Identification of an estrogen response element upstream of the human c-*fos* gene that binds the estrogen receptor and the AP-1 transcription factor

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ABSTRACT

Transcription of the proto-oncogene c-*fos* is stimulated by 17β-estradiol in estrogen responsive human and rat cells. To understand the molecular mechanisms of estrogen regulation of c-*fos* gene transcription, the human c-*fos* gene promoter, with 2.25 Kb of 5'-flanking DNA, was cloned upstream of the bacterial CAT gene and tested for estrogen regulation by transient transfection in HeLa cells. When an expression vector coding for the human estrogen receptor was co-transfected with the *fos* -CAT reporter, the promoter was found to respond to 17β-estradiol. An element responsible for estrogen induction was mapped in a 240 bp region localized 1060 to 1300 bases upstream of the startsite of transcription of the gene. Sequence analysis revealed, clustered in a 19 bp sub-region, a sequence corresponding to an imperfectly palindromic ERE: CGGCAGCGTGACC and two sequences: CTGAG and GTGAC, homologous to the core sequence of AP-1 transcription factor binding sites. A synthetic oligonucleotide reproducing this sub-region binds 'in vitro' both the estrogen receptor and AP-1 factor(s) and confers estrogen-responsivity to the HSV-*tk* gene promoter. Transcriptional activation by the estrogen receptor is prevented by mutations in the *fos* ERE that hamper binding of the receptor *in vitro*. Activation of the c-*fos* gene promoter in HeLa cells requires the DNA binding domain of the estrogen receptor, and can be achieved independently by the TAF-1 and the TAF-2 transcriptional activation functions of this molecule. A receptor mutant lacking the hormone binding domain can activate the c-*fos* gene promoter in the absence of estrogen.

INTRODUCTION

Estrogen regulates development, growth and a variety of differentiated functions of female reproductive organs of vertebrates and is a mitogen for the uterus of adult mammals, where it stimulates growth of endometrial epithelia (1−2 and references therein). The estrogen receptor is a *trans*-acting transcription enhancer factor, that binds to *cis* -acting Estrogen Response Elements (EREs), localized in the proximity of target genes,to modulate gene expression (3−5). Structural and functional analysis of estrogen regulated genes have revealed a common, palindromic ERE, with the consensus sequence 5'-GGTCANNNTGACC-3', that is sufficient to mediate hormone-dependent induction of transcription and that binds the estrogen receptor 'in vitro' (5, 6). Also, it has been shown that most EREs are imperfectly palindromic, although in this case they are less efficient in mediating transcriptional regulation by the receptor and they bind weakly the receptor (7−10). Four members of the nuclear receptors super-family (estrogen, retinoic acid, thyroid hormone and vitamin D receptors) can activate transcription through homologs of the basic sequence GGTCA-TGACC, here named Nuclear Receptors Response Element (NRRE; 11−15). Exact spacing (3 bp) between the two hexameric halves of the palyndrome is essential for estrogen receptor action, whereas it is not required by the other receptors (12; H. de Verneuil, D. Metzger and P. Chambon, personal communication). To explain the mitogenic effect of estrogen, it was assumed that this hormone regulates the expression of genes whose products control the cell cycle. In line with this hypothesis, 17β-estradiol was found to induce, both in rat uterus and in human breast cancer cells in culture, the expression of c-*fos* and c-*myc* proto-oncogenes (16−21) and, more recently, of c-*jun* and *jun*-B genes (22; M.Scalona, E.Persico, L.Cicatiello, V.Sica, F.Bresciani and A.Weisz, manuscript in preparation). Induction

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by 17β-estradiol of c-*fos* gene expression in rat uterus is due to stimulation of transcription of this gene and is not abolished by protein synthesis inhibitors, suggesting that the receptor regulates directly c-*fos* gene promoter activity (23–24).

To understand the molecular basis of c-*fos* gene regulation by estrogen, the effect of the estrogen receptor on the expression of *fos* -CAT reporter genes containing the human c-*fos* gene promoter, with its 5'-flanking DNA up to 2250 bp 5' to the startsite of transcription, was analysed by transient transfection in HeLa cells. Transcription of the *fos* -CAT reporters is stimulated significantly by the estrogen-receptor complex only when the region of c-*fos* 5'-flanking DNA localized between positions −1300 and −850 is present. Sequence analysis of this region reveals the presence of a sequence corresponding to an imperfectly palindromic ERE: CGGCAGCGTGACC, overlapping with two sequences homologous to the core sequence of AP-1 transcription factor binding sites: CTGAG and GTGAC. An oligonucleotide reproducing all these elements binds 'in vitro' the estrogen receptor and AP-1-like factors and confers estrogen responsivity to an etherologous promoter.

This is the first demonstration that expression of a proto-oncogene is directly controlled by the estrogen receptor and may explain how this hormone regulates the early phases of the cell cycle in responsive cells.

MATERIALS AND METHODS

Construction of reporter recombinants and DNA sequence determination

Standard DNA recombinant techniques were used (25). pFC1-BL was constructed inserting the Hind III-Nae I fragment from plasmid pc-fos(human)-1 (26–27), that includes the promoter and 5'-flanking region of the human c-*fos* gene (from about position −2250 to position +41), into the Hind III to Sal I (blunt-ended with Klenow) sites of the pBL-CAT 3 vector (28). pFC2-BL, pFC3-BL, pFC4-BL and pFC8-BL were generated from pFC1-BL deleting the Hind III (−2250) to Nae I (−1400), Hind III to Bam HI (−720), Hind III to Sst II (−404) or Hind III to Apa I (−224) fragments respectively. pFC2(A), pFC2(C) and pFC2(D) were obtained by partial digestion of pFC2-BL with Apa I, followed by re-ligation, and lack the DNA fragment included between the Apa I sites at positions −1300 and −850 (A), −1300 and −224 (C) or −850 and −224 (D). pFOS-*tk* -CAT I was produced cloning the Hind III to Nae I fragment of pFC-1-BL into the Hind III to Xba I (blunt-ended) sites of pBL-CAT 2 (28). pFOS-*tk* -CAT II was produced cloning the Nae I to Bam HI fragment of pFC1-BL into the Hind III (blunt-ended) to Bam HI sites of pBL-CAT 2 and pFOS-*tk* -CAT II(A), II(B) and II(C) were derived from pFOS-*tk* -CAT II by deleting the Apa I (−1300) to Apa I (−850) region, the Apa I (−850) to Bam HI (−720) region or the Apa I (−1300) to Bam HI (−720) region respectively. pFOS-*tk* -CAT V+ and V- were constructed cloning in pBL-CAT 2 the DNA fragment located between the Apa I sites at −1300 and −850, in the natural (+) or the opposite (−) orientation respect to the *tk* promoter. pFOS-*tk* -CAT VI and VII were generated respectively by cloning the Apa I (−1300) to Pvu II (−1060) and Pvu II to Apa I (−850) fragments of pc-fos (human)-1.

The sequence of both strands of the Apa I (−1300) to Apa I (−850) fragment of the human c-*fos* gene 5'-flanking DNA was determined by chain-termination DNA sequencing (29) of single stranded DNA , using Sequenase and protocols suggested by the producer of this enzyme (USB Co., Cleveland, USA).

pFOS-ERE 1 and 3 or pFOS-EREμ 1 and 3 were obtained cloning the ds oligonucleotides A or C (reported in Table III), respectively, into the Hind III to Sph I sites of pBL-CAT 2. pFOS-NRRE 1 and 3 or pFOS-NRREμ 1 and 3 were obtained cloning the ds oligonucleotides D or G, respectively, into the Bam HI to Xba I sites of pBL-CAT 2. Positive clones were controlled by determinig the sequence of the inserted oligonucleotides.

