene could be regulated by other hormones, gastrointestinal peptides or growth factors. We report here that the 5' flanking region of the pS2 gene contains also *cis*-acting elements which can respond to epidermal growth factor (EGF) (first identified as urogastrone because it inhibits gastric acid secretion, see Bower *et al.*, 1975; Gregory, 1975; Carpenter and Cohen, 1979) and to other molecules involved in the signal transduction pathway of growth factors, i.e. a phorbol ester tumour promoter [TPA or 12-O-tetradecanoyl phorbol-13-acetate which activates protein kinase(s) C (Blumberg, 1988; Nishizuka, 1988)], one oncoprotein, c-Ha-ras [an oncoprotein corresponding to a GTP-binding protein (Barbacid, 1987)] and the c-jun protein [a putative proto-oncoprotein related to the *trans*-acting factor AP1 (Bohman *et al.*, 1987; Angel *et al.*, 1988; Bos *et al.*, 1988; Imler *et al.*, 1988a; Quantin and Breathnach, 1988)].

Results

pS2 mRNA is increased by TPA treatment of MCF-7 cells

RNA was prepared from MCF-7 cells grown in the presence or in the absence of oestradiol (E2) and treated with the

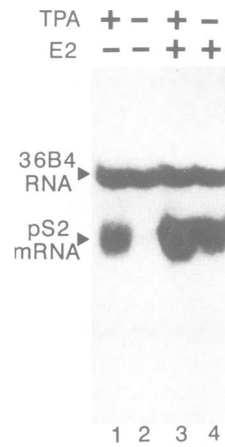


Fig. 1. RNA blot analysis of pS2 mRNA made by MCF-7 cells in response to TPA and oestradiol treatment. MCF-7 cells were grown for 1 week in phenol red-free medium, in the presence of 10% stripped FCS. On day 8, 70% confluent cells were refed with a medium containing 0.5% stripped FCS supplemented (lanes 3 and 4) or not (lanes 1 and 2) with 10^{-8} M oestradiol. They were treated with 100 ng/ml TPA (lanes 1 and 3) or mock-treated by addition of 10 μ l DMSO (lanes 2 and 4) 21 h later. Cells were collected 3 h later, RNA was prepared, electrophoresed, blotted onto DBM paper and hybridized with 36B4 and pS2 cDNA probes as described in Material and methods.

tumour promoter TPA or mock-treated with DMSO. DBM-blots were hybridized with a cDNA probe for the ubiquitously expressed 36B4 RNA and with a pS2 cDNA probe. 36B4 RNA level was not significantly modified by TPA treatment and could therefore be used as an internal control for pS2 RNA. pS2 mRNA was barely detectable in cells grown in low serum and oestrogen-stripped media (Figure 1, lane 2). As reported previously (Masiakowski *et al.*, 1982), pS2 mRNA was dramatically increased after 24 h of oestradiol treatment (Figure 1, lane 4). A 3 h treatment of hormone-withdrawn MCF-7 cells with TPA resulted in a faster and at least 10-fold, induction of pS2 mRNA corresponding to ~50–90% of the level observed after 24 h of oestradiol stimulation (Figure 1, compare lanes 1 and 4; see also Masiakowski *et al.*, 1982). Moreover pS2 mRNA inductions by TPA and oestradiol appeared to be synergistic (Figure 1, lane 3). Similar inductions by TPA were obtained with MCF-7 cells previously treated with the anti-oestrogens tamoxifen or hydroxytamoxifen, in the presence of either low (0.5%) or high (10%) serum concentration (results not shown).

Cis-acting elements responsive to oestrogens, TPA and c-Ha-ras expression are present in the 5' flanking region of the pS2 gene

The above effect of TPA and the previous demonstration that pS2 gene expression is regulated by oestradiol at the transcriptional level (Brown *et al.*, 1984) prompted us to look for the presence of oestrogen and TPA-responsive element(s) in the 5' flanking sequence of the pS2 gene. The effect of the *c-Ha-ras* oncoprotein was also studied, since it has been suggested that this protein is involved, as is protein kinase C, in the signal transduction pathway of growth factor receptors to the nucleus (for a review, see Barbacid, 1987). A reporter chimeric gene aimed at testing the possible presence of responsive elements in the 5'

