

Repression of the Interleukin-6 Promoter by Estrogen Receptor Is Mediated by NF- κ B and C/EBP β

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Bone metabolism is regulated by a balance between bone resorption caused by osteoclasts and bone formation caused by osteoblasts. This balance is disturbed in postmenopausal women as a result of lower serum estrogen levels. Estrogen, which is used in hormone replacement therapy to prevent postmenopausal osteoporosis, downregulates expression of the interleukin 6 (IL-6) gene in osteoblasts and bone marrow stromal cells. IL-6 is directly involved in bone resorption by activating immature osteoclasts. We show here that NF- κ B and C/EBP β are important regulators of IL-6 gene expression in human osteoblasts. Importantly, the IL-6 promoter is inhibited by estrogen in the absence of a functional estrogen receptor (ER) binding site. This inhibition is mediated by the transcription factors NF- κ B and C/EBP β . Evidence is presented for a direct interaction between these two factors and ER. We characterized the protein sequence requirements for this association in vitro and in vivo. The physical and functional interaction depends in part on the DNA binding domain and region D of ER and on the Rel homology domain of NF- κ B and the bZIP region of C/EBP β . The cross-coupling between ER, NF- κ B, and C/EBP β also results in reduced activity of promoters with ER binding sites. We further show that the mechanism of IL-6 gene repression by estrogen is clearly different from that of activation of promoters with ER binding sites. Therefore, drugs that separate the transactivation and transrepression functions of ER will be very helpful for treatment of osteoporosis without causing undesirable side effects.

Bone metabolism is regulated by a balance between bone resorption caused by osteoclasts and bone formation caused by osteoblasts. Osteotropic factors such as parathyroid hormone, interleukin-1 (IL-1), and tumor necrosis factor alpha do not directly activate osteoclasts but rather stimulate osteoblasts to secrete cytokines (8, 20, 33, 50, 75, 76). Activated osteoblasts secrete macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor (27, 28), tumor necrosis factor alpha (22), and IL-6 (20, 21, 30, 45, 47). These cytokines act directly on osteoclast progenitor cells and induce differentiation into mature osteoclasts (30, 45, 56).

Osteoporosis is a disease that results in accelerated bone loss. The most common form is postmenopausal osteoporosis in women after menopause or ovariectomy. Postmenopausal osteoporosis affects 1.5 million people each year, making it a major health care problem. The disease clearly correlates with the loss of estrogen production and can be prevented by early estrogen replacement therapy (19, 34, 52). These studies are supported by experiments in mice that showed that ovariectomized mice develop osteoporosis, which can be prevented by estrogen (32). To a lesser extent, older men are affected by osteoporosis, and a recent study showed that low levels of estrogen rather than of testosterone are the cause (69). The mechanism by which estrogen prevents osteoporosis is still unclear.

IL-6 is a multifunctional cytokine that exerts a variety of well-established effects, including B- and T-cell activation, stimulation of fever, and release of acute-phase response proteins (26, 36, 40, 80, 81). IL-6 also seems to play a major role in the activation process of immature osteoclasts (29, 45, 65, 66). Animal studies showed that ovariectomized mice did not develop osteoporosis if an antibody to IL-6 was administered

(32, 37). A recent in vivo study underscores the importance of IL-6 even more. IL-6 knockout mice are viable and do not express any evident phenotypic abnormality. However, ovariectomy in these mice does not cause osteoporosis, indicating that IL-6 is essential for the bone loss caused by estrogen deficiency (60).

Several recent studies indicated that estrogen downmodulates IL-6 cytokine production in osteoblasts and bone marrow stromal cells (21, 32, 59). This effect is on transcriptional activity and has been narrowed down to a 224-bp promoter fragment (61, 63). Surprisingly, the IL-6 promoter has no binding site for the estrogen receptor (ER) (61, 63) suggesting that ER may have an indirect role in IL-6 gene expression. It is possible that transcription factors that are involved in IL-6 gene regulation such as NF- κ B and C/EBP β are the target for ER. The mechanism of inhibition of these factors by estrogen could be similar to the described interaction of the glucocorticoid receptor with AP-1 or NF- κ B (35, 62, 68, 82).

We have investigated in detail the mechanism of IL-6 promoter inhibition by estrogen. We defined the transcriptional control elements in the IL-6 promoter that are involved in IL-1 β induction in osteoblasts. Further, we narrowed down the region in the IL-6 promoter that mediates inhibition by estrogen to the binding sites for NF- κ B and C/EBP. We present evidence for a physical interaction of ER with NF- κ B and C/EBP β . Further, we demonstrate that NF- κ B and C/EBP β are the target for ER in vivo, resulting in reduced IL-6 promoter activity as well as in reduced activity of promoters with ER binding sites. We also show that estrogen does not significantly affect cellular I κ B α levels. Further, we discuss a model for prevention of osteoporosis.

MATERIALS AND METHODS

Plasmid constructs. The series of IL-6 promoter luciferase constructs was generated by replacing the chloramphenicol acetyltransferase (CAT) reporter gene from the original plasmids (70) with the *XhoI-HpaI* luciferase gene fragment from pGL2-Basic (Promega). GST-HE0, the bacterial glutathione S-trans-

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ferase (GST) fusion protein expression vector for ER (HE0), was derived from pGEX1-N (Amrad) by inserting the cDNA for human ER into the *EcoRI* site. GST-p65Eco, GST-C/EBP β bZIP, and expression vectors for in vitro translation in rabbit reticulocyte lysate were described previously (74). The estrogen-responsive reporter plasmid IR3-SV40-LUC was generated by ligating an oligonucleotide composed of a consensus ER binding site, 5'-AGCTTGTCAGGTCAGC GTGACCTCCA-3', into pGL2-Promoter (Promega). The reporter plasmid A2-ERE-TK-LUC, containing two copies of the vitellogenin gene A2 estrogen response element, was generated by replacing the CAT reporter gene from the original plasmid (49) with a luciferase gene fragment from pGL2-Basic (Promega). Similarly, the CAT reporter gene of -342/+115 I κ B α -CAT (31) was replaced by the luciferase gene fragment from pGL2-Basic (Promega). MHC- κ B-SV40-LUC was generated by ligating an oligonucleotide composed of the major histocompatibility complex class I promoter NF- κ B binding site (5'-GGG GATTCCCC-3') into pGL2-promoter (Promega). Expression vectors for wild-type ER SG5-HEG0 and mutant forms are as previously described (43, 44). The variant SG5-HE0 (one amino acid exchange in position 400) and mutant forms SG5-HE11 and SG5-HE14 were generated by ligating the ER cDNA from KCR2-HE0, KCR2-HE11, and KCR2-HE14, respectively, into *EcoRI*-cut SG5 vector. SG5-HEG0:HindIII and SG5-HEG0:XcmI were generated by cutting the SG5-HEG0 plasmid with *HindIII* and *XcmI*, respectively, inserting an oligonucleotide with translation stop codons, and religating the vector. This procedure generates deletion mutants encoding amino acids 1 to 340 and 1 to 271 of human ER, respectively. The SG5 series of ER expression vectors was also used for in vitro transcription and translation. The CMV4T expression vectors for NF- κ B, C/EBP β , Jun, and I κ B α are as described previously (73, 74).