Transient transfection and CAT assay

For transient transfection, HeLa cells were plated on day 1 at about 10–20% confluency in DMEM (without phenol red) containing 5% FCS, pre-treated with dextran-coated charcoal to remove endogenous steroids (30) and 100 U/ml penicillin/streptomycin. On day 2, fresh medium was added and cells were transfected, using the calcium phosphate-DNA co-precipitation method (31), with the indicated amount of reporter and expression vectors, 3 μg of β-galactosidase expression vector pCH 110 (Pharmacia), as internal control for transfection efficiency, and carrier DNA (Bluescribe M13+) up to 20 μg total DNA/cell culture dish. After 12 to 16 hrs, cells were washed with medium for 30 min, before addition of fresh medium (with or without 10^{-8} M 17β-estradiol as indicated) and further incubated for 24 hrs. CAT enzyme assay were performed in whole-cell extracts as described (32), after normalization for β-galactosidase activity (22). Chloramphenicol acetyltransferase activity is reported as pmoles (^{14}C)chloramphenicol acetylated/hr/unit β-galactosidase activity.

Gel retardation assay and competition experiments

HeLa cells 'whole-cell' extracts were prepared as described by Kumar and Chambon (5) in 50 mM Tris-HCl (pH 7.3 at 20°C), 600 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 25% Glycerol (v/v) and protease inhibitors (2.5 μg ea/ml of antipain, aprotinin, chymostatin, leupeptin, pepstatin and 0.5 μM PMSF). Protein concentration in the extracts was determined with a colorimetric assay (33).

Gel retardation assays were performed essentially as described (34–35). Extracts (2 to 10 μg proteins) were pre-incubated with 2 μg (ERE probe) or with 0.5 μg (all other probes) of poly(dI-dC) (Pharmacia or Boehringer-Mannheim) at 0°C in a 12 μl reaction mix containing 50 mM Tris-HCl (pH 7.3 at 20°C), 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 25% Glycerol (v/v). After 10 min, the binding reaction was initiated by adding 10 to 20 fmoles (1 to 6×10^4 cpm) of [^{32}P]-5' end-labelled ds synthetic oligonucleotide probe and incubation was carried out at 20°C for 15 min. Non-denaturing PAGE was then used to separate the free from the protein-bound probe. In the competition experiments, the indicated amounts of unlabelled probes were mixed with the labelled probe (about 1 to 2×10^4 cpm) before addition to the binding reaction.

RESULTS

Identification of an estrogen-inducible enhancer activity in the 5'-flanking region of the human c-*fos* gene

A DNA fragment containing the human c-*fos* gene promoter, with about 2250 bp of 5'-flanking DNA and 45 bp of transcribed sequence, was cloned upstream of the bacterial CAT gene, to generate the reporter plasmid pFC1-BL (Figure 1 *top*). The effect of the estrogen receptor on the expression of this reporter was

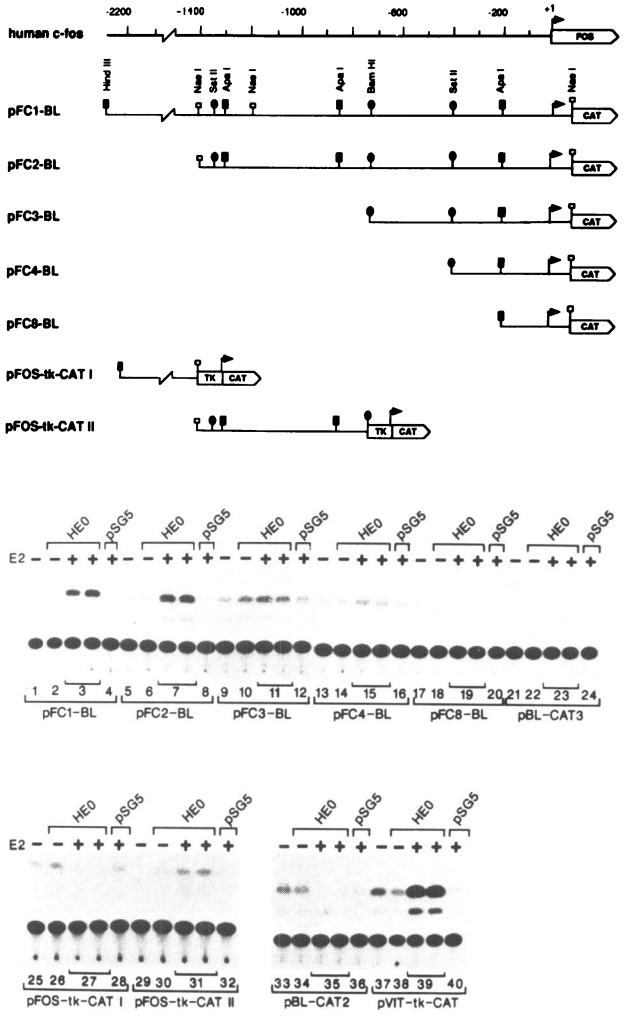

Figure 1: Identification of an estrogen-inducible enhancer activity in the 5'-flanking region of the human c-*fos* gene. *Top*, schematic description of the genomic organization of the human c-*fos* gene 5'-flanking DNA and of the *fos* -CAT reporter plasmids used. *Bottom*, effects of 17β-estradiol (E2), the estrogen receptor (HE0) or the eukaryotic expression vector pSG5 on the expression of the *fos* -CAT reporter plasmids in HeLa cells. 1 μg DNA of the indicated reporter was transfected in HeLa cells, with or without 2 μg HE0 or pSG5. CAT activity was measured in whole cell extracts from cells treated without (−) or with (+) 10^{-8} M 17β-estradiol for 24 hrs before harvesting. The data reported are representative of multiple (2 to 8), independent transfection experiments, corrected for changes in transfection efficiency as described in Methods section, and duplicate spots represent assays from two independently transfected dishes. pVIT-*tk* -CAT is an estrogen-responsive reporter that contains the ERE from the X. laevis vitellogenin A2 gene cloned upstream of the *tk* -CAT gene (39).

