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# Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence

(estradiol/breast cancer/anti-estrogen/enhancer/control of transcription)

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**ABSTRACT** **Using chimeric recombinants transfected into HeLa cells and a transient expression assay, we demonstrate that the 5′-flanking region of the pS2 gene from position −428 to position −324 exhibits both constitutive and estrogen-inducible enhancer activity. The estrogen-inducible activity, but not the constitutive activity, was inhibited by antiestrogens. ICI 164,384 behaved as a pure antagonist, whereas hydroxytamoxifen was a partial agonist–antagonist. The estrogen-responsive element of the pS2 gene has been narrowed down by site-directed deletion mutagenesis to a 13-base-pair (position −405 to position −393) imperfectly palindromic sequence, which in isolation can confer estrogen inducibility to the heterologous rabbit β-globin gene promoter. On the other hand, the sequences responsible for the constitutive enhancer activity are spread over the entire region.**

The human pS2 gene was initially characterized as a gene whose expression is specifically controlled by estrogen in the breast cancer cell line MCF-7 (1, 2). The increase in pS2 mRNA after addition of estradiol to the culture medium is a primary transcriptional event (3), suggesting that control of pS2 gene promoter activity by the estrogen receptor is mediated by a cis-acting estrogen-responsive element (ERE) that could be located in the 5′-flanking region of the pS2 gene. Using MCF-7 cells transformed with chimeric recombinants containing the promoter region of the pS2 gene controlling transcription of the gene conferring resistance to neomycin (G418), it has indeed been shown that the 5′-flanking region of the pS2 gene from position approximately −3000 to position +10 contains an estrogen-responsive promoter (4). The location of the ERE was further investigated by transient transfection into HeLa cells of an expression vector encoding the human estrogen receptor (HEO, see ref. 5) together with chimeric reporter recombinants containing various fragments of the 5′-flanking sequences of the pS2 gene fused to the bacterial chloramphenicol acetyltransferase indicator gene. Expression from pS2(≈−3000/+10)CAT, pS2(≈−1100/+10)CAT, or pS2(−428/+10)CAT, where the ends of the inserted pS2 sequences are given in parentheses, could be stimulated by addition of estradiol, whereas pS2(−90/+10)-CAT was not inducible by estradiol, suggesting the presence of an ERE within the flanking sequence from position −428 to position −90 (ref. 6 and M.B., unpublished results). To investigate whether this ERE may correspond to an inducible-enhancer element, we next constructed a series of chimeric recombinants in which the 5′-flanking sequence from position approximately −3500 to position −87, from approximately −1100 to −86, from −428 to −87, and from −332 to −87 were fused in either orientation to the heterologous rabbit β-globin gene truncated at position −109. Transfection of these recombinants together with the human estrogen receptor expression vector HEO in the presence or absence of estradiol led to the hypothesis that both constitutive and estrogen-inducible enhancer elements are located within the region of the pS2 gene from position −428 to position −332 (7).

In the present study we demonstrate that the pS2 gene sequence from position −428 to position −324 indeed contains both constitutive and estrogen-inducible enhancer elements. Furthermore, the exact location of the ERE has been determined by using both deletion and point mutagenesis. We show that the pS2 ERE sequence is related to those of characterized EREs but that it is not a perfect palindrome, which results in a weaker ERE.

