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ABSTRACT

Despite its use as an antiestrogen in the treatment of breast cancer, tamoxifen also functions as a partial estrogen agonist, protecting against postmenopausal bone loss in these patients unable to receive estrogen replacement therapy. Among the many cytokines and growth factors under investigation, insulin-like growth factor I (IGF-I) has been one of those implicated as playing a role in the pathogenesis of postmenopausal osteoporosis. IGF-I gene expression is stimulated by agents such as prostaglandin E_2 (PGE_2) that increase intracellular cyclic adenosine monophosphate (cAMP) via a protein kinase A (PKA) dependent mechanism. Treatment of primary cultures of fetal rat osteoblasts with 17β-estradiol suppresses the PGE_2 dependent stimulation of IGF-I, suggesting a role for estrogen in maintaining a basal level of bone turnover. Through preparation of cell cultures transiently transfected with estrogen receptor (ER) and the luciferase reporter plasmid controlled by promoter 1 of the IGF-I gene (IGF-I P1), we demonstrate that tamoxifen, though less potently than estrogen, also decreases PGE_2 stimulated IGF-I promoter activity. Like estrogen, tamoxifen treatment alone does not influence IGF-I expression. Tamoxifen treatment was not cytotoxic to cells, as equal concentrations weakly stimulated luciferase reporter expression under the control of a typical estrogen response element (ERE). Nuclear extracts from tamoxifen treated cells were prepared and used in gel mobility shift assays with DNA containing the atypical cAMP response element (CRE) in the IGF-I promoter required for PGE_2 activation. As

with estradiol, tamoxifen decreased the PGE_2 induced bands detected by gel-mobility shift assay. Thus tamoxifen appears to function similarly to estrogen in suppressing IGF-I in the absence of a typical estrogen response element within the IGF-I P1. These findings provide further evidence for an ERE-independent pathway of estrogen action, and help to elucidate the mechanism of the estrogen agonist effect of tamoxifen on bone.

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INTRODUCTION

Osteoporosis is a disease characterized by increased bone fragility and fracture risk resulting from a state of low bone mass and deterioration of the microstructure of bone (Johnston and Slemenda, 1995). Peak adult bone mass is attained usually in the third decade, with all individuals undergoing subsequent age-related bone loss. The incidence of osteoporotic fractures increases with age, and is higher in women than in men. Women lose approximately 50 percent of their cancellous bone and 30 percent of their cortical bone during their lifetimes (Riggs and Melton, 1986), and nearly a third to one half of all bone loss in women may be attributable to the menopause (Riggs and Melton, 1992). Estrogen therapy, if begun at the menopause, can prevent the early loss of bone and decrease the incidence of ensuing osteoporosis-related fractures by about 50 percent (Weiss *et al.*, 1980; Ettinger *et al.*, 1985). Although the efficacy of estrogen has been demonstrated through numerous clinical trials (Christiansen *et al.* 1982; Lindsay *et al.*, 1984; Ettinger *et al.*, 1987; Adami *et al.* 1989), the mechanism of estrogen action on bone has not been clearly elucidated.

Interestingly, certain partial estrogen antagonists such as tamoxifen have also been found to have anti-osteoporotic effects in bone. Among other indications, tamoxifen is used clinically as a chemotherapeutic agent to treat women with estrogen-receptor positive breast cancer. Actions of tamoxifen are, however, tissue specific, and despite opposing estrogen in breast, tamoxifen produces estrogen-like effects in other organs. Tamoxifen has been shown to reduce cardiovascular risk in post-menopausal women (Shewmon *et al.* 1994, Thanagaraju *et al.*, 1994), induce proliferation of vaginal epithelium and, in higher doses, cause endometrial hypertrophy (Fornander *et al.*, 1989, 1991). Likewise, tamoxifen protects against the postmenopausal accelerated loss of bone mineral density (Love *et al.*, 1992; Ward *et al.*, 1993). An understanding of this partial agonist/antagonist activity of tamoxifen and related compounds would provide insight

into the pathogenesis of postmenopausal osteoporosis and provide opportunities for targeting estrogenic actions to specific tissues while minimizing side effects to others.

Of much interest in the last decade of research has been the identification of local growth factors in bone and their interactions between hormones such as estrogen and other systemic factors. Central to this understanding is how these local factors effect the anabolic and catabolic balance in bone, as osteoblast and osteoclast activity are paired in a process of remodeling that occurs throughout the lifetime of an individual. In the normal adult skeleton, bone formation occurs at sites of previous resorption. Bone turnover occurs in a sequence of events known as activation-resorption-formation beginning with activation of osteoclastic precursors to become osteoclasts. During osteoclastic mediated resorption, cytokines and growth factors are released from the bone matrix which are then believed to recruit and activate osteoblasts to synthesize new bone. Osteoblasts also serve as intermediaries in the process of osteoclast activation, thereby maintaining a balance in the remodeling cycle. Building and dissolving activities are thus coupled both temporally and quantitatively, as the amount resorbed is similar to that formed. The pathogenesis of postmenopausal osteoporosis stems from an imbalance in this remodeling cycle. With each cycle, more bone is resorbed than formed and excessive bone loss occurs, particularly in early menopause when osteoclastic activity is increased resulting in excess loss of cancellous bone (Turner *et al.*, 1994). As the skeleton ages, the slow phase of osteoporosis ensues and is characterized by decreased osteoblastic activity causing a decline in both cancellous and cortical bone mass.

The coupling of osteoblast and osteoclast function is influenced by locally active factors. Among the more abundant of these growth factors found in bone are insulin-like growth factors (IGF's), isolated initially from serum and named for observations which demonstrated insulin-like effects not suppressable by anti-insulin antiserum (Centrella *et al.*, 1993). Detectable in serum as well, systemic IGF is synthesized mainly by the liver and is under tight control by growth hormone (GH). However, numerous other tissues

including bone and cartilage also synthesize IGF's active in the local environment in a paracrine or autocrine manner. Isolated bone cells in serum free cultures have been documented to produce at least two different types of IGF, termed IGF-I and IGF-II (McCarthy & Centrella,. 1993). IGF-I is identical to somatomedin-C, acting under control of GH to increase synthesis of sulfated proteoglycans in cartilage; IGF-II was found to be analogous to multiplication stimulating activity (MSA) mitogen (Centrella *et al.*, 1993). IGF-II appears to have lower biologic potency in isolated bone and bone cell cultures (McCarthy *et al.*, 1989; Centrella *et al.*, 1990). Bone cells express at least two different cell surface receptors, type 1 and type 2, preferentially binding IGF-I and IGF-II respectively. In most tissues both factors appear to bind to type 1 receptor when effects are stimulatory (Centrella *et al.*, 1993). Indeed it is believed that biological function of IGF-I and II occur via binding to type 1 receptor, supported by the finding that some organisms fail to bind IGF-II to type 2 receptor completely (McCarthy & Centrella, 1994).

Produced by osteoblasts and other bone cells, IGF-I has been shown to potently stimulate bone formation as well as to increase bone resorption. In cultures of fetal rat calvariae, IGF-I has a mitogenic effect, increasing osteoprogenitor cell replication and resulting in larger numbers of osteoblasts. Further, IGF-I has a direct effect on osteoblasts, enhancing production of type I collagen and thus bone matrix (Hock *et al.*, 1988). IGF-I also decreases bone collagen degradation (Canalis, 1993). In contrast, IGF-I influences the number of osteoclasts, recruiting immature cells to become fully functioning osteoclasts. A study by Mochizuku *et al.* (1992) demonstrated that IGF-I stimulates not only recruitment but also activation of osteoclasts in mouse bone cell cultures. IGF-I was also shown to increase production of IL-6, a resorptive cytokine, in cultures of mouse long bones and osteosarcoma cells (Slootweg *et al.*, 1992). Therefore, IGF-I appears to function as an agent that enhances bone turnover, possibly mediating

interactions between osteoblasts and osteoclasts and serving to coordinate the coupling of bone resorption and formation.

Skeletal production of IGF-I responds to several systemic factors, including parathyroid hormone (PTH). A polypeptide hormone, PTH stimulates bone resorption by indirectly affecting osteoclasts via its actions on osteoblasts. PTH also influences bone formation. In cultures of fetal rat calvariae, effects of PTH were shown to be dependent on the method of administration. Continuous treatment with PTH for 24-72 hours results in inhibition of collagen synthesis. In contrast, intermittent treatment results in stimulation of IGF-I expression and increased collagen synthesis, an effect blocked by the addition of IGF-I antibodies (Canalis *et al.*, 1989). Further experiments demonstrate a transient increase in the production of IGF-I mRNA in response to PTH treatment of osteoblast-enriched cell cultures of fetal rat bone (McCarthy *et al.*, 1988). Other systemic factors, such as growth hormone GH, cause a small stimulation of IGF-I by skeletal cells (Canalis, 1993). Long term treatment with glucocorticoid, which clinically induces osteoporosis, results in decreased collagen synthesis in bone and inhibition of IGF-I production by osteoblasts (McCarthy *et al.*, 1988; Delany & Canalis, 1995).

Of interest in understanding the pathogenesis of postmenopausal osteoporosis is the interaction of estrogen and different estrogen analogues with IGF-I. Earlier studies by Ernst et al. indicated that 17β-estradiol has a small direct stimulatory effect on levels of IGF-I mRNA and collagen mRNA while decreasing PTH-stimulated adenylate cyclase activity in primary cell cultures of neonatal rat bone (Ernst *et al.*, 1989; Ernst & Rodan, 1991). In a recent study, Watson *et al.* (1995) demonstrated that levels of IGF-I mRNA in osteoblasts are nearly identical in intact and ovariectomized (OVX) rats. The OVX rat is an accepted model for human postmenopausal osteoporosis (Kalu, 1991b). Other *in vivo* studies, however, demonstrate that in the OVX rat IGF-I levels increase in serum as well as bone. This rise in IGF-I on both circulating and local levels is prevented by daily systemic estrogen therapy (Sato *et al.*, 1993). Further, ovariectomy induces an increase

in osteoblast and osteoclast numbers and a loss of cancellous bone, effects that are all attenuated by 17β-estradiol administration (Kalu *et al.*, 1994). In a model system designed to study osteoblast activity, Yokose *et al.* (1996) concluded that bone formation is accelerated in OVX rats, a rise which is prevented by estrogen therapy. Serum from OVX rats induces proliferation and differentiation of osteoblast-like cells but the serum from OVX-estrogen treated rats does not. Furthermore, the addition of IGF-I neutralizing antibodies blocks almost completely the stimulatory effect of the OVX sera on DNA content, suggesting that IGF-I plays a role in the proliferation of osteoblasts or osteoblast precursors.