TABLE I: Identification of an estrogen-inducible enhancer activity in the 5'-flanking region of the human c-fos gene

REPORTER	EXP. VECTOR	E2	CAT ACTIVITY (pmoles/hr/U GAL)	R.A.	n
pFC1-BL	-	-	1.97	(1.0)	4
	HE0	-	2.17	1.1	
	HE0	+	10.07	5.1	
	pSG5	+	1.68	0.9	
pFC2-BL	-	-	2.14	(1.0)	5
	HE0	-	2.73	1.3	
	HE0	+	9.51	4.4	
	pSG5	+	1.98	0.9	
pFC3-BL	-	-	3.29	(1.0)	5
	HE0	-	4.71	1.4	
	HE0	+	5.79	1.8	
	pSG5	+	3.32	1.0	
pFC4-BL	-	-	3.35	(1.0)	4
	HE0	-	4.23	1.3	
	HE0	+	4.14	1.2	
	pSG5	+	3.37	1.0	
pFC8-BL	-	-	1.88	(1.0)	3
	HE0	-	1.68	0.9	
	HE0	+	1.26	0.8	
	pSG5	+	1.48	0.8	
pBL-CAT 3	-	-	0.19	(1.0)	2
	HE0	-	0.11	0.6	
	HE0	+	0.17	0.9	
	pSG5	+	0.15	0.8	
pFOS-tk-CAT I	-	-	0.78	(1.0)	3
	HE0	-	0.66	0.8	
	HE0	+	0.31	0.4	
	pSG5	+	0.56	0.7	
pFOS-tk-CAT II	-	-	0.14	(1.0)	8
	HE0	-	0.13	0.9	
	HE0	+	0.51	3.6	
	pSG5	+	0.12	0.9	
pBL-CAT 2	-	-	0.53	(1.0)	3
	HE0	-	0.51	1.0	
	HE0	+	0.22	0.4	
	pSG5	+	0.47	0.9	
pVIT-tk-CAT	-	-	1.68	(1.0)	2
	HE0	-	1.60	1.0	
	HE0	+	19.96	11.9	
	pSG5	+	1.32	0.8	

R.A.: relative activity, n: number of experiments.
For experimental details see Legend to Figure 1.

tested by transient transfection in HeLa cells. Where specified, the plasmid HE0, containing the human estrogen receptor cDNA cloned in the eucaryotic expression vector pSG5 (36), was co-transfected, since these cells lack detectable estrogen receptor. The result of a representative transfection experiment is reported in Figure 1 (*bottom*: lanes 1 to 4) to show that estrogen (+E2) stimulates significantly the expression of pFC1-BL when the estrogen receptor (HE0) is also present (compare lane 1 with lanes 3). Maximal stimulation was obtained with 1.5 to 2.0 μg of HE0 plasmid/cell culture dish (data not shown). HE0 alone, in the absence of 17β-estradiol (-E2), slightly affects expression of the test gene (compare lanes 1 and 2). This is probably due to low amount of estrogen still present in the charcoal-treated serum, although stimulation by estrogen-free receptor cannot be excluded. Changes in chloramphenicol acetyltransferase activity were quantified by measuring the amount of acetylated chloramphenicol in all samples, after correction for changes in transfection efficiency, and a summary of the results obtained in several transfection experiments are reported in Table I. This shows that the c-*fos* gene promoter in pFC1-BL is active in HeLa cells under the conditions used for this study (compare with pBL-CAT 3), and that the estrogen-receptor complex stimulates about 5 fold the expression of the reporter gene. Interestingly, this result is in line with the observation that estrogen administration is followed by an about 4 to 6 fold activation of c-*fos* gene transcription in rat uterus *in vivo* (24).

To map the location of the estrogen-inducible element(s) within the c-*fos* gene 5'-flanking DNA, a set of progressive 5' to 3' deletions of the *fos* sequence in pFC1-BL were generated, and the corresponding reporters were tested for estrogen responsivity in HeLa cells (Figure 1 and Table I). Removal of 5'-flanking DNA localized between positions −2250 and −1400 (plasmid pFC2-BL) has no effect on the response to estrogen (compare lanes 5 and 7), whereas deletion of the region localized between positions −1400 and −720 (plasmid pFC3-BL) reduces it significantly (compare lanes 9 and 11). A deletion to position −404 has no further effect (plasmid pFC4-BL; compare lanes 13 and 15), whereas a deletion to position −224 results in the complete loss of estrogen inducibility (plasmid pFC8-BL; compare lanes 17 and 19). This observation is confirmed by another series of experiments, in which c-*fos* 5'-flanking DNA

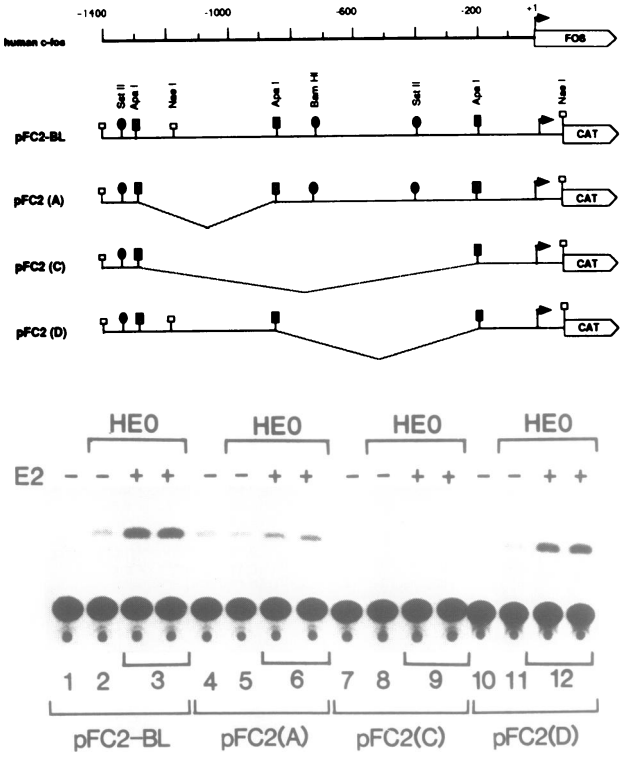

Figure 2: Localization of the estrogen-inducible enhancer activity in the 5'-flanking region of the human c-*fos* gene. *Top*, schematic description of pFC2-BL and of deletion mutants of this reporter that lack different regions of the human c-*fos* gene 5'-flanking DNA. *Bottom*, effect of the estrogen-receptor complex (HE0) on the expression of the *fos* -CAT reporter plasmids in HeLa cells. 1 μg DNA of pFC2-BL, pFC2(A) and pFC2(C) or 5 μg of pFC2(D) was transfected in HeLa cells, with or without 2 μg HE0 expression vector. Cells were treated without (−) or with (+) 10^{-8} M 17β-estradiol for 24 hrs before harvesting and CAT activity was measured in whole cell extracts, after correction for transfection efficiency. The data reported are representative of 3 to 5 independent transfection experiments, and duplicate spots represent assays from two independently transfected dishes.

TABLE II: Localization of the estrogen-inducible enhancer activity in the 5'-flanking region of the human c-fos gene

REPORTER (µg/dish)	HE0	E2	CAT ACTIVITY (pmoles/hr/U GAL)	RELATIVE ACTIVITY
pFC2-BL (1µg)	-	-	2.19	(1.0)
	+	-	3.63	1.7
	+	+	11.49	5.2
pFC2(A) (1µg)	-	-	3.81	1.7
	+	-	4.22	1.9
	+	+	4.66	2.1
pFC2(C) (1µg)	-	-	2.00	0.9
	+	-	2.11	1.0
	+	+	2.15	1.0
pFC2(D) (5µg)	-	-	1.19	(1.0)
	+	-	1.73	1.5
	+	+	4.48	3.8
pFOS-TK-CAT II (5µg)	-	-	0.65	(1.0)
	+	-	0.70	1.1
	+	+	1.90	2.9
pFOS-TK-CAT II(A) (5µg)	-	-	1.60	2.5
	+	-	1.35	2.1
	+	+	0.85	1.3
pFOS-TK-CAT II(B) (5µg)	-	-	0.85	1.3
	+	-	0.75	1.2
	+	+	1.60	2.5
pFOS-TK-CAT II(C) (5µg)	-	-	1.35	2.1
	+	-	0.50	0.8
	+	+	0.75	1.2
pFOS-TK-CAT V- (5µg)	-	-	0.75	(1.0)
	+	-	0.67	0.9
	+	+	1.89	2.5
pFOS-TK-CAT V+ (5µg)	-	-	0.71	(1.0)
	+	-	0.90	1.3
	+	+	2.43	3.4
pFOS-TK-CAT VI (5µg)	-	-	0.80	(1.0)
	+	-	0.75	0.9
	+	+	2.67	3.3
pFOS-TK-CAT VII (5µg)	-	-	0.53	(1.0)
	+	-	0.68	1.3
	+	+	0.89	1.7