Transfection of cells and analysis of luciferase activity. MCF-7 cells were cultured in phenol red-free Iscove's Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS), 500 mg of L-glutamine per liter, 10 μ g of insulin per ml, and antibiotics. U2-OS cells were cultured in phenol red-free McCoy's 5A medium supplemented with 10% FCS, 500 mg of L-glutamine per liter, and antibiotics. U2-OS cells (1.5×10^6) were transfected by electroporation (260 V, 960 μ F) and plated in phenol red-free McCoy's 5A medium supplemented with 5% dextran-charcoal-stripped FCS and antibiotics at a density of 10^5 cells per well of a 96-well flat-bottom plate. Twenty-four hours later, cells were stimulated for 8 h and then harvested in 100 μ l of lysis buffer. Luciferase activity in 50 μ l of cell lysate was measured with a Monolight 9600 Microplate Luminometer from Analytical Luminescence Laboratory, San Diego, Calif.

Protein analysis. Expression of bacterial GST fusion proteins and GST fusion protein interaction assays were performed as described previously (74). For Western blot (immunoblot) analysis whole cell extracts from $\sim 10^6$ U2-OS cells were prepared by harvesting cells in ice-cold phosphate-buffered saline and pelleting at $2,000 \times g$ at 4°C for 1 min. The cell pellet was resuspended in lysis buffer composed of 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.9)-300 mM NaCl-1.5 mM MgCl $_2$ -0.2 mM EDTA-0.5% Triton X-100-5 mM dithiothreitol-5 mM benzimidazole-1 mM phenylmethylsulfonyl fluoride-2 μ g of aprotinin per ml-2 μ g of leupeptin per ml-100 μ M calpain inhibitor I. Equal amounts of lysate were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a nitrocellulose membrane, and immunostained with antibodies specific for human I κ B α (kindly provided by Joseph DiDonato). Stained proteins were visualized by use of an Amersham enhanced chemiluminescence kit according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay ELISA. Human IL-6 cytokine levels were measured with a Cytoscreen immunoassay kit from Biosource International, Camarillo, Calif., according to the manufacturer's instructions.

Peptide aldehyde protease inhibitors. Benzylloxycarbonyl-Ile-Asp-leucinal (SynPep Corp.), benzylloxycarbonyl-Ile-Asp(*t*-butyl)-leucinal (SynPep Corp.), calpain inhibitor I (Calbiochem), and benzylloxycarbonyl-Leu-Leu-phenylalanylinal (kindly provided by Bob Sullivan) are described in reference 15. All peptide aldehydes were dissolved in 100% ethanol and used at a final concentration of 25 μ M.

RESULTS

Regulation of IL-6 gene expression by IL-1 β in osteoblasts.

To characterize the DNA elements that are involved in transcriptional regulation of IL-6 gene expression in osteoblasts, a series of 5' deletion mutants of an IL-6 promoter construct containing IL-6 gene sequences from positions -724 to +11 was created (Fig. 1). These constructs were transiently transfected by electroporation into the human osteoblast cell line U2-OS. We selected U2-OS cells for these experiments because of their higher transfection efficiency compared with those of other human osteoblast cell lines that we tested. Cells were stimulated by the addition of IL-1 β before measurement of luciferase activity. Luciferase activity of the -724 to +11 IL-6 construct was stimulated two- to threefold by IL-1 β (Fig.

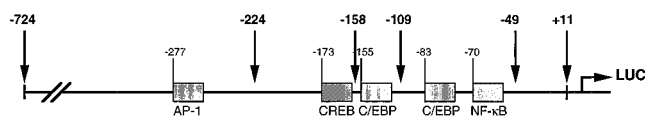


FIG. 1. IL-6 promoter constructs. Relevant transcription factor binding sites are shown. The arrows indicate the 5' deletion points of the truncated constructs. LUC, luciferase.

2). These data suggest that the promoter region from positions -724 to +11 contains important regulatory elements for IL-6 gene expression. Deletion of sequences down to position -158, which removes putative AP-1 and CREB binding sites, resulted in even greater (15- to 20-fold) stimulation. Removal of a C/EBP binding site between positions -154 to -146 (plasmid -109/+11 IL-6) had no significant effect on fold stimulation of this transfected promoter construct in U2-OS cells (Fig. 2). This finding suggests that this upstream C/EBP binding site in the context of the natural promoter is not very important for IL-6 gene regulation in osteoblasts. Further deletion of sequences down to position -49 removes a downstream C/EBP site (positions -83 to -75) as well as an NF- κ B binding site. This construct was unresponsive to IL-1 β . Similarly, IL-6 promoter constructs with point mutations in these two transcription factor binding sites did not respond to IL-1 β stimulation (data not shown). We further show that RelA and C/EBP β are strong activators of the -158/+11 IL-6 promoter construct (see Fig. 7A). We conclude that the region between positions -109 and -49 functions as a positive regulatory element in response to treatment of U2-OS cells with IL-1 β .