Estrogen also influences the anabolic demands in bone, possibly via IGF-I acting as a coupling factor. 17β-estradiol antagonizes TNF-α induced IL-6 production, an interleukin known to stimulate the recruitment and formation of osteoclasts (Girasole *et al.*, 1992; Pottratz *et al.*, 1994). Moreover, release of IL-1, another resorptive cytokine, increases in estrogen deficient states. This increase, demonstrated in blood monocytes derived from postmenopausal women and in spleen macrophages from OVX rats, returns to basal levels with systemic estrogen treatment (Sato *et al.*, 1993). Also, as mentioned above, IGF-I stimulates IL-6 production in mouse osteosarcoma cell cultures. Therefore, estrogen suppression of IL-6 may be indirect through suppression of TNF-α, IL-I and IGF-I, as well as through direct suppression of IL-6 promoter activity.

The mechanism of tamoxifen's actions and its interactions with local coupling factors are also under investigation. Abundant evidence supports the estrogen agonist properties of tamoxifen on bone in states of estrogen deficiency. *In vivo* experiments illustrate a protective effect of tamoxifen similar to estrogen in maintaining cancellous bone volume in the treatment of OVX rats (Moon *et al.*, 1991). Similarly, tamoxifen prevents resorption of endocortical and cancellous bone in the first month following ovariectomy in growing rats (Turner *et al.*, 1988; Kalu *et al.*, 1991a; Evans & Turner, 1995), as well as preventing a decrease in bone volume over long-term treatment in

mature OVX rats (Turner *et al.*, 1987). In clinical trials involving both healthy patients and women with breast cancer, tamoxifen prevents early postmenopausal bone loss at the lumbar spine and femur, as well as at the iliac crest (Ward *et al.*, 1993; Wright *et al.*, 1993). In postmenopausal women with breast cancer, tamoxifen has even been shown to significantly increase bone mineral density in the spine by 0.61% per year compared to a 1.00% decrease in patients treated with placebo (Love *et al.*, 1992). In a trial of chemoprevention of breast cancer in healthy postmenopausal patients, tamoxifen also increased the bone mineral density (BMD) in the hip by 1.71% compared to a nonsignificant loss in women administered placebo (Powles *et al.*, 1996).

Tamoxifen's role in the premenopausal state, however, is not completely understood. On the one hand, tamoxifen has been shown to have increased antiestrogenic activity in premenopausal subjects. Studies in OVX versus non-OVX rats demonstrate a small increase in uterine weight in tamoxifen treated OVX rats. In contrast, tamoxifen has an antiestrogenic effect in non-OVX rats causing a small decrease in uterine weight (Moon *et al.*, 1991). Administration of estrogen and tamoxifen to OVX rats results in slightly decreased effectiveness of the bone-sparing properties of estrogen, suggesting that tamoxifen may be competing with native estrogen for binding and activation of the estrogen receptor (Kalu *et al.*, 1991a). Furthermore, a recent study using tamoxifen as a chemopreventative agent in healthy premenopausal women revealed that it significantly decreased BMD in the hip and lumbar spine (Powles *et al.*, 1996). In the same study, however, tamoxifen increased bone mineral density in both sites in postmenopausal patients. Once again this suggests that tamoxifen may exhibit different effects depending upon existing estrogen levels. This is perhaps partially because the effects of tamoxifen may be less potent and therefore the level of activation of the estrogen receptor may be diminished in comparison to estrogen.

On the other hand, previous smaller clinical trials did not note a significant difference of tamoxifen and placebo on the BMD of the radius in premenopausal patients

with breast cancer (Gotfriedsen *et al.*, 1984). Nor was there a significant difference in the BMD of the spine or hip in premenopausal women receiving either tamoxifen or placebo for mastalgia (Fentiman *et al.*, 1989). Other *in vivo* studies also note no significant difference in bone sparing effects by tamoxifen in the treatment of OVX versus intact rats (Goulding & Gold, 1994). Tamoxifen is even reported to enhance the effects of estrogen on increasing trabecular bone mass in both OVX and non-OVX rats (Turner *et al.*, 1988), in direct conflict with results noted above. Further work is required to elucidate the interaction of endogenous estrogen and the effects of native estrogen levels with the actions of tamoxifen, particularly as premenopausal women could suffer deleterious outcomes from prolonged antiestrogen therapy.

On the local level, the actions of tamoxifen on osteoblast and osteoclast activity are similar to estrogen in states of estrogen deficiency. Tamoxifen has been shown to prevent the production of osteoclasts by inhibiting the fusion of osteoclast precursors (Turner *et al.*, 1987; Turner *et al.*, 1988; Turner *et al.*, 1993) as well as decreasing the numbers of osteoclasts in OVX rats (Isserow *et al.*, 1994). As with postmenopausal estrogen therapy, long-term tamoxifen treatment of women with breast cancer decreases the bone turnover rate, causing a longer remodeling period, lower bone formation rate, and smaller resorption cavities in cancellous bone (Wright *et al.*, 1994) Like estrogen, tamoxifen decreases serum levels of osteocalcin (Ward *et al.*, 1993; Williams *et al.*, 1991; Fornander *et al.*, 1993), an osteoblast-restricted protein that correlates positively with levels of bone formation (Brown *et al.*, 1984).

While little is known regarding tamoxifen effects on IGF-I expression in bone, tamoxifen has estrogenic effects on the liver (Evans & Turner, 1995). Serum levels of IGF-I in postmenopausal women receiving adjuvant tamoxifen therapy decrease, (Grady *et al.*, 1992; Fornander *et al.*, 1993) as do circulating levels of lipoprotein A, serum cholesterol and other lipids (Shewmon *et al.*, 1994; Thanagaraju *et al.*, 1994). Tamoxifen also reduces IGF-I levels locally in lung, a common organ for breast cancer metastasis

(Huynh *et al.*, 1993a). Tamoxifen, however, increases IGF-I gene expression in the rat uterus. Yet this action is also consistent with estrogen, for the pure anti-estrogen ICI 182780 (ICI) reduces IGF-I uterine activity (Huynh & Pollak, 1993b). With regards to bone, OH-tamoxifen decreases TNF-α induced IL-6 expression in mouse osteosarcoma cell cultures as does estrogen (Galien *et al.*, 1996). As little is known of the mechanism of tamoxifen action in bone cells, our present study aimed to elucidate the effects of tamoxifen in influencing IGF-I expression compared to previous reports of estrogen modulation of IGF-I in cultures of fetal rat osteoblasts.

Agents such as PTH and prostaglandin E_2 (PGE_2) that increase intracellular cyclic adenosine 3',5'-monophosphate (cAMP) concentrations stimulate IGF-I expression (McCarthy *et al.*, 1991; Bichell *et al.*, 1993; Pash *et al.*, 1995). An eicosanoid, PGE_2 synthesis by osteoblasts is increased by PTH, mechanical stress, and various other growth factors (Pash *et al.*, 1995). Treatment of osteoblast enriched cultures isolated from fetal rat calvariae with varying concentrations of PGE_2 results in an elevation of intracellular cAMP and a rise in IGF-I mRNA transcripts, evident within 30 minutes of treatment (McCarthy *et al.*, 1991). PGE_2 enhances IGF-I transcription via interaction with promoter 1 of the rat IGF-I gene (Bichell *et al.*, 1993; Pash *et al.*, 1995) . Similar to humans, the rat IGF-I gene consists of approximately 80 kilobases of DNA divided into six exons and five introns (Rotwein, 1991). Although the gene has two promoters, promoter 1 is active in all tissues in which IGF-I is expressed; promoter 2 is most active in the liver. Only promoter 1 is functional in fetal rat osteoblasts .

Genes regulated by cAMP typically contain a cAMP response element (CRE) within their promoters or AP-2 sites to which CRE-binding proteins (CREB's) or activator protein-2 transcription factors may respectively bind. CREB's are activated by protein kinase A (PKA) dependent phosphorylation. PGE_2 stimulated expression of IGF-I is PKA dependent, as demonstrated by experiments where co-transfection with a dominant negative mutant PKA regulatory subunit unable to bind cAMP ablates the

response of osteoblasts to PGE_2 (McCarthy *et al.*, 1995). Although the IGF-I promoter 1 contains a single, near consensus CRE and several potential binding sites for transcription factor AP-2, deletion of these regions does not block PGE_2 induced stimulation of IGF-I promoter activity. Rather, a portion of the 5'-untranslated region (UTR) of exon 1 containing a region termed HS3D (from the notation of the DNase I footprinted site), with no nucleotide sequence similarity to the consensus CRE, is integral to PGE_2 stimulation of IGF-I (Thomas *et al.*, 1996). Furthermore, gel mobility shift studies with nuclear and cytoplasmic cell extracts indicate that nuclear proteins interact with specific nucleotides within the HS3D DNA sequence, and that active factors are not present in the cytoplasm. Treatment of cultures with the protein synthesis inhibitor cycloheximide does not inhibit the effect of PGE_2 on IGF-I mRNA, and does not interfere with the PGE_2-induced binding of the nuclear proteins to the HS3D DNA sequence. These findings suggest that PGE_2 activates a pre-existing DNA-binding protein (Thomas *et al.*, 1996).

Current work by Dr. McCarthy indicates that 17β-estradiol inhibits PGE_2 induced IGF-I expression not by interfering with cAMP production but possibly by altering protein-protein interactions between the PKA activated transcription factors and the estrogen receptor (ER) (McCarthy *et al.*, 1997). Estrogen can affect cellular events by binding to an intracellular receptor, the ER, that can function as a transcription factor (Evans, 1988; Beato, 1989). Typically, the ER-ligand complex binds to DNA at estrogen response elements (ERE's) to modulate gene expression (Ham & Parker, 1989), although alternate mechanisms independent of ERE interaction are being investigated. Treatment of fetal rat osteoblast cell cultures with 17β-estradiol alone has no effect on IGF-I expression, unlike the small stimulatory effect in neonatal cultures described by Ernst *et al.* (1988), as discussed previously. Co-treatment of cells with PGE_2 and 17β-estradiol, however, results in a potent, dose-dependent suppression of IGF-I promoter 1 activity that is ER dependent and estradiol isomer selective (McCarthy *et al.*, 1997).

