\textbf{pS2 Gene Expression in HepG2 cells: Complex Regulation through Crosstalk between the Estrogen Receptor $\alpha$, an Estrogen-Responsive Element, and the Activator Protein 1 Response Element}

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\textbf{ABSTRACT}

The pS2 promoter is complex with binding sites for a number of protein factors that may participate in modulating its activity. The pS2 gene was transcriptionally activated by estrogens in HepG2 cells transformed (HepER3) to express the estrogen receptor $\alpha$ (ER$\alpha$). The phorbol ester phorbol 12-myristate 13-acetate (PMA) stimulated pS2 expression in both HepER3 and the parental, non-ER-expressing HepG2 cells, although its activity was substantially less in HepG2 cells. The use of selective protein kinase inhibitors suggested that the MAPK pathway contributes substantially to estrogen stimulation of the pS2 promoter. The activator protein 1 (AP1) site at $\text{--332 to --338 in the pS2 promoter had a dominant role in the response to both estrogens and PMA, although the estrogen response element at $\text{--393 to --405 was essential to mediate the response to estrogen. The potentiation of pS2 promoter activity by the AP1 motif in response to estrogen was dependent on the ligand binding domain of ER$\alpha$. Furthermore, the presence of an intact AP1 element in the pS2 promoter sustained suppression of pS2 promoter activity by an LXXLL peptide. In summary, the data suggest that the effect of estrogen is mediated through a cross-talk between the estrogen-responsive element and the AP1 response element and that ER$\alpha$ plays a crucial role in mediating the effect of both estrogen and PMA.}

Estrogens exert their gene regulatory function through the estrogen receptor (ER). Presently, two subtypes of the ER have been identified, the $\alpha$ subtype (ER$\alpha$) (Green et al., 1986) and the recently discovered $\beta$ subtype (ER$\beta$) (Kuiper et al., 1996). The ERs belong to a superfamily of nuclear receptors that also includes the steroid receptors for androgens, glucocorticoids, mineralocorticoids, and progestins; the receptors for vitamin A and D; the thyroid hormone receptors; and the recently discovered orphan receptors for which no ligands have yet been identified. The nuclear receptors are structurally related proteins that function as ligand-dependent transcription factors, playing a crucial role in the endocrine signaling pathways. The ligand-activated homo- or heterodimerized receptors interact specifically and with relatively high affinity with regulatory DNA sequences, so-called hormone response elements, found predominantly in the promoter region upstream of the coding sequences of target genes. After binding of ligand, the receptor 3D structure is transformed to adopt an agonist or antagonist conformation depending on the type of ligand bound (Brozowski et al., 1997; Shiau et al., 1998). The agonist structure of ER$\alpha$ has been shown to expose the activation function-2 (AF-2) (Tora et al., 1989b) in the ligand binding domain (LBD) and permits interaction with coactivators (Shiau et al., 1998), whereas, in the antagonist structure, AF-2 is translocated to a different position that may permit interaction with corepressors but not coactivators (Brozowski et al., 1997; Shiau et al., 1998; Smith et al., 1997).

The ERs are best known for their gene modulatory effect via binding to estrogen responsive elements (ERE) on DNA. Lately several alternative regulatory pathways have been described that include response elements to which the ERs do not bind directly. Genes regulated by ERs via the indirect pathway include the ovalbumin, the IGF-1 and the collagenase genes (Gaub et al., 1990; Umayahara et al., 1994; Webb et al., 1995). These genes are activated by the ERs via AP1 sites that bind the dimeric transcription factor AP1, com-

\textbf{ABBREVIATIONS:} ER, estrogen receptor; AF, activation function; LBD, ligand binding domain; ERE, estrogen-responsive element; AP1, activator protein 1; LY 294002, 2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one; PD 98059, 2-

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posed of members of the Jun and Fos families (Angel and Karin, 1991). Apparently, the ER is not in direct contact with the AP1 response element; rather, it seems to be tethered to the DNA via the transcription factor/coactivator complex that contacts the AP1 site (Kushner et al., 2000). Similar mechanisms of ER action have been described for other transcription factors (Saville et al., 2000), which, at least in part, may explain the diversity of the estrogen response of various target genes and tissues.

In addition to the conventional activation of the ERs by natural or synthetic hormones, alternative activation pathways by various effectors, including a number of mitogenic growth factors, have been described. The epidermal growth factor-factor-1 has been shown to induce phosphorylation of serine residues in the N-terminal A/B domain of ERα (Kato et al., 1995) that resulted in enhanced activity of the ligand independent activation function-1 (AF-1) within the A/B domain (Tora et al., 1989b). Recently, an AF-1 specific coactivator was described whose interaction with the AF-1 of ERα was regulated by phosphorylation of serine 118 in the A/B domain of ERα (Endoh et al., 1999). Whether a similar mechanism is important for the transcriptional response to various growth factors of complex natural promoters such as those of the estrogen-regulated pS2 and cathepsin D genes is not known. However, in the MCF-7 breast cancer cell line, growth factor stimulation of the pS2 and cathepsin D genes has been shown to be suppressed by antiestrogens, suggesting that the growth factor effect was mediated through the ER pathway (Chalbos et al., 1993).

