Cloning and Characterization of the Type I Inositol 1,4,5-Trisphosphate Receptor Gene Promoter

REGULATION BY 17β-ESTRADIOL IN OSTEOBLASTS*

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The inositol 1,4,5-trisphosphate (InsP$_3$) receptor is essential for signal Ca$^{2+}$ release from intracellular stores and for capacitative Ca$^{2+}$ entry. We have isolated the promoter and proximal DNA segments of the human type I InsP$_3$ receptor gene. Transcription initiation in human G-292 osteosarcoma and HL-60 promyelocytic leukemia cells was shown to occur predominantly from an adenine residue located 39 base pairs downstream of a consensus TATA box element. Upstream DNA including the TATA box promoted directional transcription of a chloramphenicol acetyltransferase reporter gene when transferred into G-292 cells. A negative regulatory element in the distal promoter and a positive element in the proximal region were identified by deletion mapping and transcription assays. The proximal region enhanced transcription in response to 12-O-tetradecanoylphorbol-13-acetate or serum, but conferred transcriptional repression in response to 1,25-dihydroxyvitamin D$_3$ or 17β-estradiol. The repressive effect of 17β-estradiol was mediated by the nuclear estrogen receptor, as estrogen-dependent transcriptional repression was inhibited by the antiestrogen tamoxifen and the estrogen receptor antagonist ICI 182,780. This is the first study of the type I InsP$_3$ receptor gene promoter, and the results suggest a mechanism by which estrogenic treatment of osteoblasts affects type I InsP$_3$ receptor gene expression, signal transduction, and secretion.

Inositol 1,4,5-trisphosphate (InsP$_3$) mediates the calcium-mobilizing effects of a wide range of hormones, cytokines, and neurotransmitters (1). Activation of G-protein-linked and tyrosine kinase-linked receptors stimulates phosphatidylinositol-specific phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate with the resultant generation of the second messenger molecules, diacylglycerol, and InsP$_3$. InsP$_3$ binds to specific receptors located on endoplasmic reticulum, causing the release of sequestered Ca$^{2+}$ into the cytosol and the stimulation of Ca$^{2+}$-dependent processes including secretion (1–3).

InsP$_3$ receptors have been purified as 250–313-kDa proteins and have been shown to function as tetrameric Ca$^{2+}$ channel complexes whose conductance is regulated by the binding of InsP$_3$ (4, 5). Three distinct, highly homologous (>60%) cDNAs of 8.8–10.7 kb encoding full-length InsP$_3$ receptors have been cloned from rodent, human, and other sources (6–13). Partial sequences of two additional InsP$_3$ receptor types, which are highly homologous to type II, have also been identified (14). Recent studies have shown that individual InsP$_3$ receptor types are variably expressed in different tissues, and that multiple receptor types can be expressed within a given cell and these may assemble as heterotetramers in a single Ca$^{2+}$ conductance channel (15, 16). Furthermore, InsP$_3$-induced calcium release and InsP$_3$ receptor-mediated calcium entry may be variably controlled by different InsP$_3$ receptor subtypes (17, 18).

The expression and function of InsP$_3$ receptors are hormonally regulated. Treatment of promyelocytic HL-60 cells with retinoic acid or 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) stimulates InsP$_3$ receptor gene transcription, increases the expression of functional InsP$_3$-regulated intracellular Ca$^{2+}$ channels, and up-regulates cell secretory capacity (19, 20). In pancreatic islets of fasted and then fed rats and also in insulin-secreting RINm5F cells treated chronically with high glucose, type III InsP$_3$ receptor expression is increased (21). On the other hand, stimulation of neuronal and pancreatoma cells with phosphoinositide-linked hormones causes the rapid down-regulation of the type I InsP$_3$ receptor protein (22, 23). In primary osteoblast and osteoblastic cell lines, type I InsP$_3$ receptor messenger RNA expression is decreased by 17β-estradiol and 1,25(OH)$_2$D$_3$ but increased by phorbol esters and serum (24). These studies suggest that chronic regulators of cell secretory capacity may affect these changes through regulation of InsP$_3$ receptor levels.