The IL-6 promoter is inhibited by estrogen. ERs have been previously found in human, rat, and mouse osteoblast-like cells (7, 17, 18, 38) and bone marrow stromal cells (6, 21, 32). In addition, several studies have demonstrated a downregulation of IL-6 cytokine production in osteoblastic and stromal cells by estrogen (21, 32, 59, 61, 63). Further, these studies showed that the inhibition by estrogen is on the level of IL-6 mRNA ex-

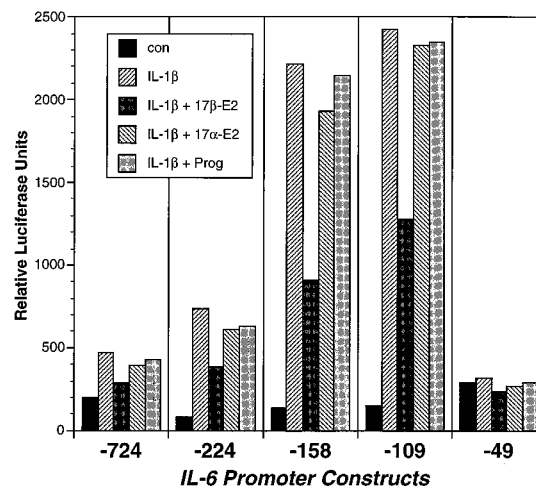


FIG. 2. Regulation of the IL-6 promoter. U2-OS cells were transiently transfected by electroporation with 10 μ g of IL-6 promoter constructs (the 5' deletion endpoints are indicated) and 2 μ g of SG5-HE0 expression vector. Twenty-four hours after transfection cells were pretreated for 30 min with 10^{-8} M 17 β -estradiol (17 β -E2), 17 α -estradiol (17 α -E2), progesterone (Prog), or the vehicle ethanol (control [con]) before the addition of 5 ng of human IL-1 β per ml. Cells were harvested 8 h later and analyzed for luciferase activity. A representative experiment is shown. All experiments were repeated at least three times and showed less than 20% deviation of luciferase activity between experiments.

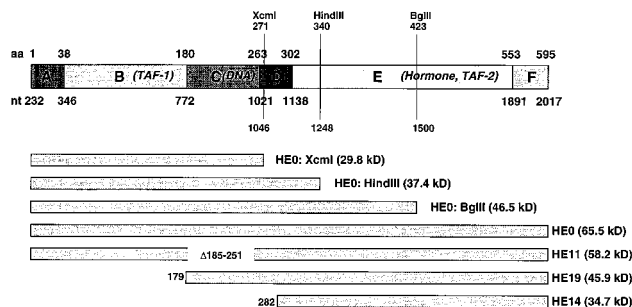


FIG. 3. Human ER. Relevant protein domains are shown. Numbers above the sequence show the amino acid positions; numbers below show the nucleotide positions of the expression vector. Restriction enzyme-cut sites used for cloning of some of the mutants are indicated. Mutant ERs are displayed with their names and molecular masses.

pression. Interestingly, the IL-6 promoter has no known binding site for ER, and two recent publications have shown the absence of any binding of ER to IL-6 promoter sequences (61, 63). This finding prompted us to define the IL-6 promoter region that mediates downregulation by estrogen.

A series of 5' deletion mutants of the IL-6 promoter was transfected together with an expression plasmid for wild-type ER into U2-OS cells. Cells were pretreated with 17β -estradiol, the biologically active form of estrogen, and then stimulated with IL-1 β before analysis of luciferase activity. In the presence of wild-type ER, estrogen repressed IL-1 β induction of promoter constructs as short as positions -109 to $+11$ by about 50% (Fig. 2). The effect of 17β -estradiol is specific, since the biologically inactive stereoisomer 17α -estradiol and progesterone, tested at identical concentrations, had no effect. The concentration for 50% inhibition of IL-6 gene expression by 17β -estradiol is about 10^{-10} M (data not shown). Estrogen had no effect on IL-6 gene expression in the absence of ER (see Fig. 4A, cotransfection with SG5). As noted above, the $-109/+11$ construct contains a C/EBP and an NF- κ B binding site. Estrogen had no effect on the transcriptional activity of the -49 deletion construct missing these two transcription factor binding sites. We obtained similar results in human MCF-7 breast cancer cells that express high levels of endogenous ER and that therefore make the cotransfection of ER plasmid unnecessary (data not shown). These data establish that both ER and hormone are required for the estrogen-mediated repression of IL-6 promoter activation by IL-1 β . The target for ER is the IL-1 response element between positions -109 and -49 .

Regions of ER necessary for repression. Human ER is a 595-amino-acid protein that has been extensively studied by mutational analyses (42, 48, 77). Additionally, it has been shown that ER exhibits two distinct transactivation domains in the N terminus (TAF-1) and the C terminus (TAF-2) involved in transactivation of estrogen-dependent reporters (77, 79). This finding prompted us to define the regions in ER that are required for transrepression of the IL-6 promoter. Expression vectors encoding a series of 5' and 3' deletion mutants of ER were constructed (Fig. 3) and cotransfected with an IL-6 promoter construct. In the presence of the empty expression vector SG5, no effect of estrogen on IL-6 gene expression was observed. Cotransfection of wild-type ER (HEG0) led to about 50% repression of phorbol ester (phorbol myristate acetate [PMA])- and IL-1 β -induced levels in the presence of estrogen (Fig. 4A). Deletion of the TAF-2 region and the hormone binding domain (HE0:XcmI) created a mutant that was not able to transrepress the IL-6 promoter (Fig. 4A). An ER mutant with a deletion of the N-terminal TAF-1 domain (HE19)

