Identification of an Estrogen Response Element in the 3'-Flanking Region of the Murine c-fos Protooncogene*

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We have used transient transfection assays with reporter plasmids expressing chloramphenicol acetyltransferase, linked to regions of mouse c-fos, to identify a specific estrogen response element (ERE) in this protooncogene. This element is located in the untranslated 3'-flanking region of the c-fos gene, 5 kilobases (kb) downstream from the c-fos promoter and 1.5 kb downstream of the poly(A) signal. This element confers estrogen responsiveness to chloramphenicol acetyltransferase reporters linked to both the herpes simplex virus thymidine kinase promoter and the homologous c-fos promoter. Deletion analysis localized the response element to a 200-base pair fragment which contains the element GGTCCAGCACGG that resembles the consensus ERE sequence GGTCCAGTCAC. This element was identified in Xenopus laevis A2 gene. A synthetic 36-base pair oligodeoxynucleotide containing this c-fos sequence conferred estrogen inducibility to the thymidine kinase promoter. The corresponding sequence also induced reporter activity when present in the c-fos gene fragment 3 kb from the thymidine kinase promoter. Gel-shift experiments demonstrated that synthetic oligonucleotides containing either the consensus ERE or the c-fos element bind human estrogen receptor obtained from a yeast expression system. However, the mobility of the shifted band is faster for the fos-ERE-complex than the consensus ERE complex, suggesting that the three-dimensional structure of the protein-DNA complexes is different or that other factors are differentially involved in the two reactions. When the 5'-GGTCA sequence present in the c-fos ERE is mutated to 5'-TTTCA, transcriptional activation and receptor binding activities are both lost. Mutation of the CAGGC-3' element corresponding to the second half-site of the c-fos sequence also led to the loss of receptor binding activity, suggesting that both half-sites of this element are involved in this function. The estrogen induction mediated by either the c-fos or the consensus ERE was blunted by the antiestrogen tamoxifen. Based on these studies, we believe the 3'-fos ERE sequence we have identified may be a major cis-acting element involved in the physiological regulation of the gene by estrogens in vivo.

The protooncogene c-fos is the cellular homologue of the oncogene of FBJ and FBR mouse osteosarcoma viruses and codes for a trans-regulatory protein that plays an important role in the control of gene expression (1, 2). The Fos protein forms a stable complex with the product of another protooncogene c-jun, and the resultant heterodimeric complex binds to specific DNA sites known as AP-1 or TPA1 responsive elements (3). The Fos protein can also complex with other members of the c-fos or c-jun families and may subsequently control gene expression in either a positive or negative fashion (4, 5). In many different systems, transcription of c-fos is rapidly and transiently induced by a variety of growth-promoting agents (6).

Estrogens play a crucial role in cell growth and proliferation of various reproductive tissues such as the uterus and mammary gland (7, 8). Estrogens induce a variety of biochemical changes within target cells including the synthesis of various enzymes, the synthesis and/or secretion of peptide growth factors, and the synthesis of growth factor receptors (9–11). Previous studies from our laboratory (12, 13) and others (14, 15) have firmly established that the c-fos protooncogene is rapidly and transiently induced in the rodent uterus, human mammary cancer cells, and rat anterior pituitary tumor cells. The effects of estrogens on target cells may thus be mediated in part via c-fos induction, since this protooncogene clearly regulates cellular growth and differentiation in many systems (6, 16).

It is now well established that transcriptional activation by estrogens occurs via interaction with an estrogen receptor (ER) that is a specific trans-regulatory protein (17). In target cell nuclei, the ER binds to a DNA response element(s) referred to as the estrogen response element or ERE, which functions as an estrogen-dependent enhancer (17). Initially it was shown that the receptor binds to a specific palindromic sequence GGTCANNTGACC (18, 19), probably as a head to head dimer. This element was identified in the Xenopus laevis vitellogenin gene and promotes transcription very efficiently in a receptor-dependent fashion. Recently several other EREs with sequences different than the consensus ERE have been identified (20–22). Regardless of their sequence most of the EREs so far identified are located 0.5–1 kb upstream of their respective promoters and are believed to regulate transcription by interacting with the RNA polymerase II transcription complex.