The pS2 gene product is a well-known estrogen inducible protein previously shown to be expressed in breast and gastrointestinal tissues (Rio et al., 1987, 1988). Its expression has been considered an indication that breast tumor cells express ER and that the tumor therefore would be responsive to antiestrogen therapy. The biological role of pS2 is not very well understood. However, a function for pS2 in stimulating to antiestrogen therapy. The biological role of pS2 is not very well understood. However, a function for pS2 in stimulating the estrogen-regulated genes has been shown to be suppressed by antiestrogens, suggesting that the growth factor effect was mediated through the ER pathway (Chalbos et al., 1993).

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peptide (αp I) expression vector has been described previously (Norriss et al., 1999).

**Cell Cultures.** HepG2 cells (American Type Culture Collection, Manassas, VA) and the HepER3 cells (Barkhem et al., 1997) were cultured in minimum essential medium supplemented with 10% FCS, 1% nonessential amino acids, 1 μM sodium pyruvate, and 2 mM l-glutamine. All cell cultures were maintained at 37°C in humidified chambers at 5% CO₂.

**Transient DNA Transfections.** All transient transfections were performed using the OptiMEM/lipofectin procedure according to the suppliers’ recommendations (Invitrogen). Transient transfections of the HepER3 cells and the HepG2 cells were performed in 48-well plates after seeding of 50 × 10⁴ cells/cm² in phenol red free Coon’s/Ham’s F12 media supplemented with 1% FCS (double dextran charcoal-stripped), 2 mM l-glutamine, and 50 μg/ml gentamycin 24 h before transfection. Cells were transfected for 6 h with 0.2 μg of DNA/well of the different pS2-ALP reporter constructs. In some experiments, 0.1 μg of DNA/well of a vector expressing the ERα variant HEO (Tora et al., 1989a) was included. The cells were then rinsed with Coon’s/Ham’s F12 media and incubated for 15 h before induction with hormone (moxestrol or tamoxifen) and/or PMA, as indicated in the figures. In all transient transfection experiments, cells were exposed to hormone or PMA for 48 h before being harvested and analyzed for reporter gene expression. All transient transfections presented were performed in triplicate and repeated at least three times. The results presented in the figures are representative for all experiments performed.

**Hormone- and Effector-Induced Expression of pS2 and SHBG in HepG2 and HepER3 Cells.** Approximately 20 × 10⁴ HepG2 or HepER3 cells were seeded per well in 96-well plates in 100 μl of Coon’s/Ham’s F12 media supplemented with 1% FCS (double dextran charcoal-stripped), 2 mM l-glutamine, and 50 μg/ml gentamycin. Twenty-four hours later, cells were rinsed and refed with the same medium supplemented with hormone (moxestrol, tamoxifen, or ICI 164,384) or PMA. Cells were exposed for hormone or PMA for 72 h (except for transiently transfected cells, which were exposed for 48 h) before being harvested and analyzed for effect on gene expression. Triplicates of each concentration of hormone or PMA were performed in all experiments, which also were repeated several times. Conditioned medium was analyzed for the levels of pS2 and SHBG was expressed using an ELSA-pS2 immunoassay and SHBG-Delfia, respectively, following the supplier’s instructions. All experiments presented were performed in triplicate and repeated at least three times. The results presented in the figures are representative for all experiments performed.

**Assay for Human Placental Alkaline Phosphatase.** The level of human ALP expressed from the various pS2-reporter constructs was determined by a chemiluminescence assay as follows. After heat inactivation of the conditioned culture medium for 15 min at 65°C, a 10-μl aliquot was mixed with 200 μl of assay buffer (10 mM diethanolamine, pH 10.0, 0.1 mM MgCl₂, and 0.5 mM CSPD) in white microtiter plates (Dynatech Laboratories In Vitro AB, Stockholm, Sweden) and incubated at 37°C for 20 min before being transferred to a 96-well luminometer (Luminoskan; Labsystems, Helsinki, Finland) for integral measurement with 1-s reading per well. The ALP activity is expressed in light units and is directly proportional to the level of ALP expressed in the cells.

**Assessment of Cytotoxicity.** To assess the content of viable cells in dose-response or transient transfection experiments, the bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymetoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (Promega, Madison, WI) to formazan by dehydrogenase enzymes found in metabolically active cells was measured at 490 nm and 650 nm (Cory et al., 1991).