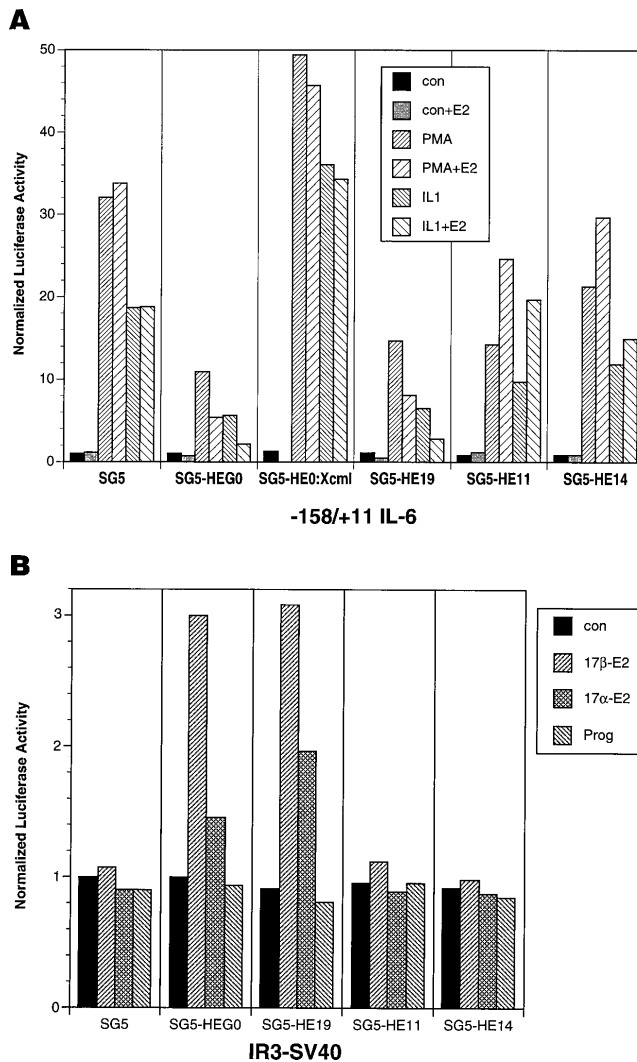


FIG. 4. Sequence requirements of the ER for IL-6 promoter inhibition. (A) U2-OS cells were transiently transfected by electroporation with $10 \mu\text{g}$ of the $-158/+11$ IL-6 promoter constructs and $2 \mu\text{g}$ of empty SG5 expression vector, the wild-type ER HEG0, or one of four mutant receptors as indicated. Twenty-four hours after transfection, cells were pretreated for 30 min with 10^{-8} M 17β -estradiol (E2) or the vehicle ethanol alone before the addition of PMA (50 ng/ml) or human IL-1 β (5 ng/ml), or left untreated. (B) U2-OS cells were transiently transfected by electroporation with $10 \mu\text{g}$ of the IR3-SV40 reporter and $2 \mu\text{g}$ of empty SG5 expression vector, the wild-type ER HEG0, or one of three mutant receptors as indicated. Twenty-four hours after transfection, cells were stimulated with 10^{-8} M 17β -estradiol (17β -E2), 17α -estradiol (17α -E2), progesterone (Prog), or the vehicle ethanol (control [con]). Cells were harvested 8 h later and analyzed for luciferase activity. Representative experiments are shown. All experiments were repeated at least three times and showed less than 20% deviation of luciferase activity between experiments.

still mediated inhibition of IL-6 gene expression by estrogen (Fig. 4A; compare SG5-HEG0 with SG5-HE19). Internal deletion of the DNA binding domain (HE11) or deletion of the N terminus up to amino acid 281 (HE14) created mutants that were no longer able to repress the IL-6 promoter. Surprisingly, cotransfection of these mutants reversed the effect of estrogen and resulted in up to twofold activation of IL-6 gene expression.

In parallel, we tested these ER mutants on a minimal estrogen-responsive reporter (IR3-SV40-LUC), composed of one copy of an ER response element in front of a minimal simian virus 40 promoter (Fig. 4B). The TAF-1 activation domain was dispensable for activation by estrogen (Fig. 4B, SG5-HE19),

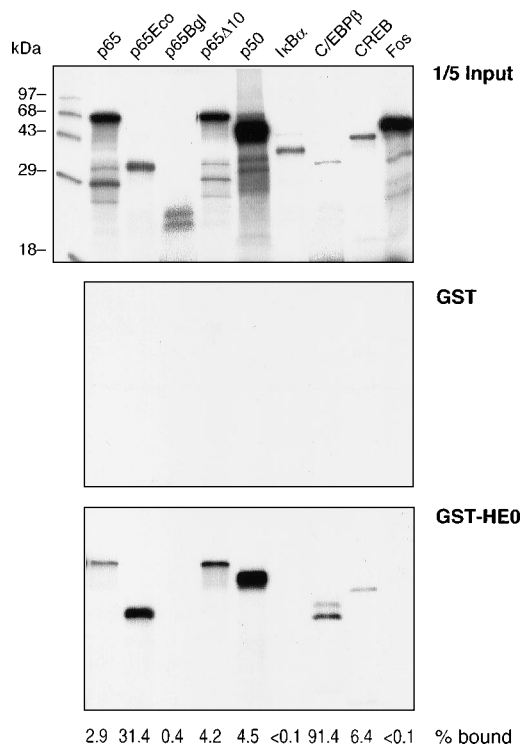


FIG. 5. In vitro association between ER and NF- κ B and C/EBP family members. Radiolabeled, rabbit reticulocyte lysate-translated proteins (5 μ l) were incubated with bacterially expressed GST protein (middle panel) or GST-HE0 wild-type ER (bottom panel) immobilized on glutathione-Sepharose 4B beads. The top panel shows one-fifth of the input amounts used for the other two panels. After washing, the bound proteins were eluted and analyzed by SDS-PAGE. Equal amounts of GST and GST-HE0 proteins were used. Numbers at the bottom represent bound protein relative to the input amount. Theoretical isoelectric points of the translated proteins as calculated by MacVector software (IBI-Kodak) are as follows: p65, 5.33; p50, 6.55; I κ B α , 4.38; C/EBP β , 8.88; CREB, 5.28; and c-Fos, 4.58.

but as expected, there was a clear requirement for the DNA binding region (Fig. 4B, SG5-HE11 and SG5-HE14). From these results together, we conclude that sequences within the DNA binding domain contribute to transrepression.

In vitro association of ER with NF- κ B and C/EBP β . Several studies demonstrated that steroid hormone receptors down-regulate genes without binding sites for the receptor proteins. One well-documented example is the association between glucocorticoid receptor and AP-1 (24, 35, 39, 68, 78, 82). Further, NF- κ B and C/EBP bind to the glucocorticoid receptor (55, 62), and AP-1 also binds to ER (16). With the data presented so far, we established that NF- κ B and C/EBP are required for activation of IL-6 gene expression and that the promoter region binding these factors mediates transrepression by estrogen. Therefore, we were interested whether the repression by estrogen is the result of an interaction of ER with one of these two transcription factors.

We were able to demonstrate the specific binding of GST-HE0 fusion protein to RelA (NF- κ B p65), NF- κ B1 (NF- κ B p50), and C/EBP β (Fig. 5). This interaction is not dependent on the presence of estrogen. The specificity of this protein-protein association is demonstrated by the lack of binding of I κ B α and Fos proteins to ER. These two proteins have an isoelectric point that is similar to those of RelA and NF- κ B1, suggesting that the lack of binding is not caused by a drastic difference in electric charges (see the legend to Fig. 5). A

shorter truncation of RelA (p65Eco) encompassing only amino acids 1 to 282 of the Rel homology domain is sufficient to bind to ER. The interaction was lost when only amino acids 1 to 194 (p65Bgl) were present. p65 Δ 10, a naturally occurring variant of RelA that has an internal 10-amino-acid deletion and that can dimerize only weakly (53, 71), still efficiently bound to ER.