Several lines of evidence indicate that c-fos induction by estrogens is a direct effect and is therefore most likely mediated by an ERE in the 3'-flanking region of the murine c-fos gene. The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; ER, estrogen receptor; ERE, estrogen response element; bp, base pair(s); kb, kilobase(s); HSV, herpes simplex virus; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; PREG, progesterone response element.
diated by the ER. First, the hormonal induction of c-fos mRNA is very rapid, increasing 20-fold within 3 h in the uterus (12, 14), and 3–5-fold in 30–60 min in estrogen-responsive cell lines (13). Second, the induction is insensitive to inhibitors of protein synthesis (12). Third, direct measurements by nuclear run-on assays show an increase in c-fos transcription rate (14). Finally, an ERE which binds the ER and confers transcriptional activation to a heterologous promoter has been reported in the 5′-flanking region of the human c-fos gene (23).

In an initial attempt to identify the murine c-fos ERE, we found that a 5′-region of the gene (~351/+44) confers a relatively weak estrogen inducibility (an approximately 2-fold induction) to a transfected reporter gene (13). Since the magnitude of this induction is similar to the induction of the endogenous c-fos mRNA in the cell line used and is also similar to the increase in transcription rate in uterine nuclei (14), we suggested that an ERE resides within this region of the gene. However, other observations are not consistent with this suggestion and raise the possibility that the putative effects of estrogen on c-fos expression are not mediated via an element(s) in this upstream segment of the gene. These include the following: 1) hormonal induction of reporter genes containing the 5′-element exhibits considerable variability; 2) we have been unable to localize the ERE to a smaller region within the promoter; 3) the ER does not bind to synthetic oligodeoxynucleotides containing candidate ERE sequences present in this 5′-element; 4) unlike other EREs which have been identified, the most likely candidate ERE sequence in the 5′-region of c-fos does not confer hormonal responsiveness to reporters containing the heterologous HSV thymidine kinase promoter (13). These observations raised the possibility that the modest responsiveness imparted by the 5′-element is due to an indirect effect involving transcription factors other than the ER. Weisz and Rosales (23) previously proposed such an indirect effect from studies of an analogous 5′-region of the human c-fos gene (23) We therefore decided to search other regions of the murine c-fos protooncogene for the presence of a more classical ERE. Initially we examined additional 5′-sequences further upstream of the promoter in the mouse gene, but could not identify potential EREs in a search extending almost 1 kb upstream of the transcription start site. This in turn led us to consider the possibility that an ERE might be present in other regions of this cellular oncogene.

In this report we describe the identification of an ERE in the 3′-flanking sequence of the murine c-fos protooncogene. This element contains sequences that bind the ER and confer hormonal responsiveness to CAT reporter genes containing either the c-fos or HSV thymidine kinase promoters. This 3′-element is located 5 kb downstream of the c-fos promoter and approximately 1.5 kb downstream from the polyadenylation signal. To our knowledge this is the first report of a hormone response element in the 3′-flanking region of a steroid inducible gene.

**MATERIALS AND METHODS**

**Plasmid Construction**—Plasmid constructions and other cloning procedures were done as previously described (24). The murine c-fos gene contained in pc-fos-3 (25) was digested with appropriate restriction enzymes and the purified fragments were ligated to a reporter plasmid (pBLCAT2) expressing chloramphenicol acetyltransferase (CAT) under the control of HSV-TK promoter (26). p8CAT, a promoterless CAT vector (27) was used in constructs containing the c-fos promoter.

Plasmid FCNco was constructed by digesting the 2.6-kb fragment from pc-fos-3 with NcoI and ligating it into the SalI site of pBLCAT2 which had been blunt-ended with Klenow. This plasmid contained sequences originating in the 5′-flanking region of c-fos and included all four exons.

Plasmid FCNco was also derived from pc-fos-3 by digesting with NotI and isolating an approximately 5-kb fragment, which originates in the fourth exon and extends to the 3′-NcoI site located in the 3′-flanking region of the gene. The NcoI sites were filled and ligated into the SalI site of pBLCAT2. This procedure recreated the SalI site. A clone representing the gene in the reverse orientation was selected and served as the parent vector for the construction of several of the recombinants described below. This plasmid contains the pSV (4) site, which is located about 1.5 kb from the end of the fourth exon.

Plasmid FCNS was created by digesting FCNco with NotI and isolating the 0.3-kb fragment, which was then ligated into the SalI site of pBLCAT2.

Plasmid FCSB was a 1.5-kb SalI-BamHI fragment digested from pc-fos-3 and ligated into pBLCAT2, which was also treated with SalI-BamHI.

