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# A Novel, Cell-Type-Specific Mechanism for Estrogen Receptor-Mediated Gene Activation in the Absence of an Estrogen-Responsive Element

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**The estrogen receptor (ER) typically activates gene transcription by binding to estrogen-responsive elements (EREs). The brain creatine kinase (BCK) promoter is responsive to estrogen but contains no ERE-related sequence. To investigate the mechanism of estrogen induction, we have introduced the estrogen receptor into HeLa cells and primary rat cardiomyocytes and fibroblasts along with 195 bp of BCK promoter linked to a chloramphenicol acetyltransferase (CAT) reporter gene. A 10-fold stimulation of CAT activity was observed in the presence of β-estradiol in both HeLa and rat primary fibroblasts, but no induction was observed in primary rat cardiomyocytes. In contrast, a control vitellogenin gene construct which contains a typical ERE was induced in an ER-dependent manner in all cell types studied. Estrogen induction in HeLa was not sensitive to cycloheximide and was blocked by the ER antagonists tamoxifen and ICI 164,384. Analysis of 5′ deletion and linker-scanning mutations indicates sequences between bp −45 and −75 including a TA-rich sequence and a CCAAT sequence to be crucial for stimulation of the BCK promoter by the ER. BCK estrogen induction is dependent on the DNA-binding domain and transactivation domain TAF2 of the ER. However, direct DNA binding is probably not required. Taken together, these results suggest a novel mechanism for ER-mediated gene activation. This mechanism is consensus ERE independent and cell type specific and requires interactions between the ER and molecules capable of interacting with the BCK promoter TA-rich region.**

Creatine kinase (CK) catalyzes reversible transfer of a phosphoryl group between creatine and ATP (25). There are two isoforms of this enzyme, the brain isoform (BCK) and the muscle isoform (MCK). Kaye and coworkers have demonstrated that estrogen induces the expression of BCK in rat uterus within an hour (34) and in diaphyseal bones of female rats (38). Progesterone at a higher concentration showed a similar effect. Conversely, for male rats, testosterone stimulated the expression of BCK in the diaphyseal bones, whereas estrogens were ineffective (38). In epiphyseal cartilage, BCK activity was stimulated by both estrogen and testosterone (37). Moreover, vitamin D metabolites modulate the effect of gonadal steroids on skeletal tissues of rats. Therefore, BCK expression is modulated by a variety of steroid hormones.

Steroid hormones modulate gene expression via intracellular receptors which belong to a class of DNA-binding, hormone-inducible transcription factors (3, 15). The estrogen receptor (ER) binds as a dimer to a 13-bp palindrome sequence, the estrogen-responsive element (ERE). The consensus ERE is 5′-GGTCANNNTGACC-3′ (3). However, no consensus ERE was identified in the proximal BCK promoter (32). We have therefore designed experiments to probe these apparently contradictory observations and to examine the mechanism by which the ER stimulates the BCK promoter in the absence of an ERE.

The BCK promoter is unique in that although there is a consensus TATA sequence (TATAAATA) at bp −66 relative to the transcription start site, the nonconsensus TATA sequence TTAA at bp −28 provides the principal TATA box function in vivo (4, 18, 19, 31). The TATAAATA sequence is flanked by two CCAAT sequences at bp −84 and −54. These sequences are highly conserved in the rat and human promoters and have been postulated to play a role in the binding of regulatory proteins (18, 19). The TATAAATA sequence binds a protein, TARP (TA-rich binding protein) (18), which is a ubiquitous protein.

In this study we demonstrate that sequences between bp −950 and −2900 of the BCK promoter are sufficient to confer strong estrogen inducibility. Although the BCK upstream region contains an ERE-like element at bp −550 (48), we also observed cycloheximide-resistant estrogen inducibility with only 195 bp of the BCK promoter even in the absence of any ERE. BCK promoter sequences from bp −37 to −195 can confer estrogen responsiveness when linked to the heterologous β-globin promoter. Mutations in the TATAAATA sequence severely attenuate the induction by estrogen. We demonstrate that the DNA-binding and ligand-binding domains of the ER are required for this induction, although direct DNA binding of the receptor to the BCK promoter is probably not required. We propose that the ER mediates this induction by interacting directly or indirectly with TARP or other accessory transcription factors.

## MATERIALS AND METHODS

**Construction of test plasmids.** The wild-type and mutant BCK promoter constructs were derived from those described previously (19, 21). The neomycin (*neo*) gene constructs were digested with *Bgl*II and *Hpa*I, and the *neo* gene was replaced by the chloramphenicol acetyltransferase (CAT) gene from *Bgl*II- and *Hpa*I-digested $pUC_{PL}CAT$ (21). The LPwtCAT construct was made by digesting pwtCAT with *Hin*dIII and *Nco*I and ligating in the *Hin*dIII-*Nco*I BCK promoter fragment from LPwtneo. The 5′ deletions were made in the LPwtCAT construct by digestion with *Stu*I (at bp −1420, to give LPΔ1420wt)

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or *Sca*I (at bp −950, to give LPΔ950wt) and *Hin*dIII, filling in with Klenow enzyme, and religation. The ER expression plasmids HEG0 (43) and HE0 (27) have been described. HE0 has a substitution of valine in place of glycine at amino acid position 400, a consequence of which gives it a lower affinity for β-estradiol at 25°C. The ER deletion constructs HE11, HE15, and HE19 have been described (27). Receptor mutant HE74 was obtained from P. Chambon. HE74 contains six amino acid substitutions within the core region, c, and does not activate an ERE-containing gene construct (30) or bind to an ERE DNA probe in a gel shift assay (27a). The vitellogenin gene-CAT reporter construct (Vit-TKCAT), which contains a consensus ERE, was obtained from P. Chambon (26).

**Gene transfer.** HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Forty-eight hours before transfection, the growth medium was changed to phenol red-free DMEM (6) supplemented with 10% fetal calf serum treated with dextran-coated charcoal to remove endogenous estrogens (22). Cells were plated at a density of $5 \times 10^5$ cells per 100-mm-diameter plate.