To investigate which sequences inside ER are required for association with NF- κ B and C/EBP β , bacterially expressed GST fusion proteins of NF- κ B and C/EBP β were tested for binding to a series of C-terminal deletions of ER. Deletion of amino acids between positions 271 and 340 strongly diminished the interaction with NF- κ B and C/EBP β (Fig. 6A; compare lanes HindIII and XcmI). The DNA binding domain is not necessary for the physical interaction, since HE11 still efficiently bound to NF- κ B and C/EBP β (Fig. 6B). Removal of amino acids from the N terminus up to position 282 (HE14) led to loss of interaction with both transcription factors. These data suggest an important role of hormone receptor region D for the physical interaction between ER and NF- κ B and C/EBP β in vitro.

Cross-coupling between ER and NF- κ B and C/EBP β results in inhibition of gene activity. The previous data led us to further analyze the mechanism of IL-6 promoter inhibition by estrogen with a series of cotransfection experiments. These experiments might determine if ER directly associates in vivo with NF- κ B and C/EBP β . Overexpression of RelA or C/EBP β transactivated the -158/+11 IL-6 promoter in U2-OS and MCF-7 cells, indicating the presence of functional NF- κ B and C/EBP binding sites (Fig. 7A). A combination of RelA and C/EBP β resulted in strong synergistic effects. As expected, NF- κ B1 and Jun were unable to stimulate the IL-6 promoter (data not shown). Estrogen effectively downmodulated RelA- and C/EBP β -induced IL-6 promoter activity in MCF-7 cells by about 50%. In U2-OS cells, we were able to demonstrate only an inhibition of C/EBP β -mediated transactivation (see Discussion). We excluded that ligand-activated ER inhibits the transcriptional activity of the cytomegalovirus enhancer used in the expression plasmids for RelA and C/EBP β by cotransfecting a cytomegalovirus-luciferase reporter plasmid with an ER expression vector. Thus, the experiments described above demonstrate that ligand-activated ER interferes with the function of NF- κ B and C/EBP β .

We then extended this observation by cotransfecting a promoter containing an ER binding site (A2-ERE-TK) with expression vectors for NF- κ B and C/EBP β in the presence of ER. A possible physical interaction between these proteins might result in inhibition of the estrogen-responsive promoter. The data presented in Fig. 7B confirm this hypothesis. RelA and to a lesser extent C/EBP β downregulated an estrogen-responsive promoter up to fivefold. This result is specific, since Jun and I κ B α did not transrepress and the inhibition depends on the presence of the ER binding site (see results with the thymidine kinase promoter alone). All proteins were expressed at similar levels, and the expression of one protein did not affect the expression of the other (data not shown). The data presented in Fig. 7 demonstrate that ER and RelA mutually repress their transactivation potentials. These findings are in line with our in vitro interaction results presented in Fig. 5 and 6.

Analysis of I κ B α levels in the presence of estrogen. NF- κ B is regulated by a family of cytoplasmic inhibitor proteins, termed I κ B. One well-characterized member is I κ B α , whose degradation is induced by various stimuli that lead to NF- κ B activation (5, 9, 13, 25, 51). Therefore, any effects of estrogen on I κ B could have indirect effects on NF- κ B levels. To explore this possibility, we first tested three different peptide aldehydes

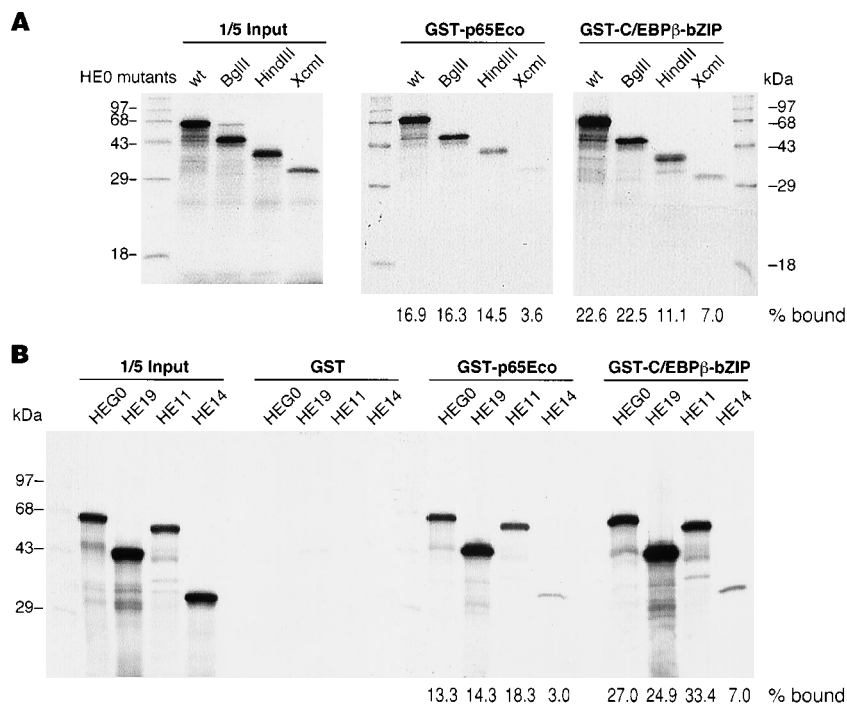


FIG. 6. Sequence requirements for the ER interaction with NF- κ B and C/EBP. Radiolabeled, rabbit reticulocyte lysate-translated ER mutants (5 μ l) were incubated with bacterially expressed GST, GST-p65Eco, or GST-C/EBP β -bZIP immobilized on glutathione-Sepharose 4B beads. (A) A series of C-terminal deletions of the ER was used. wt, HEG0 wild type; BglII, HE0:BglII (amino acids 1 to 423); HindIII, HE0:HindIII (amino acids 1 to 340); XcmI, HE0:XcmI (amino acids 1 to 271). (B) Internal and N-terminal deletions of the ER were used. See Fig. 3 for structures of these mutants. The leftmost gels in panels A and B show one-fifth of the input amounts used for the other gels. After washing, the bound proteins were eluted and analyzed by SDS-PAGE. Equal amounts of GST, GST-p65Eco, and GST-C/EBP β -bZIP proteins were used. Numbers at the bottom represent bound protein relative to the input amount.

that have been shown to prevent I κ B α degradation in HepG2 and HeLa cells by blocking the proteasome complex (15). These peptide aldehydes efficiently blocked stimulation of IL-6 promoter activity by PMA in U2-OS cells, while the control peptide benzyloxycarbonyl-Ile-Asp-leucinal had no effect (Fig. 8). These results indicate that reagents that affect I κ B levels also affect the activity of the κ B-dependent IL-6 promoter.