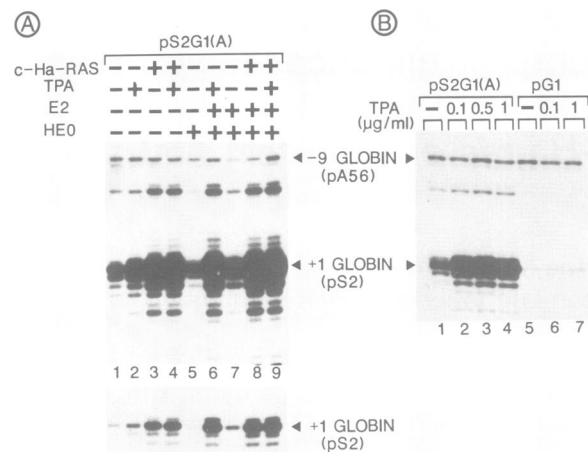


Fig. 2. (A) Oestrogen, TPA and *c-Ha-ras* responsive elements are present in the 5' flanking region of the pS2 gene. HeLa cells were co-transfected with 5 μ g of pS2G1(A), 15 μ g of pA56 (as internal control), 1 μ g of human oestrogen receptor expression vector HEO (lanes 5–9) or 1 μ g of pKCR2 (control vector for HEO) (lanes 1–4), 5 μ g of pRCBx2 (*c-Ha-ras* oncogene expression vector) (lanes 3, 4, 8, 9) or p Δ RCBx2 (control vector for pRCBx2) (lanes 1, 2, 5, 6, 7). Cells were refed with fresh medium containing 0.5% stripped FCS supplemented with oestradiol (10^{-8} M) (lanes 6–9) or 10 μ l ethanol (lanes 1–5) 24 h after transfection. TPA (100 ng/ml) (lanes 2, 4, 6 and 9) or DMSO (10 μ l) (lanes 1, 3, 5, 7 and 8) was added 21 h later. After an additional period of 3 h the cells were harvested and cytoplasmic RNA was prepared. S1 nuclease analysis was performed in the presence of an excess of 32 P-end-labelled single-stranded pS2 probe. Arrowheads indicate the position of S1 nuclease protected fragments corresponding to pS2- β -globin and pA56 specific transcripts (see Materials and methods). DMSO and ethanol treatment had no effect in this system (data not shown). The lower panel shows a shorter exposure of the +1 globin region of the same autoradiogram. (B) Effect of increasing concentrations of TPA on pS2G1(A) and pG1 transcription. HeLa cells were transfected with pS2G1(A) (5 μ g) (lanes 1–4) or pG1 (2.7 μ g) (lanes 5–7), 1 μ g of pKCR2 and 5 μ g of p Δ RCBx2. After transfection they were treated as in (A), in the absence of oestradiol, but in the presence of increasing concentrations of TPA (0.1 μ g/ml, lanes 2 and 6; 0.5 μ g/ml, lane 3; 1 μ g/ml, lanes 4 and 7) or mock-treated in the presence of 10 μ l DMSO (lanes 1 and 5).

flanking region of the pS2 gene was constructed. In pS2G1(A) (Figure 3A), the rabbit β -globin gene with its promoter sequence up to -109 (present in the parental pG1 recombinant) was placed downstream from the ~ -3500 to -86 5' flanking fragment of the pS2 gene. Any increase of RNA initiated from the globin cap site (Globin +1) of pS2G1(A) (when compared with pG1) reflects the presence of responsive element(s) in the 5' flanking region of the pS2 gene.

pS2G1(A), or pG1, was transfected into HeLa cells together with either HEO (the oestrogen receptor expression vector; parental control expression vector pKCR2) and/or pRCBx2 (*c-Ha-ras* oncoprotein expression vector; control expression vector p Δ RCBx2). pA56, a deletion mutant of pAO lacking the essential SV40 enhancer sequences that respond to TPA and *c-Ha-ras* (see Materials and methods) was also co-transfected to correct for possible variations in transfection efficiencies. Cells were subsequently treated with oestradiol or TPA, or mock-treated with ethanol or DMSO, respectively. RNA initiated from the β -globin promoter was analysed by quantitative S1 nuclease mapping (Figures 2 and 3C). The results of densitometric scanning of autoradiograms