Neonatal rat myocardial cells were prepared from the hearts of 4- to 5-day-old Sprague-Dawley rats as described by Sen et al. (36). Briefly, the minced hearts were digested with pancreatin (0.03%) and collagenase type II (0.06%) at 37°C for a total of seven digestions of 10 minutes each. The dissociated myocardial cells were combined and passed through a 150-mesh screen. Cardiomyocytes and the nonmyocyte fraction were purified on a discontinuous Percoll gradient (1.060 to 1.086 g/ml) as described by Iwaki et al. (24). To isolate nonmyocytes, another step of 1.04 g/ml was added to the Percoll gradients. The purified cardiomyocyte cells were washed and then plated in 60-mm-diameter culture dishes coated with fibronectin (0.02-mg/ml solution; Sigma F1141) at a density of $3 \times 10^6$ to $4 \times 10^6$ cells per dish in DMEM containing no phenol red, with 10% stripped serum. The nonmyocyte fraction was treated the same as the HeLa cells.

Twenty hours after plating, the medium was replaced by fresh medium. DNA was added 4 h later by the calcium phosphate precipitation method (14). Typically, 1 μg of the reporter plasmid and 1 μg of the ER expression plasmid were transfected per plate. To correct for differences in transfection and harvesting efficiencies, 2 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia) was cotransfected at the same time. Bluescript DNA (pSK+; Stratagene) was added to bring the total amount of DNA to 20 μg. After 14 to 16 h, the medium was replaced with fresh medium containing 10 nM β-estradiol (E2; Sigma) in ethanol or estrogen antagonists (100 nM final concentration). Control plates received ethanol alone. Titration experiments indicated that maximum levels of CAT activity in the presence of estrogen were obtained with 1 μg of transfected ER expression plasmid. Cells were harvested 24 to 48 h later, washed with 1× phosphate-buffered saline and resuspended in 0.1 ml of 0.25 M Tris (pH 7.4) and disrupted by four cycles of freeze-thawing. CAT assays (12) were performed (1), and β-galactosidase activity was measured by the method of Herbomel et al. (17), except that chlorophenol red β-D-galactoside (Boehringer Mannheim) was used as a substrate to increase sensitivity (9), and the reaction mixture volume was 0.2 ml. The optical density at 575 nm was measured. The protein concentration was measured by the Bradford assay (7). CAT activity was quantified with a radioanalytical imaging system (AmBis Systems, San Diego, Calif.) and/or a Molecular Dynamics (Sunnyvale, Calif.) imaging system and is expressed as percent conversion into acetylated chloramphenicol divided by the β-galactosidase activity of the same extract. The activity of pwtCAT in the presence of ER and estradiol was normalized to 1 U. Fold stimulation is the ratio of CAT activity in the presence of hormone to that without hormone. Each experiment was performed at least three times. The error bars represent the standard errors of the means.

**RNA isolation and primer extension analysis.** Cells were transfected as described above, and RNA was purified by centrifugation through a discontinuous cesium chloride gradient (2). Primer extension analysis was done by our previously published procedure (31), except that the RNA was hydrolyzed after the reverse transcriptase step. The oligonucleotide 5′-TTTACGATGCCATTGGG-3′ was used as the CAT primer (46), and 5′-GCCATGATACCCTTGC-3′ was used as the VA primer (19).

## RESULTS

**The BCK promoter is responsive to estrogen in the absence of an ERE.** To localize the region that mediates estrogen responsiveness of the BCK promoter, constructs containing 5′ deletions were cotransfected into HeLa cells with the ER expression plasmid HE0 as described in Materials and Methods. A strong estrogen-dependent stimulation (50-fold) of the 2.9-kb promoter construct (LPwt) in the presence of the ER was observed (Fig. 1a). Two 5′ deletions to bp −1420 (LPΔ1420wt) and −950 (LPΔ950wt) were also strongly inducible by estrogen (42- and 35-fold, respectively). These results are in general agreement with those of Wu-Peng et al. (48), who observed a moderate induction (sevenfold) by using 1.7 kb of BCK promoter sequence in a similar cotransfection assay with HeLa cells. The lower difference in inducibility described by these authors could be attributed to differences in the growth medium used for the cells. In our study we used charcoal-stripped serum and DMEM lacking phenol red, which in tissue culture medium is a weak agonist for the ER (6). Lower background activity in this stripped medium may have contributed to the greater apparent response to estrogen observed in our studies.

However, pWT containing only 195 bp of the BCK promoter was also induced by estrogen (10-fold) even though it contains no sequence resembling the consensus ERE. A similar result was obtained with pWT and the wild-type receptor HEG0 (Fig. 1b), except that some activity seen in the absence of hormone is due to activation of HEG0 by the low level of estrogen remaining in the charcoal-stripped serum (43). We therefore investigated the mechanism whereby the ER activates 195 bp of the BCK promoter in the absence of any known ERE.

**5′ deletion analysis of the 195-bp BCK promoter.** To locate sequences in the 195-bp promoter that confer estrogen responsiveness, a further series of 5′ deletions was tested. Deletion to −75 bp (11Δ5′, in which the distal CCAAT box has been deleted) showed 70% inducibility compared with that of pWT (Fig. 2a). Further deletion to bp −56 (13Δ5′, which deletes the TATAAATA sequence) had little further effect on basal promoter activity but completely abolished the estrogen responsiveness. Further deletion to bp −45 of the promoter (14Δ5′, which deletes the proximal CCAAT box) also showed no estrogen responsiveness. This suggested that the sequence between bp −75 and −56 which includes the TATAAATA sequence contributes to induction of the BCK promoter by estrogen.

**Analysis of the sequence requirements for estrogen induction by linker-scanning mutagenesis.** To delineate further the *cis*-acting elements required for ER-mediated stimulation of the BCK promoter, selected linker-scanning mutants were tested for their abilities to mediate a response to estrogen (Fig.