As an initial step to help understand how InsP$_3$ receptor gene expression may be regulated under different physiological or pathological situations, we have isolated genomic clones encoding the promoter and six of the initial exons of the human type I InsP$_3$ receptor gene. The major transcription start sites are identified, and it is shown that the type I InsP$_3$ receptor gene promoter functions in human osteoblastic cell lines to regulate transcription of a reporter gene. Furthermore, it is shown that several chronic regulators of osteoblasts secretory activity affect type I InsP$_3$ receptor promoter activity. Significantly, 17β-estradiol via the estrogen receptor down-regulates promoter activity, consistent with its effects on type I InsP$_3$
mRNA levels in primary osteoblasts and osteoblastic osteosarcoma cells (24). These findings are interpreted in terms of the potential mechanisms by which chronic estrogen treatment may affect osteoblast secretory capacity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cells from the G-292 human osteosarcoma cell line (ATCC CRL 1423) were cultured in McCoy’s 5a medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2 in air. Experiments with G-292 cells were conducted in phenol red-free Dulbecco’s medium (Life Technologies, Inc.) and 2% charcoal-stripped fetal bovine serum.

**Library Construction**—Cells from the human promyelocytic leukemia HL-60 cell line (ATCC CCL 240) were cultured and maintained in RPMI 1640 medium with 20% fetal bovine serum as described previously (19).

**Isolation of Human Genomic DNA Clones Containing the Type I InSp3 Receptor Gene Promoter**—A human placental genomic DNA library in bacteriophage λw85 of the Life Technologies, Inc., was screened by plaque hybridization (27) to obtain type I InSp3 receptor gene sequences, the first exon, and part of the first intron was subcloned into the pGEM-11Zf(+) plasmid vector (Promega Corp., Madison, WI), and sequenced to confirm their identities.

**DNA Sequencing**—DNA sequences, the first exon, and part of the first intron were subcloned into the pGEM-T vector (Promega Corp., Madison, WI), and sequenced to confirm their identities.

**Type I InSp3 Receptor Gene Promoter**—The eukaryotic expression vector pCAT-Basic (Promega Corp., Madison, WI), which contains the bacterial chloramphenicol acetyltransferase (CAT) gene but lacks promoter and enhancer sequences, was used as the parent vector in constructing reporter plasmids for the analysis of putative InSp3 promoter reporter and transcriptional regulatory sequences. The 1.5-kb BglII restriction fragment, containing bp 350 through 535 of the type I InSp3 receptor gene, was filled-in with the Klenow fragment of DNA polymerase I, blunt-end ligated into the filled-in, SacI-cut pCAT-Basic vector. Clones with the promoter in the forward orientation in front of the CAT gene were isolated, digested with PstI and Sau3A1, and subjected to unidirectional digestion using exonuclease III followed by mung bean nuclease. This produced a series of constructs containing progressively larger deletions starting from the Sau3A site in the vector multiple cloning site and proceeding into 5′ end of the upstream region of the promoter construct. The resultant DNAs were ligated and transformed competent DH5α E. coli. Recombinant pCAT constructs were purified by Midi Kit procedures (Qiagen), sequenced to identify the extent of the deletion, and used in transient transfection assays. In some experiments, the 0.5-kb SacI restriction fragment including bp −350 through +153 of the type I InSp3 receptor gene was cloned in front of the CAT gene of the pCAT-Basic vector and used in transient transfections.

**Transfection and CAT Expression Assays**—G-292 cells were cultured to ca. 50% confluency in 60-mm2 tissue culture dishes, washed twice with Opti-MEM (Life Technologies, Inc.), and then transfected with a DNA-LipofectAMINE (Life Technologies, Inc.) mixture according to manufacturer’s instructions. Equal molar amounts of the various recombinant pCAT-Basic expression vectors (approximately 3 μg) were transfected along with a fixed amount (1.5 μg) of the pSvβ-galactosidase control vector (Promega Corp.). Cells were incubated for 12–14 h with the DNA-LipofectAMINE mixture, washed twice, and then incubated with phenol red-free Dulbecco’s modified Eagle’s medium containing 2% charcoal-stripped fetal bovine serum. Hormones and other test reagents were then added. After 48–72 h, the cells were harvested, washed, and soluble extracts prepared by repeated freeze-thawing in 0.15 m of 0.25 m Tris, pH 8.0, and used for determination of β-galactosidase and CAT activities. β-Galactosidase activity in 20 μl of each sample was determined spectrophotometrically as described (29) using o-nitrophenyl-β-n-galactopyranoside as substrate. After normalization for β-galactosidase activities, CAT activities in the soluble extracts (usually 20–100 μl) were determined by incubation at 37 °C for 12–18 h with 3 μl of [14C]chloramphenicol (50–60 mCi/mmol, NEN Life Science Products), 5 μl of 5 mg/ml acetyl coenzyme A, and 0.25 m Tris-HCl, pH 8.0, in a volume of 150 μl. The mixtures were phase extracted and subjected to thin layer chromatography. Results were analyzed by autoradiography using Bio-Rad Phosphor Imaging and Molecular Imager Systems, version 1.4.