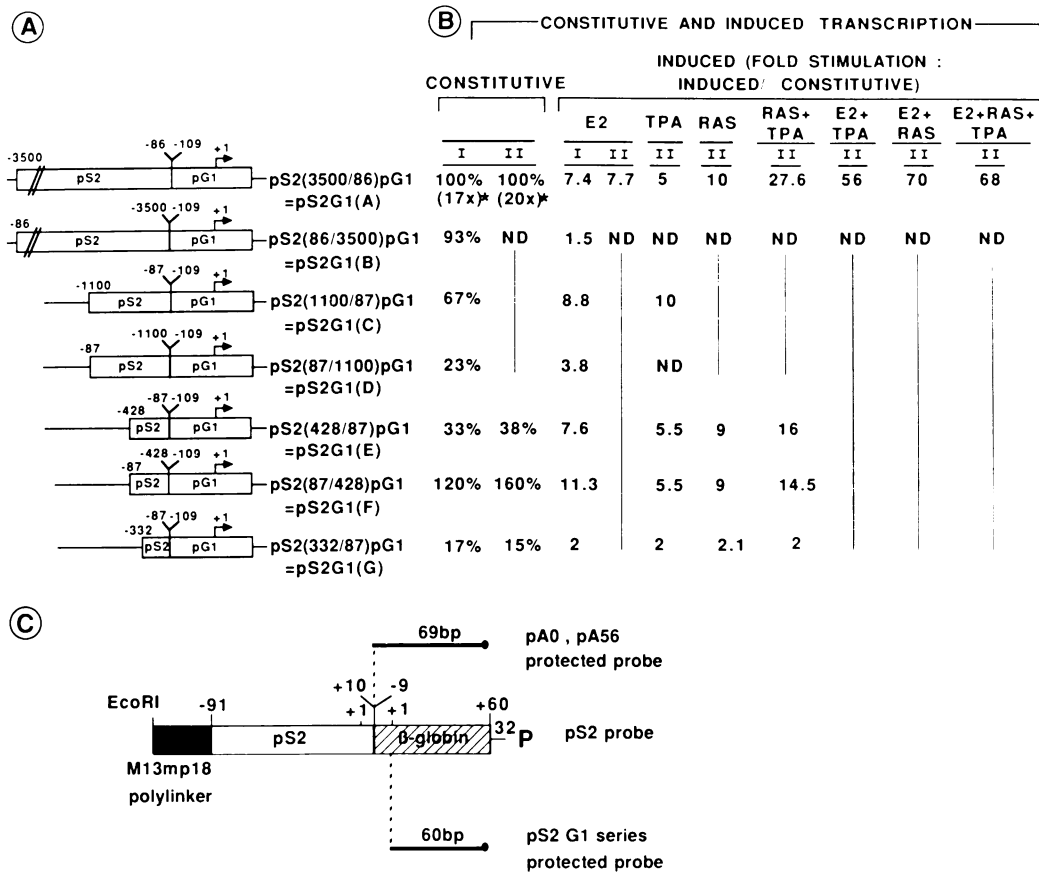


Fig. 3. Localization of oestrogen, TPA and *c-Ha-ras* responsive elements in the 5' flanking region of the pS2 gene. (A) Structure of the pS2G1 recombinants series (see Materials and methods). (B) Compilation of data representing the constitutive and the oestradiol (E2) and/or TPA and/or *c-Ha-ras* induced levels of expression of the different pS2G1 recombinants (average of at least three independent transfection experiments made with at least two different plasmid preparations). Constitutive levels are expressed as the percentage of the values obtained for pS2G1(A) taken as 100%. Induced transcription is expressed as fold-stimulation, i.e. ratios of induced/constitutive levels. (*) These numbers in parentheses correspond to the fold-stimulation with respect to pG1. ND: Not determined. I and II correspond to two different cell growth conditions. *Condition I* was used to study E2-induced-transcription. pAO was the co-transfected internal control plasmid and the cells were grown in 10% stripped FCS. *Condition II* was used to study TPA, *c-Ha-ras* and E2-induced transcription. pA56, a mutant of pAO lacking the SV40 enhancer, was used as internal control and 24 h after transfection the cells were refed with a medium containing 0.5% stripped FCS. (C) S1 nuclease probe and protected fragments (see Materials and methods).

corresponding to several independent transfection experiments similar to those illustrated in Figure 2A are presented in Figure 3B (after correction for variations in the signal of the co-transfected reference plasmid pA56).

The basal level (i.e. the level obtained in the absence of hormone, TPA, HEO or *c-Ha-ras* expression) of pS2G1(A) transcription was 17- to 20-fold higher than that of pG1 under the same conditions, irrespective of serum concentrations (Figures 2B and 3B and results not shown). This result suggests that the ~ -3500 to -86 sequence of the pS2 gene contains a 'constitutive' element(s) which may be responsible for the low basal level of pS2 gene expression observed in hormone-withdrawn MCF-7 cells. TPA and *c-Ha-ras* treatments resulted in a 5- and 10-fold increase in transcription, respectively, whereas the average stimulation by HEO/oestradiol was 8-fold. A 28-fold increase was obtained by co-stimulation with TPA and *ras* expression, whereas it was 56-fold using TPA and HEO/oestradiol, and 70-fold by co-treatment with *ras* and HEO/oestradiol. Concomitant treatment with TPA, *ras* and HEO/oestradiol resulted in a 68-fold stimulation of transcription. As previously described (Wasylyk *et al.*, 1987) pG1 transcription itself was poorly enhanced by TPA treatment (Figure 2B) or by *c-Ha-ras*