Plasmid FCNB was created by digesting FCNco with BamHI (the 5′-BamHI site is within the c-fos sequences, and the 3′-BamHI site is from the vector sequence; see Fig. 2A), isolating 1.5-kb fragment, and ligating this fragment into the BamHI site of pBLCAT2. Plasmid FCNHB was constructed by digesting an approximately 1.5-kb fragment from FCNco (the 5′-HindIII site is from the vector sequence, and the BamHI site is present in the c-fos insert of FCNco; see Fig. 2B) and ligating it to HindIII-BamHI site of pBLCAT2. This construct represents the most distal segment of c-fos that is located in the plasmid FCNco. The entire insert in FCNHB was sequenced, and the sequence data is available in another report (28) and from GenBank (accession no. M65003). A set of nested deletions was constructed with FCNHB as the parental vector. FCNHB was digested with HindIII in the polylinker, filled with α-phosphorothioate bases, then digested with SalI and deleted unidirectionally using exonuclease III as described by the manufacturer (Promega, Madison, WI). Several deletion clones were isolated and sequenced to determine the extent of deletion. Sequence data also revealed the presence of a Dral site in FCNHB, 200 bp downstream from the 5′-polylinker site within the SalI-BamHI region. This represents the construction of plasmid FCHD by digesting FCNHB with HindIII-Dral, end-filling, and ligating it to a filled SalI site of pBLCAT2.

We also constructed a chimeric plasmid by inserting the HindIII-BamHI fragment of FCNHB upstream of plasmid FC −351/+44, which has been previously described (13). The latter plasmid contains the c-fos promoter in a CAT vector. Plasmid FC −351/+44 was digested at the Smal site located in the polylinker upstream of the c-fos promoter, and end-filled HindIII-BamHI fragment of FCNHB was ligated at this site. This plasmid was abbreviated FC3′BH5′P.

Plasmid vit-ERE, which contains a consensus ERE, was a gift from Dr. Ming Tsai, Baylor College of Medicine.

All plasmids were sequenced to verify their insertion and orientation.

**Transfections and CAT Assays**—The transfection of MCF-7 cells was carried out by the calcium phosphate procedure described by Klein-Hintpass et al. (18) with certain modifications. One day prior to transfection, cells were seeded in 10-cm plates in phenol red-free DME/F12 supplemented with 10% charcoal-stripped serum. Cells (approximately 50% confluent) were fed with fresh media 3 h prior to transfection. Cells were transfected with 10 μg of cesium chloride-purified recombinant plasmid DNA. When β-galactosidase was used as an internal control for % of plasmid pSVβGal (Promega) was added. The DNA-calcium phosphate precipitate was added to the medium and the transfection allowed to proceed for 6–8 h. At the end of the transfection period, medium was removed and the cells were washed with 10% dimethyl sulfoxide. Following two washes with phosphate-buffered saline, cells were grown in media supplemented with charcoal-treated serum. The next day estradiol was added to a final concentration of 20 nM, and the cells were harvested 20–24 h later for measurement of CAT activity.

CAT assays were done as described by Gorman et al. (29). For quantitation, the acetylated chloramphenicol formed per unit of protein or per unit of β-galactosidase activity. There was no significant difference in induction when radiolabeling was normalized by protein or β-galactosidase activity (Table 3C).

**Gel-shift Assays**—All gel-shift assays were performed with incubation times of 20 min in reaction mixtures containing 10 mM Tris-
HCl, pH 7.5, 10% glycerol, 2% Ficoll-400, 50 mM NaCl, 2.5 mM MgCl₂, 0.5 mM EDTA, 2.5 mM dithiothreitol, 1.5 µg of pBR322 digested with HindIII, and 0.2-1.0 ng of specific probe which had been labeled with [γ-32P]dATP by using the T4-polynucleotide kinase. For vit-ERE 0.2 ng of probe representing 30,000-50,000 cpm was used, and for 3'-fos-ERE 1.0 ng of probe representing 300,000-500,000 cpm was used. Protein-DNA complexes were separated from protein-free labeled with [γ-'P]dATP by using the T4-polynucleotide kinase. For voltage of 160 V, and were then dried and autoradiographed. The temperature in 90 mM Tris, 90 mM boric acid, 2 mM EDTA at a constant temperature in 90 mM Tris, 90 mM boric acid, 2 mM EDTA at a constant temperature in 90 mM Tris, 90 mM boric acid, 2 mM EDTA at a constant temperature. The following oligodeoxynucleotides were used in the gel-shift experiments.