## MATERIALS AND METHODS

**Plasmid Constructions.** pS2(1100/424)pG1 and pS2(429/86)pG1 were constructed as follows. The *Pvu* II–*Bam*HI 5′-flanking fragment of the pS2 gene between positions approximately −1100 and −91 was cloned into the *Bam*HI site of the polylinker of M13mp19 by using a *Bam*HI–*Pvu* II linker, and the *Sac* I–*Bam*HI fragment from position −429 to position −91 was cloned into *Sac* I/*Bam*HI-digested M13mp19, to yield (1100/86)M13mp19 and (429/86)-M13mp19, respectively. The pS2 fragments between positions approximately −1100 and −429 and between positions −429 and −91 were then excised from these M13mp19 derivatives with *Xma* I plus *Sac* I and *Sac* I plus *Bam*HI, respectively, and inserted into the corresponding sites of the M13mp12 polylinker of pG1 (8) to yield pS2(1100/424)pG1 and pS2(429/86)pG1, respectively. pS2(332/81)pG1 was constructed by inserting in the correct orientation the *Xmn* I–*Sma* I pS2 fragment (between positions −337 and −86) into the *Sma* I site of the pG1 polylinker. pS2(428/324)pG1 and pS2(324/428)pG1 were obtained by inserting (in either orientation) the *Sac* I–*Pst* I fragment between positions −429 and −328, repaired by the Klenow fragment of DNA polymerase I, into the *Sma* I site of the pG1 polylinker. The deletion mutants pS2ApG1, pS2BpG1, pS2CpG1, pS2DpG1, pS2EpG1, and pS2FpG1 were constructed by site-directed mutagenesis using (429/86)M13mp19 and synthetic oligonucleotides (sequences available on request) as described in Grundström *et al.* (9) and Kumar *et al.* (5); the mutated fragments from (429/86)M13mp19 were then excised with *Hin*dIII and *Sac* I and inserted in the corresponding sites of the pG1 polylinker. All constructs were verified by DNA dideoxy sequencing (10). EREpG1, EREpG2, pS2-EREpG1, pS2-EREpG2, pS2-EREM1pG1, and pS2-EREM2pG1 were obtained by inserting synthetic oligonucleotides between the *Xba* I and *Xho* I sites of the M13mp12 polylinkers of either pG1 or pG2 (11), as indicated in Fig. 3*A* (note the presence

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Abbreviation: ERE, estrogen-responsive element.

of an additional *Pvu* I site at the 3′ end of the synthetic EREs). The activation vector HEO expressing the human estrogen receptor was as described (5).

**HeLa Cell Transfection and Quantitative S1 Nuclease Analysis.** HeLa cells were grown as described (6) in the presence of dextran-coated charcoal-treated fetal calf serum (stripped serum) (12) and in the absence of phenol red (13). All transfections were carried out by the calcium phosphate coprecipitate technique (14). Estradiol and anti-estrogens were added 2 hr after transfection. RNA isolation and analysis by quantitative S1 nuclease mapping using single-stranded 5′-end-labeled probes were performed as described (6). The pS2 probe was constructed by inserting the *Bam*HI–*Bam*HI fragment of the pS2 recombinant described by Kumar *et al.* (6) (pS2 fragment from position −91 to position +10 linked to the β-globin gene fragment from position −9 to position +396) into the *Bam*HI site of M13mp18. The preparation of the antisense single-stranded probe $^{32}$P-labeled on the 5′ end has been described (6). The probe covers the M13mp18 polylinker from the *Eco*RI site, the pS2 region (between positions −91 and +10), and the β-globin sequence (between positions −9 and +60). Transcription initiated at the β-globin start site protects a 60-base-pair fragment (pG1 and pG2 derived recombinants). Transcription starting at the simian virus 40 promoter [pAO (15), used as an internal reference plasmid] protects all of the globin part of the probe (69 base pairs). Autoradiograms from several independent experiments similar to those shown in the figures were scanned using a densitometer and the data were corrected for variations in transfection efficiencies by taking into account the signals given by the co-transfected reference plasmid pAO (see ref. 6).