expression (data not shown). These results suggest that TPA and the *c-Ha-ras* oncoprotein act independently from the oestrogen receptor, and probably through different responsive elements since the combined effects (TPA + E2, *ras* + E2, *ras* + TPA + E2) were synergistic. On the other hand, the stimulations brought about by TPA and *c-Ha-ras* were more additive than synergistic, suggesting that they may act through common pathway(s) and/or that their effect could be mediated by the same responsive element(s). Similar additive stimulations were also obtained with the -428 to -87 pS2G1(E) and the -87 to -428 pS2G1(F) recombinants (see Figure 5, lanes 1–8, and Figure 3B). Transcription of both constructs was stimulated ~ 5 -fold by TPA, 9-fold by *c-Ha-ras* and ~ 15 -fold by TPA + *c-Ha-ras*. Note in this respect that increasing the TPA concentration above 100 ng/ml had no further effect on the stimulation of transcription (Figure 2B), making it unlikely that insufficient TPA was used to observe a stimulation as high as that achieved with the *c-Ha-ras* oncoprotein. Note also that the expression vectors for the human oestrogen receptor (HEO) and the *ras* oncoprotein (pRCBx2) were used at optimal concentrations in these experiments (data not shown). Note finally that *c-Ha-ras* and TPA treatments have been shown

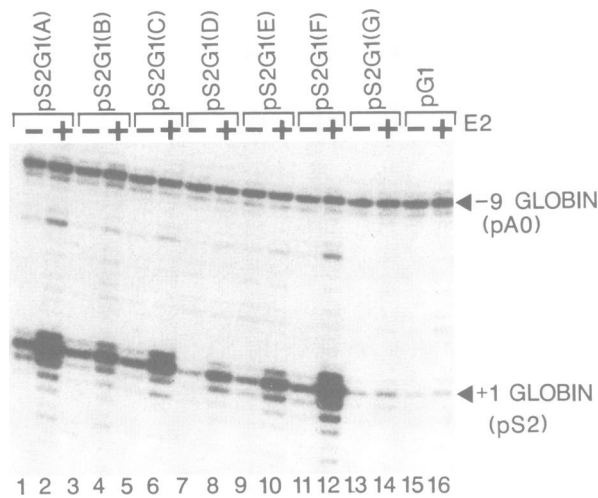


Fig. 4. The 5' flanking region of the pS2 gene contains an oestrogen responsive enhancer element. HeLa cells were transfected with either 3 pmol of a pS2G1 series plasmid or pG1 as indicated together with 1 μ g of HEO and 400 ng of pAO plasmids (see Materials and methods). The next day the cells were washed and refed with medium containing 10^{-8} M oestradiol (E2, lanes 2, 4, 6, 8, 10, 12, 14 and 16) or mock-treated with 10 μ l ethanol (lanes 1, 3, 5, 7, 9, 11, 13 and 15) for 24 h. Cytoplasmic RNA (15 μ g) from transfected cells was analysed by quantitative S1 nuclease mapping with an excess of 32 P-labelled DNA probe (Figure 3C). The arrow heads indicate the position of protected probe fragment corresponding to RNA initiated from the globin capsite [+1 globin (pS2)] or to pAO-specific transcripts.

to have little effect in HeLa cells on the SV40 promoter enhancer which is present in the HEO and pRCBx2 expression vectors (Imler *et al.*, 1988b; M.Kanno, C.Fromental and P.Chambon, unpublished results).

In summary, the 5'-flanking region of the pS2 gene can confer oestrogen, TPA and c-Ha-ras oncoprotein inducibility upon a heterologous β -globin promoter transfected into HeLa cells. Furthermore, TPA and c-HA-ras oncoprotein stimulate transcription independently from oestrogens. In addition, the same pS2 gene 5' flanking region appears to contain sequences responsible for a 'constitutive' stimulation of transcription.