**Materials—Water-soluble 17β-estradiol, 17α-estradiol, tamoxifen, retinoic acid, and 12-O-tetradecanoylphorbol-13-acetate (TPA)**, were purchased from Sigma. Retinoic acid and TPA were dissolved as stock solutions in dimethyl sulfoxide. 1,25(OH)2D3 was generously provided by Dr. J. Napoli (SUNY, Buffalo). The concentration of 1,25(OH)2D3 was determined by incubation at 37 °C for 12–18 h with 3 μl of [14C]chloramphenicol (50–60 mCi/mmol, NEN Life Science Products), 5 μl of 5 mg/ml acetyl coenzyme A, and 0.25 m Tris-HCl, pH 8.0, in a volume of 150 μl. The mixtures were phase extracted and subjected to thin layer chromatography. Results were analyzed by autoradiography using Bio-Rad Phosphor Imaging and Molecular Imager Systems, version 1.4.

**Cloning and Sequence Analysis of the 5′-Region of the Human Type I InSp3 Receptor Gene**—To study the regulatory elements governing the tissue-specific expression of the type I InSp3 receptor, a human placental genomic DNA library in a 325-mer oligonucleotide, p190a, corresponding to the antisense sequence of bases +109 through +85 of the reported mouse type I InSp3 receptor cDNA (7) (+37 through +13 of the later reported human sequence; Ref. 11) was end-labeled using γ32P-ATP (7,000 Ci/mmol, ICN Biomedical Inc, Costa Mesa, CA) and T4 polynucleotide kinase and purified using Bio-spin 6 columns (Bio-Rad). The labeled primer (50 fmol, 4 × 105cpm/reaction) was allowed to anneal to 5 μg of total cellular RNA derived from either G-292 osteosarcoma or HL-60 promyelocytic cells by incubation for 1 h at 62 °C in Superscript II reverse transcriptase buffer (Life Technologies, Inc.) containing RNasin. The reactions were cooled to 46–48 °C, and then extended for 25 min by addition of dNTPs and Superscript II reverse transcriptase. The mixtures were subjected to RNAase A digestion, phe-nochloroform extraction, and ethanol/sodium acetate precipitation. Samples were analyzed by electrophoresis on denaturing 6% polyacrylamide gels alongside DNA sequencing reactions of the 0.5 kb human type I InSp3 receptor SacI genomic subclone using the same p190a type I InSp3 receptor oligonucleotide as used for the extension.

**Type I InSp3 Receptor Gene Promoter-CAT Reporter Construct**—The eukaryotic expression vector pCAT-Basic (Promega Corp., Madison, WI), which contains the bacterial chloramphenicol acetyltransferase (CAT) gene but lacks promoter and enhancer sequences, was used as the parent vector in constructing reporter plasmids for the analysis of putative InSp3 promoter reporter and transcriptional regulatory sequences. The 1.5-kb BglII restriction fragment, containing bp −1150 through +350 of the type I InSp3 receptor gene, was filled-in with the Klenow fragment of DNA polymerase I, blunt-end ligated into the filled-in, SacI-cut pCAT-Basic vector. Clones with the promoter in the forward orientation in front of the CAT gene were isolated, digested with PstI and Sau3A1, and subjected to unidirectional digestion using exonuclease III followed by mung bean nuclease.