We then prepared protein extracts from U2-OS cells that have been transfected with ER and stimulated with various combinations of estrogen and IL-1 β as indicated (Fig. 9A). We analyzed I κ B α (MAD-3) levels in these extracts by Western blotting. Estrogen slightly augmented the IL-1 β -induced degradation of I κ B α (Fig. 9A; compare lanes 2 to 5 with lanes 7 to 10) and slightly reduced I κ B α levels by itself (Fig. 9A; compare lane 1 with lane 6). To confirm that the transfection efficiency for ER was high enough to detect changes on I κ B levels in Western blots and that estrogen actually inhibited the IL-6 gene, we measured IL-6 cytokine release by ELISA in the same experiment (Fig. 9B) and found that estrogen reduced IL-6 levels by more than 50%. Additionally, we analyzed the transcriptional regulation of the human I κ B α promoter in this cell line. U2-OS cells were transfected with a promoter construct containing sequences from positions -342 to +115. This region contains three NF- κ B binding sites, shown to be necessary for tumor necrosis factor alpha induction in K562 cells (31). Our results showed an about 1.5-fold stimulation of basal level and less than 20% inhibition of IL-1 β -induced level by estrogen. Further, computer sequence analysis did not detect any potential ER response elements within the published sequence from positions -1275 to +115. Thus, we conclude that estrogen is not able to significantly modulate I κ B α levels.

DISCUSSION

In this report, we demonstrate the interaction of members of two distinct transcription factor families, NF- κ B and C/EBP, with ER. This interaction leads to reduced transcriptional activity at NF- κ B-, C/EBP-, and ER-dependent promoters. The inhibition depends on the DNA binding domain of ER and the DNA binding and dimerization domains of NF- κ B and C/EBP. More importantly, the results presented here give detailed insight how estrogen inhibits the cytokine IL-6 at the transcriptional level.

Transient transfection experiments with human osteoblasts demonstrate that osteotropic factors such as IL-1 β elevate IL-6 levels by an increase in transcription mediated by the transcription factors C/EBP β and NF- κ B. We measured upregulation of IL-6 in U2-OS and four other human osteoblast cell lines. IL-1 is also a potent stimulator of IL-6 production in other cell types such as monocytes and fibroblasts (54, 84). Interestingly, deletion of IL-6 promoter upstream sequences to position -158 increases the fold induction by IL-1 β . This is possibly due to removal of negative regulatory sequences responsible for downmodulation of transcription. Several other potential transcription factor binding sites upstream of position -158 seem to have no effect on IL-1 β inducibility of the IL-6 promoter, at least in U2-OS cells. The -158/+11 IL-6 construct harbors two C/EBP binding sites and one NF- κ B binding site that have been suggested to be involved in IL-6 promoter regulation in human monocytes (26, 46, 83). We confirm these results for osteoblasts and clearly show that C/EBP β and the NF- κ B subunit RelA (p65) alone are potent activators of the

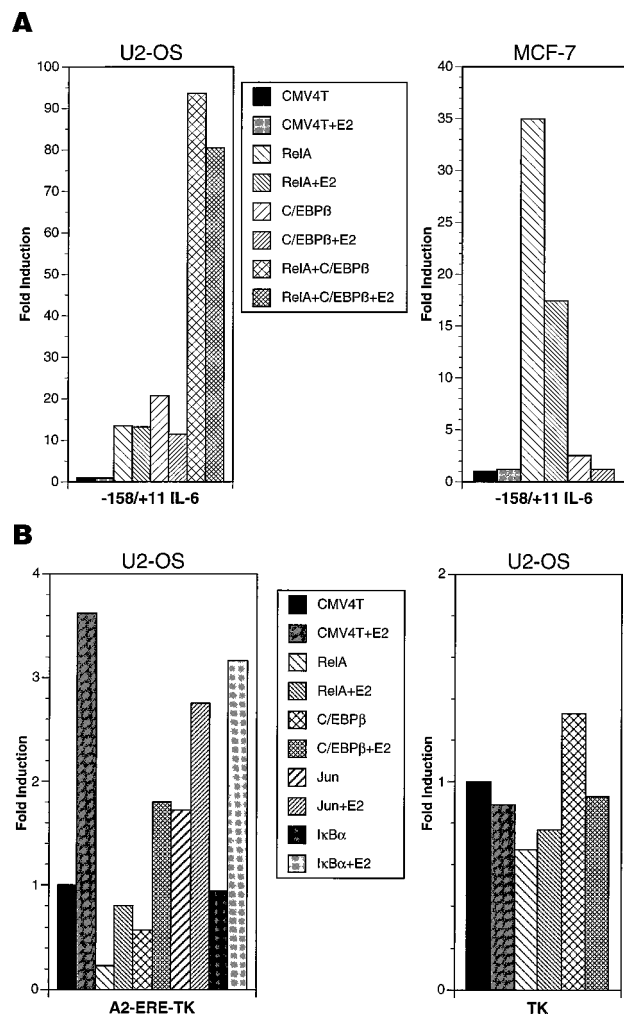


FIG. 7. Cross-coupling between ER and RelA and C/EBP β results in inhibition of gene transcription. (A) U2-OS and MCF-7 cells were transiently transfected by electroporation with 10 μ g of the $-158/+11$ IL-6 promoter construct, 2 μ g of SG5-HE0 expression vector (omitted for MCF-7 cells), and 25 ng (U2-OS) or 1 μ g (MCF-7) of expression vector for RelA or 100 ng (U2-OS) or 1 μ g (MCF-7) of expression vector for C/EBP β as indicated. E2, 17 β -estradiol. (B) U2-OS cells were transiently electroporated with 10 μ g of the A2-ERE-TK or thymidine kinase (TK) promoter construct, 2 μ g of SG5-HE0 expression vector, and 100 ng of expression vectors for RelA, C/EBP β , Jun, and I κ B α as indicated. Twenty-four hours after transfection, cells were treated with 10^{-8} M 17 β -estradiol (E2) or the vehicle ethanol alone. Cells were harvested 8 h later and analyzed for luciferase activity. Representative experiments are shown. All experiments were repeated at least three times and showed less than 20% deviation of luciferase activity between experiments.