In order to further elucidate the mechanism of estrogen action and to begin to understand the tissue selective effects of antiestrogens, we have investigated the actions of tamoxifen on IGF-I promoter activity. Consistent with the anti-osteoporotic effects of tamoxifen on bone, we hypothesized that tamoxifen would also suppress the PGE_2 stimulation of IGF-I expression. Primary cell cultures of fetal rat osteoblasts were transfected with IGF-I promoter 1 constructs with a luciferase reporter gene and human ER. A luciferase reporter was used to determine the activity of the IGF promoter very specifically, as luciferase is not native to osteoblasts. Although osteoblasts contain ER's, (Eriksen *et al.*, 1988; Komm *et al.*, 1988; Ernst *et al.*, 1991b) cells are routinely transfected with ER in order to ensure the expression of active estrogen receptors. Transfected osteoblasts were then treated with vehicle, PGE_2, or with 17β-estradiol or 4-hydroxy-tamoxifen (OH-tamoxifen) (an active metabolite of tamoxifen found in the serum of tamoxifen treated patients) both with and without PGE_2. Nuclear extracts of cells treated in a similar manner were prepared and used in gel-mobility shift assays with a ^{32}P-labeled oligonucelotide probe comprising region HS3D of the IGF-I promoter, containing the nonconsensus CRE required for PGE_2 stimulation. Our results indicate that, although tamoxifen behaves similarly to estrogen, it is not as potent at influencing cAMP activated IGF-I expression. Gel-mobility shift studies provide evidence of tamoxifen suppression of IGF-I promoter function via a similar mechanism of action as estrogen. Further work is required to elucidate tamoxifen's and estrogen's apparent non-genomic suppression of PGE_2 induced IGF-I expression.

MATERIALS AND METHODS

Cell Cultures Primary osteoblast cell cultures were prepared from parietal bones of 22-day old Sprague-Dawley rat fetuses (Charles River Breeding Laboratories, Raleigh, NC). All animals were euthanized in accordance with guidelines set by the Yale University Animal Care and Use Committee. Cranial sutures were removed by dissection, and bones were digested for five 20-minute intervals with collagenase. The last three populations of cells were pooled and plated at 6,250-8,000 per cm^2 in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.2), 0.1 mg/ml ascorbic acid, penicillin and streptomycin (all from Life Science Technologies, Gaithersburg, MD), and 10% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO). During and following transfection, cultures were maintained in phenol-red free (PRF) DMEM. Likewise, after transfection, cells were grown in charcoal stripped FBS containing PRF-DMEM and all treatments were carried out in serum-free PRF-DMEM. To maintain consistency throughout the laboratory, cell cultures were routinely prepared by Sandra Casinghino and plated by Dr. Thomas L. McCarthy or Sandra Casinghino.

Plasmids A recombinant luciferase reporter construct containing two tandem estrogen response elements (ERE from the vitellogenin gene) along with a segment of the prolactin promoter ($(ERE)^2$-PRL-Luc) and a Rous sarcoma virus promoter expression plasmid containing the human estrogen receptor (pRSV-hER) were provided by Dr. Stuart Adler (Washington University School of Medicine). Plasmid pGL3 control is a reporter vector that utilizes the promoter and enhancer of Simian virus 40 to allow for high level expression of the luciferase reporter gene, thereby serving as a positive control for the transfections. Plasmid pϕLuc is a parental, promoterless luciferase reporter vector into which the IGF-I promoter 1 segment was cloned. IGF-1711b-Luc plasmid contains 1711 base pairs (bp) of 5'upstream sequence of the IGF-I promoter 1, along with 328 bp of 5'transcribed and untranslated sequence from exon 1. All plasmids were propagated in

E. coli strain DH5α with ampicillin selection and purified by Sandra Casinghino using a Wizard Plus Maxiprep DNA Purification Kit according to the manufacturer's recommended protocol. Recombinant human ER plasmid was prepared by Kristina Crothers, by modification of the alkaline extraction method (Nicoletti & Condorelli, 1993). Briefly, *E. coli* cultures transformed with hER plasmid were grown with ampicillin selection, harvested, and washed in STE (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Cells were then pelleted and resuspended in 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA solution. NaOH-SDS solution (200 mM NaOH, 1% SDS) was used to lyse the cells on ice and denature *E. coli* genomic DNA; the solution was neutralized with the addition of sodium acetate. After centrifugation, the supernatant was filtered and the plasmid was ethanol precipitated (2 volumes ethanol added). The pellet was resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and RNA digested with RNase. Proteins were removed by a phenol:chloroform:isoamyl alcohol extraction followed by a chloroform:isoamyl alcohol extraction to reduce phenol contamination of the aqueous phase. The DNA was ethanol precipitated, resuspended in TE and the degraded RNA eliminated by 40% PEG-6000 (30 mM $MgCl_2$) precipitation. Two 70% ethanol washes to remove the residual PEG followed. The pellet was air dried and resuspended in TE and the plasmid DNA quantified by optical density absorbance at 260 nM using a UV spectrophotometer.

Transfection Studies Cultures were grown to approximately 50% confluent density, rinsed in serum-free PRF-DMEM and exposed to plasmids in the presence of 0.5% Lipofectin™ (Life Science Technologies). Each plasmid was used at a final concentration of 1.25 μg/9.6 cm^2 culture well in the following combinations: pGL3 control co-transfected with hER, pϕLuc co-transfected with hER, $(ERE)^2$PRL-Luc with and without hER, and IGF-1711b-Luc co-transfected with hER. Plasmid combinations and individual treatment conditions were performed in replicates of three in every experiment. After 3 hours of incubation, the plasmid solutions were aspirated and

replaced with growth medium containing PRF-DMEM with 5% charcoal stripped FBS. Cultures were allowed to grow for 48 hours. Prior to treatment with test agents, cultures were rinsed with serum-free PRF-DMEM and then treated for indicated times with either vehicle (ethanol at 1/1000 dilution), PGE2 (1 μM), 17β-estradiol (10 nM) either alone or in combination with PGE_2, or 4-hydroxy-tamoxifen (OH-tamoxifen) (in concentrations as indicated) either alone or in combination with PGE_2. At the end of the treatment interval, cultures were rinsed with phosphate buffered saline (PBS), and then lysed in 100 μl of Cell Lysis Buffer (Promega Corp.) containing 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol (DTT), 2 mM 1,2-diaminocyclohexane-N, N, N', N'-tetraacetic acid, 10% glycerol (to stabilize enzymes for freezer storage), 1% Triton-X-100 (to lyse cell membranes). Lysates were collected and centrifuged at 12,000 x g for 5 minutes to pellet nuclei. Supernatants were collected and stored at -75°C until assayed. Luciferase reporter enzyme activity was measured in a Packard scintillation spectrometer by single channel photon counting employing a commercial kit from Promega Corp. according to manufacturer's instructions. Protein content of each cell extract was determined by Bradford dye binding assay, using bovine serum albumin to generate a standard curve. Luciferase activity was corrected for protein content. Most transfections were performed by Kristina Crothers; experimental results were corroborated by Sandra Casinghino and Dr. Thomas L. McCarthy.

Nuclear Protein Extracts Cultures were grown to 40% confluent density, rinsed in serum-free PRF-DMEM and exposed to 20 μg hER plasmid per 177 cm^2 culture plate (150 mm) in the presence of 15 ml containing 0.5% Lipofectin™ (Life Science Technologies). After 3 hours, the plasmid solutions were aspirated and replaced with PRF-DMEM containing 5% charcoal stripped FBS and allowed to grow for 48 hours. Prior to treatment with test agents, cultures were rinsed with serum-free PRF-DMEM and then treated for 4 hours with either vehicle (ethanol at 1/1000 dilution), PGE_2 (1 μM), 17β-estradiol (10 nM) either alone or in combination with PGE_2, or OH-tamoxifen (1

μM) either alone or in combination with PGE_2. Previous experiments in our laboratory demonstrated that 17β-estradiol suppressed PGE_2 induced binding to HS3D, the atypical CRE, within 4 hours as determined by gel mobility shift assay. The treatment solutions were then aspirated and cultures were rinsed twice with PBS at 4°C; all subsequent steps were performed on ice. Using a sterile cell scraper, cells were harvested in PBS and gently pelleted at 3000 rpm, washed with PBS and repelleted. Cells were then resuspended in hypotonic buffer containing 1% Triton X-100, 10 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM sodium orthovanadate, and protease inhibitors (0.5 mM phenyl methylsulfonyl fluoride, 1 μg/ml pepstatin A, 2 μg/ml leupeptin, and 2 μg/ml aprotinin, all from Sigma Chemical) to gently lyse the cells. Nuclei were pelleted at 3500 rpm. The lysates were resuspended in hypotonic buffer as above, and repelleted to achieve complete lysis and to wash the nuclei in order to reduce the level of membrane bound alkaline phosphatase in the nuclear fraction. Nuclear proteins were released in hypertonic buffer containing 0.42 M NaCl, 0.2 mM disodium ethylenediamine tetraacetic acid, 25 % glycerol, and sodium orthovanadate and protease inhibitors as above. After 30 minutes of incubation, the nuclear debris was pelleted at 13,000 rpm for 5 minutes and the supernatant containing the nuclear protein extracts were aliquoted and stored at -75°C until gel-shift analysis was performed. Protein concentrations were determined by Bradford dye binding assay. Buffer solutions were provided by Chang-Hua Ji; all nuclear extracts were prepared by Kristina Crothers.

Electrophoretic Mobility Shift Assay Gel-shift assays were performed as described previously (Revzin, 1989; Carey, 1991; Thomas, 1996). Briefly, a radiolabeled double-stranded oligonucleotide probe (sequence shown below) (Universal DNA Inc., Tigard, OR) was prepared by annealing complimentary nucleotides in 10 M Tris-Cl (pH 8.0), 1 mM EDTA, and 5 mM $MgCl_2$ by heating to 95°C then cooling to room temperature over a one hour time period. Using the Klenow fragment of *E. coli* DNA polymerase I, single-stranded overhangs were filled in with dCTP, dGTP, dTTP, and [α-^{32}P]dATP at room

temperature. The probe was then gel purified prior to use. 5 μg of each nuclear protein extract was incubated in binding buffer (25 mM HEPES pH 7.5, 50 mM KCl, 2 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, and 12.5% glycerol) containing 1.25 μg poly(dI/dC) (Sigma) on ice. After 10 minutes, 0.1 ng (approximately 3 x 10^4 cpm) of ^{32}P-labeled HS3D DNA probe was added for a total reaction volume of 20 μl and incubated on ice for an additional 30 minutes. A 5% nondenaturing polyacrylamide gel was preelectrophoresed for 30 minutes at 12.5 V/cm at 25°C in 45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3. Samples were then loaded onto the gel and electrophoresis proceeded under identical conditions for 2.5 hours. The gel was then dried and exposed to X-ray film at -75°C with an intensifying screen. Labeled HS3D probe was prepared by Chang-Hua Ji; gel-shifts were performed by Kristina Crothers.

