Insulin-like growth factor-I (IGF-I) is a key factor in bone remodeling. In osteoblasts, IGF-I synthesis is enhanced by parathyroid hormone and prostaglandin E\(_2\) (PGE\(_2\)) through cAMP-activated protein kinase. In rats, estrogen loss after ovariectomy leads to a rise in serum IGF-I and an increase in bone remodeling, both of which are reversed by estrogen treatment. To examine estrogen-dependent regulation of IGF-I expression at the molecular level, primary fetal rat osteoblasts were co-transfected with the estrogen receptor (hER, to ensure active ER expression), and luciferase reporter plasmids controlled by promoter 1 of the rat IGF-I gene (IGF-I P1), used exclusively in these cells. As reported, 1 \(\mu\)M PGE\(_2\) increased IGF-I P1 activity by 5-fold. 17\(\beta\)-Estradiol alone had no effect, but dose-dependently suppressed the stimulatory effect of PGE\(_2\) by up to 90% (ED\(_{50} = 0.1\) nM). This occurred within 3 h, persisted for at least 16 h, required ER, and appeared specific, since 17\(\alpha\)-estradiol was 100–300-fold less effective. By contrast, 17\(\beta\)-estradiol stimulated estrogen response element (ERE)-dependent reporter expression by up to 10-fold. 17\(\beta\)-Estradiol also suppressed an IGF-I P1 construct retaining only minimal promoter sequence required for cAMP-dependent gene activation, but did not affect the 60-fold increase in cAMP induced by PGE\(_2\). There is no consensus ERE in rat IGF-I P1, suggesting novel down-regulation of IGF-I expression is in part responsible for the anabolic effect of IGF-I. Although postmenopausal osteoporosis is one of the most prevalent age-related skeletal disorders, our understanding of the role of estrogen, which has a critical role in maintaining bone mass, remains incomplete. The principal laboratory animal model of postmenopausal osteoporosis is the ovariectomized (OVX)\(^1\) rat. One consistent observation with this model is an increase in the rate of bone remodeling after OVX-induced estrogen depletion (1–5). Furthermore, consistent with a cause and effect relationship, estrogen administration re-establishes a reduced rate of bone remodeling in the OVX rat. Bone remodeling consists of two opposing events, i.e. bone resorption and formation (6). Skeletal integrity in adults relies on closely coupled remodeling where there is a balance between these catabolic and anabolic processes. Net bone loss in postmenopausal osteoporosis is thought to result from bone resorption that exceeds formation, even though both processes are accelerated in this condition (7–9). Recent data indicate the involvement of several interleukins (IL-1\(\beta\) and IL-6) in estrogen deficiency-induced bone resorption (10–13). IL-6 has emerged as a pivotal factor in accelerated bone resorption in the estrogen-depleted state, due in part to the apparent absence of bone loss in OVX mice carrying a knockout of the IL-6 gene (14). While progress has been made in understanding the interactions of estrogen and interleukins in regulating bone resorption, relatively little is known about factors involved in bone formation in the estrogen-depleted state. Recently, several groups reported elevated serum IGF-I levels that returned to normal following estrogen supplementation in OVX rats (15, 16). Similarly, postmenopausal women given estrogen experience a drop in serum IGF-I levels (17). The endocrine influence of elevated levels of circulating IGF-I is likely to include a positive effect on bone formation (18–21).

Local osteoblast-derived IGF-I also increases bone cell activity (18, 19, 22–26). The osteotropic hormone, parathyroid hormone (PTH), while stimulating resorption, also stimulates IGF-I synthesis by osteoblasts through a mechanism involving cAMP-dependent protein kinase A (22, 23, 27). PTH administered intermittently in vivo or in vitro increases osteoblast replication and matrix collagen synthesis (23, 28–32). An increase in IGF-I expression is in part responsible for the anabolic effect of PTH. In fetal rat calvarial bone explants, a brief exposure to PTH increases the rate of collagen synthesis, and neutralizing antibodies to IGF-I dramatically reduce this effect (23). Therefore, IGF-I appears to couple this aspect of bone remodeling.