## RESULTS

**The 5′-Flanking Region of the pS2 Gene Between Positions −428 and −324 Contains Both Constitutive and Estrogen-Inducible Enhancer Activities.** We have shown elsewhere that the 5′-flanking region of the pS2 gene between positions −428 and −87 contains both constitutive and estrogen-inducible enhancer activities capable of stimulating transcription from the heterologous rabbit β-globin gene promoter truncated at position −109 (pG1 recombinant) (ref. 7 and Fig. 1*A*). A series of additional reporter genes were constructed to further delineate the pS2 gene 5′-flanking sequences responsible for these activities (see Fig. 1*A*). These reporter genes were transfected into HeLa cells together with the estrogen receptor expression vector HEO (5) and a reference recombinant pAO (15) to normalize the results for possible variations in transfection efficiency. The enhancing effect of the pS2 gene 5′-flanking sequence was measured by quantitative S1 nuclease analysis of RNA initiated from the β-globin gene promoter of pG1 (+1 globin signal in figures). RNA initiated from the cotransfected reference gene pAO that contains the simian virus 40 early promoter region located upstream from the rabbit β-globin gene sequence truncated at position −9 was measured with the same S1 nuclease probe (−9 globin signal in figures). The autoradiograms shown in the figures are typical of several similar transfection experiments that, in each case, were carried out with at least two different plasmid-DNA preparations. Transcription initiated at the globin cap site (+1 globin) was quantitated by densitometry of appropriately exposed autoradiograms and, after correction for transcription from the reference plasmid pAO, expressed as fold stimulation relative to transcription from the parental recombinant pG1.

In agreement with results published elsewhere (7), transcription from the pG1 globin promoter of pS2(429/86)pG1 was stimulated ≈20-fold and 190-fold in the absence and in the presence of estradiol, respectively, whereas no or very

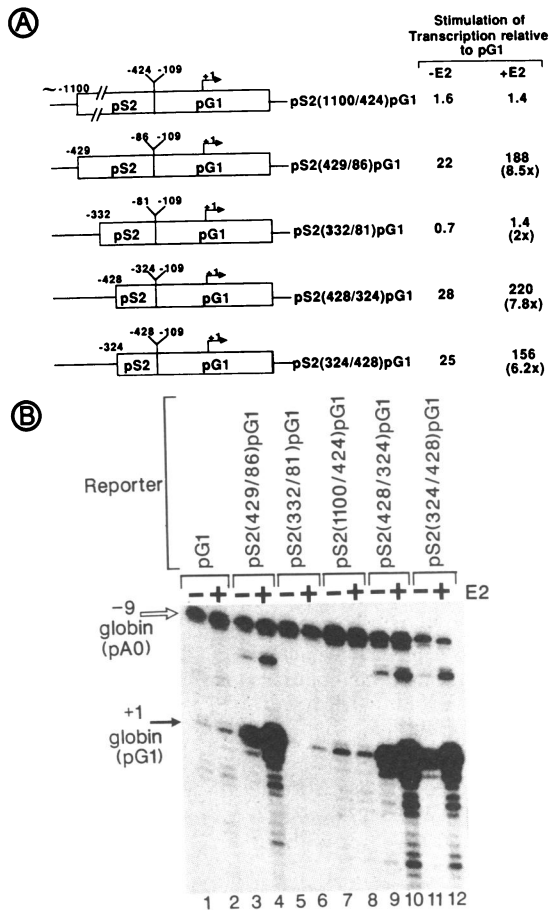


FIG. 1. Constitutive and estrogen-inducible enhancer activity in the 5′-flanking region of pS2 gene. (*A*) Schematic representation of reporter plasmids derived from the 5′-flanking region of pS2 gene. The rabbit β-globin gene transcription start site (+1) is indicated with an arrow. The constitutive and estrogen (E2)-inducible transcriptional activity of the chimeric reporter plasmids, relative to pG1 (average values from several experiments similar to that shown in *B*) is given, as fold stimulation. The estrogen inducibility is indicated in parentheses. (*B*) A representative quantitative S1 nuclease analysis of transcription from the chimeric reporter and reference (pAO) plasmids. Either pG1 or pS2pG1 reporter gene series (5 μg) was transfected with 1 μg of HEO and 0.2 μg of the internal reference plasmid pAO. HeLa cells were maintained for 48 hr in the absence (−) or presence (+) of estradiol (E2, 10 nM). Cytoplasmic RNA was prepared and hybridized with the pS2 probe 5′-end-labeled with $^{32}$P, and the protected fragments were analyzed on an 8% sequencing gel. The open arrow indicates the protected fragment for the cotransfected reference plasmid pAO. The solid arrow refers to the protected fragment for the reporter plasmids (pG1 and pS2pG1 series).