The sense strand oligonucelotide sequence of HS3D with response element underlined is shown below.

5'-GAGCAGATAGAGCCTGCGCAATCGAAATA-3'

Reagents Cell culture reagents were obtained from Life Science Technologies (Gaithersburg, MD) and Sigma Chemical Co. (St. Louis, MO). 17β-estradiol and PGE_2 were obtained from Sigma Chemical Co. OH-Tamoxifen was obtained from Research Biochemical International (Natick, MA).

Statistical Analysis Data from transfections were reported in triplicate for each condition; results are reported as means +/- S.E. Statistical analysis was performed using the SigmaStat computer software package, using the one-way analysis of variance (ANOVA) to determine significance. Results of the electrophoretic mobility shift assay were analyzed by computer-assisted densitometry using the SigmaGel computer software package.

RESULTS

17β-estradiol effects on IGF-I activity

Primary cultures of fetal rat osteoblasts were transfected with the IGF-I promoter 1-luciferase reporter construct, IGF1711b/Luc. This construct contains an atypical cAMP response element (CRE) required for the PGE_2 stimulated rise in IGF-I via a cAMP dependent mechanism (Thomas *et al*, 1996). Cells were co-transfected with hER expression vector, required for estrogen as well as tamoxifen action. Previous work by Dr. McCarthy illustrated that, at a concentration of 1 μM, PGE_2 stimulates IGF-I transcription an average of 4- to 5-fold after 6 hours of exposure. Furthermore, this effect is dose-dependently suppressed by 17β-estradiol, and sustained over 16 hours of treatment (Fig. 1, adapted from McCarthy *et al.*, 1997). Inhibition is hER dependent, as it is not observed in the absence of hER co-transfection. This effect is estradiol isomer specific, as 17α-estradiol is 100-300 fold less potent. Experiments repeated in this thesis confirm that PGE_2 produces nearly 6-fold stimulation of IGF-I promoter activity which can be reduced by 10 nM 17β-estradiol after 6 hours of co-treatment, with no effect on IGF-I activity by estrogen alone (Fig. 2). Thus 17β-estradiol served as an internal control in appropriate experiments.

OH-tamoxifen effects on IGF-I activity

To investigate the effects of tamoxifen on IGF-I expression, all experiments reported in this thesis used 4-hydroxy-tamoxifen (OH-tamoxifen), in agreement with other investigations of tamoxifen (Galien *et al.*, 1996). This is an active metabolite of tamoxifen formed *in vivo* in both animal models and in the serum of breast cancer patients treated with tamoxifen, and is reported to be 100- to 1000-fold more potent than tamoxifen (Borgna & Rochefort, 1980; Rochefort, 1991; Pujol *et al.*, 1995). Cell cultures were treated for 6 hours with 1 μM PGE_2 with or without equal concentrations of OH-tamoxifen or estrogen (both 10 nM). Neither 17β-estradiol nor OH-tamoxifen alone had any effect on IGF-I promoter activity when compared to vehicle (ethanol diluted 1/1000).

The 6-fold PGE_2 stimulation was significantly reduced by the addition of 10 nM 17β-estradiol but was unaffected by the addition of 10 nM OH-tamoxifen (Fig. 2).

Given that tamoxifen is a therapeutic agent and not a physiologic hormone as is estrogen, pharmacologic doses of OH-tamoxifen were next used to determine if higher doses might suppress the PGE_2 induction of IGF-I as effectively as 17β-estradiol. Overall, concentrations from 0.1 nM to 1 μM were tested. Results from five experiments were pooled (Fig. 3). Like estrogen, tamoxifen suppressed PGE_2 induced IGF-I promoter activity in a dose dependent manner. At concentrations of 1 μM, OH-tamoxifen significantly ($p<0.05$) reduced promoter activity by 70%. Once again, 1 nM concentrations of tamoxifen were not significantly inhibitory, compared to 64% inhibition seen with the same concentration of 17β-estradiol (Fig. 3). OH-tamoxifen alone at these higher doses had no significant effect on IGF-I promoter activity. These initial findings indicate that tamoxifen, while capable of suppressing IGF-I activity, appears to be less potent in its behavior on osteoblasts than 17β-estradiol.

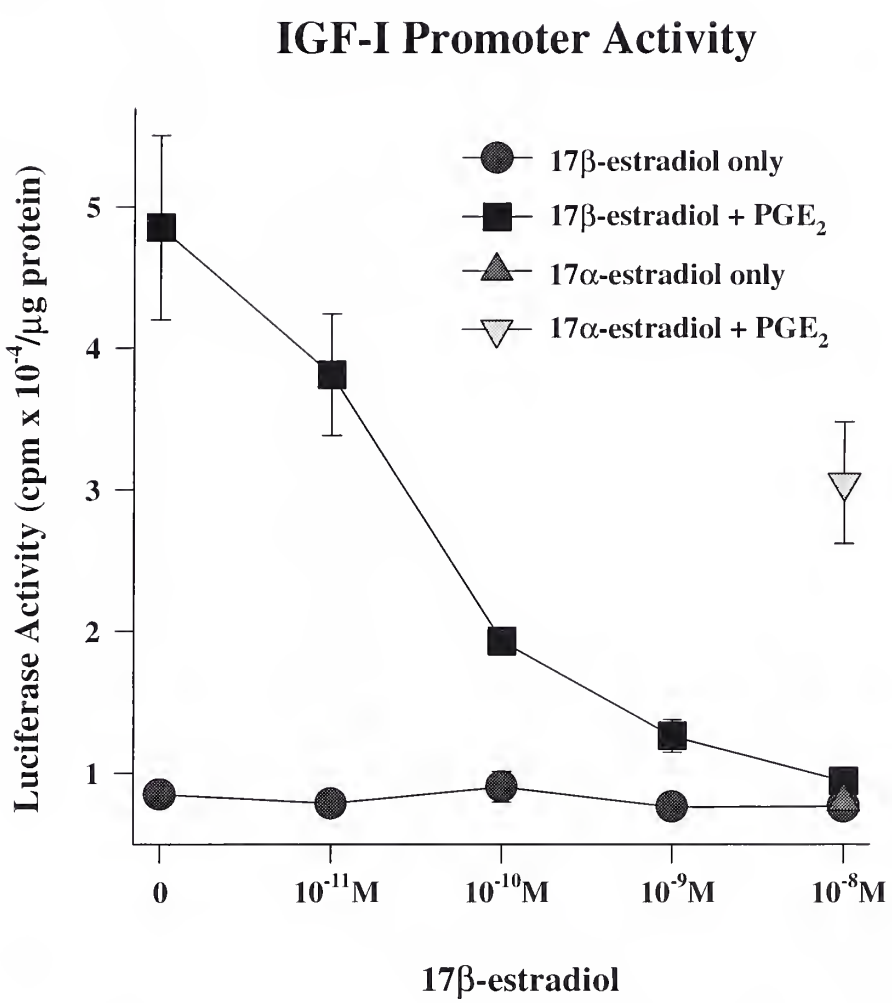

Figure 1. 17β-estradiol dose dependently suppressed PGE_2 stimulated IGF-I promoter 1 activity in osteoblast cell cultures expressing hER. IGF-I promoter 1 luciferase reporter plasmid, IGF1711b-Luc, was co-transfected with hER into osteoblast cultures. Cultures were grown in phenol red-free DMEM containing 5% charcoal-stripped FBS for 48 hours. The growth medium was aspirated, and cultures rinsed with phenol red-free, serum-free DMEM. Cultures were treated with control medium (ethanol at 1/1000 dilution), PGE_2 (1 μM), 0.01 to 10 nM 17β-estradiol, or both agents for 16 hours. 17α-estradiol (10 nM) was used both with and without PGE_2 (1 μM) to test for estrogen isoform specificity. Cytoplasmic extracts were prepared and luciferase activity determined. Data are corrected for protein content. Transfections were performed in triplicate, and results are representative of 3 separate experiments. The mean +/- SE for luciferase expression (cpm per μg protein) are shown.

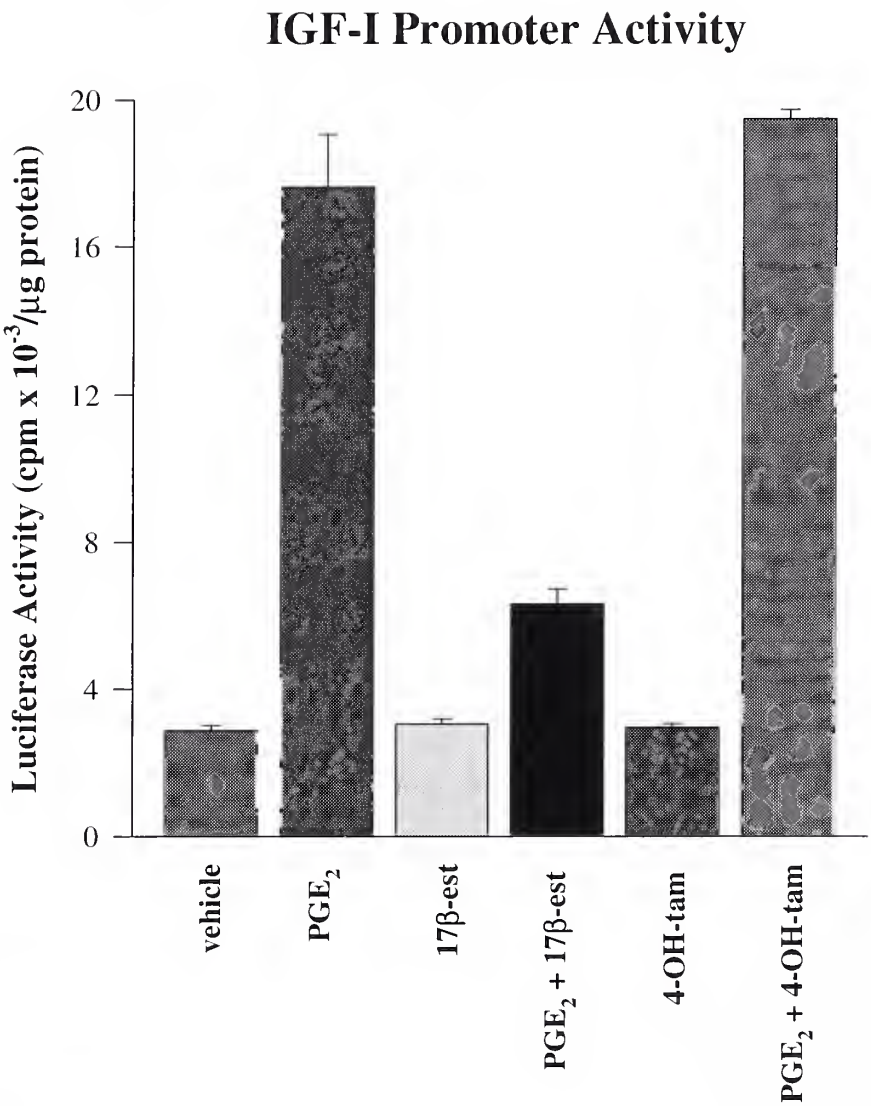

Figure 2. As with 17β-estradiol treatment, 4-OH-tamoxifen treatment of osteoblast cell cultures had no effect on IGF-I promoter activity. Unlike estrogen, an equal concentration of 4-OH-tamoxifen did not suppress PGE_2 stimulation of the IGF promoter 1. Osteoblast cell cultures were transfected as described in Figure 1 and Materials and Methods. Cultures were treated with vehicle (1/1000 ethanol), 1 µM PGE_2, 10 nM 17β-estradiol in the absence or presence of PGE_2, or 10 nM 4-OH-tamoxifen in the absence or presence of PGE_2. After 6 hours, cell extracts were prepared and luciferase activity determined. Data are corrected for protein content. Transfections were performed in triplicate. Results of a representative experiment, +/- SE, are shown.