Representative of results obtained in 2 to 5 transfection experiments.
For a description of pFC2(A), pFC2(C) and pFC2(D) deletion mutants see Figure 2.
For a description of pFOS-tk-CAT V-, pFOS-tk-CAT V+, pFOS-tk-CAT VI and pFOS-tk-CAT VII see Figure 4.
pFOS-tk-CAT II(A), pFOS-tk-CAT II(B) and pFOS-tk-CAT II(C) are deletion mutants of pFOS-tk-CAT II obtained by deleting the Apa I (-1300) to Apa I (-850) region, the Apa I (-850) to Bam HI (-720) region or the Apa I (-1300) to Bam HI (-720) region respectively.

from position −1400 to position −720 was cloned upstream of the HSV-*tk* gene promoter-CAT in pBL-CAT 2 (28), to generate the reporter pFOS-*tk* -CAT II (Figure 1 *top*). Activity of the *tk* promoter from this test gene is stimulated by the estrogen-receptor complex 3 to 4 fold (compare lanes 29 and 31 in Figure 1 *bottom* and see Table I). This relatively low level of induction was reproducible and confirmed in several, independent experiments. For comparison, in Figure 1 and Table I are also reported the results obtained with the estrogen-responsive reporter pVIT-*tk* -CAT, containing the ERE from the X. laevis vitellogenin A2 gene (6, 37). This test gene is also inducible by the estrogen-receptor complex under the experimental conditions used (10 to 12 fold, compare lanes 37 with 39 in Figure 1). When 1μg of pVIT-*tk* -CAT or 5 μg of pFOS-*tk* -CAT II was used, maximal stimulation was obtained with 25 to 50 ng and 250 to 500 ng of HE0 respectively (data not shown), suggesting a different sensitivity to the estrogen receptor of the putative *fos* ERE, compared with the vitellogenin ERE. The response to HE0 of the reporters containing the c-*fos* 5'-flanking region from position −404 to position −224 is somehow erratic and not always reproducible, is low and only weakly enhanced by 17β-estradiol in HeLa cells (Table I) and is better seen when higher concentrations of HE0 (up to 12 μg plasmid/dish) are used. It is possible that this effect is artifactual, due to interference by

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                10                  20                  30                  40                  50                  60
GGGCCCGAAG AGTGGAGAAG GGAAGACCGG GGCTGTGCGG GGACATGCGT CTTCGCGCCC

                70                  80                  90                 100                 110                 120
TGGAGGTGGC CAGCGCGCTG GGCTGAGCCG GCAGCGTGAC CCCGGCTGTC CTACGCAGCA

               130                 140                 150                 160                 170                 180
GGGCAGGAGA TTGGGGGGCG TGGCACACTC TGGAGCACCT TGCCTCCCCA AAGCCCCGTG

               190                 200                 210                 220                 230                 240
TTCCAGGACG TGGAGCCGCG CCTGGGGTCC CAGCAGTCGA GGTATTCCGC CCAGGCGCAG

               250                 260                 270                 280                 290                 300
CTGGACACTG TCCTTCCAGC CCCCGTCCTC CACCCTCCAA GTCCGCGCTG GAAAATCACC

               310                 320                 330                 340                 350                 360
CGCGTCGGCT CCCGTAAGCA CAGCTTCTGG CGGGACCGAA CCAGCCCTCA GCGCAGATTT

               370                 380                 390                 400                 410                 420
GAGTTCCCCG CAGGAAGCAC ACCCCGCCTT GTCATCCCGA ACTGACCACC CTGCCCACAT

               430                 440                 450
AACCACACCT CGCACTCCCT ACCCCTGGGG CCC
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Figure 3: Nucleotide sequence of the −1300 to −850 region of the human c-*fos* gene. The sequence of the sense strand is reported in the 5' to 3' direction (left to right). Nucleotide 1 correspond to about position −1300. The nucleotides corresponding to imperfectly palindromic sequences homologous to the Estrogen Response Element (nucleotides 90 to 101) and to Nuclear Receptors Response Elements (nucleotides 391 to 407) are overlined; the sequences homologous to the 'core' sequence of AP-1 binding sites are underlined. Boxed bases correspond to the Pvu II restriction site used for further cloning.

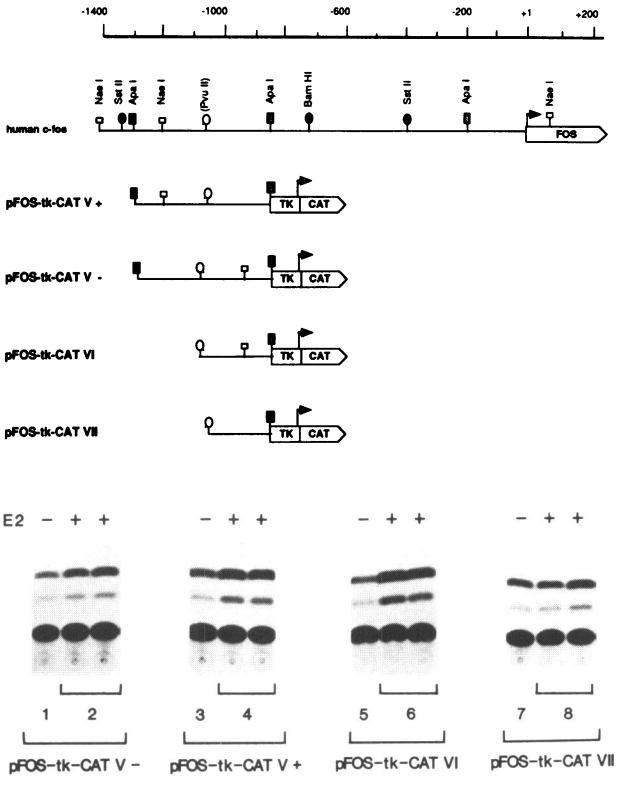

Figure 4: The ERE of the human c-*fos* gene is localized between about positions −1300 and −1060. *Top*, schematic description of the genomic organization of the human c-*fos* gene 5′-flanking DNA and of the *tk* promoter-CAT reporter plasmids used. Respect to the promoter, the DNAs from c-*fos* are in the natural orientation in pFOS-*tk* -CAT V+ and pFOS-*tk* -CAT VI and in the reverse orientation in pFOS-*tk* -CAT V- and pFOS-*tk* -CAT VII. *Bottom*, effect of the estrogen-receptor complex on the expression of these CAT reporter plasmids in HeLa cells. 5 μg DNA of the indicated reporter was transfected in HeLa cells, with 0.5 μg of HE0 expression vector. Where specified (+E2) cells were treated with 10^{-8} M 17β-estradiol for 24 hrs before harvesting and CAT activity was measured in whole cell extracts. The data reported are representative of 4 to 5 independent transfection experiments, corrected for changes in transfection efficiency, and duplicate spots represent assays from two independently transfected dishes.