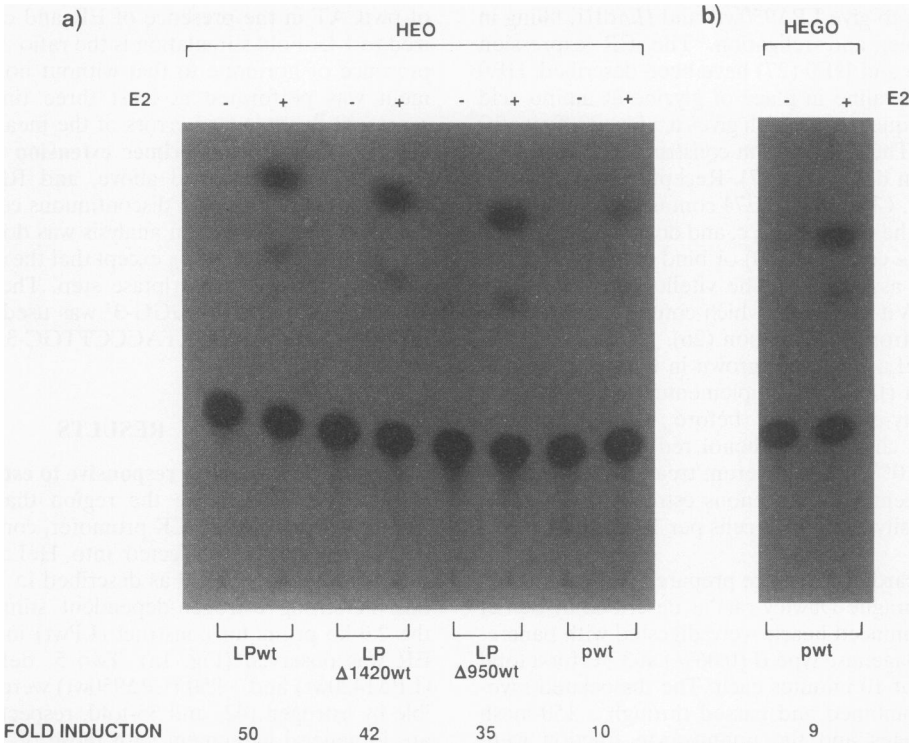


FIG. 1. Sequence requirements for estrogen induction of the BCK promoter region by deletion analysis. LPwt, LPD1420wt, LPD950wt (1.5 μg), or pWT (1 μg) was transfected into HeLa cells with 1 μg of ER expression plasmid and 2 μg of PCH110. HE0 (a) or HEG0 (b) was used as the ER expression plasmid. CAT and β-galactosidase assays were performed as described in Materials and Methods. The results shown are those of a typical experiment. The fold induction is the average of three experiments.

3). The wild-type promoter (pWT) showed a 10-fold induction in the presence of estrogen. Mutations in the upstream CCAAT box (LS11) and the TTAA sequence (LS16 and LS17) were strongly induced by estrogen (8-, 10-, and 17-fold, respectively). However, mutating the TATAAATA sequence (LS13) caused a severe attenuation of estrogen responsiveness. Mutations in the downstream CCAAT box (LS14) showed a less severe attenuation. A similar result was obtained with HEG0 (data not shown). These results are consistent with the results of 5′-deletion analysis (Fig. 2) and further suggest that the sequences in the region bp −75 to −45 play a role in the estrogen-induced stimulation of the 195-bp BCK promoter. No estrogen induction was observed with $pUC_{PL}CAT$, which lacks the BCK promoter sequences (data not shown). Hence, the observed induction was not mediated through cryptic EREs in the vector sequences.

We note that estrogen-mediated CAT induction is higher with LS17 compared with that of pwt. We do not know the reason for this at present. The transcription start site with this mutation is shifted downstream by a few nucleotides (16, 19). Therefore, one possibility is that this RNA is translated better or is more stable in the presence of estrogen.

**BCK promoter sequences from bp −195 to −37 can confer estrogen responsiveness to a heterologous promoter.** To determine whether TARP binding to the sequence TATAAATA was mediating the ER-induced stimulation of the BCK promoter, the TATAAATA sequence was replaced by the sequence TAAAAATA, a TARP binding site in the MCK enhancer (20, 21), to give E3 replace. This TARP-binding site no longer serves as a possible TATA box (31). E3 replace is inducible by the ER in the presence of the hormone, as is pWT (Fig. 4a). This suggests that replacement of the BCK TARP binding site with another non-TATA box TARP binding site from the MCK enhancer maintains estrogen inducibility.

To determine whether BCK promoter sequences from bp −195 to −37 could confer estrogen-induced stimulation by the ER on a heterologous promoter, the TTAA sequence and the CAP site in the BCK promoter were replaced by the mouse β-globin TATA box and CAP site in pWT, LS13, and E3 replace to give pWTβ, LS13β, and E3β, respectively. We observe induction by estrogen with pWTβ and E3β similar to that with pWT and E3 replace (Fig. 4b). Thus, BCK promoter sequences from bp −195 to −37 can confer estrogen responsiveness to a heterologous β-globin promoter. Mutations in the TARP binding sequence (LS13 and LS13β) severely decreased this induction in both cases. This indicates a possible role for TARP in mediating the estrogen response.

**The estrogen-dependent induction of pWT is not sensitive to cycloheximide.** To determine whether a protein(s) was induced by the ER which in turn activated the BCK promoter, the protein synthesis inhibitor cycloheximide was used. HeLa cells were cotransfected with HEG0 and the reporter plasmids LPwt (lanes 1 to 3) and pWT (lanes 5 to 7) and treated with estrogen and cycloheximide as indicated in Fig. 5. Primer extension analysis was performed with RNA isolated from these cells as described in Materials and Methods. Transcription from these constructs initiated from the downstream start site (19, 31). This was confirmed by a sequence ladder run in parallel (data not shown). Lanes 4 and 8 show the result of primer extension performed with control RNA isolated from cells transfected with Bluescript plasmid alone.

A clear stimulation of transcription of CAT RNA is observed with both the Lpwt and the pWT promoter constructs in the presence of estrogen (compare lanes 1 with 2 and 5 with 6).