little stimulation was seen when the sequence between positions −429 and −332 was deleted [pS2(332/81)pG1] and no stimulation was observed in either condition with the pS2 gene 5′-flanking sequence between positions approximately −1100 and −424 (Fig. 1*A* and compare Fig. 1*B*, lanes 1–8). The results obtained with the two constructions pS2(428/324)pG1 and pS2(324/428)pG1 in which the isolated pS2 segment from position −428 to position −324 was inserted in the two orientations indicate that this 5′-flanking sequence contains the essential elements responsible for the constitutive and estrogen-induced stimulations of transcription. Furthermore, these elements have enhancer characteristics, since they activated transcription irrespective of their orientation relative to the stimulated β-globin promoter and their stimulatory activity was not critically dependent on their distance from the promoter [compare pS2(429/86)pG1 with either pS2(428/324)pG1 or pS2(324/428)pG1 in Fig. 1]. In all

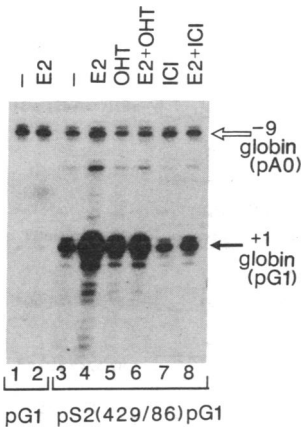


FIG. 2. Effect of estradiol, 4-hydroxytamoxifen, and ICI 164,384 on the enhancer activity of the pS2 gene 5′-flanking region. The effects of estradiol (E2), 4-hydroxytamoxifen (OHT), and ICI 164,384 (ICI) were tested on the reporter plasmid pS2(429/86)pG1 by quantitative S1 nuclease analysis, as described in Fig. 1. pG1 or pS2(429/86)pG1 (5 μg) was cotransfected into HeLa cells grown in hormone-stripped medium, in the absence of phenol red, with 1 μg of HEO and 0.2 μg of pAO (reference plasmid) by using the calcium phosphate co-precipitation technique. E2, OHT, and ICI were added as indicated in the following concentrations. Lanes: 2 and 4, E2 alone (10 nM); 5, OHT alone (10 nM); 6, E2 (10 nM) plus OHT (1 μM); 7, ICI alone (100 nM); 8, E2 (10 nM) plus ICI (1 μM).

cases, as expected, no stimulation of transcription was observed when the parental expression vector pKCR2 (16) was transfected together with either of the various reporter genes, instead of the estrogen receptor expression vector HEO (data not shown)

**Differential Effect of Estradiol and Antiestrogens on pS2 Gene Enhancer Activity.** The effect of two antiestrogens, 4-hydroxytamoxifen and ICI 164,384, was compared with that of estradiol (Fig. 2). Interestingly, hydroxytamoxifen at 10 nM acted as a partial agonist, stimulating transcription from pS2(429/86)pG1 by ≈2- to 3-fold under conditions where estradiol stimulated transcription 8- to 10-fold (compare lanes 3–6). On the other hand, ICI 164,384 behaved as a pure antagonist, since no stimulation was observed when it was added to the culture medium of the transfected HeLa cells, and its addition at 1 μM suppressed almost completely the stimulation by 10 nM estradiol (compare lanes 3, 4, 7, and 8 in Fig. 2), in contrast with the effect observed with 1 μM hydroxytamoxifen (lane 6).

**Localization of the ERE in the 5′-Flanking Region of the pS2 Gene.** A series of deletions (deletions A–F, as indicated in Fig. 3*A*, pS2ApG1 to pS2FpG1) were created in pS2(429/86)pG1 using synthetic oligonucleotides, to localize the ERE within the pS2 gene 5′-flanking region from position −428 to position −324. Deletions A and B were detrimental to the estrogen-induced enhancer activity [compare pS2(429/86)-pG1 with pS2ApG1 and pS2BpG1 in Fig. 3], with deletion B resulting in a complete loss of the estrogen-induced stimulation brought about by the region between positions −429 and −332 [compare pS2(332/81)pG1 in Fig. 1 with pS2BpG1 in Fig. 3]. On the other hand, the constitutive enhancer activity appears to be scattered throughout the region between positions −419 and −322, since four deletions (A, D, E, and F) clearly adversely affected this activity (Fig. 3).