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\begin{align*}
\text{vit-ERE} & \quad 5'\text{-GATCCGTCAAGTCACACTGACATG-3'} \\
\text{3'-fos-ERE} & \quad 5'\text{-TCGACCTTTATCCACCTCACCACAGCAGGCCATG-3'} \\
\text{m13'-fos-ERE} & \quad 5'\text{-TCGACCTTTATCCACCTCACCACAGCAGGCCATG-3'} \\
\text{m123'-fos-ERE} & \quad 3'\text{-GAAATACGGTCAAGGTGTCAGTCGCGTACAGCT-5'} \\
\text{m133'-fos-ERE} & \quad 3'\text{-GATCCGTCAAGTCACACTGACATG-3'} \\
\text{PRE} & \quad 5'\text{-GATCCGTCAAGTCACACTGACATG-3'} \\
\text{3'-NcoI digest} & \quad 3'\text{-GAAATACGGTCAAGGTGTCAGTCGCGTACAGCT-5'} \\
\text{FCNaeI} & \quad 1.4-kb region which showed similarity to the consensus ERE. The estrogen-dependent induction, this placed the ERE in the distal 180-bp region of the murine c-fos oncogene. Since plasmid FCNaeI fragment fused upstream of the TK promoter (FCNHb). This fragment was consistently induced by estrogen in the MCF-7 cells (4.0 ± 0.7, mean ± S.E., n = 8) (Fig. 2B). We conclude from these results that a functional ERE is located in the 3'-flanking region of the murine c-fos oncogene.

The ERE Located in the 3'-Flanking Region of Murine c-fos Induces CAT Expression When Linked to Its Own Promoter—It was of interest to determine whether the 3'-ERE could induce transcription from the homologous c-fos promoter, and if so, if there was any synergism in constructs containing 5'-flanking sequences of the gene. We therefore placed the BamHI-NcoI fragment (obtained from FCNco) upstream of the c-fos promoter in a construct containing the -351/+44 region of the c-fos promoter linked to the CAT gene (FC3'-HB5/P). In this experiment, estradiol expression of the transfected construct to the same level as that observed with FCNHB alone (Fig. 2C). In this particular series, negligible induction was observed with the FC -351/+44 region. We observed that the basal level of CAT activity increased when the c-fos promoter region was linked to the reporter.

A 200-bp Region from the Distal Region of c-fos Responds to Estrogen in an Orientation-independent Manner—In our attempt to fine map the ERE in the 3'-region of the murine c-fos gene we sequenced the entire insert in FCNHB (see Fig. 2 for the location) (28) and noted several regions within the 1.4-kb region which showed similarity to the consensus ERE. Several additional deletions were thus constructed from FCNHB, and these were transfected into the MCF-7 cells to more precisely locate the ERE. The estrogen-dependent induction was lost with the first deletion, which lacked the initial 180 bp of FCNHB (data not shown). Since plasmid FCNHB contained the c-fos sequences in the reverse orientation, this placed the ERE in the distal 180-bp region of the
c-fos sequence, next to the distal 3'-NcoI site of pc-fos-3.

A convenient DraI site was located about 200 bp downstream of the 5'-polylinker in PCNH8. We therefore digested PCNH8 with HindIII-DraI, ligated the blunt-ended fragment back into pBLCAT2, and selected for clones which contained the insert in both orientations. Using transfections with MCF-7 cells, constructs containing the insert in either orientation showed increased CAT activity following estrogen treatment that was similar to the induction obtained with PCNH8 (Fig. 3A).

Within this 200-bp region (Fig. 3A, FCHD) the most likely candidate for the ERE was 5'-GGTACCCAGGCC-3'. We therefore synthesized a 36-bp oligomer containing this ERE-like sequence, ligated the oligonucleotide into the polylinker region of pBLCAT2, and transfected the resultant construct into MCF-7 cells. The oligonucleotide directed estradiol-dependent transcription of the CAT gene (Fig. 3B).

To further test this candidate sequence, another oligodeoxynucleotide was synthesized in which we mutated the potential ERE to 5'-ttTCACCAGGCC-3', i.e. the two 5'-guanosine residues were replaced with thymines. There was no induction of CAT activity when this construct was transfected into the MCF-7 cells (Fig. 3C), which supports this sequence as the ERE and further indicates the importance of the two guanosine residues for estrogen responsiveness.