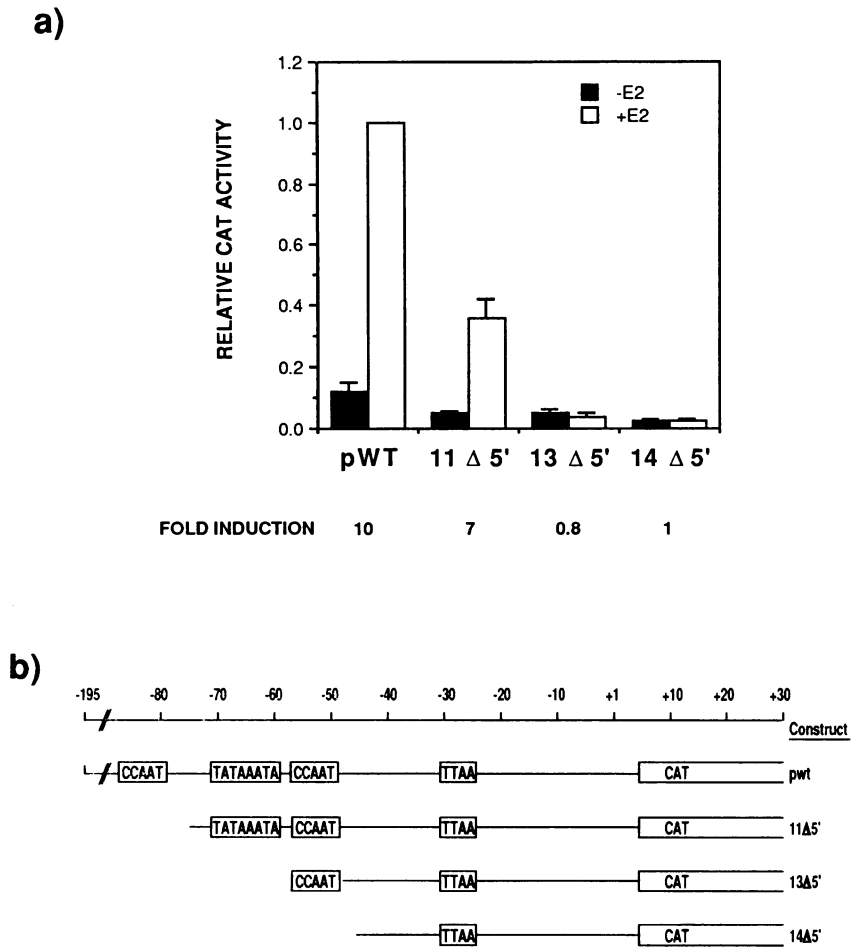


FIG. 2. Sequence requirements for estrogen induction of the 195-bp BCK promoter region by deletion analysis. The 5′ deletion mutants of the 195-bp promoter region are described elsewhere in detail (19) and in Materials and Methods. Reporter constructs (1 μg) were transfected into HeLa cells with 1 μg of HE0 and 2 μg of pCH110. (a) Relative CAT activity of the 5′ deletion constructs; (b) schematic representation of the promoter region of the 5′ deletion constructs. Potential control regions are shown boxed.

This stimulation was not decreased when cycloheximide was added to the medium together with estrogen (compare lanes 2 with 3 and 6 with 7). Synthesis of VA RNA which serves as an internal control was not increased in the presence of estrogen and is slightly decreased in the presence of cycloheximide. VA and CAT transcripts are not observed with control RNA from cells transfected with Bluescript DNA alone (lanes 4 and 8). Cycloheximide at this concentration completely blocked estrogen-inducible synthesis of CAT protein from pWT, as measured by a CAT assay (data not shown), proving that protein synthesis is indeed blocked. This experiment indicates that estrogen induction of the BCK promoter occurs at the RNA level, that the transcription start site remains unchanged, and that de novo protein synthesis is not required for the estrogen-dependent induction of the BCK promoter. Note that with LPwt, a small fraction (<10%) of the transcripts initiates from the upstream start site (31) in the presence of estrogen and is also not decreased in the presence of cycloheximide. This transcript (indicated by the arrow) is not observed in the absence of estrogen or with the 195-bp promoter construct (pWT) and presumably represents transactivation of the upstream TATA box by EREs upstream of bp −195.

**Domains of the ER required for induction of the BCK promoter.** To determine the domains of the ER necessary for induction of the BCK promoter, truncated ER mutants HE11, HE15, and HE19 (27) were analyzed (Fig. 6). The receptor DNA-binding domain is required because a mutation removing this domain (HE11) abolishes induction. HE15 has the N-terminal transcription activating function (TAF1) domain (44) and DNA-binding domain but lacks the C-terminal ligand-binding domain. Some basal activity is observed in the presence of HE15 but is not induced in the presence of the hormone. This is not surprising, since HE15 lacks the ligand-binding domain but has the constitutively acting transactivating domain TAF1 which is weakly active in HeLa cells (5, 44). Conversely, HE19 contains the DNA-binding domain, the ligand-binding domain, and the hormone-inducible transactivating function (TAF2) which is active in HeLa cells (5, 42, 44, 45). In the presence of HE19 and estrogen, the BCK promoter was strongly induced. Thus, the DNA-binding and ligand-binding domains of the ER are required for the estrogen-mediated induction of the BCK promoter.

**Induction of the BCK promoter by estrogen can be selectively blocked by an estrogen antagonist.** To determine the specificity of the estrogen induction of the BCK promoter, we tested whether two specific estrogen antagonists, 4-hydroxytamoxifen and ICI 164,384, could block this response. The presence of these antagonists added simultaneously with es-