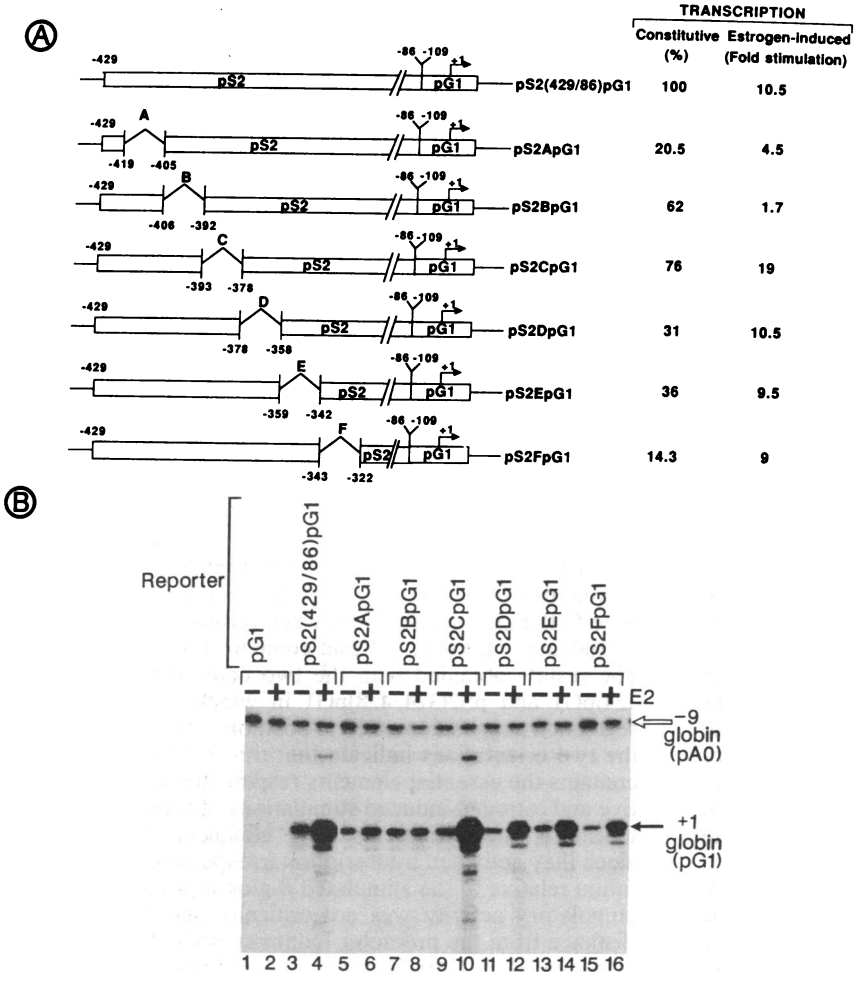


FIG. 3. Localization of the ERE in the pS2 gene 5′-flanking region. (*A*) Schematic representation of chimeric reporter recombinants containing the wild-type [pS2(429/86)pG1] and deleted (pS2A to pS2FpG1) pS2 5′-flanking region from position −429 to position −86. The 5′ and 3′ bases bordering the deletion sites are indicated in the figure. The rabbit β-globin gene transcription start site (+1) is indicated with an arrow. The constitutive transcriptional activity of the various deletion mutants pS2ApG1 to pS2FpG1 is given relative to pS2(429/86)pG1 activity, taken as 100%, which corresponds to a 28-fold stimulation over pG1 (see Fig. 1). The estrogen induction is expressed in fold stimulation of transcription upon addition of estradiol to 10 nM. (*B*) A representative quantitative S1 nuclease analysis of cytoplasmic RNA synthesized from HeLa cells transfected with pS2(429/86)pG1 and the pS2 deletion plasmids (pS2A to pS2FpG1). Reporter plasmid (5 μg) was cotransfected with 1 μg of HEO along with 0.2 μg of pAO as a reference plasmid. The cells were kept in either absence (−) or presence (+) of 10 nM estradiol. S1 nuclease analysis was carried out as described in the legend to Fig. 1.