**RESULTS**

Cloning and Sequence Analysis of the 5′-Region of the Human Type I InSp3 Receptor Gene—To study the regulatory elements governing the tissue-specific expression of the type I InSp3 receptor, a human placental genomic DNA library in a 325-mer oligonucleotide, p190a, corresponding to the antisense sequence of bases +109 through +85 of the reported mouse type I InSp3 receptor cDNA (7) (+37 through +13 of the later reported human sequence; Ref. 11) was end-labeled using γ32P-ATP (7,000 Ci/mmol, ICN Biomedical Inc, Costa Mesa, CA) and T4 polynucleotide kinase and purified using Bio-spin 6 columns (Bio-Rad). The labeled primer (50 fmol, 4 × 105cpm/reaction) was allowed to anneal to 5 μg of total cellular RNA derived from either G-292 osteosarcoma or HL-60 promyelocytic cells by incubation for 1 h at 62 °C in Superscript II reverse transcriptase buffer (Life Technologies, Inc.) containing RNasin. The reactions were cooled to 46–48 °C, and then extended for 25 min by addition of dNTPs and Superscript II reverse transcriptase. The mixtures were subjected to RNAase A digestion, phe-nochloroform extraction, and ethanol/sodium acetate precipitation. Samples were analyzed by electrophoresis on denaturing 6% polyacrylamide gels alongside DNA sequencing reactions of the 0.5 kb human type I InSp3 receptor SacI genomic subclone using the same p190a type I InSp3 receptor oligonucleotide as used for the extension.
enzyme mapping, Southern blotting, DNA subcloning, and DNA sequencing (Fig. 1). Three of these clones (λ1-5, λ3-1-2, and λ5-2-1) were selected by their hybridization to a human probe corresponding to bp 489–970 of the type I InsP3 receptor cDNA (numbering is in reference to the homologous mouse sequence) and were shown to overlap as indicated. Three separate exons were found by restriction enzyme digestion and Southern hybridization of these overlapping clones. A fourth independent clone (λ124) encoding two exons was isolated using a human cDNA probe derived from bp 85–513 of the cDNA sequence (mouse numbering). The more 5′ of these exons, denoted exon 3, is at least the third exon in the gene and begins at base 241 of the human cDNA sequence (11). The ATG initiation methionine codon at nucleotides 257–259 is included within this exon. The clone λ5-1-7 was isolated using a human 174-bp probe derived from bp 85–187 (mouse cDNA numbering) of the 5′-untranslated region of the type I InsP3 receptor cDNA. Subclones of this λ5-1-7 DNA, including a 0.5-kb SacI fragment and a 3.2-kb XbaI/HindIII fragment were isolated by their hybridization to the same 174-bp probe. The 1.5-kb BglII internal fragment of the 3.2-kb XbaI/HindIII subclone was sequenced completely (Fig. 2). This BglII fragment contains −1.2 kb of upstream nontranscribed DNA including a consensus TATA box element, the first exon, and part of the first intron of the human type I InsP3 receptor gene.

Identification of the Transcription Start Site of the Human Type I InsP3 Receptor Gene—The transcription start site was determined by primer extension and rapid amplification of cDNA ends (5′-RACE). The antisense primer, p109a, corresponding to bases +109 through +85 of the mouse type I InsP3 receptor cDNA (7) (+37 through +13 of the human type I InsP3R cDNA; Ref. 11) was hybridized to and extended by reverse transcription of RNA from human G-292 osteosarcoma cells and human HL-60 promyelocytic cells. The primer extension (Fig. 3) identified an adenine (A) residue as the predominant transcription start site. This adenine is located 39 residues downstream of a consensus TATA box motif (TATATAA) and is referenced in the sequence as position +1 (Fig. 2). Results from three separate experiments using independently isolated RNAs from G-292 and HL-60 cells indicated the same major transcription start site in both cell types. A minor start site (C), also identified in both cell types, was identified at position −26 relative to the major start site and 14 bp downstream of the TATA box. Interestingly, the 5′-RACE analysis performed at 37 °C consistently yielded products shorter (indicated by asterisks in Fig. 2) than expected from the published human and rat cDNAs or from the observed primer extension products. Inspection of the DNA sequence immediately upstream and encompassing the 5′-RACE terminated sites indicates a conserved GGA-rich region, which may impart an unusual secondary structure on the DNA and prevent full-length extension. Successful primer extension through this region required temperatures of 46–48 °C; extensions at 37 °C were terminated prematurely.