The -428 to -332 sequence of the pS2 gene contains enhancer elements critical for the response to oestrogens, TPA, EGF, c-Ha-ras, c-jun and a fraction of the 'constitutive' activation

A series of recombinants containing various pS2 5' flanking segments in either their natural or inverted orientation, but all similarly inserted upstream from the β -globin promoter in pG1 (Figure 3A), were constructed. The effect of oestradiol (E2), TPA, c-Ha-ras, and also c-jun and epidermal growth factor (EGF) on transcription of these recombinants was tested (Figures 3B, 4 and 5; and data not shown). Transcription of pS2G1 (E) which contains the -428 to -87 5' flanking region of the pS2 gene was induced by oestradiol, TPA, c-Ha-ras, c-jun and EGF, as efficiently as that of pS2G1(A) and pS2G1(C), whereas transcription of the -332 to -87 pS2G1(G) recombinant was barely stimulated. The maximal level of stimulation observed was ~8-fold for oestradiol, ~5-fold for TPA and ~10-fold for c-Ha-ras, c-jun and EGF. Thus, the -428 to -332 5' flanking region of the pS2 gene appear to contain elements

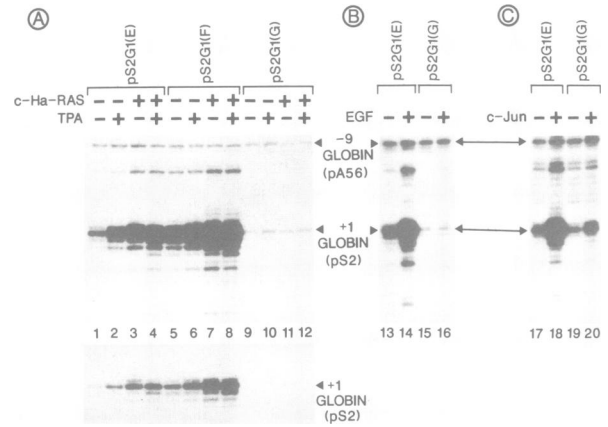


Fig. 5. The (-428 to -332) region of the pS2 gene contains TPA, c-Ha-ras, c-jun and EGF responsive enhancer element(s). In (A) HeLa cells were transfected with 1 pmol of a pS2G1 series recombinant as indicated, together with 15 μ g of the internal control pA56, and either 5 μ g of pRCBx2 [c-Ha-ras(+), lanes 3 and 4, 7 and 8, 11 and 12] or 5 μ g of p Δ RCBx2 [c-Ha-ras(-), lanes 1 and 2, 5 and 6, 9 and 10]. In (B), transfection was as in panel (A), but without pRCBx2 or p Δ RCBx2. In panel (C), HeLa cells were similarly co-transfected with a pS2G1 series recombinant, pA56 and either 5 μ g of pSG-c-jun (lanes 18 and 20) or of pSG1 [control vector for c-jun (lanes 17 and 19)]. At 24 h following transfection, the cells were refed with phenol red-free medium containing 0.5% stripped FCS. The cells were treated with TPA (100 ng/ml) (lanes 2, 4, 6, 8, 10 and 12) or mock-treated with 10 μ l DMSO (lanes 1, 3, 5, 7, 9 and 11) or treated or not with EGF (lanes 13-16) 24 h later. Cells were collected after an additional period of 3 h and cytoplasmic RNA was prepared and analysed by quantitative S1 nuclease mapping as described in legend to Figure 4. The arrowhead at -9 globin (pA56) corresponds to RNA initiated from the SV40 early start site of the internal control pA56. The lower panel (A) shows a shorter exposure of the +1 globin (pS2) region of the same autoradiogram.

necessary for the stimulation of transcription by the oestrogen receptor, TPA, c-Ha-ras, c-jun and EGF.

The data displayed in Figures 3-5 also demonstrate that these pS2 gene-responsive elements have the characteristics of inducible enhancers. First, stimulation by oestradiol, TPA, c-Ha-ras, c-jun and EGF can indeed be conferred by the -428 to -87 pS2 segment to a heterologous promoter, i.e. the β -globin gene promoter. Secondly, the same segment of the pS2 gene can stimulate transcription efficiently in either orientation [compare pS2G1(E) and pS2G1(F) in Figures 3B, 4 and 5, and results not shown]. The lower oestrogen stimulation obtained with pS2G1(B) and pS2G1(D), and the higher stimulation observed with pS2G1(F), when compared with pS2G1(E), are in agreement with previous results indicating that the efficiency of stimulation of transcription by enhancers decreases with increasing distances (Wasylyk *et al.*, 1983, 1984).