Although postmenopausal osteoporosis is one of the most prevalent age-related skeletal disorders, our understanding of the role of estrogen, which has a critical role in maintaining bone mass, remains incomplete. The principal laboratory animal model of postmenopausal osteoporosis is the ovariectomized (OVX)\(^1\) rat. One consistent observation with this model is an increase in the rate of bone remodeling after OVX-induced estrogen depletion (1–5). Furthermore, consistent with a cause and effect relationship, estrogen administration re-establishes a reduced rate of bone remodeling in the OVX rat. Bone remodeling consists of two opposing events, i.e. bone resorption and formation (6). Skeletal integrity in adults relies on closely coupled remodeling where there is a balance between these catabolic and anabolic processes. Net bone loss in postmenopausal osteoporosis is thought to result from bone resorption that exceeds formation, even though both processes are accelerated in this condition (7–9). Recent data indicate the involvement of several interleukins (IL-1\(\beta\) and IL-6) in estrogen deficiency-induced bone resorption (10–13). IL-6 has emerged as a pivotal factor in accelerated bone resorption in the estrogen-depleted state, due in part to the apparent absence of bone loss in OVX mice carrying a knockout of the IL-6 gene (14). While progress has been made in understanding the interactions of estrogen and interleukins in regulating bone resorption, relatively little is known about factors involved in bone formation in the estrogen-depleted state. Recently, several groups reported elevated serum IGF-I levels that returned to normal following estrogen supplementation in OVX rats (15, 16). Similarly, postmenopausal women given estrogen experience a drop in serum IGF-I levels (17). The endocrine influence of elevated levels of circulating IGF-I is likely to include a positive effect on bone formation (18–21).

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\(^{1}\) The abbreviations used are: OVX, ovariectomized; IL, interleukin; IGF-I, insulin-like growth factor-I; IGF-I P1, promoter 1 of the rat IGF-I gene; PTH, parathyroid hormone; PKA, cAMP-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; ER, estrogen receptor; ERE, estrogen response element; DTT, dithiothreitol; bp, base pair(s); CRE, cAMP response element.

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Earlier studies examining the influence of estrogen on IGF-I expression by primary cultures of neonatal rat calvarial osteoblasts indicated a small stimulatory effect (~2-fold), thought to result from changes in the rate of gene transcription (33, 34). However, OVX increases IGF-I mRNA expression in the calvariae of growing rats, and this effect can be reversed by the estrogen agonist diethylstilbestrol (35, 36). These latter observations agree with the elevated serum IGF-I levels seen in OVX rats, indicated earlier (15, 16). Therefore, in vivo and in vitro findings together predict that estrogen may suppress IGF-I expression in skeletal tissue and that this may be focused on the stimulatory effect of agents that induce cAMP. This interpretation is consistent with the lower rate of bone remodeling seen in the estrogen replete versus estrogen-depleted animals.

In this study we wished to evaluate the effect of 17β-estradiol on IGF-I promoter activity. Because we previously demonstrated a PKA-dependent increase in IGF-I transcription after exposure to PGE$_2$ in fetal rat osteoblasts (37–40), we used this model to examine the influence of 17β-estradiol alone and in combination with PGE$_2$ on IGF-I promoter function. Furthermore, because other studies suggested that estrogen can suppress an increase in cAMP in response to PTH (33), we also examined the effect of 17β-estradiol on PGE$_2$-stimulated CAMP in these cultures. Although we find a potent regulatory effect of 17β-estradiol on CAMP-dependent IGF-I expression, it occurs downstream of CAMP accumulation.