**Calculation of Normalized Response.** In Fig. 9A, HepG2 cells were cotransfected with ERα (HEO) together with the pS2-ALP reporter vector and increasing amounts of the expression vector for the Gal4DBD-LXXLL-peptide fusion. Normalized response was obtained by dividing the ALP activity of the reporter vector with the value obtained in the cytotoxicity assay described above, which measures viable cell activity. In Fig. 9B, HepG2 cells were cotransfected with ERα (HEO) and pS2(EREvit)-ALP or pS2(EREvit) mutAP1-ALP together with increasing amounts of the expression vector for the Gal4DBD-LXXL-peptide fusion or the Gal4DBD expression vector. Normalized response was obtained as in Fig. 9A. However, the responses are expressed as the ratio of the normalized ALP activity evoked by cells transfected with Gal4DBD-LXXL fusion and cells transfected with an equivalent amount of the empty Gal4DBD expression vector, respectively.

**Results**

**Induction of the pS2 and SHBG Genes in HepG2/HepER3 Cells.** The response to the synthetic estrogen moxestrol or the phorbol ester PMA on the expression of the endogenous pS2 and SHBG genes in HepG2 cells, in the presence or absence of estrogen receptor expression, was examined. We have used the human hepatocarcinoma cell line HepG2, which lacks endogenous expression of ERα and -β (data not shown) and HepG2 cells stably transformed to express ERα (HepER3) (Barkhem et al., 1997) to assess the role of ERα on SHBG and, in particular, pS2 gene regulation. In the presence of ERα, both moxestrol and PMA showed a stimulatory effect on pS2 gene expression but with different potency and efficacy (Fig. 1A). PMA displayed the most efficacious response on pS2 expression, exceeding the response to moxestrol. In the non–ER-expressing HepG2 cells, only PMA had a stimulatory effect on pS2 gene expression (Fig. 1B). The response to PMA in the HepG2 cells was at least 10-fold lower compared with its effect in the ERα expressing HepER3 cells, suggesting that the presence of ERα potentiates the effect of PMA. Only moxestrol, in contrast to PMA, stimulated expression of the estrogen-responsive SHBG gene in HepER3 (Fig. 1C). Thus, ligand-independent activation of estrogen inducible genes in these liver cells does not seem to be a general phenomenon; rather, it is restricted to specific genes (e.g., the pS2 gene). We also examined the combined effect of moxestrol and PMA in HepER3 cells, which resulted in strong synergism with a pS2 expression level severalfold higher than would be predicted by the sum of the expression levels induced by PMA or moxestrol alone (Fig. 1D). The synergistic effects were effectively blocked by the pure antiestrogen ICI 164,384, which also antagonized the individual responses of the effectors on pS2 expression (Fig. 1D), emphasizing the importance of ERα in both events. As expected, no synergism was observed in the parental ERα-deficient HepG2 cells (data not shown).

**Effects of Selective Protein Kinase Inhibitors on pS2 Gene Expression.** To dissect the role of different signal transduction pathways for estrogen or PMA activation of pS2 expression in HepER3 cells, selective protein kinase inhibitors were used. The mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor PD 98059 blocked moxestrol stimulated pS2 expression, suggesting a role of the extracellular-signal regulated family of mitogen-activated protein kinases (MAPK) in the estrogen effect on pS2 gene expression (Fig. 2). Furthermore, the phosphoinositide 3-kinase (PI3K) inhibitor LY 294002 also suppressed pS2 expression in response to moxestrol, whereas the protein kinase C inhibitor bisindolylmaleimide had only a modest effect, if any. In contrast, PMA induction of pS2 was effectively blocked by bisindolylmaleimide, which, as anticipated, sug-
gests that PMA acts via PKC in stimulating pS2 gene expression. However, PD 98059 and LY 294002 also had an inhibitory effect on PMA-stimulated pS2 expression, reducing the activity by approximately 50% (Fig. 2). Interestingly, the effect of MAPK seems to be cell-specific with respect to estrogen induction of the pS2 gene because PD 98059 did not affect estrogen stimulation of the pS2 gene in the breast cancer cell line, ZR-75-1 (data not shown). To exclude that the effects of the different inhibitors were caused by unspecific cytotoxicity, the concentrations of inhibitors were chosen such that a cytotoxicity marker (Cory et al., 1991) was unaffected at the doses used in the experiments (data not shown). Furthermore, SHBG protein levels were not reduced by any of the different inhibitors (data not shown).

Role of the ERE and AP1 Response Elements in Response to Estrogen or PMA. Because both PMA and MAPK signal transduction pathways may converge on AP1 response elements, we decided to investigate the function of the AP1 motif adjacent to the ERE in the pS2 promoter. To examine the individual and combined importance of the ERE and the AP1 motif in the response to hormones and PMA, a mutational analysis at the promoter level was carried out. Oligonucleotides containing mutated ERE and/or AP1 motifs were ligated into the pS2 promoter, replacing the corresponding wild-type sequences. To retain distances between different response elements within the pS2 promoter, the wild-type ERE and AP1 motifs, respectively, were substituted by the same number of nonsense nucleotides. The wild-type pS2 promoter fragment and the different mutated variants were fused to the ALP-reporter gene.