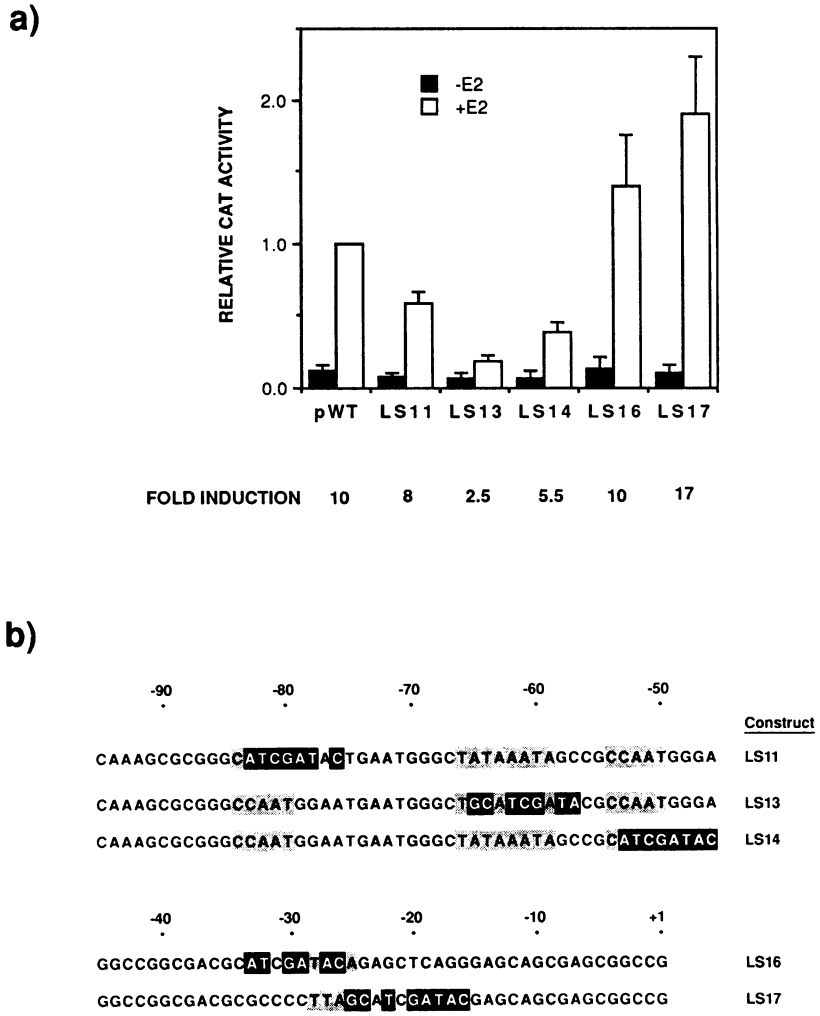


FIG. 3. Analysis of sequence requirements for estrogen induction by linker-scanning mutagenesis of the 195-bp BCK promoter region. The indicated linker-scanning mutants (1 μg) were transfected into HeLa cells along with the HE0 and pCH110 plasmids as described in the legend to Fig. 2. (a) Relative CAT activity of the various linker-scanning mutants. (b) Sequences that have been mutated in the linker-scanning mutants. Mutated sequences are indicated by black boxes, and potential control regions are shown by gray boxes.

trogen completely blocked the estrogen-induced stimulation of the BCK promoter (Fig. 7). These estrogen antagonists themselves do not stimulate the BCK promoter. The same result was obtained with HeLa cells grown in regular DMEM supplemented with fetal bovine serum not treated with charcoal (data not shown). In this case, hormone or antihormone was added before transfection. These data suggest that the induction of the BCK promoter by the ER specifically requires estrogen.

**Estrogen induction of the BCK promoter is cell type specific and does not require direct binding of the ER to DNA.** Although ER-mediated activation of the BCK promoter requires the ER DNA-binding domain, we were unable to demonstrate direct binding of the ER to the BCK promoter region by using electrophoretic mobility shift assays. These experiments were performed under conditions that promote ER binding to an ERE (31a). To address this problem, we tested the ability of a mutant ER (HE74), which cannot bind to and activate ERE-containing promoters (30), to activate the BCK and vitellogenin promoters in HeLa cells (Fig. 8). Despite the mutation in HE74, estrogen-dependent activation of the BCK promoter is still observed. The level of induction when HE74 is cotransfected with pWT is similar to the level of induction observed with cotransfected HE0 (Fig. 8). In addition, the responsiveness to HE74 maps to the same BCK promoter sequence elements responsive to HE0 expression (data not shown). The HE74 receptor has been reported to be capable of activation via binding to glucocorticoid response elements (30). The BCK promoter is unresponsive to activation mediated via the glucocorticoid receptor, although a control glucocorticoid-responsive construct (MMTV-CAT) is activated under similar experimental conditions (data not shown). In contrast, activation of the vitellogenin-ERE-driven promoter (vitTKCAT) is observed only with coexpression with HE0 and not with the HE74 ER mutant. These results demonstrate that the BCK promoter region does not contain a functional glucocorticoid response element and HE74 transactivation does not result from a change in the binding site specificity of the HE74 receptor. More importantly, these results suggest that although the DNA-binding domain of the ER is required for estrogen-dependent activation of the BCK