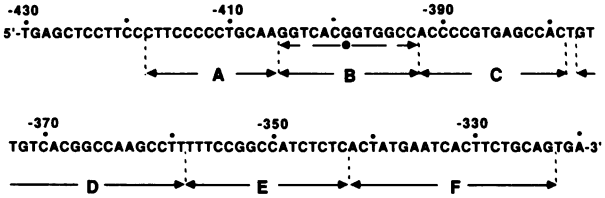


FIG. 4. pS2 gene 5'-flanking sequence from position −430 to position −320. The pS2 gene 5'-flanking region (between positions −429 and −86) was cloned in M13mp19. The pS2 gene fragments, both wild type and the mutants (deletions A–F, see Fig. 3*A*) were sequenced by dideoxy sequencing (10). We found several mistakes in the published sequence of pS2 gene from position −430 to position −320 (18) and the corrected sequence is shown here. The imperfectly palindromic ERE (from position −405 to position −393) is underlined as well as deletions A–F (see Fig. 3*A* and text).

The loss of estrogen inducibility in the mutant bearing deletion B prompted us to look in the sequence of the corresponding region for a motif similar to the consensus ERE (17). No such sequence was present in the previously reported (18) pS2 gene 5'-flanking sequence. However, we found that this published sequence contains a number of mistakes that occurred when it was entered in the computer. The corrected pS2 gene sequence from position −430 to position −320 is displayed in Fig. 4. When comparing this sequence with that of the consensus palindromic ERE (5'-GGTCANNNTGACC-3', see ref. 17), it was apparent that a closely related sequence (between positions −405 and −393, 5'-GGTCACGGTGGCC-3') was overlapping with deletion B, the only difference being an adenosine to guanosine transition in the 3' stem of the palindrome.

**The Isolated Imperfect Palindromic Sequence from Position −405 to Position −393 Is an ERE.** The imperfectly palindromic putative ERE sequence of the pS2 gene was chemically synthesized and inserted upstream to the β-globin gene promoter in either pG1 (pS2-EREpG1) or pG2 (pS2-EREpG2) (Fig. 5*A*). Its ability to mediate estrogen induction was compared with that of the perfectly palindromic consensus ERE sequence (17) (EREpG1 and EREpG2 reporter genes in Fig. 5*A*). The results of quantitative S1 nuclease analysis of several transfection experiments similar to those present in Fig. 5*B* were averaged and are presented in Fig. 5*A* (numbers are given in parentheses) as fold stimulation of transcription by the estrogen receptor upon addition of estradiol to the culture medium of the transfected HeLa cells. The imperfectly palindromic pS2 sequence is acting as an ERE, albeit with a stimulatory activity lower than that of the perfectly palindromic ERE (Fig. 5). This lower efficiency was particularly noticeable when the EREs were inserted in pG2 at position −425 with respect to the globin cap site (i.e., at a position corresponding to the natural position of the pS2 ERE). The requirement for a palindromic sequence to achieve maximal estrogen stimulation was further supported by introducing additional point mutations in the 3' stem of the pS2 ERE (pS2-EREM1pG1 and pS2-EREM2pG1 in Fig. 5). Finally, we note that the estrogen stimulation (≈4.5-fold) brought about by the presence of the pS2 ERE inserted at position −425 in pG2 is approximately half of that observed when the pS2 ERE is present in its natural location in pS2(429/86)pG1, which indicates that this imperfectly palindromic ERE is largely responsible for the estrogen-inducible enhancer activity of the pS2 gene 5'-flanking sequence.

## DISCUSSION

We have demonstrated that the 5'-flanking region of the pS2 gene from position −428 to position −324 contains a complex enhancer region that contains an ERE as well as constitutive enhancer elements that appear to be scattered throughout the region and are further characterized in a separate report (7).