Initially, the significance of the ERE and the AP1 motifs in pS2 gene regulation in the presence of PMA was evaluated. In transient transfection experiments the various pS2 promoter constructs (Fig. 3A) were introduced into the HepER3 cells. PMA stimulated transcription from the wild-type pS2-ALP construct in the HepER3 cells (Fig. 3B). Mutation of the ERE caused only a modest reduction in the response to PMA. However, absence of a functional AP1 motif (mutAP1) was deleterious to stimulation of gene expression by PMA. The results of ALP expression from wild-type and mutated versions of the pS2 promoter in response to moxestrol in transient transfections of HepER3 cells is shown in Fig. 4. As expected, in the absence of a functional ERE (mutERE) the effect of moxestrol was extinguished. Mutation of only the

![Fig. 1. Dose-response of endogenous pS2 and SHBG gene expression to moxestrol (Mox) and PMA, respectively. pS2 response in HepER3 cells (A) and HepG2 cells (B). Effect on SHBG expression in HepER3 cells (C). The x-axis expresses the molar effector concentration. D, endogenous pS2 expression in HepER3 cells in response to 10^{-10} M moxestrol (Mox), 10^{-7} M PMA, 10^{-10} M moxestrol + 10^{-7} M PMA in the presence or absence of 10^{-7} M ICI 164,384. The concentration of 10^{-15} (M) on the x-axis in A, B, and C represents no effector/ligand added, only medium and solvent. Values are the means of triplicate determinations for each concentration of the various hormones or PMA added, with error bars (S.D.) indicated for each value. For some concentrations error bars are not visible because they were smaller than the symbol.]
effect in the presence of ER
the presence of both sites results in more than an additive
activity of the pS2 promoter in response to estrogen and that
that both the ERE site and the AP1 site are important for full
intact AP1 response element. Thus, the data in Fig. 4 suggest
that the ER response via the ERE is also dependent on an
same extent as the mutation of the ERE alone, suggesting
reporter gene expression in response to moxestrol to the
AP1 motif (mutAP1) also had a dramatic effect, blocking
cells were induced with $10^{-9}$ M moxestrol or $10^{-7}$ M PMA. HepER3
Fig. 2. Effects of bisindolylmaleimide, PD 98059, or LY 294002 on endoge-
nous pS2 protein in HepER3 cells induced with moxestrol or PMA. HepER3
cells were induced with $10^{-9}$ M moxestrol or $10^{-7}$ M PMA in the presence or
absence of $5 \times 10^{-7}$ M bisindolylmaleimide, $2.5 \times 10^{-8}$ M PD 98059 or $5 \times
10^{-8}$ M LY 294002. The level of inhibition is expressed as a percentage of the
full response to moxestrol or PMA, respectively. Values are the means of
triplicate determinations for each concentration of the various hormones and
effectors added, with error bars (S.D.) indicated for each value. For some
determinations, error bars are too small to be visible.

AP1 motif (mutAP1) also had a dramatic effect, blocking
reporter gene expression in response to moxestrol to the
same extent as the mutation of the ERE alone, suggesting
that the ER response via the ERE is also dependent on an
intact AP1 response element. Thus, the data in Fig. 4 suggest
that both the ERE site and the AP1 site are important for full
activity of the pS2 promoter in response to estrogen and that
the presence of both sites results in more than an additive
effect in the presence of ERα.

In transient transfections of HepER3 cells with the wild-
type pS2 promoter construct, both moxestrol- and PMA-in-
duced reporter gene expression were blocked with tamoxifen
(Fig. 5). In the absence of a functional ERE, tamoxifen was
still able to suppress PMA-stimulated reporter gene activity.
Furthermore, tamoxifen alone displayed no agonistic activity
on the pS2 gene on either intact or ERE-mutated promoter
(Fig. 5). Tamoxifen blocked the effect of PMA also in the
parental HepG2 cells that lack ERα; however, doses at least
100-fold higher than in HepER3 cells were required, indicating
a non-ERα-mediated mechanism (data not shown).

To further examine the role of the AP1 motif in estrogen-
dependent pS2 promoter activation, moxestrol was titrated
in transient transfection experiments in which the ERα vari-
ant HEO (Tora et al., 1989a) together with wild-type or AP1
mutated promoter constructs were used. The reason for using
HEO, which is unstable in the absence of ligand, was simply
to reduce background expression levels and thereby obtain a
better signal-to-noise ratio. The pS2 promoter-reporter
showed the same characteristic bell shaped titration curve in
response to moxestrol (Fig. 6A) as observed for endogenous
pS2 protein (Fig. 1A). Interestingly, also the AP1 mutated
construct showed a weak but significant response to moxes-
trol (Fig. 6B). Furthermore, the AP1 mutated construct dis-
played a 10-fold lower potency for moxestrol compared with
the wild-type promoter construct. Thus, the presence of the
AP1 motif adjacent to the ERE potentiated pS2 promoter
activity in response to estrogen with respect to both potency
and efficacy. We have noticed in electrophoretic mobility shift
assay experiments that the pS2 ERE has a much lower
affinity for ERα than the consensus ERE from the vitelloge-
nin gene (EREvit) (data not shown). To investigate whether
potentiation from the AP1 motif was restricted to imperfect
EREs that bind ERα with low affinity, the ERE of the pS2
promoter was mutated into the high-affinity EREVit (Fig.
6E). The pS2 promoter construct containing the EREVit dis-
played 4-fold higher amplitude than the wild-type pS2 pro-
moter-reporter construct and a 10-fold increase in the po-
tency of moxestrol (Fig. 6C). However, the high-affinity
EREvit was also markedly potentiated by the presence of the
AP1 motif in the pS2 promoter context (Fig. 6D). In the
absence of a functional AP1 motif, the EREVit construct
displayed a decrease in reporter gene activity similar to the
decrease observed for the wild-type pS2 promoter (10-versus
15-fold) and the potency of moxestrol was in both cases re-
duced 10-fold.