EXPERIMENTAL PROCEDURES

Cell Cultures—Primary osteoblast-enriched cell cultures were prepared from the parietal bones of 22-day-old Sprague-Dawley rat fetuses (Charles River Laboratories, Raleigh, NC). Animals were housed and euthanized by methods approved by the Yale University Animal Care and Use Committee. Cranial sutures were eliminated during dissection, and the bones were digested with collagenase for five sequential 20-min intervals. The cell population released during the last three digestions exhibits biochemical characteristics associated with differentiated osteoblasts, including PTH receptors, type I collagen synthesis, and a rise in osteocalcin expression in response to dithiothreitol (41, 42). Histochemical staining demonstrates that approximately 80% of the cells express alkaline phosphatase, although this itself is not entirely specific for osteoblasts. However, using these criteria, as well as differential sensitivity to transforming growth factor-β, bone morphogenetic protein-2, various prostaglandins, and the ability to form mineralized nodules in vitro (43, 44), these cells are well distinguished from the less differentiated cells released during earlier collagenase digestions. Cells from the last three digestions were pooled and then plated at 4,800 per cm$^2$ in Dulbecco’s modified Eagle’s medium (DMEM) containing 20 mM HEPES (pH 7.2), 0.1 mg/ml ascorbic acid, penicillin and streptomycin (all from Life Technologies Inc.) and 10% fetal bovine serum (Sigma). At the time of transfection, cultures were rinsed twice with phenol red-free DMEM, with (growth medium) or without (treatment medium) 10% charcoal-stripped serum, prepared as described by Ernst and Rodan (43). Primary osteoblast-enriched cell cultures were preincubated for 20 min on ice with 2 g of poly(dI-dC) with or without 9 mM Na$_2$EDTA, 25% glycerol, and the phosphatase and protease inhibitors (1 mM sodium orthovanadate, 10 mM sodium fluoride), protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 g/ml pepstatin A, 2 g/ml leupeptin, 2 g/ml aprotinin, Sigma), and 1% Triton X-100. Nuclei were gently pelleted. Cells were resuspended in hypertonic buffer containing 10 mM HEPES, pH 7.4, 0.1 mM EDTA, 2% glycerol, 0.2 mM Na$_2$EDTA, 25% glicerol, and the protease and phosphatase inhibitors indicated above. Soluble proteins released by a 30-min incubation at 4 °C were collected by centrifugation at 12,000 × g for 5 min, and the supernatant was stored at -75 °C.

Cell Preparation—Cultures were isolated from the linear portion of a standard curve and is expressed as picomoles of CAMP/2-cm$^2$ culture, as reported previously (27).

Nuclear Protein Extracts—Control or pRSV-hER-transfected cultures were rinsed with serum-free medium and exposed to treatment solution for 4 h. Medium was aspirated, and cultures were rinsed twice with phosphate-buffered saline at 4 °C. All subsequent steps were performed on ice or at 4 °C. Cells were harvested with a cell scraper and gently pelleted, and the pellets were washed with phosphate-buffered saline. Nuclear extracts were prepared by the method of Lee et al. with minor modifications (39, 47). Cells were lysed in hypotonic buffer (10 mM HEPES (pH 7.4), 1.5 mM MgCl$_2$, 10 mM KCI, 0.5 mM DTT) with phenolphosphate inhibitors (1 mM sodium orthovanadate, 10 mM sodium fluoride), protease inhibitors (0.5 mA phenylmethylsulfonyl fluoride, 1 g/ml pepstatin A, 2 g/ml leupeptin, 2 g/ml aprotinin, Sigma), and 1% Triton X-100. Nuclei were gently pelleted. Nuclei were resuspended in hypertonic buffer containing 10 mM HEPES, pH 7.4, 0.1 mM EDTA, 5 mM DTT, and 0.025% bovine serum albumin. After the addition of 5 × 10$^4$ cpm of DNA probe (0.1–2 ng) for 30 min on ice, samples were applied to a 5% nondenaturing polyacrylamide gel that was pre-electrophoresed for 30 min at 12.5 V/cm at 25 °C in 45 mM Tris, 45 mM boric acid, 1 mM EDTA. Electrophoresis proceeded for 2.5 h under identical conditions. Dried gels were exposed to x-ray film at -75 °C with intensifying screens. Electrophoretic mobility shift assays were performed in select experiments using normal rabbit serum as a negative control and monoclonal anti-hER antibody (Santa Cruz Biotechnologies Inc., Santa Cruz, CA) for its ability to produce a supershift of hER-bound probe. Sequences of sense strands of oligonucleotides used in the electrophoretic mobility shift assays are as follows: HSID, 5'-GGAGCAGGGATGAGATCGTCCCAAGATGAGATAA-3' (the minimal binding sequence of the atypical estrogen response element in HSID); HSID footprint site of the rat IGF-I promoter 1 is underlined (40) and ERE, 5'-GATCCAAATCGTGCTACGTCGGTGAAGA-3' (the estrogen response element is underlined in this sequence). The statistical analysis was performed by T. L. McCarthy and M. Centrella, unpublished data.
variance with SigmaStat® software, with the Student-Newman-Keuls method for post hoc comparison.