A detailed functional deletion analysis led us to localize the ERE within a region containing a 13-base-pair DNA fragment (from position −405 to position −393) consisting of an imperfectly palindromic sequence 5'-GGTCACGGTGGCC-3', which deviates from the canonical consensus palindromic ERE, 5'-GGTCANNNTGACC-3' (17), by 1 bp in the 3' stem

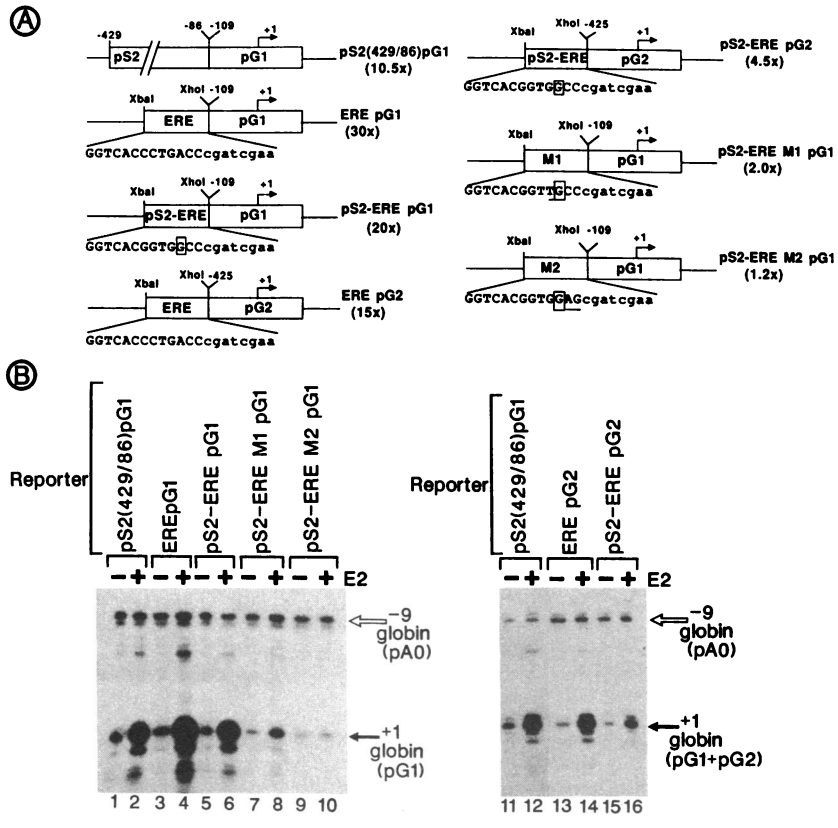


FIG. 5. Synthetic pS2 ERE confers estrogen inducibility on the heterologous rabbit β-globin gene promoter. (*A*) Schematic organization of the reporter recombinants pS2(429/86)pG1, EREpG1, pS2EREpG1, pS2EREM1pG1 (mutant with a single base change in the pS2 ERE), and pS2EREM2pG1 (mutant with two base changes in the pS2 ERE). The synthetic oligonucleotides whose sequences are depicted in the figure were flanked by *Xba* I and *Xho* I restriction enzyme sites. In the pS2 ERE sequence, the base that is different from that present in the consensus ERE is boxed, while the bases substituted in the mutants are underlined. The rabbit β-globin gene transcription start site (+1) is indicated with an arrow. The average transcriptional induction by estradiol of the various reporter recombinants is indicated in parentheses as fold stimulation. (*B*) A representative quantitative S1 nuclease analysis of transcription from the various EREpG1 and EREpG2 reporter plasmids. HeLa cells were transfected with 5 μg of reporter gene and 1 μg of HEO, along with 0.2 μg of the internal reference plasmid pAO. The transcriptional activity was assayed by quantitative S1 nuclease mapping in the absence (−) or presence (+) of 10 nM estradiol (E2) (see legend to Fig. 1).