The 3'-fos-ERE Responds to Physiological Concentrations of Estradiol and Is Inhibited by Tamoxifen—To determine if the 3'-fos-ERE responds to physiological levels of estradiol, we transfected MCF-7 cells with this construct and then exposed the cells to various concentrations of the hormone. As seen in Fig. 4A, estradiol concentrations as low as 10^{-11} M induced CAT activity.

To determine if this induction is mediated through the estrogen receptor, we also tested the effect of the estrogen antagonist tamoxifen on the hormonal induction of CAT activity. Previous studies have shown that tamoxifen binds to the estrogen receptor and the tamoxifen-receptor complex binds to the vit-ERE (32). However, the transcriptional activation domain of the receptor is inactive when tamoxifen is bound (32).

In the presence of tamoxifen alone there was no induction of CAT activity and the induction by estrogen was blunted (Fig. 4A). Similarly, constructs containing the vit-ERE did not respond to tamoxifen, but the antiestrogen also diminished the estrogenic response in these constructs (Fig. 4B). These results again indicate that the induction observed with the 3'-fos-ERE is most likely mediated by the binding of the estrogen receptor to this sequence.

Estradiol has been shown to induce both c-fos and c-jun expression, and the resultant heterodimer activates gene transcription through AP-1 sites (33). To insure that the induction of CAT activity was not a secondary result of estrogen induction of endogenous c-fos and c-jun in the MCF-7 cells, we investigated the possibility that the 3'-fos-ERE construct contained functional AP-1 sites. For this purpose, we treated transfected cells with TPA (2 × 10^{-7} M), and measured the resulting level of CAT activity. In this experiment, we observed no induction of the 3'-fos-ERE by TPA (data not shown).

The Estrogen Receptor Binds Directly to the Response Element Present in the 3'-Flanking Region of c-fos—In order to determine if the ER binds to the response element present in the 3'-fos-ERE we performed band-shift experiments. For these studies we utilized a full-length human estrogen receptor expressed in yeast as previously described (34). Labeled oligodeoxynucleotides containing sequences representing the

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**Fig. 2.** Further localization of ERE in the 3'-flanking region of murine c-fos gene. A, several constructs as indicated in the figure were transfected into the MCF-7 cells and treated with estradiol. CAT assays following the preparation of cell extracts were performed as described under “Materials and Methods.” All c-fos constructs except FCNco were negative for estrogen induction indicating that the response element must reside in the distal BamHI-NcoI fragment of the c-fos gene. B, c-fos construct representing the distal BamHI-NcoI fragment (FCNH8) was transfected into the MCF-7 cells. The construct FCNco was also transfected in parallel as control. The expressed CAT activity was monitored as previously described. FCNH8 contained the ERE. C, the ERE located in the 3'-flanking region of c-fos oncogene induces CAT expression when linked to its own promoter. The BamHI-NcoI fragment from the 3'-region which exhibited the ERE activity was ligated upstream of the c-fos promoter (-351/+44) which was linked to the reporter CAT. These constructs were then transfected into the MCF-7 cells treated with estradiol, collected, and processed for the expression of CAT activity as described under “Materials and Methods.” The BamHI-NcoI fragment was able to induce CAT activity via its own promoter.
ERE was mutated to 5'-ttTCA to generate the mt3'-fos-ERE. These constructs were transfected into the MCF-7 cells and treated with estradiol. Cells were harvested, and the extracts were normalized to equal β-galactosidase activity. CAT assay was performed as described under "Materials and Methods," and the results are plotted as fold induction over the untreated controls. Induction of both the 3'-fos ERE and the vit-ERE was reduced by 10^-6 M tamoxifen. The values shown represent mean ± S.E. of the number of determinations indicated.

Fig. 4. The 3'-fos-ERE responds to physiological concentrations of estradiol and the induction is blunted by estradiol. The 3'-fos-ERE (A) or the vit-ERE (B) was transfected into the MCF-7 cells and treated with estradiol. Cells were harvested, and the extracts were normalized to equal β-galactosidase activity. CAT assay was performed as described under "Materials and Methods," and the results are plotted as fold induction over the untreated controls.