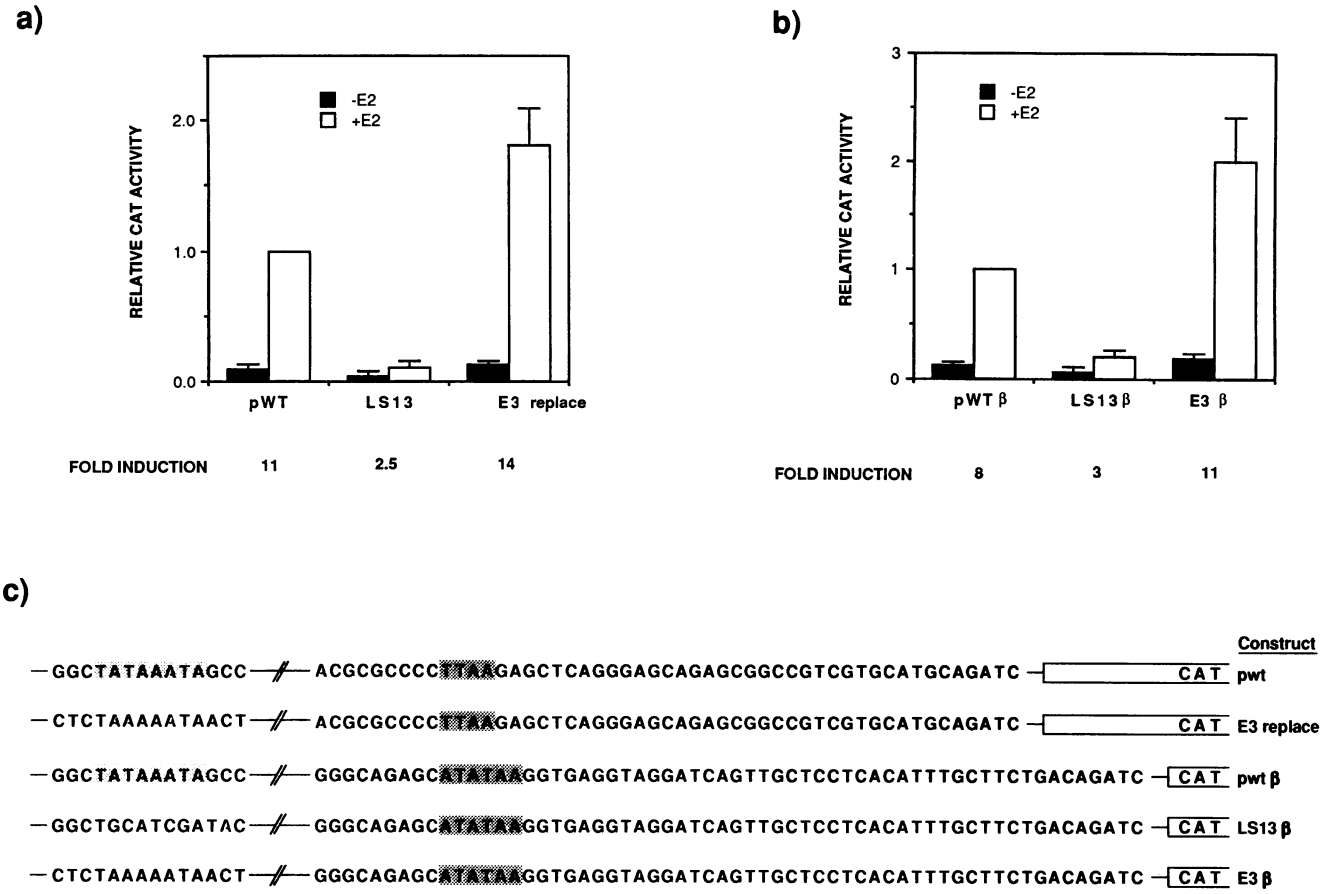


FIG. 4. BCK promoter sequences from bp −195 to −37 can confer estrogen responsiveness to a heterologous promoter. Cotransfections were performed as described in the legend to Fig. 2. The reporter plasmids pWT, LS13, and E3 replace (a) and pWTβ, LS13β and E3β (b) were used. (c) Schematic diagram of the various replacement constructs. Potential regulatory regions are shown in a shaded box. The sequence of the E3 element in E3 replace and E3β is shown but is not in a shaded box.

promoter, direct binding of the ER to the BCK promoter is probably not.

We next tested the ER-dependent activation of the BCK promoter in primary neonatal rat cardiomyocytes by cotransfecting the CAT reporter constructs pWT or vitTKCAT with HE0 or HE74. In primary rat cardiomyocytes, we observed no estrogen-dependent activation of the BCK promoter with either HE0 or HE74 (Fig. 9). Other transactivators are capable of activating the BCK promoter in these cells, indicating that this result does not result from an intrinsic inability of this promoter to function in cardiomyocytes (40). This result is in contrast to the vitellogenin gene construct which was activated in cardiomyocytes when coexpressed with HE0 but not HE74. To determine if this effect was specific to primary cells, the primary rat fibroblast fraction was also tested for pWT induction. In the primary neonatal rat fibroblasts, pWT was induced by both HE0 and HE74 to levels similar to those observed with HeLa cells (data not shown). These results suggest a cell-type-specific mechanism for the ER-dependent activation of the BCK promoter.

## DISCUSSION

The BCK gene has been shown to be estrogen responsive in vivo and was identified as the major estrogen-induced protein in rat uterus (34). Subsequently, the BCK gene has been shown to respond to a variety of steroid hormones in multiple cell types (34, 37–39). In fact, the ability of this gene to respond to such a wide range of steroid hormones suggests that this induction occurs by a mechanism distinct from that involving standard steroid hormone response elements.

Transactivation of the BCK promoter in response to estrogen can be reproduced in vitro upon transient gene transfer in cell culture (39, 48). We have mapped the elements required to mediate the estrogen response, using a series of BCK promoter deletion CAT reporter constructs and linker-scanning mutants. These elements turn out to differ significantly from a consensus ERE. Instead, the response maps to sequences between bp −195 and −37, which include a TA-rich sequence at −66 and two adjacent CCAAT sequences. Mutations at either site compromise the estrogen response. Mutations in the TA-rich sequence have the most severe effect on the estrogen response, and this response can be restored by a similar TA-rich sequence from the MCK enhancer which has been shown in some assays to be functionally equivalent (21). However, mutations in either of the adjacent CCAAT sequences, particularly the 3′-CCAAT sequence, also reduce the estrogen response. This suggests that either or both of these sequences also contribute to the estrogen effect and that the effect is not mediated by the TA-rich region alone. In support of this conclusion, preliminary experiments using three tandem TA-rich binding sites from the BCK promoter fused to a thymidine

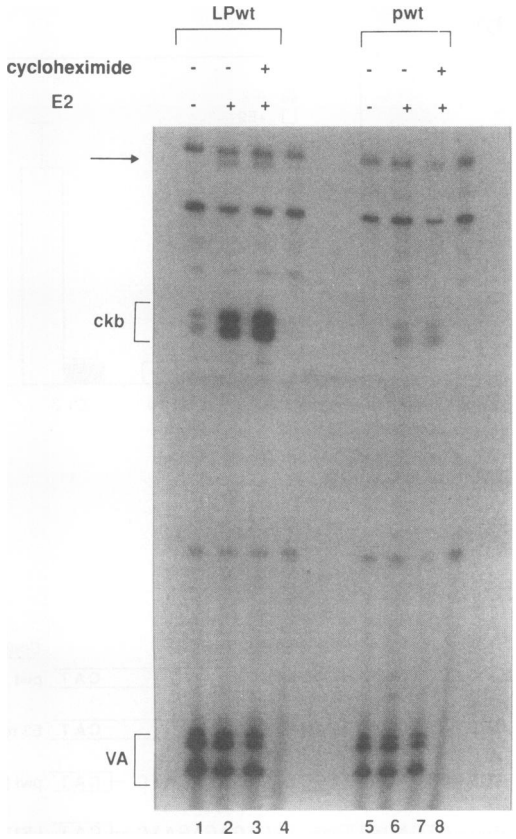


FIG. 5. Estrogen stimulation of the BCK promoter is resistant to cycloheximide. HeLa cells were transfected with 15 μg of LPwt or 10 μg of pWT reporter plasmids and 2 μg of HEG0 expression plasmid. Cycloheximide was added to a final concentration of 10 mg/ml along with β-estradiol where indicated. RNA was isolated, and primer extension assay was performed as described in Materials and Methods. The primer extension products of the CAT and VA RNA are indicated by ckb and VA, respectively. The arrow indicates BCK promoter-driven CAT transcription initiated from the upstream start site. The results of a typical experiment are shown.