The 5′-Flanking Region of the Type I InsP3 Receptor Gene Functions as a Promoter in Transient Transfection Assays—To determine if the 5′-flanking region of the InsP3 receptor gene could function as a promoter and regulate transcription in target cells, the 0.5-kb SacI genomic fragment (−359 bp through +153 bp) was subcloned into the promoterless pCAT Basic expression vector as described under “Experimental Procedures.” After transient transfection of G-292 osteosarcoma cells with reporter constructs containing the SacI fragment inserted in the forward orientation in front of the CAT gene,
CAT activity averaged 4-fold greater \((n = 3)\) than activity in cells transfected with constructs in the reverse orientation (Fig. 4). In G-292 cells transfected with the forward promoter construct, CAT activity was further increased by treatment with 1 nM phorbol ester (TPA) but decreased in cells treated with either 10 nM \(17\beta\)-estradiol or 10 nM 1,25(OH)\(_2\)D\(_3\). Both \(17\beta\)-estradiol and 1,25(OH)\(_2\)D\(_3\) decreased CAT expression when added together with TPA when compared with TPA alone.

Transcription promoting activity of the 5′-flanking region of the type I InsP\(_3\) receptor gene promoter was also determined by transient transfection of G-292 cells with pCAT-Basic constructs containing the 1.5-kb BglII fragment (−1152 through +350 bp) or 5′-deletions of this fragment. These data (Fig. 5) have been normalized and are reported as percent of activity relative to the −174 bp construct, which consistently exhibited the highest CAT activity. The results summarized in Fig. 5 show that the longer promoter constructs (−1150 bp, −911 bp) had less CAT promoting activity that shorter constructs (−691 bp through −134 bp), and that even shorter constructs (−110 bp through −99 bp) had less CAT promoting activity than the −174 bp promoter construct. Significantly, CAT expression from deletion constructs including just the TATA box, as in the −39 construct, was greater than expression from the pCAT-Basic parental vector, whereas the slightly longer constructs (−54, −134, and −174 bp) that included a consensus NF-κB binding element and a serum response element had greater transcription promoting activity than constructs containing only the TATA box \((p < 0.05,\) paired Student’s \(t\) test).

\(17\beta\)-Estradiol Represses Expression of the Type I InsP\(_3\) Receptor Promoter in G-292 Cells—We have reported that treatment of human G-292 osteosarcoma cells and rat primary calvarial osteoblasts with estrogen decreases steady state expression of type I InsP\(_3\) receptor mRNA \((24)\). That data were consistent with mechanism involving reduced gene transcription. Using the human type I InsP\(_3\) receptor promoter CAT reporter constructs, the regions of the promoter that might confer transcription repression by \(17\beta\)-estradiol were investigated. Following transient transfection of G-292 cells with various deletion constructs of the type I InsP\(_3\) receptor gene promoter-pCAT plasmid, cells were incubated with or without 10 nM \(17\beta\)-estradiol for 48 h and assayed for soluble CAT activity. At top is the autoradiogram of the CAT assay, and below is the quantified results normalized to the activity of the forward construct. Two separate experiments for the untreated cells are shown. The unlabeled, last lane of the autoradiogram is a positive control for CAT activity. The experiments shown are representative of two additional experiments showing similar results.

**Fig. 3.** Primer extension analysis on the 5′ end of the human type I InsP\(_3\) receptor gene. Twenty \(\mu\)g of total RNA from human G-292 osteosarcoma cells and human HL-60 promyelocytes were subjected to primer extension using the p109a antisense oligonucleotide corresponding to bases +109 through +85 of the published mouse type I InsP\(_3\) receptor cDNA \((+118\) through +95 of the genomic DNA). The location of the major extended product is shown by the arrow. The corresponding DNA sequence was determined using the p109a primer and the pGEM11/SacI-0.5-kb subclone identified in Fig. 1. The asterisk indicates the adenine identified as the major start site. The putative TATA box sequence is also shown.

**Fig. 4.** The 5′ region of the human type I InsP\(_3\) receptor gene functions as a promoter. The pCAT-Basic/SacI-0.5-kb plasmid \((3\ \mu\text{g})\) containing the −359 bp through +153 bp fragment of the type I InsP\(_3\) receptor gene inserted in the forward or reverse orientation in front of the bacterial CAT gene was transfected into G-292 osteosarcoma cells as described. Cells were then treated with hormones or TPA as indicated for 48 h and assayed for soluble CAT activity. At top is the autoradiogram of the CAT assay, and below is the quantified results normalized to the activity of the forward construct. Two separate experiments for the untreated cells are shown. The unlabeled, last lane of the autoradiogram is a positive control for CAT activity. The experiments shown are representative of two additional experiments showing similar results.
mimic this response. However, tamoxifen did block the effect of 17β-estradiol. In this experiment, a slight inhibitory effect of 17α-estradiol was seen but this was not reproduced in the other experiments. In a separate series of experiments, the pure estrogen receptor antagonist, ICI 182,780, was used. In these experiments (Fig. 7B), 10 nM 17β-estradiol reduced CAT expression from the −357 bp type I InsP3 receptor promoter construct to 87 ± 2% (mean ± S.E., p < 0.01) of control, whereas CAT expression in cells treated with 17β-estradiol plus 20 nM ICI 182,780 was 122 ± 9%. Although cells treated