The human estrogen receptor bound to the 3'-fos-ERE (Fig. 5A). The mobility of the complex was further retarded by incubation with an antibody raised against the ER (Fig. 5A), indicating that the original band shift was due to binding of receptor present in the yeast extract. This interaction is specific since an oligodeoxynucleotide containing the progesterone response element failed to compete with the fos-ERE for binding of the ER, but an excess of unlabeled oligomer containing the 3'-fos-ERE completely abolished the receptor binding. Similar results were obtained when the band-shift experiments were performed with the vic-ERE (Fig. 5A). Interestingly, the mobility of the shifted band was slower for the interaction between the ER and the vit-ERE than for the interaction between the ER and the 3'-fos-ERE. This observation suggests either that different factors are involved in the vit-ERE and 3'-fos-ERE complexes or that the ER forms complexes with different conformations with the two DNA sequences, e.g. protein binding may cause differential bending of oligonucleotides containing the vit-ERE and the 3'-fos-ERE.

Since mutation of the 5'-GGTCA-3' sequence in the 3'-fos-ERE to 5'-ttTCA-3' (mt3'-fos-ERE) destroyed the transcriptional response to estradiol (Fig. 3C), it was of interest to determine the effect of this alteration on ER binding. As shown in Fig. 5B, this change leads to the loss of ER binding, which again suggests that this region plays a role in mediating hormonal induction.

To further explore sequences involved in receptor binding, we mutated bases both downstream, in the second half-site of fos-ERE (mt23'-fos-ERE) and in a similar location upstream of the 3'-fos-ERE (mt33'-fos-ERE) element (see "Materials containing the vitellogenin ERE fused to TK-CAT was also transfected into MCF-7 cells and the effect of estradiol on CAT activity was assessed.
other bands which migrated faster than the specific band when this particular yeast preparation was used as the source of ER. However, this more rapidly migrating bands were clearly due to nonspecific interaction(s) since these are not supershifted by the antibody against the ER.

In the final set of experiments, we sought to assess the relative affinities of the 3'-fos-ERE and the vit-ERE for the ER. For this purpose we compared the ability of either unlabeled vit-ERE or the unlabeled 3'-fos-ERE to compete for the binding of ER to the labeled vit-ERE sequence (Fig. 5C). Our results demonstrated that the ER bound the 3'-fos-ERE with an affinity at least 5-10 times less than the vit-ERE. It is particularly interesting to note that the relative difference in affinity corresponds approximately to the relative activation potential of the two sequences, i.e. the induction obtained with constructs containing the two EREs (see Fig. 3C).

**DISCUSSION**

The protooncogene c-fos is a representative of a class of genes termed the immediate early growth response genes. The expression of this gene is correlated with entry of quiescent cells into the cell cycle and the onset of proliferation (16, 35, 36). The product of the c-fos protooncogene serves as both a repressor and as an inducer of a variety of growth responsive genes (4, 5, 37). A number of growth promoting agents rapidly induce the transient synthesis of the Fos protein (6). The transient expression of the gene serves as a stringent regulatory process since overexpression of this protein leads to cell transformation (38).

We and others have shown previously that c-fos is rapidly and transiently induced by estradiol in the uterus of ovariectomized rats and estrogen responsive cell lines (12-15). Nuclear run-on assays (14) and the insensitivity of this process to puromycin (12) indicate that c-fos induction is most likely a primary hormonal response. We therefore searched for the cis-regions of c-fos gene for the presence of an ERE, and our previous studies focused on the 5'-flanking sequence of the gene (13).

We initially suggested that the -273/-135 region of c-fos contains an ERE since this region gave a modest induction following estrogen treatment (13). However, we have subsequently found this response to be erratic (see, for example, Fig. 2C) as has been reported by others for an analogous 5'-region of the human c-fos gene (23). More recently we have found that an ERE like palindrome represented by GGTCTNNNA-GACC (consensus GGTCANNNTGACC), does not bind the ER. In the final set of experiments, we sought to assess the competition of ER in band-shift assays? For these and other reasons (see Introduction) we therefore searched additional c-fos sequences for the presence of a functional ERE.

Since we had previously analyzed the available murine c-fos genomic sequences which extended up to 1 kb upstream of the transcription start site (13), we elected to explore the intragenic and the 3'-flanking region of the gene. An additional impetus for these studies was that several other genes have recently been shown to contain regulatory elements in both the intragenic and the 3' regions (39, 40), and c-fos contains at least two intragenic regions which regulate levels of its mRNA. One of these is involved in the turnover of the c-fos message (41), and the other affects the rate of transcriptional progression (42).