IL-6 promoter and together mediate strong synergistic activation.

Bone metabolism is regulated by a balance between bone resorption caused by osteoclasts and bone formation caused by osteoblasts. Osteoporosis is a disease that results in accelerated bone loss. The cytokine IL-6 plays a major role in the activation process of immature osteoclasts. Several recent studies indicated that estrogen downmodulates IL-6 cytokine production in osteoblasts and bone marrow stromal cells (21, 32, 59). This finding prompted us to analyze the possibility that transcription factors that are involved in IL-6 gene regulation such as NF- κ B and C/EBP β are the target for ER.

For these studies, it was necessary to cotransfect wild-type ER since all human osteoblast cell lines tested had lost the

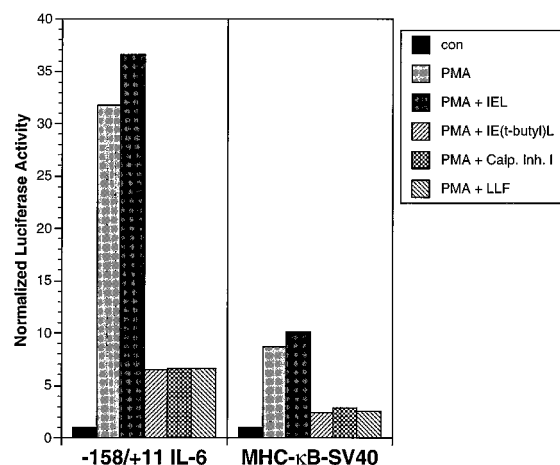


FIG. 8. Peptide aldehydes inhibit NF- κ B activity. U2-OS cells were transiently transfected by electroporation with 10 μ g of $-158/+11$ IL-6 promoter construct or MHC- κ B-SV40 reporter. Twenty-four hours after transfection, cells were pretreated for 30 min with 25 μ M the indicated peptide aldehydes (dissolved in ethanol) before the addition of PMA or left untreated (con). Cells were harvested 8 h later and analyzed for luciferase activity. A representative experiment is shown. All experiments were repeated at least three times and showed less than 20% deviation of luciferase activity between experiments. IEL, benzoyloxycarbonyl-Ile-Asp-leucinal; IE(t-butyl)L, benzoyloxycarbonyl-Ile-Asp(t-butyl)-leucinal; Calp. Inh. I, calpain inhibitor I; LLF, benzoyloxycarbonyl-Leu-Leu-phenylalaninal.

estrogen response. This can be attributed to a downregulation of ER in more differentiated cells (6) and to the fact that the ER gene is located in an unstable chromosomal region (41). Our data demonstrate that estrogen inhibits the IL-6 promoter through the region between positions -158 and -49 . Interestingly, ER does not bind to this DNA region (61, 63), and binding sites for NF- κ B and C/EBP are the only responsive elements present in this area. Therefore, it is likely that estrogen interferes with the transcriptional activity of at least one of these two transcription factor families. Inhibition of transcription factors by steroid hormones has been reported. One well-known example is the inhibition of AP-1 activity by glucocorticoid hormones through a direct physical interaction between the glucocorticoid hormone receptor (GR) and Jun or Fos (35, 62, 68, 82). Other described examples are the interaction of Jun with the ER (16), RelA with GR (10, 62), and C/EBP β with GR (55). Further, in support of our findings, these studies demonstrate a physical interaction between the steroid hormone receptor and the respective transcription factor. This association may introduce conformational changes in both proteins, altering their transcriptional activation potential. A ligand-induced conformational change is required for efficient activation of genes with ER response elements (4).

The inhibition of the IL-6 promoter by estrogen parallels above-mentioned studies in several aspects. The IL-6 promoter has no binding site for ER (61, 63). The interaction between ER and NF- κ B and C/EBP β occurs in the absence of DNA. The bZIP domain of C/EBP β and the Rel homology domain of RelA are sufficient for binding to ER. This finding suggests that NF- κ B and C/EBP β use the same domains for dimerization within their families and with ER. Similar results have been reported for the NF- κ B-AP-1 and NF- κ B-C/EBP interaction (72-74). Our mutational analyses of ER showed that amino acids 251 to 282 are critical for binding to NF- κ B and C/EBP β . Further, part of the DNA binding region C and the adjacent region D of ER are involved in transrepression as well as transactivation. This observation is consistent with other

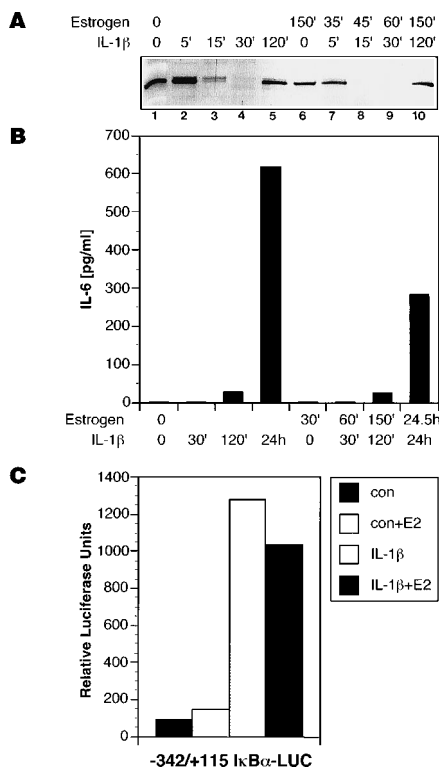


FIG. 9. Estrogen does not significantly affect $I\kappa B\alpha$ levels. (A) U2-OS cells were transiently transfected by electroporation with 5 μ g of SG5-HE0 expression vector. Twenty-four hours later, cells were pretreated for 30 min with 10^{-8} M estrogen before the addition of 5 ng of human IL-1 β per ml. At the times (minutes) indicated, whole cell extracts were prepared, and equal aliquots were analyzed for $I\kappa B\alpha$ amounts by Western blot analysis with $I\kappa B\alpha$ -specific antibodies. (B) The supernatants from the cells used for panel A were analyzed for IL-6 cytokine release by ELISA. (C) U2-OS cells were transiently transfected by electroporation with 10 μ g of the -342/+115 human $I\kappa B\alpha$ promoter construct and 2 μ g of SG5-HE0 expression vector. Twenty-four hours later, cells were pretreated for 30 min with 10^{-8} M estrogen (17 β -estradiol [E2]) before the addition of 5 ng of human IL-1 β per ml. Cells were harvested 8 h later and analyzed for luciferase activity. A representative experiment is shown. All experiments were repeated at least three times and showed less than 20% deviation of luciferase activity between experiments. con, control.