FIG. 5. Effects of 5′ deletions of type I InsP3 receptor promoter function. Deletion constructs containing different lengths of the human type I InsP3 receptor gene promoter upstream of the CAT reporter gene in the pCAT-Basic plasmid were transiently transfected into G-292 osteosarcoma cells along with the pSV-β-galactosidase vector. CAT activities are reported relative to the −174 bp construct. Numbers indicate the upstream limit of the promoter sequence in each deletion construct. Locations of consensus regulatory sites are shown at the top. Bars represent means ± S.E. (stippled bar) of three to five separate experiments or means of duplicate experiments (−911 bp, −134 bp, −110 bp, −94 bp, and pCAT-Basic-alone).

FIG. 6. The effects of 17β-estradiol on the function of type I InsP3 receptor promoter deletion constructs. Deletion constructs containing the indicated lengths of the human type I InsP3 receptor gene promoter upstream of the CAT gene in the pCAT-Basic plasmid were transiently transfected into G-292 osteosarcoma cells along with the pSV-β-galactosidase vector. Cells were treated for 48 h with (stippled bars) or without (solid bars) 10 nM 17β-estradiol as indicated and normalized CAT activities determined as described. At top is the autoradiogram of the CAT assay, and below are the quantified results. The experiment is representative of two experiments showing similar results.

with 17β-estradiol plus ICI 182,780 had elevated CAT activity, this was not significantly different from cells treated with ICI 182,780 alone (124 ± 8%). In these series of experiments, 17α-estradiol was again inactive as an inhibitor.

DISCUSSION

The effects of many bone resorptive agents are mediated through their actions on osteoblasts to stimulate the production and secretion of osteoclast-activating factors, including IL-6 (30, 31). Parathyroid hormone, parathyroid hormone-related peptide, bradykinin, prostaglandins, and endothelins induce bone resorption, require osteoblasts for this action, and stimulate IL-6 secretion from osteoblasts (32). These hormones all increase phosphoinositide turnover, InsP3 formation, and calcium mobilization in osteoblasts, suggesting that the InsP3 receptor may be important in osteoblast signal transduction and may be an essential component in the regulation of IL-6 secretion. On the other hand, estrogens have antiosteoporotic effects mediated in part by their effects on osteoblasts to reduce IL-6 secretion (33). The mechanism for this action of estrogen in osteoblasts has been attributed to the repression of IL-6 gene transcription, despite the lack of consensus estrogen response elements in the IL-6 promoter. Indeed, additional experimental evidence suggests that 17β-estradiol affects estrogen receptor-dependent IL-6 gene repression in osteosarcoma cells by direct inhibitory interaction with the activator transcription factors NF-κB and C/EBPβ (34). The present study indicates that an additional mechanism may contribute to the reduced IL-6 secretory capacity of osteoblasts following estrogen treatment, viz. that chronic estrogen treatment reduces the expression of the type I InsP3 receptor gene resulting in diminished osteoblast secretory activity.

In the present investigation, the 5′ region of the human type I InsP3 receptor gene including its putative promoter was cloned and its regulation in osteoblasts by estrogen and other agents is described. Three overlapping genomic clones and two

FIG. 7. The effects of 17β-estradiol, 17α-estradiol, tamoxifen, and ICI 182,780 on type I InsP3 receptor promoter function. The −357 bp construct of the human type I InsP3 receptor gene promoter upstream of the CAT reporter gene in the pCAT-Basic plasmid was transiently transfected into G-292 osteosarcoma cells along with the pSV-β-galactosidase vector. In A, cells were treated for 48 h with or without 1 nM TPA, 10 nM 17β-estradiol, 10 nM 17α-estradiol, 100 nM tamoxifen, or the indicated combination. In B, cells were treated for 48 h with or without 10 nM 17β-estradiol, 20 nM ICI 182,780, or the combination. Extracts were normalized for β-galactosidase activities, and CAT activities were determined as described. The experiments are representative of two (A) and three (B) such experiments. Means ± S.E. for all experiments are reported under “Results.”