Our results demonstrated that the entire coding sequence and the introns lacked an ERE, but a strong estrogen-dependent enhancer was located in the 3'-untranslated region.
We subsequently localized this activity to a 200-bp region which demonstrated enhancer properties when tested in transient transfection studies. This region is located 5 kb downstream of the c-fos promoter and about 1.5 kb downstream of the poly(A) signal. Within this 200-bp region, the most likely ERE appeared to be the sequence 5'-GGTCACACAGCC-3', which closely resembles the consensus response element 5'-GGTTCGAGGTCC-3'. A 36-bp oligonucleotide containing this sequence conferred estradiol responsiveness when ligated to homologous or heterologous promoters. The induction of reporter genes containing this sequence displays specificity for estradiol, occurs at physiological levels of hormone, and is inhibited by the antiestrogen tamoxifen. The ability of this 3'-fos sequence to function as an ERE was further demonstrated by experiments in which estrogen responsiveness was completely lost when the 5'-GGTCA was mutated to 5'-ttTCA. The importance of the G residues has been previously recognized in other ERGs (43).

In the c-fos genomic sequence the proposed ERE is at a relatively long distance (about 5 kb) from the transcriptional start site. Therefore, if this sequence is an ERE in the physiological context, it should be able to enhance transcription in a setting where it is placed a considerable distance from a promoter. The experiments in Fig. 1 and 2 illustrate that this is the case. The circular 7.5-kb FCNco plasmid used for these studies was constructed by linking a 3'-fos fragment, 3 kb in length, to the parental pBLCAT2 vector which is 4.5 kb. Thus, the 3'-fos-ERE contained in the inserted fragment is located 3 kb in the counterclockwise direction and 4.5 kb in the clockwise direction from the TK promoter. In this position the 3'-fos-ERE clearly functions as a strong transcriptional activator.

It is also interesting to note that the 3'-fos-ERE sequence imparts the same estrogen inducibility when placed 3 kb from the TK promoter (Fig. 1) or when ligated as a synthetic oligonucleotide sequence just a few bases upstream of the same promoter (Fig. 3). This suggests that other regions in the FCNHB construct do not have a major influence on the ERE activity of this sequence, at least in the MCF-7 test system.

In addition to transfection analysis, band-shift experiments also provide evidence that the 3'-fos sequence can function as an ERE. A 36-bp synthetic oligonucleotide with the 5'-GGTCACACAGCC-3' sequence binds the human ER, and this binding is abolished if the 5'-sequence is mutated to 5'-ttTCA. The importance of these G residues for both hormone inducibility and receptor binding has previously been reported (43). To further investigate the nature of the ER interaction with this c-fos sequence, we also examined the effects of other base changes. A mutation upstream of the 5'-GGTCA sequence did not affect receptor binding (Fig. 5B), but mutation of -GGTTCAGGCGGTGTCACCACAGCC to -GGTTCACAGGCGGTGTCACCACAGCC led to the loss of specific ER binding (Fig. 5B). Taken together, these results indicate that 2 half-sites (i.e. 5'-GGTCA and CAGCC-3') are involved in the ER binding seen in Fig. 5B. This argues that our results are due to the binding of an ER dimer (either a homo- or heterodimer) to two half-sites rather than the binding of an ER monomer to the single 5'-GGTCA half-site as recently observed in the ovalbumin gene (44). Because the CAGCC-3' half-site is similar to the consensus ERE in only two of five positions, our results are most consistent with the binding of an ER heterodimer to the c-fos ERE, although this possibility remains to be rigorously established by further experimentation.

In these studies we also noted that the mobility of the complex formed with the ER and the 3'-fos-ERE was faster than that of the complex formed when the consensus vit-ERE was used. To our knowledge this observation has not previously been made for ER binding to different ERE sequences. This indicates that the three-dimensional structures of the two protein-DNA complexes are different, and this in turn could occur for one of several reasons. Since receptor binding may produce DNA bending, ER binding to the two sequences may produce different types of bending. Alternatively, another protein(s) present in the yeast extract could be differentially bound (either specifically or non-specifically) to the two complexes, since the antibody supershift experiments do not rule out the presence of other non-receptor factors in the shifted bands (45). For example, the ER could bind to the vit-ERE as a homodimer (18, 19) but bind to the fos-ERE as a heterodimer. Additional studies will clearly be required to distinguish between these possibilities. Based on the data provided in Fig. 5B, it is unlikely that the receptor binds to the 3'-fos sequence as a monomer.