The ERE mutated pS2 promoter (mutERE) was basically
unresponsive to estrogen (Fig. 4), even when ERα was over-
expressed (data not shown). However, we asked whether
ERα could interact directly or indirectly with factors at the
AP1 motif. Using a one-hybrid system, we demonstrated that
a chimera of the transcriptional activator protein VP-16 and
full-length ERα stimulated gene expression from the ERE
mutated construct to some extent (i.e., via the AP1 motif),

Fig. 3. Effect of PMA on the pS2 promoter-
ALP reporter gene and mutated pS2 pro-
moter versions thereof. HepER3 cells were
transiently transfected with pS2-ALP, pS2
mutERE-ALP (mutERE), pS2 mutAP1-
ALP (mutAP1), and pS2 mutERE mutAP1-
ALP (mutERE mutAP1), respectively (A).
The level of secreted ALP reporter protein
was determined 48 h postinduction with
$10^{-7}$ M PMA (B). The columns are means
of triplicate determinations with the S.D.
indicated. For some determinations, error
bars are too small to be visible.
whereas VP16 alone had no effect (Fig. 7). Thus, ERα seems
to bring the chimera to the pS2 promoter despite the absence
of a functional ERE, possibly by interacting with the protein
complex that stimulates transcription via the AP1 motif.

The AP1 Motif Potentiates pS2 Gene Expression via
the Ligand-Binding Domain of ERα. The pS2 promoter
variants containing the high-affinity EREvit and an intact or
a mutated AP1 response element were cotransfected together
with A/B- or EF-domain truncated ERα (Fig. 8, A and B) to
assess the relative influence of AF-1 and AF-2 on pS2 promoter
activity. In these experiments, we preferred to use pS2
promoter variants encompassing the EREvit to obtain more
robust responses; however, similar results have been ob-
tained using constructs that contain the natural ERE of the
pS2 promoter (pS2-ALP and mutAPI) (data not shown). The
ERα AD construct, which lacks the LBD but retains the
N-terminal AF-1 and the DNA binding C-domain, did not
respond to moxestrol in the context of the pS2 promoter-
reporter that contained an intact API response element (Fig.
8A). Furthermore, it displayed a significantly reduced hor-
monedependent activity, suggesting that AF-1 alone does
not participate in pS2 promoter activation. The N-termi-
nally truncated ERα CF variant encoding the DNA- and LBD was
able to stimulate the pS2 promoter in the presence of moxes-
trol, although with a reduced activity compared with the
full-length receptor (Fig. 8A). The data suggest that the AF-2
but not AF-1 is necessary and sufficient to stimulate pS2
promoter activity although AF-1, in the context of the full-
length receptor, may synergize with AF-2 in activating
the pS2 promoter to a full response. Full-length ERα stimu-
lated the AP1-mutated pS2 promoter to a degree approximately
20% of the wild-type promoter (Fig. 8B). Interestingly,
nether of the truncated versions of ERα (ER AD and ER CF)
was able to stimulate the pS2 promoter in which the AP1
motif was mutated (Fig. 8B), suggesting that the potentia-
tion of the pS2 promoter through the API motif is mediated by
the LBD of ERα.

Suppression of pS2 Promoter Activity by an LXXLL-
Containing Peptide Is Modulated by the AP1 Motif.
The transcriptional activity of the AF-2 of ERα is depend-
on its ability to interact with the p160 family of coactivators
via conserved LXXLL motifs in the central region of the
coactivator. To further investigate the importance of AF-2 in
estrogen stimulation of the pS2 promoter, the pS2 promo-
ter-reporter-construct was cotransfected together with increas-
ing amounts of a vector expressing an LXXLL peptide. The
LXXLL peptide, which has been isolated from a phage-dis-
played peptide library because of its ability to interact with
E2 activated ERα (Paige et al., 1999), was fused to the DNA
binding domain of the yeast protein Gal4 (Gal4DBD). Over-
expression of the LXXLL peptide suppressed ALP expression
from the wild-type pS2 promoter in a dose-dependent fashion
(Fig. 9A), indicating that the LXXLL peptide was able to
compete with and displace coactivators from the ERα AF-2
surface, resulting in repression of the transcription. Next, we
investigated the effect of the LXXLL peptide in the context of
the pS2 promoter in which the API motif had been mutated.
In these experiments, we preferred to use pS2 promoter
variants encompassing the EREvit to obtain more robust
responses. We cotransfected the pS2/EREvit-ALP reporter
construct or the pS2/EREvit mutAPI1-ALP reporter devoid of
a functional API1 element together with the full-length ERα
(HEO) expression vector and the expression vector for
the LXXLL peptide. Interestingly, larger amounts of vector
expressing the LXXLL peptide were required to suppress the
intact pS2 promoter compared with the construct that lacks
an intact API element (Fig. 9B). In fact, the API1 defective
pS2 promoter showed approximately 30% less activity than
intact pS2 promoter at an equal amount of the LXXLL peptide.