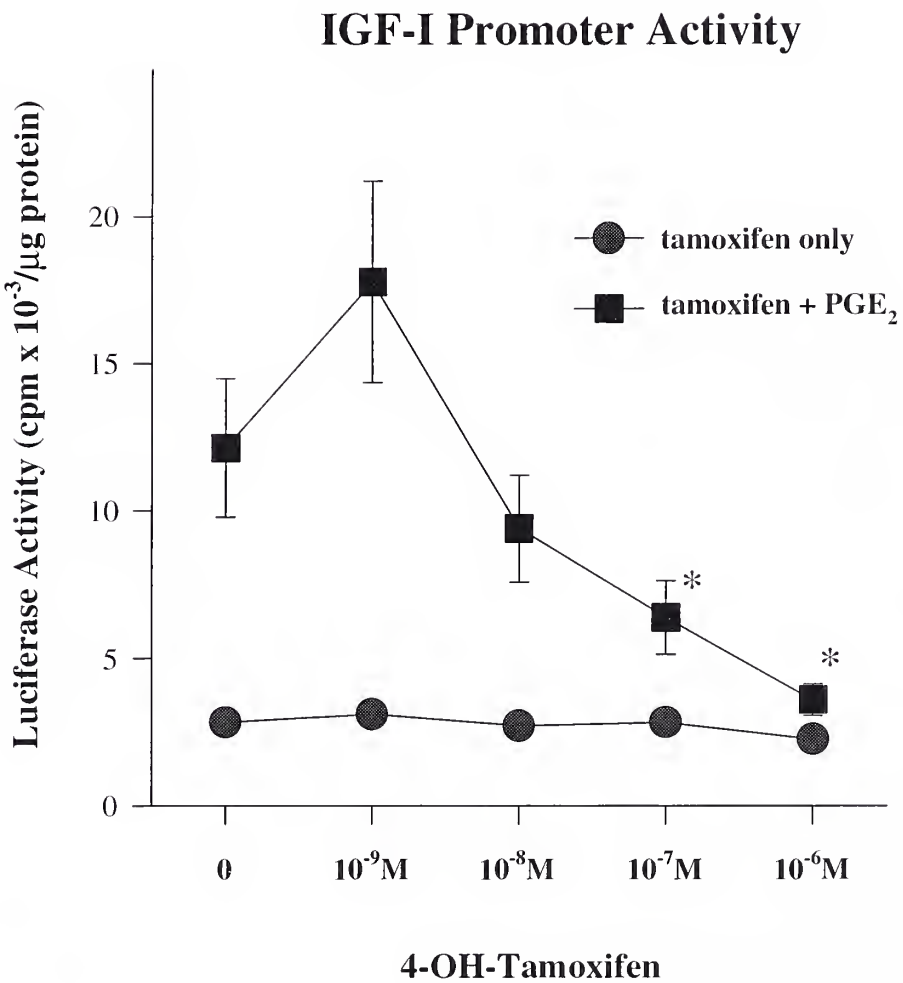

Figure 3. 4-OH-tamoxifen dose dependently suppressed PGE_2 stimulated IGF-I promoter 1 activity in osteoblast cultures expressing hER. Osteoblast cell cultures were transfected as described in Figure 1 and Materials and Methods. Cells were treated for 6 hours with varying concentrations of 4-OH-tamoxifen both with and without PGE_2 (1 µM). Luciferase activity of cytoplasmic extracts was determined, and data were corrected for average protein content. Data represent pooled results from 5 experiments (n = 9-15). At 1 µM and 0.1 µM concentrations, tamoxifen suppression of PGE_2 induced IGF-I expression was statistically significant (*, $p < 0.05$).

Comparison of OH-tamoxifen to 17β-estradiol stimulation of the ERE

To address whether the inhibitory effects of the higher doses of OH-tamoxifen could be due to toxic effects on the fetal rat osteoblasts, cultures were co-transfected with hER and a recombinant reporter plasmid containing two tandem copies of the consensus estrogen response element (ERE) and a segment of the prolactin promoter controlling the expression of the luciferase reporter ($(ERE)^2$PRL-Luc). Previously, a 6 hour treatment with 10 nM 17β-estradiol of cultures transfected with ERE^2PRL-Luc demonstrated on average a 5-fold stimulation (McCarthy *et al.*, 1997). It was unclear initially whether OH-tamoxifen would behave similarly. As with 17β-estradiol stimulation, OH-tamoxifen activation of the ERE required co-transfection with hER, as cells not transfected with the hER expression vector were unable to respond to either OH-tamoxifen or 17β-estradiol (Fig. 4). However, the stimulation by tamoxifen appeared much weaker than that observed with estrogen.

To determine the dose response of the ERE activation, transfected cultures were treated for 6 hours with varying concentrations of OH-tamoxifen, ranging from 1 μM to 0.1 nM. These experiments consistently reveal that OH-tamoxifen was weakly stimulatory of the ERE containing plasmid only at the highest doses tested. While 10 nM 17β-estradiol enhanced transcription of this construct on average 3- to 5-fold, OH-tamoxifen was 200- to 350-fold less potent, only increasing activity 1.3 fold at 1 μM concentration (Fig. 5). This weak stimulation was statistically significant ($p < 0.05$), but became indistinguishable from control treatment at lower concentration of OH-tamoxifen. Prolonged treatment of $(ERE)^2$PRL-Luc transfected cultures with 1 μM OH-tamoxifen resulted in a nearly 50% increase in activity, from a stimulation of 1.3 fold compared to control values after a 6 hour treatment to a nearly 1.9 fold enhancement of activity after 24 hours of treatment. This weak activation of the ERE-construct demonstrates that higher concentrations of OH-tamoxifen were likely not toxic to the cell cultures, even after 24 hours of exposure. These data support the conclusion that

tamoxifen acts as a partial estrogen agonist in osteoblasts, but is less potent than 17β-estradiol.

17β-estradiol and OH-tamoxifen effects on the parental, promoterless luciferase reporter construct

Cells were transfected with the parental luciferase reporter construct that contained no promoter or ERE, and only the luciferase reporter gene (pϕLuc). Cell cultures were then treated for 6 hours with vehicle, PGE_2, 17β-estradiol, or OH-tamoxifen, both with and without PGE_2. Luciferase values were at background levels with each treatment condition (Fig. 6), confirming that luciferase activity is promoter dependent, and requires control by either the IGF-I promoter or $(ERE)^2$PRL construct transfected into the cell cultures to detect any response to these test agents.

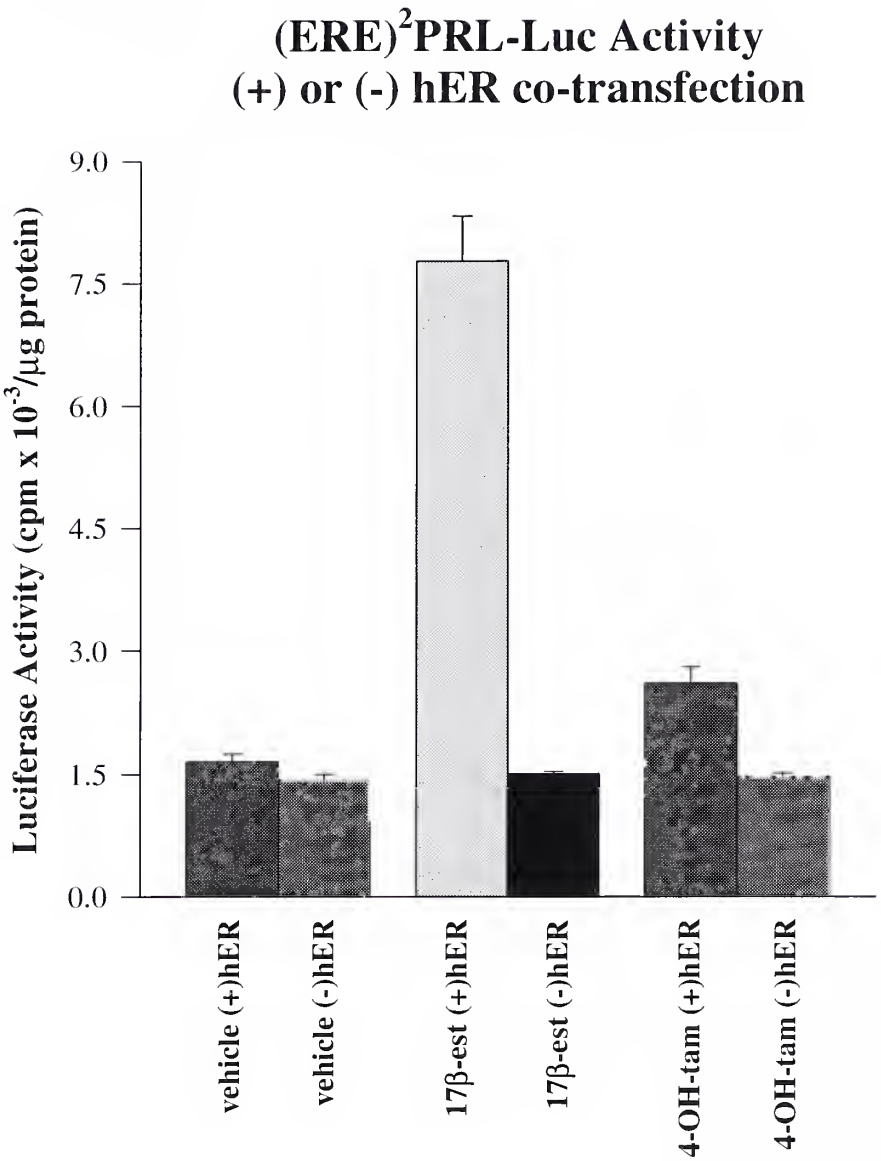

Figure 4. As with 17β-estradiol, tamoxifen action is dependent on expression of hER. However, 4-OH-tamoxifen stimulation of the ERE is much less potent than 17β-estradiol. Cells were transfected with a recombinant luciferase reporter construct containing tandem copies of the consensus ERE and a segment of the prolactin promoter, ((ERE)^{2}PRL-Luc), both with and without hER. Cultures were treated with vehicle (ethanol at 1/1000 dilution), 17β-estradiol (10 nM), or 4-OH-tamoxifen (1 µM). After 6 hours, cytoplasmic extracts were prepared and luciferase activity determined. Data are corrected for protein content. Transfections were performed in triplicate, and results shown are representative of one experiment. The mean +/- SE for luciferase expression (cpm per µg protein) are shown.