RESULTS

The IGF-I gene has two promoters, and promoter 1 (P1) is used exclusively in cultures of fetal rat osteoblasts (38). Initial studies were conducted with osteoblast cultures transiently transfected with the longest IGF-I P1-luciferase reporter construct, IGF1711b/Luc (containing 1711 bp of upstream sequence, and the initial 328 bp of transcribed but untranslated exon 1), which we previously found to be most responsive to treatment with the cAMP-inducing agent PGE2 (39). To ensure expression of active estrogen receptors, cultures were co-transfected with the hER expression vector pRSV-hER. Incubation with 17β-estradiol at 0.01–10 nM for 16 h did not alter basal IGF-I P1 activity (Fig. 1, left panel). As a positive control, parallel cultures were co-transfected with pRSV-hER and a recombinant reporter plasmid containing tandem copies of the consensus estrogen response element (ERE) from the vitellogenin gene, and a short segment of the prolactin promoter (plasmid (ERE)2PRL-Luc). Unlike the IGF-I P1 recombinant plasmid, treatment with 17β-estradiol enhanced expression of this ERE-containing reporter gene up to 10-fold (Fig. 1, center panel). Co-transfection with pRSV-hER was required for all 17β-estradiol-dependent responses, including activation of the (ERE)2PRL-Luc construct (data not shown).

Fetal rat osteoblasts respond to PGE2 with a rapid increase in IGF-I expression, mediated by PKA-dependent activation of IGF-I P1 (39). This occurs through an atypical CRE located at bp +202 to +209 of exon 1 (40), and PTH similarly requires this element to enhance IGF-I promoter function (data not shown). As we reported previously, PGE2 (1 μM, 16 h) increased IGF-I P1 activity 5-fold (39). While 17β-estradiol alone was ineffective, co-treatment with PGE2 led to a dose-dependent suppression of IGF-I promoter activation over a concentration range of 0.1–10 nM (ED50 = 0.2 nM) (Fig. 1, left panel). This response required expression of hER (Fig. 1, right panel), and an active form of estradiol, since 17α-estradiol was 100–300-fold less potent than 17β-estradiol at blocking PGE2-mediated IGF-I P1 activity (Fig. 1, left panel). By contrast, expression of the ERE-driven reporter gene was not inhibited by co-treatment with 17β-estradiol and PGE2 (see Fig. 3).

Time course experiments next were performed to examine the kinetics of inhibition of IGF-I P1 activity by 17β-estradiol. Cells were treated with PGE2 (1 μM) or 17β-estradiol (10 nM), alone or in combination for 3, 6, or 16 h. PGE2-activated expression of the IGF-I P1 reporter gene was rapidly blunted by 17β-estradiol. Both the stimulatory effect of PGE2 on IGF-I promoter activity and the opposing, suppressive influence of 17β-estradiol were observed within 3 h of hormone treatment. Both responses were maintained throughout a 16 h incubation, while the ERE-containing plasmid (ERE)2PRL-Luc was activated progressively by 17β-estradiol (Fig. 2).

IGF-I P1 lacks a consensus ERE, and yet 17β-estradiol suppressed cAMP-dependent promoter activation. Therefore, an effort was made to localize the region(s) of the promoter responsible for the opposing effect of estradiol on cAMP-mediated gene activation. Deletion of 5′ flanking DNA from nucleotides –1711 to –123 (with respect to the most 5′ transcription start site of rat IGF-I exon 1 (45)) did not alter either PGE2-stimulated promoter activation (39), or the opposing inhibitory effect of 17β-estradiol (Fig. 3). All three of the plasmids tested retained the same 328 bp of exon 1, which preserves the unconventional CRE (located at bp +202 to +209) required for the stimulatory effect of PGE2 (40).

Next, several approaches were taken to determine the mechanism by which estrogen might suppress IGF-I promoter activation by PGE2. Previous conflicting reports suggested a possible inhibitory effect by estrogen on cAMP accumulation in response to PTH in osteoblasts (33, 50). We examined cAMP levels in cells that were treated with 1 μM PGE2 alone or in combination with 10 nM 17β-estradiol (as in the promoter/reporter assays) and in cells preincubated with 17β-estradiol.
Estrogen Suppresses cAMP-activated IGF-I Expression