The pS2 promoter construct, in which the AP1 motif is preserved but the ERE has been mutated (mutERE), is unresponsive to estrogen (Fig. 4 and 5). Furthermore, overexpression of the LXXLL peptide did not have any significant effect on the background expression of the mutERE reporter with respect to estrogen effects. However, as shown in Fig. 3, PMA stimulates the mutERE reporter construct. We have investigated the effect of the LXXLL peptide on PMA stimulated pS2 promoter. Neither intact nor ERE-mutated pS2 promoter was suppressed by overexpression of the LXXLL peptide (data not shown). Thus, LXXLL motifs do not seem to play a major role in transcriptional activation of the pS2 promoter via the AP1 motif.

**Discussion**

Two different findings encouraged us to examine the function of the AP1 response element located near the ERE in the pS2 promoter. The first was our observation that an inhibitor of the MAPK cascade, the intracellular signal transduction pathway of which may target AP1 response elements on DNA, was able to block estrogen stimulation of the pS2 gene (Fig. 2). Second, a previous report demonstrated, with photo–cross-linking experiments using a fragment of the pS2 promoter and protein extracts from MCF-7 cells, the participation of both ERα and a c-fos related protein in two multiprotein complexes associated with the promoter segment that spans the ERE (Schuh and Mueller, 1993).

We have studied the regulation of the pS2 gene in HepG2 cells. The investigation was focused on the role of ERα because ERβ has not been found to be expressed in hepatocytes (Kuiper et al., 1997; Taylor and Al-Azzawi, 2000). Our parental HepG2 cells lack estrogen receptors; however, stable integration of ERα (HepER3) does not seem to have changed the cells except with regard to estrogen response, because HepG2 and HepER3 cells showed identical gene expression profiles in the absence of estrogen when analyzed with a cDNA array displaying more than 1200 genes (data not shown).

Initially, we confirmed that an “AP-1 like” complex also interacts with the pS2 promoter in liver cells. With the use of the electrophoretic mobility shift assay, a specific complex that immunologically resembles AP1 was formed using a nuclear extract of HepER3 cells and an oligonucleotide that spans the AP1 motif of the pS2 promoter (data not shown).

![Fig. 6. Dose-response of pS2-ALP, pS2 mutAP1-ALP (mutAPI), pS2(EREvit)-ALP and pS2(EREvit) mutAP1 to moxestrol (A-D). The pS2 promoter variants were cotransfected together with ERα (HEO) into HepG2 cells. The concentration of 10^(-11) M on the x-axis represents no ligand added, only medium and solvent. Values are the means of triplicate determinations for each concentration of moxestrol added, with error bars (S.D.) indicated for each value. For some concentrations, error bars are not visible because they were smaller than the symbol. E, ERE sequences of the pS2-ALP and the pS2(EREvit)-ALP, respectively. The ERE half-sites are capitalized and nucleotides that differ from the EREvit are underlined.](image_url)
We dissected the contribution of the ERE and the adjacent AP1 response element, respectively, in response to estrogen (Fig. 4). That both a mutated ERE and an AP1 site, respectively, blunted the pS2 response to the synthetic estrogen molexestrol told us two things: that both sites play an important role in pS2 expression in response to estrogens and that the AP1 element plays a dominant role in the regulation of pS2 gene expression in HepG2 cells.

By fusion of a strong activation domain (VP16) to ERα, we showed that ERα was able to interact not only with the ERE but also somehow with the AP1 motif in the context of the pS2 promoter (Fig. 7). Furthermore, cotransfection of ERα devoid of its A/B domain demonstrated that this construct was able to stimulate the pS2 promoter in response to estrogen but that the stimulatory effect was dependent on an intact AP1 motif. A potential explanation could be that the LBD domain of ERα is able to interact directly or indirectly with factors at the AP1 element, which results in the significant potentiation of the transcriptional response of the pS2 promoter (Fig. 8, A and B). Conflicting data exist regarding whether LBD of ERα interacts directly with different components of the AP1 complex (Webb et al., 1995; Teyssier et al., 2001). However, the p160 coactivator SRC-1 has been shown to interact with both c-jun and c-fos (Lee et al., 1998). The c-jun and c-fos binding sites were shown to be localized in the C-terminal subregion of SRC-1. Thus, SRC-1 may constitute a potential bridging factor between the AP1 motif and ERα bound to the ERE by its LXXLL motif interacting with AF-2 of ERα and via its C-terminal region with the AP1 complex.