The results presented in Figures 3-5 indicate also that about one third of the 'constitutive' stimulatory activity exhibited by pS2G1(A) is located between coordinates ~ -3500 and ~ -1100, whereas another third is located between ~ -1100 and -428, and the rest is distributed between the -428 to -332 and -332 to -86 segments. The high 'constitutive' activity of pS2G1(F), when compared to pS2G1(E), suggests that the -428 to -332 segment contains a constitutive stimulatory element(s) that could be either more active in the reverse orientation or more efficient when brought nearer to the globin promoter.

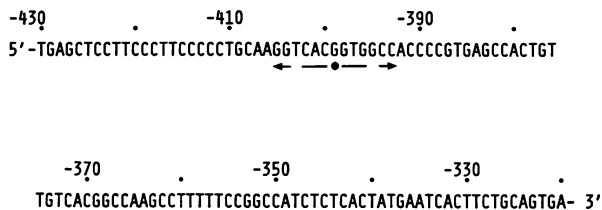


Fig. 6. Nucleotide sequence of the -430 to -320 region of the pS2 gene. The imperfect palindromic ERE is underlined. The sequence published previously by Jeltsch *et al.* (1987) had a number of mistakes in the -400 to -360 region which have been corrected here (see Berry *et al.*, 1989).

Discussion

Transcription of pS2 gene in breast cancer cells MCF-7 is inducible by oestrogens (Brown *et al.*, 1984), and there is an oestrogen-responsive element (ERE) in its 5' flanking promoter region (Kumar *et al.*, 1987; Roberts *et al.*, 1988). We report here that the level of pS2 mRNA can also be increased in MCF-7 cells by treatment with a tumour promoter, the phorbol ester TPA, and we have identified a complex enhancer region which contains elements responsive to oestradiol (ERE), TPA (TRE), the *c-Ha-ras* oncoprotein (RRE), the *c-jun* protein (jun-RE) and EGF (EGF-RE). We note in this respect that treatment of MCF-7 cells with EGF results in an increase of pS2 mRNA (Cavaillès *et al.*, 1988, and our unpublished results).

The pS2 ERE which has been further characterized in a separate study (Berry *et al.*, 1989), is an imperfect 13 bp palindromic sequence located between pS2 gene coordinates -405 and -393 and differing from the canonical perfect palindromic ERE (5'-GGTCANNNTGACC-3') by 1 bp in its 3' stem (see Figure 6). What is then the nature of the DNA elements responsive to TPA, EGF, *c-Ha-ras* and *c-jun*? Enhancer elements interacting with the factors AP1 (Angel *et al.*, 1987; Lee *et al.*, 1987) /PEA1 (Imler *et al.*, 1988b; Martin *et al.*, 1988), AP2 (Mitchell *et al.*, 1987; Imagawa *et al.*, 1987), AP3 (Chiu *et al.*, 1987), serum-responsive factor (SRF, Treisman, 1986; Gilman, 1988; Phan-Dinh-Tuy *et al.*, 1988), prolactin site 1-P factor(s) (Elsholtz *et al.*, 1986; Nelson *et al.*, 1988) and NF- κ B (Sen and Baltimore, 1986; Nabel and Baltimore, 1987) have been reported to respond to TPA. Examination of the pS2 gene -428 to -332 sequence (Figure 6) does not reveal the presence of any of the corresponding cognate consensus sequences. However, the *c-jun* protein may correspond to the enhancer factor AP1 or to a very closely related factor and the sequence recognized by AP1 can mediate *c-jun* transactivation of transcription (Bohman *et al.*, 1987; Angel *et al.*, 1988; Bos *et al.*, 1988; Imler *et al.*, 1988a). Moreover, the same *cis*-acting DNA element can mediate the effect of TPA, *c-Ha-ras* and *c-jun* (Wasylyk *et al.*, 1987, 1988; Imler *et al.*, 1988a,b; Schönthal *et al.*, 1988). Therefore one or several degenerated AP1-like binding sites may be present in the pS2 enhancer region, and mediate the effects of *c-jun*, TPA and *c-Ha-ras*. That the effects of TPA and *c-Ha-ras* were more additive than synergistic is in agreement with this possibility. This common element(s) may also mediate the effect of EGF. In this respect we note that the prolactin gene also contains an element which responds to both EGF and TPA (Elsholtz *et al.*, 1986; Nelson *et al.*, 1988) and that EGF and TPA can induce *c-jun* expression

(Quantin and Breathnach, 1988; Lamph *et al.*, 1988). Similarly the Moloney murine leukemia virus (Elsholtz *et al.*, 1986) and the *c-fos* oncogene (Sheng *et al.*, 1988) appear to contain elements which respond to both TPA and EGF. Clearly, a detailed mutagenesis of the enhancer region of the pS2 gene is required to investigate whether one or several elements mediate the effect of TPA, *c-Ha-ras*, *c-jun* and EGF.