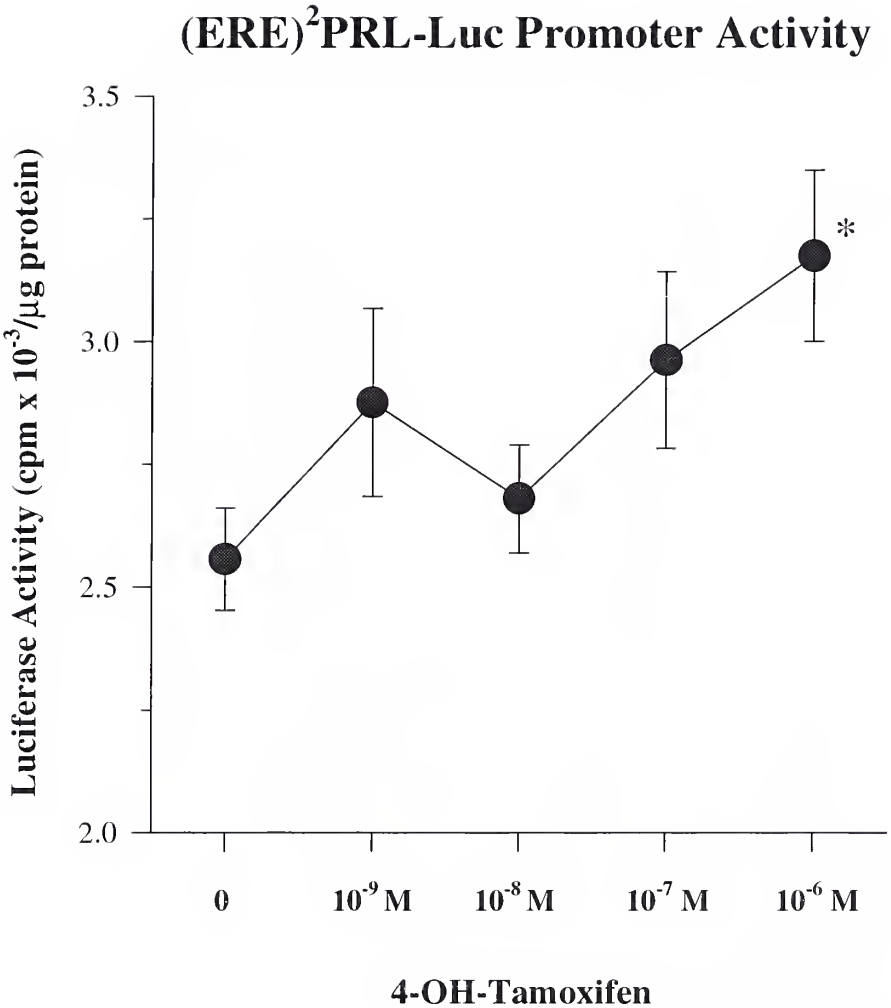

Figure 5. 1 μM 4-OH-tamoxifen weakly stimulated the (ERE)²PRL-Luc promoter. Osteoblasts were transfected as described in Figure 4 and Materials and Methods with the (ERE)²PRL-Luc construct and hER. Cells were treated for 6 hours with 1 nM to 1 μM 4-OH-tamoxifen. Cytoplasmic extracts were prepared and luciferase activity determined. Data are corrected for average protein content. Results represent data pooled from 8 experiments (n = 12-24). Stimulation of the ERE promoter construct by 1 μM tamoxifen was statistically significant (*, $p < 0.05$).

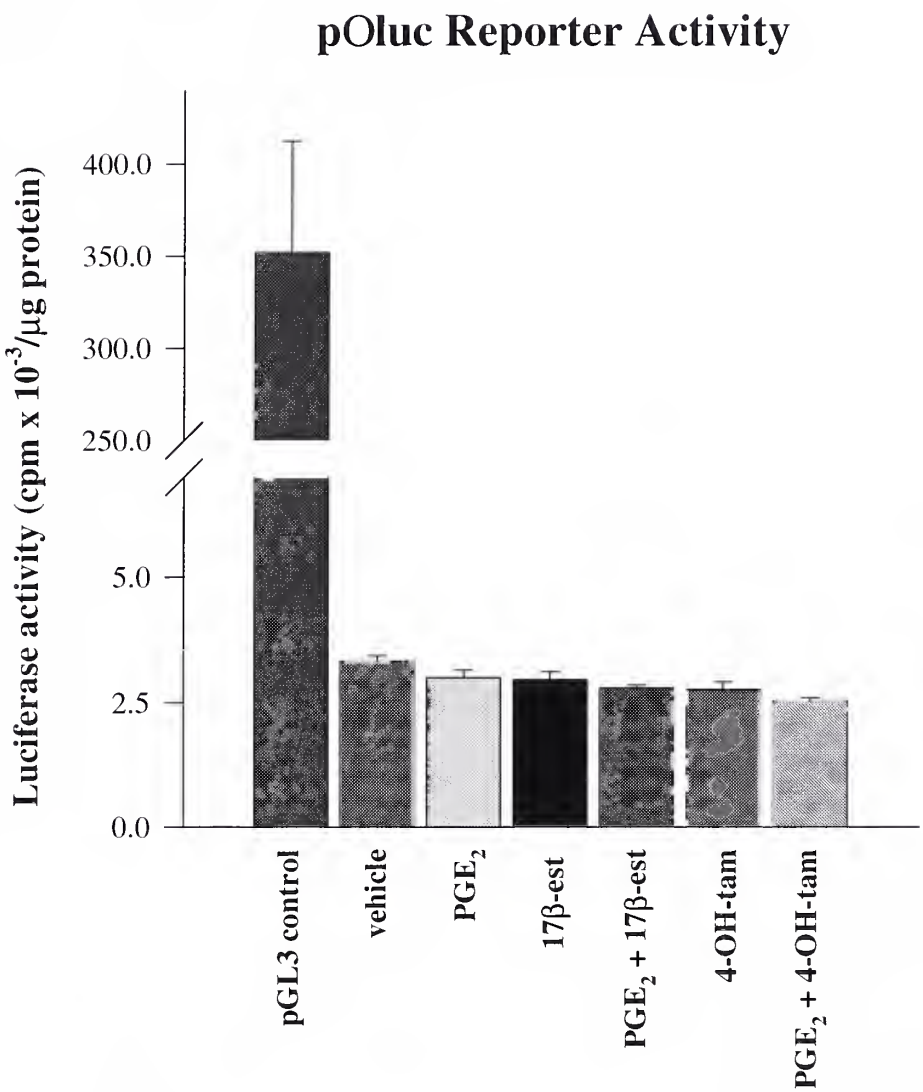

Figure 6. PGE_2 stimulation was specific for the IGF-I promoter 1; estrogen and tamoxifen stimulation was specific for the ERE. Osteoblasts were transfected with the promoterless, parental expression vector pOluc or pGL3 control vector as described in Materials and Methods. Cells were treated for 6 hours with vehicle, 1 µM PGE_2, 10 nM 17β-estradiol with and without PGE_2, or 1 µM 4-OH tamoxifen, both with and without PGE_2. Cytoplasmic extracts were prepared, and luciferase activity determined. Transfections were performed in triplicate. Results of a representative experiment are shown.

Nuclear protein extractions tested in electrophoretic mobility shift assays

A gel mobility shift assay was conducted using a ^{32}P-labeled oligonucleotide containing the IGF-I CRE, termed HS3D, and nuclear extracts of cell cultures transfected with hER. Nuclear extracts from cultures treated with 1 μM PGE_2 caused an inducible gel-shift, demonstrating cAMP activated transcription factor binding to the nonconsensus CRE in the IGF-I promoter 1 with no detectable interference by hER expression (McCarthy *et al.*, 1997). Consistent with previous findings in the laboratory, co-treatment of cells with PGE_2 and 17β-estradiol (1 nM) caused a reduction in the intensity of PGE_2 induced gel shift bands, with no alteration in the banding pattern (Fig. 7). Treatment of cells with either 17β-estradiol or OH-tamoxifen alone did not produce an appreciable change compared to control cultures. A preparation of nuclear extract from cells co-treated with both PGE_2 and OH-tamoxifen (1 μM) for 4 hours tested with the ^{32}P-HS3D oligonucleotide also induced a gel-shift, with reduced band intensity when compared to that induced by PGE_2 treatment alone. Analysis of band density indicates that OH-tamoxifen reduced the PGE_2 induced bands by 32%, while co-treatment with 17β-estradiol and PGE_2 resulted in a decrease in band density by 47%. These results further confirm that OH-tamoxifen is less potent than 17β-estradiol at inhibiting PGE_2 induced activation of transcription factors that bind to this CRE element within the IGF-I promoter.