TABLE III: Double-stranded oligonucleotides used for 'in vivo' and 'in vitro' studies

A	hFOS ERE	5′ - AGCTTGGGCTGAGCCGGCAGCGTGACCCCGCATG - 3′ ACCCGACTCGGCCGTCGCACTGGGGC
B	hFOS ERE μ I	5′ - AGCTTGGGCTGAGCCGGCAGCGACACCCCGCATG - 3′ ACCCGACTCGGCCGTCGCTGTGGGGC
C	hFOS ERE μ II	5′ - AGCTTGGGCCGAGCCGGCAGCGTTACCCCGCATG - 3′ ACCCGGCTCGGCCGTCGCAATGGGGC
D	hFOS NRRE	5′ - CTAGAGCCTTGTCATCCCGAACTGACCACCCTG TCGGAACAGTAGGGCTTGACTGGTGGGACCTAG - 5′
E	hFOS NRRE μ I	5′ - CTAGAGCCTTGTCATCCCGAACACACCACCCTG TCGGAACAGTAGGGCTTGTGTGGTGGGACCTAG - 5′
F	hFOS NRRE μ II	5′ - CTAGAGCCTTGTGTTCCCGAACTGACCACCCTG TCGGAACACAAGGGCTTGACTGGTGGGACCTAG - 5′
G	hFOS NRRE μ III	5′ - CTAGAGCCTTGTGATCCCGAACGGACCACCCTG TCGGAACACTAGGGCTTGCCTGGTGGGACCTAG - 5′
	ERE (xVIT A2)	5′ - GGGAGTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTTACCC CCCTCAGGTTTCAGTCCAGTGTCACTGGACTAGTTTCAATGGG - 5′
	AP-1 (hMT IIA)	5′ - TCGGGGAAGTGACTCAGCGC CCTTCACTGAGTCGCGAGCC - 5′
	AP-1 μ	5′ - TCGGGGAAGTGCAGCAGCGC CCTTCACGTCGTCGCGAGCC - 5′

hFOS oligonucleotides: numbers refer to the position within the sequence reported in Figure 3.
xVIT A2: X. laevis vitellogenin A2 gene; hMT IIA: human metallothionein IIA gene. Negative numbers refer to the position respect to the startsite of transcription (+1).
Sequences corresponding or homologous to Estrogen Response Elements (EREs) are overlined.
Sequences corresponding or homologous to AP-1 binding sites are underlined.
Boxed base pairs are mutations.

the receptor on the activity of factors that affects the function of this promoter.

To further define the upstream elements responsible for estrogen regulation of the *fos* -CAT hybrid gene in HeLa cells, the reporters pFC2(A), pFC2(C) and pFC2(D), with different internal deletions of the *fos* sequence present in pFC2-BL (deletion from position −1300 to −850, −1300 to −224 or −850 to −224 respectively (Figure 2, *top*) were constructed and tested for estrogen responsivity. The results from a representative transfection experiment are reported in Figure 2 (*bottom*) and the data, resumptive of results obtained in several independent transfections, are shown in Table II. The major estrogen-inducible enhancer activity can be mapped in the 5′ flanking DNA localized between positions −1300 and −850, since only the reporters pFC2-BL and pFC2(D), that include this region, are clearly responsive (compare lanes 1 and 3, 10 and 12 in Figure 2 and see Table II). This result is confirmed with the *fos* DNA in the reporter pFOS-*tk* -CAT II, since internal deletions that remove the −1300 to −850 region result, here also, in the loss of estrogen inducibility (see pFOS-*tk* -CAT II(A) and II(C) in Table II).

The experiments reported in Figures 1 and 2 and in Tables I and II reveal also that the human c-*fos* gene 5′-flanking DNA localized between position −1300 and position −850 has a negative effect on the activity of the *fos* promoter in HeLa cells, when present in the *fos*-CAT reporters. In fact, the basal activity of pFC3-BL, pFC4-BL or pFC2(A) is about the double of that of pFC1-BL or pFC2-BL (compare lanes 9 and 13 with lanes 1 and 5 in Figure 1, lane 1 with lane 4 in Figure 2 and see Tables I and II). This negative effect on promoter activity is reproducible in a large number of separate transfection experiments and is more evident when the DNA from −850 and −224 is deleted, since the basal expression decreases in this case more than 8 fold, under comparable conditions (1μg reporter/dish, compare pFC2(D) with pFC2(C) in Table II and with pFC8-BL in Table I). The same is true for the *tk* promoter, whose basal activity is reduced 2.5 to 4 fold (compare lanes 29 and 33 in Figure 1, pFOS-*tk* -CAT II with pBL-CAT 2 in Table I and compare pFOS-*tk* -CAT II with pFOS-*tk* -CAT II(A) and II(C) in Table II). The negative effect on *tk* promoter activity of the c-*fos* sequences located between positions −1300 and −850 is specific, since it do not occurs with the c-*fos* DNA regions from position −2250 to −1400, −1400 to −1300 or −850 to −720 (see pFOS-*tk* -CAT I, II(A) and II(C) in Tables I and II) or with the vitellogenin ERE DNA (pVIT-*tk* -CAT in Table I), and is independent of the site of insertion within the pBL-CAT 2 polylinker and from the orientation respect to the promoter (see pFOS-*tk* -CAT V+ and V− in Table II). The possible significance of this finding is discussed below.

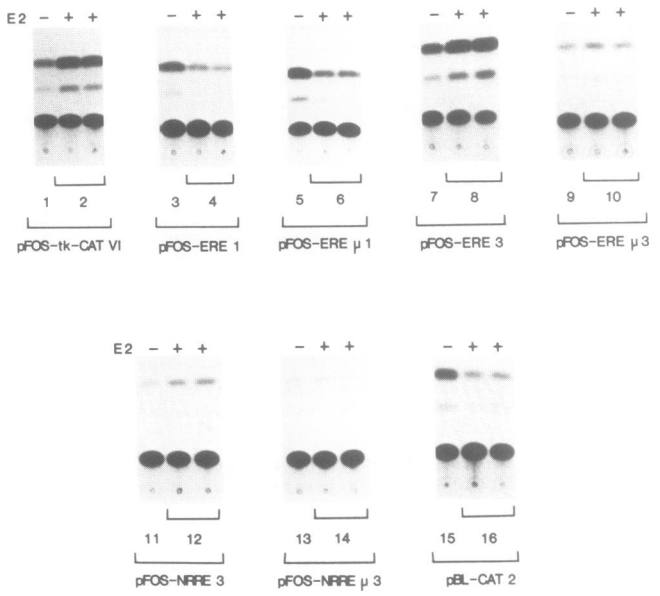

Figure 5: Multiple copies of oligonucleotides reproducing the ERE of the human c-*fos* gene confer estrogen inducibility to the HSV-*tk* gene promoter. The structure of pFOS-*tk* -CAT VI is reported in the legend to Figure 4. pFOS-ERE 1, pFOS-EREμ 1, pFOS-NRRE 1 or pFOS-NRREμ 1 contain each one copy of oligonucleotides A, C, D or G reported in Table III, cloned upstream of the *tk* promoter in the vector pBL-CAT 2. pFOS-ERE 3, pFOS-EREμ 3, pFOS-NRRE 3 or pFOS-NRREμ 3 contain three, head-to-tail, copies of the same oligonucleotides. For transfection, 5 μg DNA of the indicated reporter was transfected in HeLa cells with 0.5 μg of HE0 expression vector. Where indicated (+E2) cells were treated with 10^{-8} M 17β-estradiol for 24 hrs before harvesting and CAT activity was measured in whole cell extracts. The data reported are representative of 2 to 3 independent transfection experiments and are corrected for changes in transfection efficiency. Duplicate spots represent assays from two independently transfected dishes.