of the palindrome (see Fig. 4). That this imperfectly palindromic sequence is in fact a functional ERE was conclusively shown by associating it, in isolation of any other pS2 gene 5′-flanking sequence, with the rabbit β-globin promoter. This association indeed conferred estrogen inducibility to the globin promoter, whether the synthetic pS2 ERE was inserted at position −109 or position −425 upstream of the globin gene. We have no explanation for the previous observation of Klein-Hitpass *et al.* (19) who reported that the imperfectly palindromic element 5′-GGTCACAGTGGCC-3′ did not confer estrogen inducibility on its own, although it is very similar to the pS2 ERE. We note, however, that a duplication of their imperfectly palindromic element led, under their assay conditions, to an inducibility very close to that of a duplicated perfectly palindromic ERE (20). We note also that under these assay conditions, their perfectly palindromic ERE, which was located immediately upstream of the herpes simplex virus thymidine kinase promoter, mediated only a 6-fold induction by estrogen, whereas we observed a much higher induction level under our conditions. The discrepancy between their results and our results may possibly reside in the amount of estrogen receptor present in the transfected cells, which was higher in our cells (HeLa cells transfected with a human estrogen receptor expression vector) than in their cells (natural human estrogen receptor present in MCF-7 cells).

The pS2 ERE is the first example of an imperfectly palindromic element that can function on its own, albeit with an efficiency 3–4 times lower than that of a perfectly palindromic ERE, when positioned 425 base pairs upstream from the activated promoter (compare EREpG2 and pS2-EREpG2 in Fig. 5). We note in this respect that the affinity of the estrogen receptor for the pS2 ERE is ≈5 times lower than for a perfectly palindromic ERE (21). All previously reported natural imperfectly palindromic elements were inactive on their own, and duplication or association of such elements was shown to be necessary to generate a functional ERE (20, 22, 23). This indicates clearly that not all of the bases of the palindrome (see also Fig. 5, pS2-EREM1 and pS2-EREM2) are equally important for estrogen responsiveness—i.e., estrogen receptor binding (21). Moreover, at a given position within the palindrome, the nature of the base that deviates from the consensus sequence is also important, since the presence of a thymidine instead of a guanosine at the deviating position of the pS2 ERE generates an inactive ERE (22). Deletions of sequences located upstream (deletion A in Fig. 4) and downstream (deletion C in Fig. 4) from the pS2 ERE (deletion B in Fig. 4) led to a 2 times decrease and a 2-fold increase in estrogen inducibility, respectively. Further studies are required to investigate whether these small variations reflect an effect of the new flanking sequences generated by the deletions or the existence of true positive (for the upstream sequence A) and negative (for the downstream sequence B) modulators of the pS2 ERE activity.

Finally, we note that the antiestrogens hydroxytamoxifen and ICI 164,384 have a different effect on the enhancer activity of the 5′-flanking region of the pS2 gene from position −429 to position −86 in the chimeric recombinant pS2(429/86)pG1 (Fig. 2). Transcription from the β-globin gene promoter was stimulated by hydroxytamoxifen, albeit with a lower efficiency than with estradiol, whereas ICI 164,384 did not stimulate, but fully antagonized the effect of estradiol. This observation is in agreement with the observation of May and Westley (24) who showed that pS2 mRNA was induced in MCF-7 cells treated with hydroxytamoxifen with an efficiency 10 times lower than with estradiol. In contrast, Westley *et al.* (25) have reported that tamoxifen did not induce pS2 gene expression in MCF-7 cells. In this latter study, tamoxifen induction may have been masked by the use of a culture medium containing phenol red, which behaves like a weak estrogen (13). We note, however, that hydroxytamoxifen did not induce the secretion of the pS2 protein by MCF-7 cells grown in a phenol red-free culture medium (26). Irrespective of these present discrepancies, it will be interesting to investigate whether the hydroxytamoxifen induction observed here can also be achieved with an isolated ERE and, therefore, reflects an intrinsic property of the human estrogen receptor in human cells, or whether it requires the enhancer/promoter "environment" present in the 5′-flank-ing region of the pS2 gene.

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