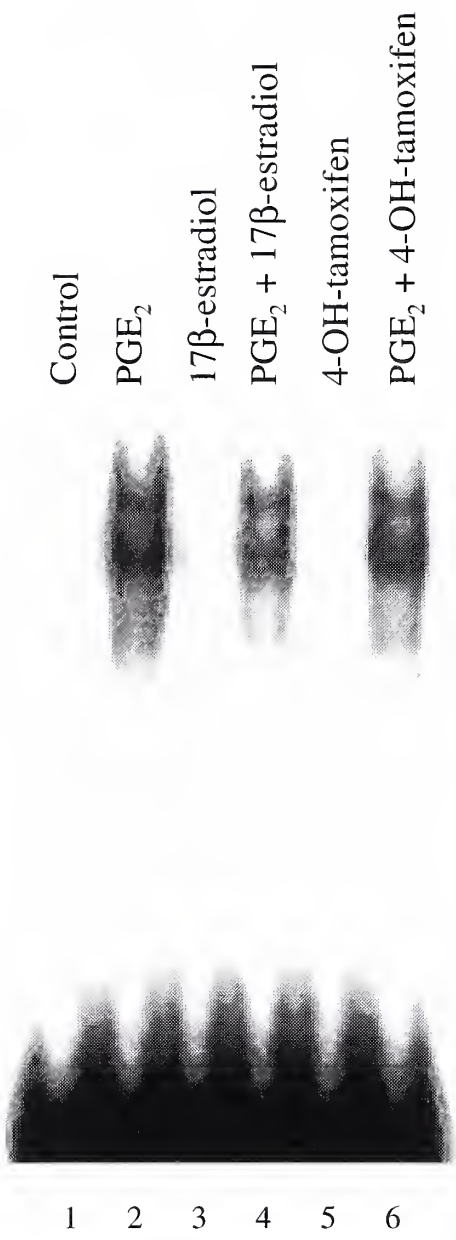

Figure 7. 4-OH-tamoxifen was not as potent as 17β-estradiol in suppressing the PGE_2 induced binding of nuclear protein(s) to the IGF-I promoter 1 atypical CRE. Electrophoretic mobility shift experiments were performed as described in Materials and Methods with nuclear extracts from osteoblast cultures. All osteoblast cultures were transfected with hER; cultures were treated with PGE_2, 10 nM 17β-estradiol with or without PGE_2, or 4-OH-tamoxifen with or without PGE_2 for 4 hours, as indicated above each lane. By analysis of band density, estrogen suppressed the PGE_2 induced bands by 47%, while tamoxifen inhibition of the PGE_2 induced bands was only 32%.

DISCUSSION

Although estrogen replacement therapy has been clinically proven to prevent the accelerated postmenopausal loss of bone, its mechanism of action remains incompletely understood. Accumulating evidence suggests that estrogen may act as a suppressive agent to maintain bone turnover at physiologically appropriate levels. Estrogen antagonizes IL-6 production (Girasole *et al.*, 1992; Pottratz *et al.*, 1994), as well as returning the release of IL-1 to premenopausal levels (Sato *et al*, 1993). On the other hand, estrogen also counters the postmenopausal or post-OVX rise in IGF-I both systemically and locally in bone tissue, as well as decreasing the rate of bone formation in OVX rats (Sato *et al*, 1993; Kalu *et al.*, 1994; Yokose *et al*, 1996). Although it is a potent stimulator of bone formation, IGF-I also enhances bone resorption by recruitment of osteoclasts ((Mochizuki *et al.*, 1992). Interestingly, IGF-I was shown to increase IL-6 production in cultures of mouse long bones and osteosarcoma cells (Slootweg *et al.*, 1992). In combination with results from our laboratory demonstrating estrogen mediated suppression of PGE_2 induced activation of the IGF-I promoter, these pieces of information suggest a role for estrogen in maintaining a basal rate of bone turnover which then becomes accelerated in states of estrogen deficiency. This tonic balance of anabolic and catabolic activity may be mediated in part by IGF-I, a known coupling factor for bone remodeling.

Estrogen antagonists/agonists are becoming increasingly studied as possible therapeutic agents to target estrogenic effects to select tissues such as bone in order to prevent postmenopausal osteoporosis while minimizing side effects to other organ systems. Although used clinically as an antiestrogen to treat women with breast cancer, tamoxifen does not accelerate bone loss in these patients. Indeed, tamoxifen behaves as an estrogen agonist in bone tissue, but whether its mechanism of action is the same as that of estrogen is unclear. To begin answering these questions, this study investigated

the effects of tamoxifen in comparison to estrogen on IGF-I promoter function in primary cell cultures of fetal rat osteoblasts.

Current work in the laboratory of Dr. McCarthy investigates the mechanism by which estrogen modulates the PGE_2 stimulation of IGF-I gene promoter activity. This study confirms earlier results and demonstrates that treatment of fetal rat osteoblast cell cultures with 17β-estradiol alone has no effect on IGF-I expression, contrary to previous *in vitro* studies showing a small stimulatory effect of estrogen on IGF-I mRNA levels in neonatal rat-derived bone cells (Ernst *et al.*, 1989; Ernst *et al.*, 1991a). Co-treatment of osteoblasts with PGE_2 and 17β-estradiol, however, results in a potent, dose-dependent suppression of IGF-I promoter activation that is ER dependent and estradiol isomer selective (McCarthy *et al.*, 1997). Our results are consistent with *in vivo* studies in rats that reveal a rise in IGF-I levels in bone tissue post-OVX, an effect that is attenuated with systemic estrogen treatment (Sato *et al.*, 1993).

As with 17β-estradiol, in these studies OH-tamoxifen alone has no effect on IGF-I promoter activity over a range of concentrations from 0.1 nM to 1 μM. Also similar to estrogen, OH-tamoxifen is capable of suppressing PGE_2 induced IGF-I activity, although it is significantly less potent. Only a partial estrogen agonist, tamoxifen has been reported in other studies to be less potent than estrogen. Work by Galien *et al.* (1996) demonstrating estradiol repression of TNFα-induced IL-6 secretion also investigated the effects of OH-tamoxifen. As with results reported here, tamoxifen displayed only intermediate agonism (approximately 50% the potency of estrogen) in cultures of human osteosarcoma cells, despite displaying full antagonism in breast cancer cell cultures (Pottratz *et al.*, 1994). Interestingly, this reflects partial agonist and antagonist actions seen *in vivo* in bone and breast tissue. Similarly, while both tamoxifen and estrogen cause an increase in trabecular bone in OVX rats, estrogen was 25% more effective than a 50-fold higher dose of tamoxifen (Turner *et al.*, 1988). In another study, the increase in cancellous bone apposition rate in the OVX rat was suppressed 63% by estrogen.

Tamoxifen, though significantly decreasing the apposition rate, did so only by 18% (Kalu *et al.*, 1991a). Likewise, tamoxifen was not as potent as estrogen at inhibiting radial growth in OVX rats, nor did tamoxifen increase uterine weight as much as estrogen (Moon *et al.*, 1991). Both 17β-estradiol and OH-tamoxifen increase intracellular cAMP in uterine cell cultures, but once again the response to a 100-fold higher dose of tamoxifen was less dramatic (Aronica *et al.*, 1994). Therefore evidence derived from OVX rats and cell culture systems supports the conclusion that tamoxifen, while behaving as an estrogen agonist in certain tissues, is less potent.

Nonetheless, comparing cell culture to *in vivo* results poses a degree of difficulty. Therapeutic serum levels of tamoxifen in patients and rats is reported at 5×10^{-7} M, while concentrations of 10^{-4} to 10^{-6}M may be necessary to achieve *in vitro* effects, and such high concentrations can even prove cytostatic to cells (Turner *et al.*, 1988). This may be explained partially by the fact that compared to estrogen, OH-tamoxifen has been reported to have lower availability in serum-containing medium and more restricted entry into cells (Aronica *et al.*, 1994). Also, while many cell culture experiments have used 4-OH-tamoxifen, and was the form used in experiments for this study, many animal studies have employed systemic administration of tamoxifen. Although 4-OH-tamoxifen is a 100- to 1000-fold more potent active metabolite of tamoxifen that is found in the serum of breast cancer patients, it is unclear whether this particular metabolite is the locally active form in bone. Tamoxifen is metabolized to numerous different compounds, and many of these have different estrogenic or antiestrogenic potential (Jordan & Murphy, 1990). Certain forms may be concentrated in different tissues, and this may contribute to the tissue specific actions of tamoxifen. Thus, while the effects of OH-tamoxifen on reducing PGE_2 stimulated IGF-I expression are consistent with *in vivo* studies, conclusions regarding its absolute potency can only be surmised.

Next, we sought to compare the mechanism of tamoxifen action to what we understand of the actions of estrogen and its influences on IGF-I expression. IGF-I

transcription is stimulated by agents such as PGE_2 and PTH that increase intracellular cAMP. PGE_2 stimulated expression of IGF-I is PKA dependent (McCarthy *et al.*, 1995). Although the IGF-I promoter 1 contains a single, near consensus CRE and several potential binding sites for transcription factor AP-2, deletion of these regions does not block PGE_2 induced cAMP stimulation of IGF-I promoter activity. Rather, the DNA sequence HS3D, located in a the 5'-untranslated region (UTR) of exon 1, is integral to PGE_2 stimulation of IGF-I although it has no nucleotide sequence similarity to the consensus CRE (Thomas *et al.*, 1996). The active factors are not present in the cytoplasm, and cycloheximide treatment does not interfere with the PGE_2 induced rise in IGF-I mRNA. PKA is thought to activate nuclear pre-existing DNA binding proteins, which may then interact with specific nucleotides within the HS3D DNA sequence (Thomas *et al.*, 1996).

Recent data generated in our laboratory indicate that 17β-estradiol does not inhibit PGE_2 induced IGF-I expression by interfering with cAMP production, but rather may alter protein-protein interactions between transcription factors (McCarthy *et al.*, 1997). cAMP levels rise with PGE_2 treatment, and are unaffected by co-treatment or pre-treatment with estrogen. A gel-mobility shift assay of ^{32}P-labeled oligonucleotide containing HS3D using nuclear extracts of cells revealed reduced intensity of the PGE_2 induced gel shift band when extracts were prepared with cultures co-treated with PGE_2 and estrogen. The IGF-I promoter 1 contains no ERE, and no modification was seen in the gel mobility shift pattern of the HS3D probe with nuclear extracts from estrogen treated cultures. This suggests that estrogen may act via an ERE-independent mechanism, possibly affecting protein-protein interactions between the ligand activated ER and the cAMP activated transcription factor(s) involved in IGF-I expression (Fig. 8). Such an interaction may limit the binding ability of the activated transcription factor(s) for the HS3D element.