reports that although portions of the DNA binding domain of a steroid hormone receptor are required for transrepression, specific binding of the steroid hormone receptor to the respective promoter is not required. Surprisingly, ER mutants missing the DNA binding domain (HE11 and HE14) display the reverse phenotype of wild-type ER, mediating estrogen-dependent transactivation of the IL-6 promoter. This finding indicates that the mechanism of transrepression of the IL-6 promoter is different from the mechanism of transactivation of promoters with ER binding elements. This topic is being addressed in more detail (71). The apparent fivefold-higher activation levels of the IL-6 promoter with SG5-HE0:XcmI (and the empty SG5 vector) are caused by the missing TAF-1 region, which causes squelching effects on many mammalian promoters (data not shown).

The results here presented extend previous studies on the inhibition of the IL-6 promoter by estrogen. We clearly define the region that mediates inhibition by estrogen and show the involvement of NF- κ B and C/EBP β . Further, we define the sequences inside ER required for inhibition of IL-6 promoter activity. Specificity of this observation is shown by the inability of the biologically inactive stereoisomer 17 α -estradiol and pro-

gestosterone to transrepress the IL-6 promoter. The transrepression by estrogen appears to involve the formation of heteromeric ER-NF- κ B and ER-C/EBP β complexes. Data obtained with bacterially expressed and in vitro-translated proteins support a direct physical interaction. We cannot exclude the possibility that this interaction is stabilized or mediated by accessory proteins (11, 23). The interaction appears to be specific, since $I\kappa B\alpha$ and Fos proteins were unable to bind to ER. The lack of binding of these two proteins is not due to a drastic difference in electrical charge, as shown by their isoelectric points. A truncated form of RelA encompassing only the Rel homology domain (NF- κ B p65Eco) interacts as well as wild-type RelA, while a further truncation of the Rel homology domain (NF- κ B p65Bgl) obliterates this interaction. Interestingly, NF- κ B p65 Δ 10, a naturally occurring isoform of RelA with an internal deletion of 10 amino acids that cannot efficiently dimerize, still binds to ER. This finding suggests that a monomer of RelA can interact with ER. The interaction does not depend on the presence of estrogen, which is similar to the interaction of RelA with GR (67).

How does estrogen inhibit the IL-6 promoter in vivo? In this report, we demonstrate the functional and physical association between ER, RelA, and C/EBP β . The observed mutual transcriptional inhibition between ER, RelA, and C/EBP β is likely to be mediated by a physical interaction between these proteins. Hormone-activated ER directly interferes with the RelA- and C/EBP β -mediated activation of the IL-6 promoter. The fold inhibition by estrogen depends on the ratio of ER to RelA and C/EBP β . The lack of inhibition of RelA-induced promoter activity in U2-OS cells might be explained by an unfavorable ratio between RelA and ER expression plasmids. In MCF-7 cells, in which the ER/RelA ratio is presumably higher, we did observe inhibition of RelA-dependent transactivation by estrogen. Similarly, Caldenhoven et al. (10) showed that repression of an NF- κ B-dependent promoter by GR depends on the ratio of GR to RelA. Further, it is possible that the ligand-dependent activation of ER is too slow to counteract the strong transactivation potential of RelA. In the reverse situation, transcription from a minimal promoter containing an ER binding site is specifically downmodulated by RelA and C/EBP β but not by Jun or $I\kappa B\alpha$. Overall, these in vivo results are best explained by a physical interaction between ER, NF- κ B, and C/EBP β .

Nevertheless, we wanted to analyze the possibility of additional mechanisms. First, in a recent report, an estrogen-dependent increase of cyclic AMP (cAMP) levels in breast cancer and uterine cells has been demonstrated (2). We excluded such a pathway for the estrogen-dependent downmodulation of the IL-6 promoter in U2-OS, since cAMP had no inhibitory effect on a transfected IL-6 promoter construct (data not shown). Second, NF- κ B is regulated by cytoplasmic inhibitor proteins termed $I\kappa B$. Any effect of estrogen on $I\kappa B$ will modulate NF- κ B activity. Careful analysis of $I\kappa B\alpha$ levels in the presence and absence of estrogen did not reveal any significant differences in amounts. Further, estrogen did not interfere with the IL-1 β induction of the human $I\kappa B\alpha$ promoter in U2-OS cells. Therefore, we conclude that estrogen has no demonstrable effect on $I\kappa B\alpha$.

No cytoplasmic inhibitor proteins have been described for C/EBP β ; thus, ER most likely transrepresses C/EBP β activity by a direct protein-protein interaction as suggested by our in vitro and in vivo experiments. We can exclude that estrogen causes the preferential translation of C/EBP β from the third AUG on its mRNA and thereby creates a transdominant negative inhibitor protein (14). Western blot analysis of MCF-7

cells treated with estrogen did not reveal any changes in the amount of the truncated C/EBP β form (data not shown).

The biological relevance of estrogen in bone metabolism is evident from a large collection of clinical data (references 1, 3, 12, 32, 57, 58, 64, and 69 and references therein). We provide further insight into the mechanism of prevention of osteoporosis by estrogen. We hope to find compounds that will be able to transrepress the IL-6 promoter but do not have the undesirable hormonal side effects that are associated with the currently used estrogen replacement therapy. In support of our goal, glucocorticoid hormone analogs that are able to distinguish between transrepression of AP-1-dependent promoters and transactivation of promoters with binding sites for GR have been described (24). Our findings may have important implications for development of antiosteoporosis drugs.

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