![Figure 2](image1.png)  
**FIG. 2.** 17β-Estradiol suppression of PGE$_2$-induced IGF-I promoter 1 activity occurred within 3 h of co-treatment and was sustained for 16 h. Osteoblast cultures were co-transfected with IGF1711b/Luc and pRSV-hER, as described under "Experimental Procedures" and in the legend to Fig. 1. Parallel cultures were transfected with a recombinant luciferase reporter construct (ERE)$_2$PRL-Luc, along with pRSV-hER. Cultures were grown 48 h, the growth medium was aspirated, and the cultures were rinsed with phenol red-free, serum-free DMEM. Cultures were treated with control medium (containing vehicle), PGE$_2$ (1 μM), 10 nm 17β-estradiol, or both agents for 3, 6, or 16 h. Cytoplasmic extracts were prepared and luciferase activity determined. Data are corrected for protein content. Transfections were performed in triplicate, and results are representative of three separate experiments. The mean ± S.E. for luciferase expression (counts/min/μg of protein) are shown. * indicates luciferase levels significantly different (p < 0.05) from control, and ** indicates levels significantly different (p < 0.05) from PGE$_2$ treatment alone.

![Figure 3](image2.png)  
**FIG. 3.** Co-localization of cAMP response element and estrogen inhibitory function within the shortest IGF-I promoter 1 construct. Osteoblast cultures were co-transfected with IGF1711b/Luc (containing 1711 bp of upstream sequence and 328 bp of 5'-transcribed and untranslated exon 1), IGF823/Luc (containing 823 bp of upstream sequence and 328 bp of 5'-transcribed and untranslated exon 1), or IGF122/Luc (containing 122 bp of upstream sequence and 328 bp of 5'-transcribed and untranslated exon 1). Each construct is diagramatically shown in A), along with pRSV-hER, as described under "Experimental Procedures." Parallel cultures were transfected with recombinant luciferase reporter construct (ERE)$_2$PRL-Luc, along with pRSV-hER. Cultures were grown 48 h, the growth medium was aspirated, and the cultures were rinsed with phenol red-free, serum-free DMEM. Cultures were treated with control medium (containing ethanol vehicle), PGE$_2$ (1 μM), 10 nm 17β-estradiol, or both agents for 16 h. Cytoplasmic extracts were prepared and luciferase activity determined. Data are corrected for protein content. Transfections were performed in triplicate, and results are representative of three separate experiments. The mean ± S.E. for luciferase expression (counts/min/μg of protein) are shown in B. * indicates luciferase levels significantly different (p < 0.05) from control, and ** indicates levels significantly different (p < 0.05) from PGE$_2$ treatment alone.

for 30 min followed by PGE$_2$ treatment. All cultures were transfected 48 h earlier with pRSV-hER and pretreated with isobutylmethylxanthine for 5 min to inhibit phosphodiesterase activity. In all experiments, cAMP accumulation increased by 60-fold after 10-min treatment with PGE$_2$, even in the presence of 17β-estradiol co-treatment or pretreatment (Fig. 4). In addition, regardless of the presence of 17β-estradiol, PGE$_2$-induced accumulation of cAMP was accompanied by pronounced morphological changes, as seen previously with these cells (data not shown). These results indicate that the adenylate cyclase pathway was intact and functional in cells exposed to 17β-estradiol.

While cAMP levels were not altered by 17β-estradiol, estrogen may interfere with other steps in the process of PKA-dependent IGF-I gene activation that lie downstream of the generation of cAMP. One possibility is that the ligand-activated ER may modulate the binding of cAMP-dependent transcription factor(s) to the IGF-I CRE. To examine this possibility, nuclear extracts prepared from control and PGE$_2$-treated cells were tested in the electrophoretic mobility shift assay using a 32P-labeled double-stranded oligonucleotide containing the IGF-I CRE (designated HS3D, based on the notation for the footprinted site (45)). In agreement with our earlier observations, a gel-shifted doublet band was seen with this oligonucleotide using nuclear extracts from PGE$_2$ (1 μM, 4 h)-treated cultures (40). The PGE$_2$-inducible gel shift that occurred for extracts from cells previously transfected with the hER expression plasmid was identical to that for untransfected cultures (Fig. 5). In cells expressing hER, incubation with 17β-estradiol alone did not induce nuclear protein binding to the HS3D oligonucleotide probe. However, the amount of nuclear protein complex that occurred with extract from hER transfected cells co-treated with PGE$_2$ and 17β-estradiol was dose-dependently reduced in intensity compared with PGE$_2$ treatment alone, but there was no change in the banding pattern. In contrast 17α-estradiol was not similarly inhibitory.