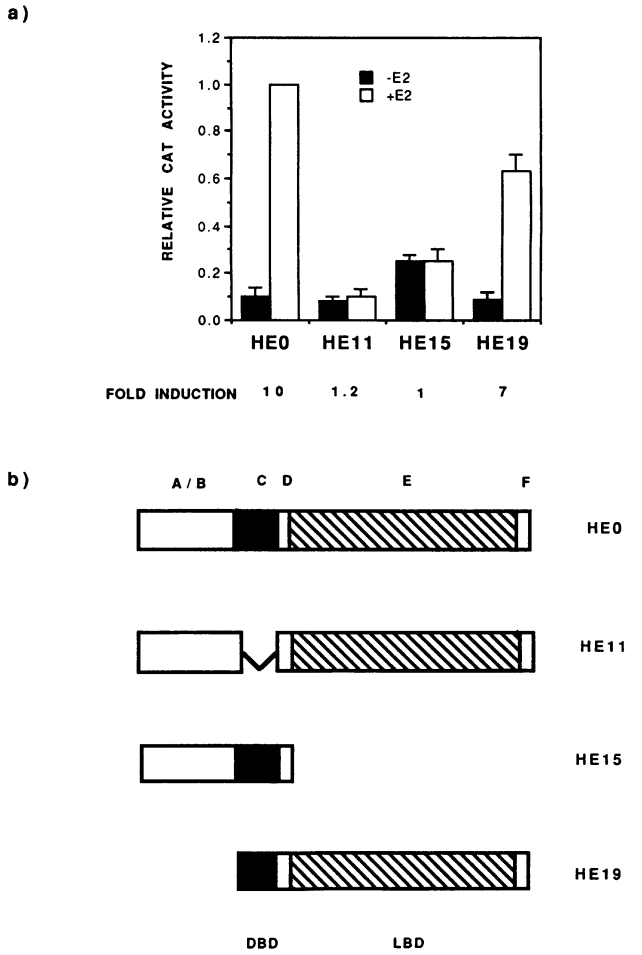


FIG. 6. Domains of the ER required for stimulation of the BCK promoter. Truncated mutants of the ER used are shown at the bottom. These expression plasmids (1 μg) were cotransfected into HeLa cells with pwtCAT and pCH110 and assayed as described in the legend to Fig. 2. (a) Relative CAT activity with the various ER deletion mutants. (b) Schematic representation of the various ER mutants. The various domains (A through F) are from reference 45. The DNA-binding domain (DBD) and the ligand-binding domain (LBD) are indicated.

kinase promoter-luciferase reporter construct cotransfected with HE0 into HeLa cells has demonstrated no estrogen induction (40). In contrast, BCK promoter sequences from bp −195 to −37 can confer estrogen responsiveness to a heterologous promoter (Fig. 4b). These results, taken together with our mutational analysis of the BCK promoter region, suggest that both the TA-rich and the CCAAT DNA sequence elements and possibly the protein complexes bound to these sites play an important role in the estrogen induction of the BCK promoter. We have previously shown that these DNA sequences bind discrete protein complexes in a gel shift assay (18, 19).

The BCK promoter TA-rich sequence at bp −66 has been shown to be an important *cis*-acting element for expression driven by the nonconsensus TTAA element at bp −30 (18, 19, 21). However, the TA-rich sequence is also a perfect consensus TATA box that can bind TATA binding protein and support transcription in vitro by both polymerase II and polymerase III (31). Despite the fact that it is not normally functional in vivo, we felt it important to show that estrogen stimulation did not allow transactivation of this upstream promoter. Mapping of the BCK transcript start sites following estrogen induction on the short (195-bp) BCK promoter confirms that estrogen stimulates initiation from the normal downstream start site and that the role of the TATA box at bp −66 is to serve as a *cis*-acting mediator of this activation. This conclusion is also supported by the observed ability of the MCK enhancer TA-rich region to substitute for the BCK TA-rich region and mediate estrogen stimulation.

The ability of the ER to support BCK promoter activation through an element apparently unrelated to a consensus ERE suggests that this activation occurs via a novel mechanism different from that driven by a standard ERE. This conclusion is further supported by several observations. First, the BCK response to estrogen appears to be cell type specific. Whereas transactivation can be observed in proliferative HeLa cells and primary cardiac fibroblasts it is absent in nonproliferative primary cardiomyocytes. By contrast, transactivation of a vitellogenin gene construct driven by a conventional ERE is observed in all three cell types. The ability of estrogen to support receptor-mediated activation in fibroblasts argues that the result observed with HeLa cells is not an artifact of adaptation to cell culture or a problem with interspecies (rat versus human) variation.

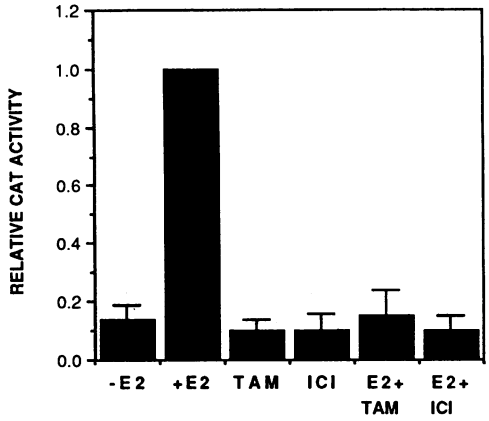


FIG. 7. Induction of the BCK promoter by estrogen can be selectively blocked by an estrogen antagonist. HeLa cells were transfected with pWT, HE0, and pCH110 plasmids and assayed as described in the legend to Fig. 2. Sixteen hours after transfection, the medium was replaced with medium containing β-estradiol (10 nM) or the estrogen antagonists 4-hydroxytamoxifen (TAM) or ICI 164,384 (ICI) (100 nM).

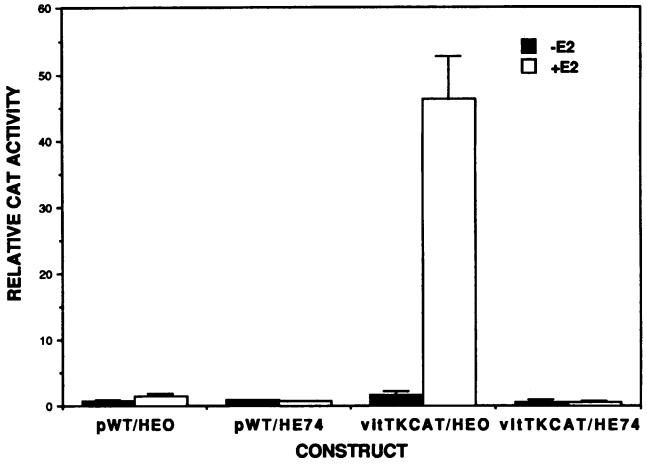


FIG. 9. Estrogen induction of the BCK promoter is cell type specific. BCK promoter construct pWT or the vitellogenin gene construct vitTKCAT was cotransfected into primary neonatal rat cardiomyocytes with wild-type ER (HE0) or mutant ER (HE74), as described in Materials and Methods. Relative CAT activities are shown and represent the averages of at least three independent experiments. Standard errors are indicated by the error bars.