The observation that a larger amount of the GAL4DBD-LXXLL-peptide fusion expression vector was required to disrupt ERα-mediated transcriptional activity on the pS2 promoter in the presence of an intact AP1 motif may further support the assumption that a p160 coactivator protein is involved in the interplay between the AP1 motif and the ERE (Fig. 9B). A potential mechanistic explanation could be that an interaction between a protein factor at the AP1 motif and the p160 coactivator has a stabilizing effect on the interaction between the AP-2 of ERE-bound ERα and the LXXLL motif of the coactivator. Furthermore, as shown in Fig. 6, the presence of an intact AP1 motif had a significant effect on the potency of moxestrol, increasing it by 10-fold. It has recently been shown that an elevated concentration of the coactivator SRC-1 in cells caused an increase in the potency of estradiol (Gee et al., 1999), explained by coactivator-mediated stabilization of the ER-ligand complex. Perhaps the AP1 motif of the pS2 promoter functions in a similar manner; i.e., an interaction between protein factors at the AP1 motif and ERα on the ERE, via a coactivator, causes a similar decrease in the off rate of moxestrol and thus an enhanced potency in the transcriptional response to moxestrol. In addition, the type of ERE in the pS2 promoter had a significant effect on its activity (Fig. 6) and it is therefore possible that the presence of the AP1 motif also affects the strength of the ER/ERE interaction. Thus, the presence of the AP1 motif in the pS2 promoter may enhance the transcriptional activity in response to estrogen in several possible ways. Perhaps the potentiation of the pS2 promoter activity that originates at the AP1 motif, is a result of a combination of events that results in an additive or synergistic effect on the transcriptional response to estrogen.

The MAPK pathway is well known to converge on AP1 response elements (Karin, 1995; Duan et al., 2001). That an inhibitor of PI3K also blocked the estrogen effect on the pS2 gene is consistent with previous reports that have documented inhibition of the MAPK pathway by pharmacological inhibitors of PI3K, suggesting that MAPK may serve as a downstream effector of PI3K (Marra et al., 1995). However, the nature of the exogenous signal that, in the present study, stimulates the PI3K and MAPK pathways and eventually converges on the AP1 motif of the pS2 gene promoter is intriguing. A body of evidence suggests the existence of a plasma membrane estrogen receptor (Pappas et al., 1995; Razandi et al., 1999). We have been unable, however, to demonstrate rapid activation of the MAPK pathway in response to estrogen, suggesting that activation of the pS2 gene is probably not mediated via a cell membrane ER. We favor instead a model in which serum factors present in cell me-

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**Fig. 7.** Effect of a chimeric VP16ERα fusion protein on pS2 promoter variants. HepG2 cells were transiently transfected with pS2-ALP, pS2 mutERE-ALP (mutERE), pS2 mutAP1-ALP (mutAP1), and pS2 mutERE mutAP1-ALP (mutERE mutAP1) together with vectors expressing VP16ERα, VP16, and T7T319 control DNA and induced with 10^{-8} M moxestrol. The columns are means of triplicate determinations with the S.D. indicated.
dium stimulate intracellular signal transduction pathways, including the MAPK pathway, resulting in maintenance of a basal AP1 level sufficient to potentiate the transcriptional activity of the pS2 promoter in response to estrogen.

ERα seems to constitute a key factor also in ligand-independent stimulation of the pS2 gene because PMA had a substantially reduced activity in the absence of ERαs (Fig. 1B). Furthermore, such antagonists as ICI 164,384, tamoxifen, and raloxifene (Figs. 1D and 5, and data not shown) suppressed the stimulatory activity of moxestrol and PMA alone or in combination. Ligand-independent ERα action has been studied extensively and shown to be associated with MAPK-dependent phosphorylation of serine 118 in the AF-1 of ERα (Kato et al., 1995). However, transient transfections of serine 118 mutated ERα demonstrated that this mutant and the wild-type ERα responded equally well to PMA in stimulating the pS2 promoter reporter gene (data not shown). Recently, it was demonstrated that the PI3K dependent pathway may act directly on ERα by phosphorylation of serine 167 in the N-terminal part of the receptor, resulting in increased activity of the ligand-independent AF-1 function (Campbell et al., 2001). We therefore cannot exclude that serines other than serine 118 of ERα have been phosphorylated in the HepER3 cells, which may explain the ligand-independent involvement of ERα in the regulated expression of the pS2 gene. Moreover, PMA was shown to mediate its effect primarily via the AP1 element of the pS2 promoter (Fig. 3B). In contrast to estrogen, PMA was able to stimulate the transcriptional activity of the pS2 promoter in the absence of an intact ERE. Taken together, ERα seems to have an indispensable role in the activation process through the AP1 motif because tamoxifen blocked PMA-stimulated reporter gene activity also in the absence of a functional ERE (Fig. 5).