We then examined the possibility that ER directly associates with the IGF-I CRE and therefore competes with cAMP-stimulated transcription factor(s) for binding at this element. First, we tested recombinant hER (rhER) for its ability to bind a 32P-labeled consensus CRE oligonucleotide as a positive control and to determine if excess unlabeled HS3D oligonucleotide could compete for rhER binding. Without rhER, no gel-shifted band was detected with 32P-labeled CRE oligonucleotide. However, the association of rhER with 32P-labeled CRE oligonucleotide produced strong gel shift complexes that were significantly reduced in intensity by 50-fold molar excess of unlabeled homologous CRE, but were unaffected by excess unlabeled HS3D oligonucleotide. To confirm the specificity of the hER/ERE in-
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**FIG. 4.** 17β-Estradiol does not alter PGE2-stimulated cAMP levels in cultures transfected with hER. Osteoblast cultures were transiently transfected with hER expression vector pRSV-hER. Forty-eight hours later, cultures were rinsed twice with serum-free phenol red-free DMEM and exposed to control medium or 10 nM 17β-estradiol for 30 min. Cultures were treated for 5 min with 0.5 mM isobutylmethylxanthine (phosphodiesterase inhibitor) prior to a 10-min treatment (at room temperature) with control (ethanol vehicle), PGE2 (1 μM), 17β-estradiol (0.1–10 nM), alone or in combination. Cultures were extracted with n-propyl alcohol and the cAMP content determined by radioimmunoassay. These data are the pooled results from two independent experiments. * indicates comparably elevated cAMP levels that are significantly different (p < 0.05) from control.

Interaction further, a monoclonal anti-hER antibody was used in a supershift assay. As shown, normal rabbit serum was ineffective, while anti-hER antibody altered the mobility of the hER-dependent gel-shifted band (Fig. 6, left panel). Next, to examine binding of hER to the IGF-I promoter directly, two 32P-labeled double-stranded DNA probes generated from the −122 bp to +328 bp portion of rat IGF-I P1 were combined with recombinant hER. No gel shift complex was seen with either fragment. These findings indicate that no direct interactions occur between hER and the regions of IGF-I P1 that contain or flank HS3D (Fig. 6, right panel), even though HS3D is required for stimulation of reporter gene expression by PGE2 and for the opposing influence of 17β-estradiol.

**DISCUSSION**

Estrogen deprivation has long been associated with a decline in skeletal integrity, but its role in the normal maintenance of bone mass is still poorly understood. Several lines of evidence indicate that estrogen deficiency increases the total process of bone remodeling in such a way that the rate of bone resorption significantly outpaces formation and results in osteoporosis (1–5). Studies with OVX rats, where surgical estrogen depletion incurs a decrease in bone mass, strongly connect IL-1 and IL-6 to accelerated bone resorption. In *vivo* studies using an IL-1 receptor antagonist demonstrate a bone sparing effect similar to that observed with estrogen replacement therapy, while IL-6 knockout mice do not experience the OVX-induced bone loss seen in their heterozygous littersmates (14, 51). *In vitro* studies indicate that both lymphokines are associated with activation of mature osteoclasts and stimulation of osteoclastogenesis. These results have increased our understanding of the high rates of bone resorption that follow the loss of endogenous estrogen. Nevertheless, little is still known about the mechanisms by which bone formation rates also increase, albeit to a lesser extent, under similar conditions.