Second, we have mapped the domains of the ER required for estrogen induction. The ligand- and DNA-binding domains of the ER are required for the estrogen-mediated induction of the BCK promoter. However, receptor N-terminal sequences containing the TAF1 activation domain (44) appear dispensible (Fig. 6). Not surprisingly, HE19, which contains the TAF2 activation domain but lacks the TAF1 domain, activates the BCK promoter in the presence of β-estradiol. This is consistent with the observed inhibition by the estrogen antagonists tamoxifen and ICI 164,368 (Fig. 7). However, deletion of the DNA-binding domain destroys receptor activity, suggesting that direct DNA binding might also be required. This simple explanation is negated by the observation that an ER mutant (HE74) that retains this domain but is unable to bind an ERE still supports efficient BCK estrogen activation. This same mutant is unable to support estrogen-dependent activation via the vitellogenin gene promoter ERE (vitTKCAT) in both HeLa and primary neonatal rat cardiomyocytes. These results strongly indicate that although the DNA-binding domain of the ER is required for BCK activation, direct DNA binding to an ERE is probably not. Instead, we suggest that activation occurs via interaction of the ER with other regulators capable of binding the BCK promoter in a manner that requires an intact ER DNA-binding domain. These interactions occur in a cell-type- and/or proliferation-specific manner.

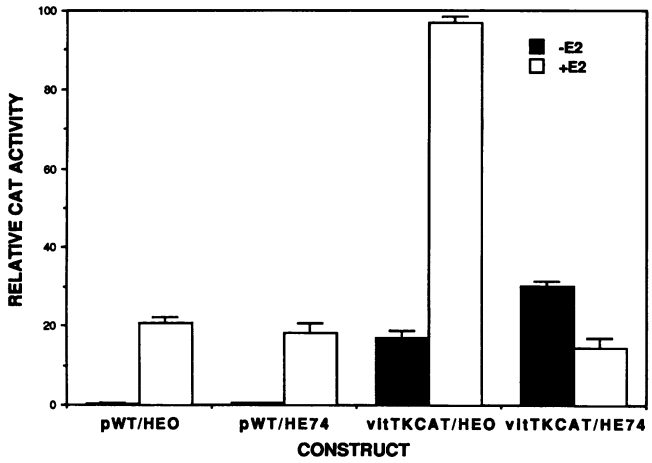


FIG. 8. DNA binding of the ER is not required for estrogen-dependent induction of the BCK promoter. BCK promoter construct pWT or the vitellogenin construct vitTKCAT, which contains a consensus ERE, was cotransfected into HeLa cells with wild-type ER (HE0) or a mutant ER with 6 amino acid substitutions within the DNA-binding domain (HE74), as described in Materials and Methods. Relative CAT activities are shown and represent the averages of at least three independent experiments. Standard errors are indicated by the error bars.

We have tested the potential contribution to this effect of several proteins known to interact with the ER. First, like many other steroid hormone receptors (35, 47, 49, 51), the ER interacts with transcription factors of the AP-1 family (10, 47). The region of the BCK promoter demonstrated here to support estrogen induction contains no consensus AP-1 binding sites. However, the promoter can be transactivated upon transient gene transfer by members of the AP-1 family (41). This response maps to sequences different from those mediating the estrogen response. For the BCK promoter, AP-1 and the ER appear to have additive activation effects (40).

Putative binding sites for AP-1 have been identified within the backbone of pUC plasmids and have been shown to have unique regulatory effects on transcription (29). It was therefore possible that vector sequences could mediate interactions with AP-1, and this in turn could bring the ER into proximity with regulators bound to the BCK promoter region. We therefore repeated the experiments with the BCK promoter in the background of the pGL2 luciferase reporter vector which lacks the unusual AP-1 recognition sequence described for pUC plasmids. The estrogen response was maintained, suggesting that these vector sequences are not responsible for the effect (data not shown).

We have identified at least two other proteins that interact with the TA-rich region of the BCK promoter. One is the TATA binding protein which binds the TA-rich element in the BCK promoter (31). We have demonstrated that the MCK

enhancer TA-rich region can also support BCK estrogen induction. As this MCK sequence cannot bind TBP (31), this argues against TBP binding to this element as a mediator of the estrogen effect.

Another factor that recognizes the BCK promoter is represented by the MADS box family of proteins, including rSRF and MEF2 (13, 33, 50). Although there is a controversy about the cell type specificity of these regulators, they are at least believed to be expressed in certain tissues in which BCK is also expressed, i.e., muscle and brain (50), and are therefore candidates for mediators of these transactivation effects. We have demonstrated that MADS box domain peptides can bind the BCK promoter TA-rich region (3a).

One activity that can interact with the TA-rich regions of both the BCK promoter and MCK enhancer is the activity we have previously reported as TARP (18, 19). TARP is present in all cell types and is therefore potentially distinct from the MEF2 family of MADS box proteins (8, 13, 50), although there are indications that TARP may be at least in part antigenically related to the rSRF family of factors (33). Currently the molecular identity of TARP is obscure, but it remains an attractive candidate for interacting with the ER.

Nevertheless, the observation of estrogen induction via an element capable of binding factors important for cellular growth control (the MADS family) is intriguing. For example, it has been reported that vitamin $D_3$ receptor activation can initiate a signalling cascade involving *raf* kinase and protein kinase C (28), and the existence of a common pathway for growth factor and steroid hormone receptor signaling has already been proposed for epidermal growth factor and the ER (23). Thus, it is possible that the ER can initiate a signaling cascade similar to that initiated by growth factors. MADS box family proteins like SRF are known to be modulated by phosphorylation in response to such signaling pathways (11) and therefore represent attractive downstream targets for growth factor stimulation.

In summary, the BCK promoter responds to estrogen in an ER-dependent manner. This response requires the receptor DNA-binding domain but not that the receptor be able to bind directly via a typical ERE. The required interactions appear cell type specific in that they are not functional in cardiomyocytes. The response is mediated by a region of the BCK promoter that contains a TA-rich region and a CCAAT box. Regulatory proteins binding to these regions may interact directly with the ER or potentially may respond indirectly to ER-mediated signaling cascades. BCK induction often accompanies cellular proliferative responses, and estrogen is known to have proliferative effects on cells, the mechanistic basis of which are unknown. This raises an additional intriguing possibility that BCK estrogen induction is sensitive to the proliferative capacity of the cell. These possibilities will provide impetus for further study on the specific mechanism whereby estrogen can induce the BCK promoter.

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