One exciting but speculative mechanism could be that ERα activated ligand independently enhances stimulation of pS2 gene expression by participating in the transcriptionally active complex that targets the AP1 motif. The capability of the VP16ERα chimera to stimulate transcription via the AP1 element indicates that ERα somehow may interact with the AP1 motif (Fig. 7). ER has been proposed previously to serve as a coactivator for the transcription factor Islet-1 (ISL1) in certain promoter contexts containing ISL1 binding sites (Gay et al., 2000). Thus, ERα may function as a coactivator at transcriptional activation of the pS2 gene via the AP1 motif in response to such factors as PMA. A similar role for ER has been suggested at estrogen induction of the collagenase gene.

**Fig. 8.** Effect of N- or C-terminally truncated ERαs on the pS2 promoter in the presence/absence of an intact AP1 motif. HepG2 cells were cotransfected with pS2/EREvit-ALP (A) or pS2/EREvit mutAP1 (B) and vectors expressing full-length ERα or the truncated ERα variants expressing the AD or CF domains, respectively. The cells were induced with 10^{-9} M moxestrol. The columns represent the mean of triplicate samples with the S.D. indicated. As control, cells were transfected with T7T319 DNA. For some determinations, error bars are not visible because they were smaller than the symbol.

**Fig. 9.** Suppression of pS2 promoter activity by overexpression of the LXXLL-peptide (a8I). A, HepG2 cells were cotransfected with ERαs (HEO), the pS2-ALP reporter vector and 0, 100, 200, or 400 ng of the Gal4DBD-LXXLL-peptide fusion and induced with 10^{-9} M moxestrol. The normalized response was obtained as described under Experimental Procedures. The value obtained for cells transfected with ERα and reporter vector alone is set as 100% activity. Control represents the transcriptional activity evoked by moxestrol activated ERαs on the pS2-ALP reporter in the presence of 400 ng of the empty Gal4DBD expression vector. The columns represent the mean of triplicate samples with the SD indicated. B, HepG2 cells were cotransfected with ERαs (HEO) and pS2/EREvit-ALP or pS2/EREvit mutAP1-ALP together with 0, 50, 100, 200, or 400 ng of the Gal4DBD-LXXLL-peptide fusion or the empty Gal4DBD expression vector. The normalized response was obtained as described under Experimental Procedures. The responses are expressed as the ratio of the normalized ALP activity evoked by cells transfected with Gal4DBD-LXXLL fusion and cells transfected with an equivalent amount of the empty Gal4DBD expression vector. The columns represent the mean of four separate experiments with the S.E.M. indicated.
that is mediated through an AP1 response element in its promoter region ( Webb et al., 1995 ). However, the AP1 sites of the collagenase- and pS2-promoters seem to function differently because the pS2 promoter is unresponsive to estrogen via its AP1 motif in the absence of an intact ERE (Fig. 4 ). Moreover, tamoxifen and raloxifene are potent activators of the collagenase gene ( Webb et al., 1998 ), whereas they function as pure antagonists at the AP1 site of the pS2 gene ( Barkhem et al., 1997 ; Fig. 5 ). However, the discrepancy between estrogen and such effectors as PMA in the mode of signaling via the AP1 motif of the pS2 promoter is intriguing. Perhaps it could be explained by differences in the pattern of post-translational modifications of ERα and/or cofactors induced by estrogen and PMA, respectively.

We believe that our data on the functional interaction between the ERE and AP1 response element in the pS2 promoter-reporter constructs reflect what would be observed on the endogenous pS2 promoter. The presence of an intact AP1 response element in the pS2 promoter dramatically potentiated the estrogen-induced expression of the pS2 promoter-reporter in HepG2 cells, both when ERα was transiently overexpressed (Fig. 6 ) and in HepG2 cells ( HepER3 ) stably transformed to express physiological levels of ERα (Fig. 4 ). Furthermore, PMA was a more efficacious activator of the endogenous pS2 gene than moxestrol in HepER3 cells (Fig. 1A ), which was also the case when the effect of PMA or moxestrol was assessed at transient transfections of the pS2 promoter-reporter (Fig. 5 ). In fact, the relative difference in amplitude between the PMA and moxestrol responses observed on the endogenous pS2 gene was almost identical to the relative difference between the responses evoked by PMA and moxestrol on the pS2 promoter-reporter (Figs. 1A and 5 ), supporting the notion that the activity of the pS2 promoter-reporter reflects the correct characteristics of the endogenous pS2 gene.

In conclusion, and in accordance with the proposed model ( Fig. 10 ), the data presented indicate that ERα plays a crucial role in mediating the effect not only of estrogen but also of PMA and that the AP1 motif in the pS2 promoter is an essential target on DNA through which various signals converge to modulate pS2 gene expression in the HepG2 cells.