Estrogen has a positive influence on the growth of some tissues, such as uterine tissue. In rats, OVX causes weight gain and increased longitudinal bone growth, both of which are reversed by estrogen treatment. Consistent with this, OVX increases the circulating levels of IGF-I, and estrogen treatment can normalize this change (15, 16). Similar results were observed in postmenopausal women (17). Liver is a primary source of serum IGF-I. In this regard, estrogen suppresses growth hormone-stimulated hepatic IGF-I synthesis in hypophysectomized and OVX animals, even though it increases serum growth hormone levels in OVX animals (52, 53). These latter findings may help explain some *in vivo* results with OVX rats. Interestingly, estrogen treatment is also associated with accelerated skeletal maturation and closure of growth plates in both rats and humans (54). Local IGF-I synthesis by bone cells is also induced by growth hormone and contributes significantly to longitudinal bone growth (4), predicting that estrogen can oppose its stimulatory effect on IGF-I synthesis in liver as well as peripheral tissue. OVX also alters several markers of osteoblast activity in intramembranous bone such as calvariae (the source of osteoblastic cells used in our studies). Within 1 week of OVX, transcript levels for osteocalcin, osteonectin, and IGF-I increase at this site. Subsequent treatment with the estrogen analog diethylstilbestrol rapidly decreases the appearance of these mRNAs, reduces periosteal mineral apposition rate, and also suppresses osteoblast number (8, 35, 36, 55).
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presses PKA-dependent IGF-I gene activation in a rapid and sustained, estrogen receptor-dependent, isoform-specific, and dose-dependent way. The EC₅₀ of 17β-estradiol was ~0.1 nM, within its physiological concentration range (60), while 17α-estradiol was 100–300-fold less effective. This suppressive effect was not due to hormone toxicity, since the same doses of 17β-estradiol stimulated expression of a reporter gene containing a consensus CRE. Furthermore, neither estrogen pretreatment nor co-treatment altered cAMP accumulation, indicating that its effect on IGF-I expression occurs downstream of cAMP itself. Reporter gene expression, under control of the smallest fragment of IGF-I promoter DNA, which retains PKA sensitivity, was completely suppressed by 17β-estradiol. This observation co-localizes the responsive sequences for each event to one element or a small region of DNA. The rat IGF-I promoter 1 contains no consensus CRE or CRE DNA sequences. However, electrophoretic mobility shift assays using the HS3D region of the IGF-I promoter, where the atypical IGF-I promoter CRE resides, revealed that 17β-estradiol reduced the intensity of the cAMP-inducible gel shift. No additional bands or changes in relative mobility occurred with estrogen treatment. Therefore, interactions between hER and the cAMP-activated nuclear factor(s) or co-activator(s) appear to limit nuclear factor binding to the atypical IGF-I CRE within the HS3D region itself. Complex kinetics for the suppressive effect of 17β-estradiol on PKA-activated IGF-I promoter function may indicate multiple actions of 17β-estradiol on activation, binding, and synthesis of the responsible cAMP-activated transcription factor. Once we have identified with certainty the cAMP-activated transcription factor responsible for this effect, we will be better positioned to resolve the mechanism by which its binding is reduced at this element.

In contrast to our findings, earlier studies found that estrogen alone may enhance IGF-I mRNA levels ~2-fold in neonatal rat osteoblasts in vitro (33, 34). We do not yet understand this obvious difference with our results in fetal bone cells or with other in vivo studies where IGF-I mRNA in intramembranous bone increases after OVX (35, 36). Our findings clearly indicate that estradiol reduces nuclear factor binding to the atypical CRE found in the IGF-I promoter, which is much less evident in the basal state. Consequently, the ~2-fold stimulation by estrogen on basal IGF-I expression that was reported previously differs in context from our studies that focus on the ability of this hormone to suppress the more potent 5–10-fold increase in IGF-I expression that occurs in response to PKA activation. Transfection-mediated hER expression was required to observe an effect of estrogen in the studies we present here, including reporter gene expression by the positive estrogen responsive (ERE)²-PRL-Luc, indicating low to negligible levels of ER in our fetal osteoblast cultures. Unlike the earlier results of Ernst and Rodan (34), we have been unable to detect an effect of 17β-estradiol on IGF-I transcript or promoter function in newly confluent or confluent and aged untransfected primary fetal rat osteoblasts. The reasons for these discrepancies are unclear, but may reflect differences in neonatal bone used by Ernst and Rodan (34) versus fetal bone used in our studies to prepare the primary cell cultures or subsequent culture conditions.

Other earlier studies identified the importance of an AP-1 binding site for the hER-dependent stimulatory effect of 17β-estradiol on chicken IGF-I promoter activity in human hepatoma cells (61). Again, we could not detect any effect by 17β-estradiol alone on rat IGF-I promoter activity with or without co-expression of hER in fetal rat bone cells. The minimal region of the IGF-I promoter, which is sensitive to both PGE₂ and 17β-estradiol, does not contain an identifiable AP-1 site (45). We further examined agents such as phorbol myristate acetate,
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