Treatment of MCF-7 cells with oestrogens increase the secretion of several growth factors such as TGF- α (Bates *et al.*, 1988), TGF- β (Knabbe *et al.*, 1987), IGF-I (Huff *et al.*, 1988) and PDGF (Bronzert *et al.*, 1987). In view of the increase of pS2 mRNA level in MCF-7 cells treated with EGF (the present study and Cavaillès *et al.*, 1988), insulin (Cavaillès *et al.*, 1988) and of the known activation of the EGF receptor by TGF- α (Derynck *et al.*, 1988), the question arises as to whether induction of pS2 transcription by oestrogen may be indirectly mediated by an autocrine loop. This possibility can be excluded. First, oestrogen-induced activation of transcription of the pS2 gene is not inhibited by cycloheximide (Brown *et al.*, 1984) in contrast to the activation brought about by EGF (unpublished results from our laboratory). Secondly, deletion of the pS2 ERE abolishes oestrogen response, but not TPA, *c-Ha-ras*, *c-jun* or EGF responses (our unpublished results). Thirdly, a synthetic pS2 ERE (inserted in a β -globin promoter-based reporter gene) can mediate oestrogen response on its own (Berry *et al.*, 1989). However, the mechanism of induction of transcription of the pS2 gene by oestradiol appears to be more complex than initially thought, since, in addition to the primary induction by the hormone, the oestrogen-induced secretion of growth factors may result in a further increase in the rate of transcription. Note that oestrogens may also induce the transcription of the *c-fos* proto-oncogene (Wilding *et al.*, 1988) which is known to cooperate with the AP1/*c-jun* protein in enhancing transcription (Chiu *et al.*, 1988; Sassone-Corsi *et al.*, 1988; Schönthal *et al.*, 1988; Wasylyk *et al.*, 1988). Thus, in addition to its primary response to oestrogens, the pS2 enhancer region responds to several protein factors which are known to mediate the mitogenic activity of oestrogens.

That expression of the activated *c-Ha-ras* in MCF-7 cells can activate pS2 gene transcription raises the question as to whether a similar activation may occur in breast cancers. The *in vitro* growth of MCF-7 cells transformed with an activated *ras* exhibit oestrogen-independence, whereas *ras* transformation may not be sufficient to overcome the *in vivo* hormonal dependence of MCF-7 cells for inducing tumours in nude mice (Kasid *et al.*, 1985; Sukumar *et al.*, 1988). Amplified, but not activated *N-ras* gene, has been found in MCF-7 cells (Fasano *et al.*, 1984). It may be responsible for part of the 'constitutive' activity of the pS2 enhancer region. *c-Ha-ras* activation has been found in some breast cancer cell lines, e.g. in the oestrogen receptor (ER)-negative and EGF receptor-positive Hs578T cell line (Davidson *et al.*, 1987; Kraus *et al.*, 1988). However, in breast carcinomas, the presence of pS2 mRNA and/or protein is highly correlated with the presence of ER (Rio *et al.*, 1987). Thus the pS2 gene enhancer/promoter region may have to be 'poised' by an oestrogen-dependent mechanism to be able to respond to oncogenes and/or growth factors. The very rare cases of ER-negative breast carcinomas in which the pS2 gene is expressed (Rio *et al.*,

1987) may reflect the rare occurrence of an oestrogen-independent pS2 gene 'poising'.

Finally, the present findings could provide an explanation for the oestrogen-independent expression of the pS2 gene in stomach mucosa cells of individuals of both sexes (Rio *et al.*, 1988), since EGF is known to have an effect on the stomach where it inhibits acid secretion (Carpenter and Cohen, 1979). Thus, in these cells, the EGF-responsive element of the complex enhancer region may control expression of the pS2 gene, which may be one of the EGF target genes in the stomach.

Materials and methods

Cell cultures

HeLa cells were grown as previously described (Kumar *et al.*, 1987) in the presence of dextran-coated charcoal (DCC)-treated fetal calf serum (stripped FCS) (Horwitz and McGuire, 1978) and absence of phenol red (Berthois *et al.*, 1986). Oestrogen-withdrawn MCF-7 cells were obtained by culture for 1 week in phenol red free medium containing 10% stripped FCS (Nunez *et al.*, 1987).