TABLE IV: Activity of different human estrogen receptor mutants on the Estrogen Response Element of the human c-fos gene

hER MUTANT (deleted A-As)	A/B D.	D.B.D.	H.B.D.	E2	CAT ACTIVITY (pmol/hr/U GAL) pFC2-BL (1μg/dish)	pFC2(D) (5μg/dish)	pFOS-tk-CAT II (5μg/dish)
HE0	+	+	+	-	2.63	1.42	0.85
				+	14.52	4.45	2.05
HE11 (185 - 251)	+	-	+	-	2.73	1.87	0.80
				+	2.22	1.19	0.51
HE15 (282 - 595)	+	+	-	-	7.06	3.04	1.00
HE19 (1 - 178)	-	+	+	-	2.40	1.04	0.85
				+	6.88	3.25	2.45
HE72 (1 - 159 & 316 - 595)	-	+	-	-	-	0.81	-
ER-GR CAS 1 (185 - 250*)	+	GR-R*	+	-	-	0.67	-
				+	-	0.55	-

Representative of results obtained in 2 to 3 transfection experiments.
For a description of reporters pFC2-BL, pFC2(D) and pFOS-tk-CAT II see Figures 1 and 2.
For a detailed description of the human estrogen receptor mutants see Ref. 37.
A/B D.: A and B domains, D. B. D.: DNA binding domain, H. B. D.: hormone binding domain.

The 5'-flanking region of the human c-*fos* gene comprises an imperfectly palindromic Estrogen Response Element that is functional in HeLa cells

The nucleotide sequence of the estrogen-responsive 5'-flanking region of the human c-*fos* gene, localized between the two Apa I restriction endonuclease sites at about positions −1300 and −850 (see Figure 1), was determined and is reported in Figure 3. Analysis of this sequence reveals two elements homologous to known EREs (overlined in Figure 3). The first, localized

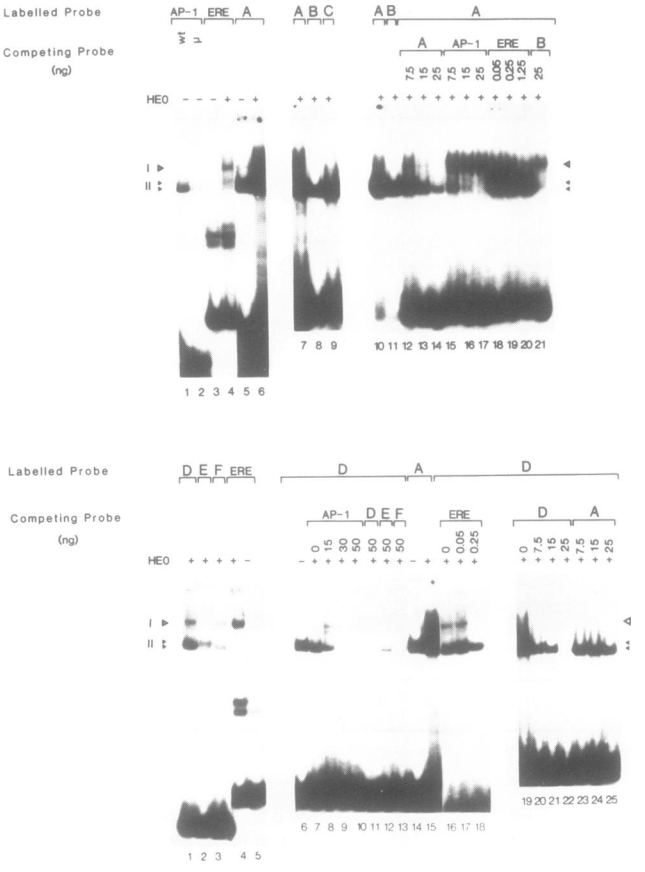

Figure 6: The human c-*fos* gene ERE binds 'in vitro' the estrogen receptor and AP-1 transcription factors. Whole-cell extracts of HeLa cells, transfected with the human estrogen receptor expression vector HE0 (+) or with the parental expression vector pSG5 (−) and treated with 17β-estradiol as reported in Methods, were prepared and incubated with the indicated radiolabelled probes. The sequence of the oligonucleotide probes used is reported in Table III. The free and bound oligonucleotides were resolved in a low ionic strength, non denaturing 4% polyacrylamide gel and visualized by autoradiography. The bands visible at the bottom of each autoradiograph correspond to the free probe, whereas the bands corresponding to specifically retarded probe are indicated. Where specified, the indicated amounts of unlabelled competitor probe were also added during the binding reaction.

between nucleotides 89 and 101, corresponding approximately to positions −1211 and −1199, is characterized by an imperfect palindrome with a 3 bp spacing between its two halves. The second element, localized between nucleotides 390 and 407 (about −910 to −893), is also an imperfect palindrome but with 8 bp in between. Based on the number of spacing nucleotides, the first element is the site for regulation by the estrogen receptor (ERE), whereas the second element is a potential site for regulation by other members of the nuclear receptors super-family (NRRE). The DNA sequence around and within each of these two palyndromic elements shows another distinctive feature: two sequences, in each case, homologous of the core sequence of AP-1 transcription factors binding sites (38−40; underlined in Figure 3).

To define the contribution of each of these two potential REs to the stimulation of *tk* promoter activity by the estrogen receptor, the two c-*fos* upstream sub-regions, from position −1300 to −1060 (sub-region D) or from position −1059 to −850 (sub-region P), were cloned, together or separately, upstream of the *tk* -CAT gene in pBL-CAT 2, to generate the reporters pFOS-*tk*-CAT V +, V −, VI and VII respectively (Figure 4 *top*). The

results, representative of multiple transfection experiments performed with these reporters, are reported in Figure 4 (*bottom*), to show that the upstream region identified previously mediates estrogen induction independently of its orientation respect to the promoter (compare lanes 1 and 2, 3 and 4 and pFOS-*tk*-CAT V+ and V− in Table II). Moreover, the element within sub-region D can mediate estrogen induction to the same extent with or without that in sub-region P (compare lanes 5 and 6 with lanes 1 and 2 in Figure 4 and pFOS-*tk*-CAT VI with pFOS-*tk*-CAT V in Table II), whereas this last is only weakly responsive to estrogen (compare lanes 7 and 8 with lanes 1 and 2 in Figure 4 and pFOS-*tk*-CAT VII with pFOS-*tk*-CAT V in Table II).

Oligonucleotides reproducing the sequence of the putative ERE or NRRE from the c-*fos* gene were synthesized (A and D in Table III) and cloned upstream of the *tk* -CAT gene, either in single copy (pFOS-ERE 1), in three, head-to-tail, copies (pFOS-ERE 3). When tested for estrogen responsivity (Figure 5), only the hybrid genes containing multiple copies of the ERE-homologous, imperfectly palindromic element from sub-region D are responsive to estrogen (2.5 to 4.5 fold induction). The fact that only multiple copies of this ERE respond to the estrogen receptor is not surprising, since it is known that imperfectly palyndromic EREs can be un-responsive to the estrogen receptor, but cooperate efficiently with each other when present in multiple copies upstream of test promoters (7, 10; M. Ponglikitmongkol, J. H. White and P. Chambon, personal communication) and that, in general, single copies of oligonucleotides reproducing enhancer elements are much less efficient than multiple copies of the same in activating transcription. Mutation of one of the 5 nucleotides identical to the vitellogenin A2 ERE half palyndrome (oligonucleotide C in Table III) results in the complete loss of estrogen responsivity (pFOS-EREμ 3).