Tamoxifen may work in a similar manner. Using the gel-mobility shift assay with ^{32}P-labeled HS3D probe and nuclear extracts from tamoxifen treated cells, no detectable change in gel-shift pattern was observed, making it unlikely that tamoxifen alone can significantly elevate cAMP levels in these cultures. Therefore, tamoxifen, like estrogen, does not alter DNA binding, in agreement with results from cell cultures revealing no change in activity of the IGF-I promoter by estrogen or tamoxifen treatment. However, treatment of cells with tamoxifen plus PGE_2 and electrophoresis of nuclear extracts with the ^{32}P-labeled HS3D probe demonstrates that tamoxifen decreases the intensity of the PGE_2 inducible gel-shift bands compared to extracts from cells treated with PGE_2 alone. Just as tamoxifen was not as potent as estrogen in cell cultures, inhibition detected with the gel-shift assay was not as marked.

Interestingly, the actions of estrogen and tamoxifen may be enhanced in the presence of elevated cAMP levels, perhaps explaining in part why estrogen and tamoxifen have detectable effects only in the presence of PGE_2. Protein kinase activators have been shown to have a synergistic effect on estrogen-dependent ER-mediated transcription functioning through a consensus ERE (Cho & Katzenellenbogen, 1993). Observations in our laboratory illustrate that estrogen dependent stimulation of the ERE was elevated in the presence of PGE_2, suggesting additional and alternate interactions between estrogen action and cAMP from those observed for the IGF-I promoter. Furthermore, preliminary results from our experiments indicate that tamoxifen stimulation of the $(ERE)^2$PRL-Luc was likewise enhanced when given in combination with PGE_2.

Although data suggest that tamoxifen acts via a similar ER-dependent mechanism as estrogen, estrogen and tamoxifen differentially activate transcription through the ERE, depending on the cellular context. While estrogen strongly activates the classical ERE, tamoxifen does so only weakly, indeed if at all. In our studies, OH-tamoxifen consistently and significantly stimulated the ERE to a very low degree at the highest

concentration tested (1μM), thereby confirming that this concentration of tamoxifen is likely not cytotoxic. Other studies similarly report no or very weak tamoxifen activation of the ERE, ranging from 3% to 30% of estrogen induction, depending upon species and cell type (Webb *et al.*, 1995). In *in vitro* studies, tamoxifen bound to ER recognizes an ERE specifically (Berry *et al.*, 1990), indicating that tamoxifen does allow ER binding to ERE, unlike the pure antiestrogen ICI which inhibits ER dimerization and prevents its interaction with the ERE (Webb *et al.*, 1995).

The weak interaction of tamoxifen with the ERE may be due to differential activation of the ER (Rochefort & Borgna, 1981; Tzukerman *et al.*, 1994). The molecular structure of the ER consists of discrete domains which can function independently; two of the most highly conserved regions are the DNA binding domain (DBD) (region C) and the hormone binding domain (HBD) (region E). The ER exhibits hormone-induced conformational changes that appear to be compound specific, as the antiestrogen ICI induced a different conformation of the HBD than estrogen (Beekman *et al.*, 1993). Two independent transcriptional activation functions, TAF-1 and TAF-2, of the ER have been characterized (Lees *et al.*, 1989; Danielian *et al.*, 1992). The activity of TAF-1, located in the amino-terminus, is constitutive, while TAF-2, in the carboxyl-terminal within the HBD, is hormone inducible (Kumar *et al.*, 1987). The activity of TAF-1 and TAF-2 depend upon cell specificity and target gene promoter context (Tasset *et al.*, 1990). It has been reported that OH-tamoxifen, in contrast to estradiol, cannot induce TAF-2 activity, and thus the partial agonist characteristics of tamoxifen may stem from selective, and consequently limited, TAF-1 activity (Berry *et al.*, 1990; Tzukerman *et al.*, 1994).

Alternately, the partial estrogenic properties of tamoxifen have also been proposed to arise via activation of an ERE-independent pathway. The ER can stimulate transcription from a promoter that contains an AP-1 site, the binding site for transcription factors Jun and Fos (Gaub *et al.*, 1990; Umayahara *et al.*, 1994). Since this function does

not depend on an intact ER DBD, this pathway may act via protein-protein interactions (Webb *et al.*, 1995). Additional support for a non-genomic, ERE-independent pathway was provided by *in vitro* experiments demonstrating that protein kinase activators synergistically enhance estrogen-dependent ER-mediated transcriptional activation without altering the DNA binding properties of the ER (Cho & Katzenellenbogen, 1993). Estrogen repression of the IL-6 promoter was also shown to be ERE-independent, mediated instead by interactions with the DBD of the ER and DNA binding and dimerization domains for the transcription factors NF-κB and C/EBP involved in regulation of IL-6 (Pottratz *et al.*, 1994; Stein & Yang, 1995).

Data from our laboratory likewise support the ERE-independent pathway of estrogen action within the IGF-I promoter 1 in osteoblasts. Furthermore, tamoxifen, like estrogen, suppressed PGE_2 induced IGF-I promoter activation, and this effect was limited to a DNA sequence that lacked both AP-1 and ERE binding sites. We demonstrate for the first time the effects of tamoxifen in osteoblast cultures on IGF-I activity both alone and in conjunction with PGE_2. Co-treatment of cells with PGE_2 and tamoxifen or estrogen results in decreased binding to HS3D, the 5'-UTR region of IGF-I P1 containing an atypical CRE crucial for PGE_2 stimulation. Further studies are ongoing in our laboratory to characterize the nature of potential inhibitory protein-protein interactions.

The discovery of alternate pathways of estrogen action in various tissues other than the classical ERE helps to elucidate the seemingly contradictory actions of partial estrogen antagonists such as tamoxifen. As our knowledge of these different mechanisms increases, the possibilities for future therapeutic interventions grow. Postmenopausal hormone replacement therapy could be targeted to bone and cardiovascular systems to maximize the beneficial effects of estrogen on these tissues while minimizing actions on uterine tissue, thus obviating the need to combine an estrogen with progesterone. Additionally, in premenopausal or postmenopausal patients with increased risk of breast cancer, antiestrogen therapy specific to breast tissue would have application as a

chemoprophylactic agent while maintaining bone mineral density in the skeleton. Estrogen analogues specific to uterine and ovarian tissue could have potential use as fertility agents. Undoubtedly, the future ability to target estrogenic or antiestrogenic effects to isolated tissues will have profound clinical relevance.

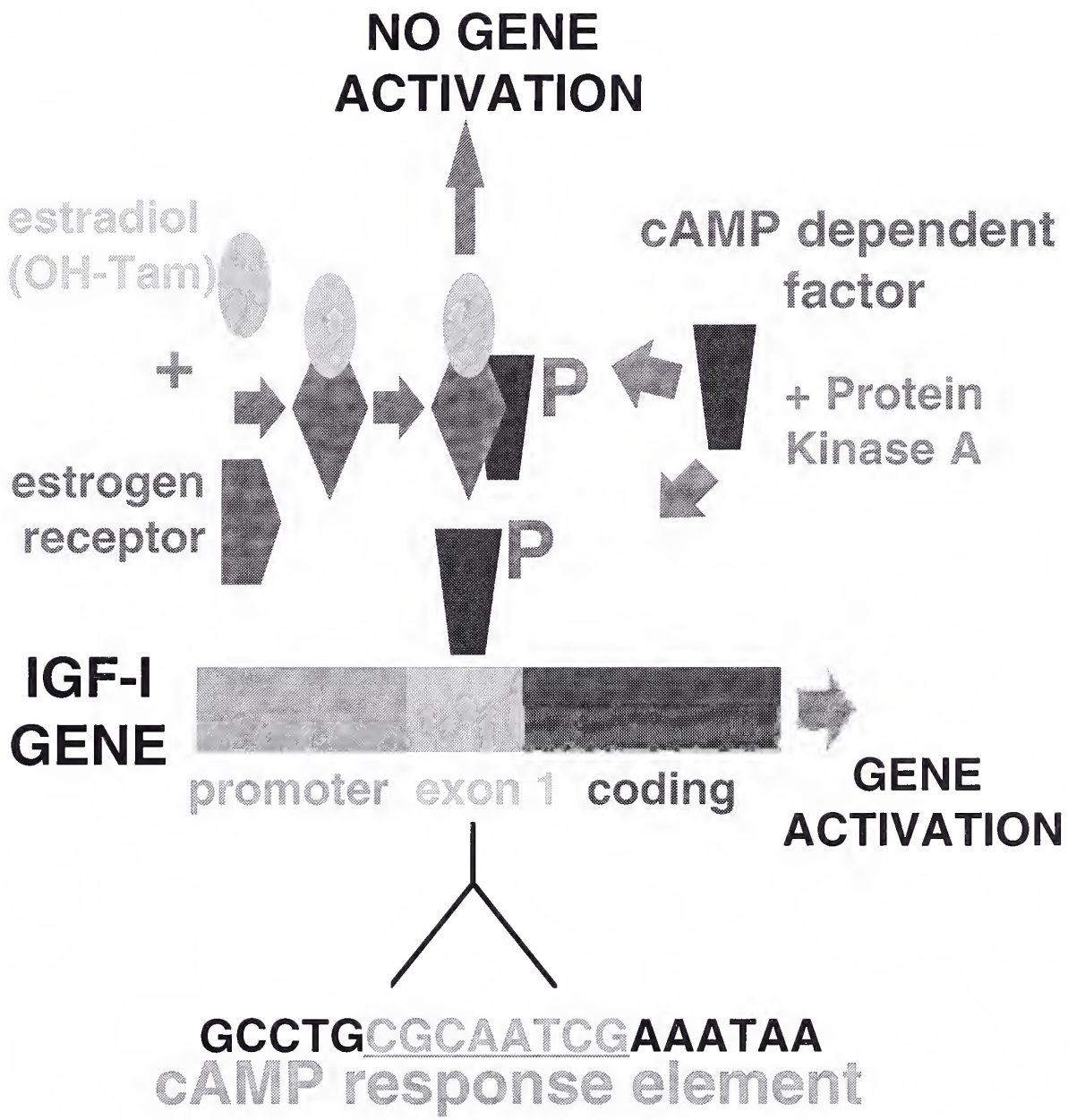

Figure 8. Proposed model for the interaction of 17β-estradiol (and 4-OH-tamoxifen) with cAMP mediators of IGF-I promoter activation. Protein kinase A activation results in enhanced IGF-I promoter transcription. While estrogen (or tamoxifen) alone do not affect IGF-I promoter activity, these steroid hormones suppress PKA activation possibly through protein-protein interactions between ligand bound estrogen receptor and the cAMP activated transcription factor(s), resulting in steric hindrance of binding to the IGF-I cyclic response element.

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