Construction of reporter recombinants

Standard DNA recombinant techniques were used (Maniatis *et al.*, 1982). The pS2pG1 series (also termed pS2G1 for sake of simplicity) (see Figure 3A) was constructed by inserting different fragments of the 5' flanking region of the pS2 gene (Jeltsch *et al.*, 1987) into the *Hind*III site of the pG1 vector (Wasylyk and Wasylyk, 1986). pG1 contains the rabbit β -globin sequences from -109 to +1650 and the M13mp12 polylinker with the *Hind*III site closest to the β -globin promoter. For pS2G1(A) and pS2G1(B), the *Bam*HI-*Bam*HI (~ -3500 to -86) fragment of the pS2 gene was cloned with *Bam*HI-*Hind*III adaptors into the *Hind*III site of pG1. pS2G1(C) and pS2G1(D) were constructed by inserting the DNA polymerase I (Klenow) repaired *Pvu*II (~ -1100) - *Bam*HI(-87) fragment of the pS2 gene into the Klenow repaired *Hind*III site of pG1. pS2G1(E) and pS2G1(F) were similarly obtained by insertion of the repaired pS2 gene *Sac*I(-428) - *Bam*HI(-87) fragment, whereas pS2G1(G) was constructed by blunt-end ligation of the pS2 fragment *Xmn*I(-332) - *Bam*HI(-87) into the repaired *Hind*III site of pG1. pAO (Zenke *et al.*, 1986) was used in transfections as a co-transfected reference plasmid to correct for variations in transfection efficiencies, whereas pA56 (Zenke *et al.*, 1986), a deletion mutant of pAO lacking the SV40 enhancer sequences that respond to TPA and c-Ha-ras (M.Kanno, C. Fromental and P. Chambon, unpublished results), was used as a reference plasmid in experiments involving TPA, c-Ha-ras or c-jun. Note that the pS2 coordinates are as in Jeltsch *et al.* (1987) except for the -430 to -320 region which has been corrected as indicated in Figure 6.

Activation vectors

Vectors expressing the human oestrogen receptor (HEO) (Green *et al.*, 1986), the c-Ha-ras oncogene (pRCBx2) (Wasylyk *et al.*, 1987), the c-jun oncogene pSG-c-jun (Quantin and Breathnach, 1988; R. Breathnach, personal communication) and the control expression vectors [pKCR2 for HEO (Breathnach and Harris, 1983), p Δ RCBx2 for pRCBx2 (Imler *et al.*, 1988b), pSG1 for pSG-c-jun (Green *et al.*, 1988)] have been previously described. pRCBx2 expresses the human T24 bladder carcinoma p21 c-Ha-ras protein which carries a point mutation in codon 12 and represents the activated oncogenic counterpart of the normal c-Ha-ras proto-oncoprotein (for a review, see Barbacid, 1987). The control expression vector p Δ RCBx2 contains a deletion preventing expression of ras. The c-jun oncogene was obtained from a human tumour cDNA library screened with a v-jun specific probe (Quantin and Breathnach, 1988). The sequence (10-1444) (Angel *et al.*, 1988) was cloned into the *Eco*RI site of pSG1 (R. Breathnach, personal communication).

Transfections, RNA isolation and quantification by S1 nuclease analysis

All transfections were carried out with the calcium phosphate co-precipitation technique (Gorman, 1985). RNA isolation and analysis by quantitative S1 nuclease mapping using single-stranded 5' end-labelled probes were performed as described previously (Kumar *et al.*, 1987). The pS2 probe (Figure 3C) was constructed by inserting the *Bam*HI-*Bam*HI fragment of the pS2 recombinant described in Kumar *et al.* (1987) (pS2 fragment from -91 to +10 linked to the β -globin gene fragment from -9 to +396) into the *Bam*HI site of M13mp18. The preparation of the ³²P-5'-end-labelled

antisense single-stranded probe has been described (Kumar *et al.*, 1987). The probe extends from M13mp18 polylinker (*Eco*RI site) through the pS2 region (-91 to +10) and the β -globin gene sequence (-9 to +60). RNA initiated at the β -globin gene start site (+1) protects a 60 base fragment (pS2G1 series), whereas RNA initiated at the SV40 early start sites of pAO and pA56 (used as internal controls) protects all of the globin part of the probe (69 bp fragment).

Northern hybridization

MCF-7 cell RNA transfer to diazobenzyloxymethyl paper (DBM paper) and hybridization with ³²P-labelled nick-translated cDNA probes (~ 10⁸ c.p.m./ μ g of DNA) was performed as described (Masiakowski *et al.*, 1982; Jakowlev *et al.*, 1984), using the ubiquitously expressed 36B4 mRNA (Masiakowski *et al.*, 1982) as an internal control.

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