The oligonucleotide D, corresponding to the NRRE-homologous element from sub-region P (pFOS-NRRE 3) is, instead, inefficient in mediating a transcriptional response to estrogen, even when present in three copies in the reporter, confirming that the major, functional ERE of the c-*fos* gene is localized in sub-region D, between about positions −1211 and −1199.

Activation of the c-*fos* gene promoter by the estrogen receptor requires the DNA Binding Domain and can be achieved independently by the Transcriptional Activation Functions 1 and 2 of the receptor molecule

The estrogen receptor comprises three, characterized functional domains: the first, within the A/B region of the receptor molecule (between aminoacid residues 1 and 180, A/B D.), contains a constitutive Transcriptional Activation Function (TAF-2) that exhibits cell specificity and, in HeLa cells, promoter specificity; the second, within the C region (residues 181 to 263), is the DNA Binding Domain (D.B.D.), responsible for recognition of the ERE; the third, within the E region (residues 303 to 552), is the Hormone Binding Domain (H.B.D.), responsible for binding of estrogen and for hormone-inducible transcriptional activation mediated by the TAF-1 (41 and references therein). To define the domain(s) of the estrogen receptor molecule involved in transcriptional activation of the c-*fos* gene, the activity of deletion mutants of the human estrogen receptor (37) lacking either the A/B domain (HE19), the D.B.D. (HE11) or the H.B.D. (HE15), was tested on pFC2-BL, pFC2(D) and pFOS-*tk* -CAT II reporters. The results, reported in Table IV, show that expression of all reporters is inducible only when the D.B.D. of the estrogen receptor is present in the mutant, since HE11 is unable to activate transcription. This is confirmed by the mutant ER-GR-CAS 1, where the D.B.D. of the estrogen receptor is substituted for that of the human glucocorticoid receptor, since this mutant is also unactive on these reporters. The D.B.D. alone is, however, not sufficient to activate transcription (see receptor mutant HE 72), suggesting that the transcriptional activation functions of the receptor molecule are also necessary. Both the A/B and H.B. domains can activate transcription from the c-*fos* gene promoter, independently from each other, but both are required to achieve full transcriptional stimulation (compare in Table IV the activity of HE0 with that of HE15 and HE19 on pFC2-BL or pFC2(D)). On the contrary, only the H.B.D. can activate transcription from the *tk* promoter (compare the activity of the mutants HE15 and HE19 on pFOS-*tk* -CAT II). Taken together, these results suggest that binding of the estrogen receptor to the ERE is a prerequisite for activation of transcription of the c-*fos* gene and that both transcriptional activation functions of the receptor enhance the activity of this promoter in HeLa cells.

The human c-*fos* gene ERE binds *in vitro* the estrogen receptor and AP-1 transcription factors

The interaction of the estrogen receptor with the *fos* ERE was studied *in vitro* using a gel retardation (band-shift) assay and ^{32}P-labelled oligonucleotides reproducing the *fos* sequence between about positions −1221 and −1196 (nucleotides 79 to 104 in Figure 3; ds-oligonucleotide A in Table III) as binding probes. As a source of estrogen receptor, HeLa cells were transfected with HE0 and whole cell extracts were made from transfected cells exposed for 1 hr to 17β-estradiol prior to collection. Receptor-free extracts, made from cells transfected with the expression vector pSG5 and treated the same way, were used as a control. The results reported in Figure 6 (top panel) shows that two retarded bands (I and II) are present when labelled oligonucleotide A is incubated with extracts from HE0-transfected cells (+HE0; lanes 6, 7 and 10), whereas only one retarded band (band II) is found with control extracts (−HE0, lane 5), suggesting that two different complexes can form with this probe under these conditions, one of which (corresponding to band I) is due to the presence of the estrogen receptor. The intensity of band I is variable when extracts from different transfections are used (compare it in lanes 6, 7 and 10), whereas the intensity of band II is more constant (compare it in lanes 5, 6, 7 and 10). The size of the complex corresponding to band I is identical to that of the complex formed by the estrogen receptor with an oligonucleotide reproducing the vitellogenin ERE (probe ERE in Table III; upper band in lane 4 of Figure 6). These data suggests that complex I could be due to the estrogen receptor bound to probe A. To further investigate this possibility, two mutants of the ERE-homologous sequence present in this probe were tested: the first has two base changes in the *fos* ERE-like sequence, within the 5 nucleotides identical to the vitellogenin ERE (probe B), whereas the second (probe C) has only one base change in this sequence and is unresponsive to the estrogen receptor *in vivo* (see Figure 5 and Table IV). With either mutant, only the faster-migrating band II is still present (lanes 8, 9 and 11) and excess of unlabelled mutant B oligonucleotide prevents formation of band II but not of band I (lane 21) with labelled probe A. A competition experiment was also performed using increasing concentrations of un-labelled ERE oligonucleotide against a fixed concentration of labelled probe A and the result shows that the ERE probe competes efficiently for band I but not for band II (lanes 18 to 20), demonstrating that the complex corresponding to band I is due to estrogen receptor bound to the

fos probe. Comparison of the amount of ERE oligonucleotide required for reducing by about 50% the intensity of band I and the amount of A oligonucleotide required to achieve the same result, shows that the relative affinity of the receptor for the *fos* ERE is about 1/8 to 1/10 of that for the vitellogenin ERE (compare lanes 12 and 13 with lanes 19 and 20). This result was confirmed using radioactive ERE and cold A probes (data not shown). Analysis of the sequence present in probe A revealed two areas of homology with the core sequence of AP-1 transcription factors binding sites (underlined in Table III). Since AP-1 are present in HeLa cells, the question was asked if the complex corresponding to band II is actually due to AP-1 interacting with this oligonucleotide. The size of this complex corresponds, in fact, to that formed by AP-1 with an oligonucleotide containing the TRE from the human metallothionein IIA (MT IIA) gene (39; probe AP-1, lane 1). Competition between labelled probe A and un-labelled AP-1 probe (lanes 15 to 17) shows that the AP-1 oligonucleotide can disrupt the complex corresponding to band II, but not that corresponding to band I, and that the AP-1 oligonucleotide binds about 3 to 5 fold better than the *fos* oligonucleotide the protein(s) within band II (compare lanes 12 to 14 with lanes 15 to 17). When a reverse competition (labelled AP-1 vs. un-labelled probe A) was done, a comparable result was obtained (data not shown). These results support the hypothesis that the complex in band II is due to binding of AP-1, or closely related factors, to the *fos* upstream sequence reproduced in oligonucleotide A. It is worth noting that 3 mutations in the AP-1 binding site of the MT IIA TRE (oligonucleotide AP-1μ) do not abolish completely binding of AP-1, since about 20% of the retarded band is still present (compare lanes 1 and 2). Moreover, a careful examination of band II allows to distinguish two, closely spaced, bands of different intensity (marked with two small arrows in Figure 6 and better visible in lane 2). These data are in agreement with previously published results that suggests that recognition by this transcriptional regulator of its cognate DNA element 'in vitro' do not have a strict sequence requirement and that different AP-1s can interact with variants of this sequence (42–43 and references therein). This could explain the fact that single mutations within each of the two AP-1-like sequences (as in oligonucleotide C), or a double mutation within one of these sequences (as in oligonucleotide B) do not prevent completely formation of the retarded band II with the *fos* derived oligonucleotide (lanes 8, 9 and 11).