In addition to the structural differences in ER interactions implied from the band-shift studies, there are functional differences between the 3'-fos-ERE and vit-ERE sequences. Thus, the vit-ERE has roughly a 5-10-fold greater affinity for the ER, and constructs containing this ERE also show about a 5-10-fold greater response to estrogenic stimulation. It will be particularly interesting to determine in future studies whether these functional differences are related to the subtle differences in tertiary structure suggested by the gel retardation studies.

A number of observations suggest that the 3'-fos-ERE we have identified in this work is intimately involved in the physiological regulation of the endogenous gene. 1) This 3'-sequence confers hormonal responsiveness to transfected reporters containing both homologous and heterologous promoters. 2) The response mediated by this element occurs at physiological levels of estradiol, is blocked by an antiestrogen, and exhibits the same hormonal specificity observed for c-fos induction in the uterus (12). 3) This element also binds the ER in band-shift experiments. Nevertheless, additional experiments are clearly needed before we can unequivocally ascribe the in vivo estrogenic regulation of uterine c-fos expression to this element.

In addition, the 3'-element we have identified activates transcription when located at a considerable distance from the TK promoter in an experimental system (see Fig. 1). Since this is roughly analogous to the location of the sequence in the mouse genome, i.e. 5 kb from the c-fos promoter, one could interpret this observation as support for a physiological role for the 3'-fos-ERE. While cis-regulatory sequences have been found in the downstream flanking regions of other genes (40), this is nevertheless an unusual position based upon results with most steroid responsive genes. Consequently, we also considered the possibility that this ERE might regulate the in vivo transcription of other sequences located on its downstream side (i.e. on the 3'-side). In other words, this sequence might mediate estrogenic regulation of another gene downstream of c-fos, since this would place the element in the more traditional 5'-regulatory region of such a second gene.

To test this possibility we used plasmid FCHD to prepare random primed duplex DNA probes spanning the 200-bp mouse genomic sequences on the immediate downstream side of the 3'-fos-ERE. These probes were then used to analyze RNA blots for the presence of complementary mRNA sequences in uterine RNA prepared from control or estrogen-treated animals. This study failed to detect the hybridization of any transcripts in uterine RNA. This experiment does not rule out the regulation or co-regulation of another gene by
the 3'-fos-ERE, but it does indicate that no mature uterine transcripts are derived from either strand of this 200-bp sequence under basal or estrogen stimulated conditions. We are currently initiating studies to investigate the possible transcriptional regulation of additional genomic sequences farther than 200 bp downstream of the 3'-fos-ERE.

As discussed above, it seems likely that the 3'-fos-ERE we have identified plays an important role in the physiological control of c-fos expression by estrogenic hormones. However, the overall in vivo control of c-fos may be considerably more complex and may involve additional cis- and trans-acting factors. For example, Weisz and Rosales have identified an ERE in the 5'-flanking region of the human c-fos gene, which corresponds to our FCSB construct. However, this construct is transcriptionally inactive when placed upstream of the TK promoter (24). We have recently observed that progesterone can inhibit c-fos induction in the uterus by estrogen levels of c-fos mRNA (33). Furthermore, we have recently observed ER binding to a 3'-SalI-BamHI fragment of the murine c-fos gene, which corresponds to our FCSB construct. However, this construct is transcriptionally inactive when placed upstream of the TK promoter (see Fig. 2A). At present, it is unclear how the human or mouse sequences respectively identified by Weisz and Rosales (23) and Weisz et al. (46) are related to the mouse 3'-fos-ERE we have identified in this work, or if multiple EREs (e.g. both 5' - and 3' ) are present in the human and/or murine c-fos genes.

Furthermore, the overall regulation of c-fos expression in the uterus is very likely to be even more complex and to involve other trans-acting factors. For example, it is well known that c-fos is induced in many systems by TPA (2); Weisz and Rosales (23) have shown that the AP-1 transcription factor binds to the human 5'-ERE they identified, and we have shown that in vivo TPA treatment elevates uterine levels of c-fos mRNA (38). Furthermore, we have recently observed that progesterone can inhibit c-fos induction in the uterus by estrogen (47). It is thus clear that multiple factors can exert positive and negative control over c-fos expression in estrogen responsive tissues. Additional studies will be required to understand the interrelationships between these various factors, the signaling pathways involved, and the precise role of c-fos expression in cell growth and proliferation.

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