The lower panel in Figure 6 shows also the results of gel retardation analysis of the complexes formed with the NRRE-homologous sequence from sub-region P (oligonucleotides D in Table III). Even in this case two retarded bands are visible, that migrate in the gel at the same position of bands I and II observed with oligonucleotide A (compare lanes 1, 7, 15 and 19 with lane 15). The complex corresponding to band I is due to binding of the estrogen receptor to probe D, since this band is present, with variable intensity (compare lanes 1, 7, 16 and 19), only when extracts from HE0-transfected cells are used (compare lane 6 with lane 7 or 16), is competed by un-labelled ERE and A oligonucleotides (compare lane 16 with 18 and lane 19 with 23) and is abolished by mutations within the 5 nucleotides identical to the vitellogenin ERE half palyndrome (probe E; lane 2). The relative affinity of the receptor for this sequence is about 20 to 30 fold lower than that for the vitellogenin ERE, and 3 to 5 fold lower than that for the *fos* ERE (compare lane 19 with 20 and lane 16 with 18, and data not shown). Also this oligonucleotide binds AP-1, since band II can be efficiently competed by excess unlabelled AP-1 probe (compare lane 7 with lanes 8 to 10). Separate mutations of each of the two sequences homologous to AP-1 binding sites (oligonucleotides E and F) reduces the intensity of band II and allows to distinguish, within this band, two components of different size (compare lanes 1, 2 and 3).

DISCUSSION

Estrogen is a mitogen for target cells of female reproductive organs, where it stimulates transcription of the proto-oncogene c-*fos* , followed by DNA synthesis and cell proliferation (22 and references therein). We now present evidence that the transcriptional effect of the hormone is, at least in part, due to direct interaction of the estrogen-receptor complex with a regulatory element of c-*fos* . This is supported by the following evidences: a) it can be reproduced *in vitro* using the *fos* promoter and cloned estrogen receptor; b) it is mediated by an ERE localized within the 5'-flanking region of this gene, between about positions −1221 and −1196, that has enhancer characteristics since it confers estrogen responsivity to an heterologous promoter irrespective of its distance and orientation; c) the ERE binds the estrogen receptor *in vitro*; d) mutations within the ERE that abolish binding of the receptor *in vitro* prevent transcriptional activation by estrogen *in vivo*; e) mutants of the human estrogen receptor that lack the DNA Binding Domain fail to activate transcription of this gene. The *fos* ERE is an imperfectly palyndromic element, different from the perfectly palindromic ERE of the X. laevis vitellogenin A2 gene in that it requires about 10 times more receptor (HE0) to activate transcription *in vivo* and binds 8 to 10 times less strongly the estrogen receptor *in vitro*, showing also a lower transcriptional response to the hormone. A similar observation was made with the human pS2 gene ERE, also imperfectly palindromic and less sensitive to the receptor in transfection experiments (9, 37). The presence of a low affinity ERE regulating the *fos* gene is probably related to the type of transcriptional response to estrogen that is required in this case. In fact, the response of c-*fos* to estrogen stimulation differs from that of other estrogen-responsive genes. In rat uterus, in the presence of continous estrogenic stimulus after a first increase in transcription, during the early G1 phase of the cell cycle, the gene become refractory to the hormone (24). At the same time, the concentration of transcriptionally active estrogen-receptor complexes increases within the first 2 hrs and than decreases, to a level that is about 1/4 of the peak level (44). This could result in the dissociation of the weakly bound receptor from the c-*fos* ERE, with the consequent reduction of transcription of the gene.

Functional analysis of imperfectly palindromic EREs have shown that often they are virtually inactive in isolation, but they can synergize with each other (7, 10) or with other enhancer factors, like for example JUN and AP-1 in the case of the pS2 gene (45). The *fos* region that includes the ERE shows two areas of homology with the core sequence of AP-1 transcription factors binding sites, one of which overlaps with the ERE, and oligonucleotides reproducing these sequences binds *in vitro* AP-1 factors. AP-1 is a composite transcription factor, that can be formed by a repertoire of protein complexes including also FOS, JUN-C and JUN-B (43, 46). We have shown that also c-*jun* and *jun* -B gene transcription is rapidly and transiently stimulated by estrogen in rat uterus at the same time of c-*fos* (22 and M. Scalona, E. Persico, L. Cicatiello, V. Sica, F. Bresciani and A.

Weisz, manuscript in preparation). Since FOS and JUN-C, separately or in combination with each other, are able to bring about transcriptional activation and transcriptional repression of c-*fos* gene promoter activity (46 and references therein; 47), and JUN-B can be a transcriptional repressor (48–49), one can postulate that the AP-1 binding sites identified near and within the *fos* ERE are involved, perhaps all and with opposite effects, in the regulation of transcription of this gene by estrogen. The interaction of multiple factors, having opposite transcriptional effects, with this ERE is the probable explanation for the relatively low level of induction observed in transfection experiments (this work) and *in vivo* (21, 24), since the overall induction depends on the equilibrium reached between the different factors and, in the transfection experiments reported, cellular AP-1 could quench the stimulatory effect of the transfected estrogen receptor on its Response Element. Schule et al. (50) have recently described a similar antagonism between the vitamin D or the retinoic acid receptors and AP-1 on the human osteocalcin gene NRRE. The *fos* upstream region includes also a putative NRRE, that is unresponsive to the estrogen receptor but that could be recognized by other members of the nuclear receptors super-family (retinoic acid, vitamin D and thyroid hormone receptors). In support of this possibility, vitamin D_3 was found to induce rapidly (within 3 minutes) and transiently c-*fos* gene transcription in U937 and HL-60 cells (51); also, during retinoic acid-induced differentiation of P19 and HL-60 cells, expression of the *fos* gene is increased (52–53).

The basal activity of both *fos* and *tk* promoters is reduced by element(s) present within the −1300 to −850 region of the human c-*fos* gene. This effect is independent of the distance and of the orientation respect to the promoter, suggesting that it could be the result of the interaction of negative-acting factor(s), present in HeLa cells, with regulatory element(s) of the c-*fos* gene upstream region. These negative acting element(s) cannot be clearly mapped in one of the two sub-regions D and P defined above, since both sub-regions exert the same effect on the activity of the *tk* promoter. Comparison of the sequence of the two sub-regions have revealed two distinct features: the high GC content (71% and 65% respectively) and the presence of AP-1 binding sites. A human factor, GCF, that binds to GC-rich sequences and represses transcription, have been identified and cloned (54) and is a possible candidate for *trans* -repression of the reporters containing these *fos* upstream sequences. For the reasons stated above, more important could be the fact that AP-1 binds to these regions, since this factor, at least in one of its molecular forms (that includes JUN-B), could mediate the transcriptional repression.

In conclusion, upstream of the human c-*fos* gene we have identified a functional ERE that could account for induction of transcription of this gene by estrogen during the early phases of the cell cycle and, perhaps, in other instances. This ERE is located within a newly identified regulatory region that includes different elements responsive to nuclear receptors, to AP-1 and possibly to other *trans*-acting factors.

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