The effects of 17β-estradiol, 17α-estradiol, and ICI 182,780 on the human type I InsP3 receptor promoter function.
additional neighboring clones of the human type I InsP$_3$ receptor gene were isolated by hybridization with cDNA probes. Approximately 50 kb of genomic DNA was isolated and determined to encode eight of the promoter proximal exons within the first 10% (through bp 880) of the 10-kb human type I InsP$_3$ receptor cDNA. The relative large size of this portion of the gene suggests that the entire human type I InsP$_3$ receptor gene may be as large as 500 kb. An interesting feature of the promoter proximal region of the gene is that the exon containing the translation start site is preceded by at least two noncoding exons. Examination of the exon-intron boundaries, as illustrated for the first such boundary at bp +246 included in the sequence reported in Fig. 2, showed them all to be classical AGgt...agG sequences. Another interesting feature of the first exon is the repetitive GGAGG(ATT) sequence from +45 through +94. This sequence is found with high homology in several other signal transduction genes including those for c-Myc, c-ErbB2, epidermal growth factor receptor P1, IGF II, and snRNA U1 (NCBI). An unusual RNA secondary structure may be imparted by this sequence since the 5'-RACE analyses performed at 37°C were arrested within this sequence, and the primer extension required temperatures between 46–48°C to traverse through this region.

The identification of the major transcription start site by primer extension locates the initiation site 40 bp downstream from a TATATTA sequence, suggesting that the latter serves as a functional TATA box. Indeed, the transcription reporter constructs showed that inclusion of the DNA through and including the TATA box conferred significantly greater transcription promoting activity than the promoterless CAT parental vector. This TATATTA stretch is surrounded by GC-rich sequences, also typical of consensus TATA elements. With this orientation, examination of the upstream sequence reveals several consensus transcription regulatory sequences that potentially could control type I InsP$_3$ receptor gene expression in a cell-specific and hormonally regulated fashion. Included among these are recognition sequences for AP-1, serum response element (SRE)-binding factors, NF-xB, C/EBP, and nuclear receptors (see Fig. 2).

Paired AP-1 sequences are identified at −334 and −261 in the upstream region. AP-1 sites can act as positive regulatory elements for phorbol ester-induced transcription through activation of c-Jun/c-Fos signaling (35). In G-292 cells transfected with the −357 bp type I InsP$_3$ receptor promoter pCAT construct, TPA stimulated 3 times more CAT expression compared with untreated cells. An SRE-like sequence is found at −159 and maximal CAT activity was stimulated by 2% charcoal-stripped serum with the −174 construct (Fig. 5). However, the −134 construct, which lacks the consensus SRE, still exhibited CAT activity comparable to the −174 construct. Consensus regulatory sequences for NF-xB at positions −274 and −52, as well as for C/EBP at −382, are also identified, although a role for these elements in regulating transcription in osteoblasts is not immediately apparent.

Paired steroid receptor half-sites separated by a 4-base spacer are found at −746. Previous studies have demonstrated that retinoic acid induces InsP$_3$ receptor mRNA expression and increases the rate of type I InsP$_3$ receptor gene transcription in HL-60 cells (19). The nuclear receptor half-sites found at −746 (CCGTCAgactTGAGGT) have a 4-base spacer and are in an inverted repeat orientation (IR-4); however, the IR-4 configuration of the hormone response half-site is not an optimal arrangement for retinoids or other nuclear hormones (36). Interestingly, consensus vitamin D-responsive elements or estrogen-responsive elements were not identified, even though previous studies have shown that levels of type I InsP$_3$ receptor mRNA are positively regulated by vitamin D in HL-60 cells (20) and negatively regulated by estrogens and vitamin D in primary osteoblasts and osteosarcoma cell lines (24).

The effects of 17β-estradiol on type I InsP$_3$ receptor promoter activity were investigated in greater detail. Our previous studies of human G-292 osteosarcoma cells and rat calvarial osteoblasts have indicated that estrogens decrease type I InsP$_3$ receptor mRNA levels without an effect on mRNA stability, suggesting an inhibitory effect on the rate of gene transcription (24). The data presented here are consistent with this contention. In G-292 cells transfected with InsP$_3$ receptor promoter constructs, exposure to 10 nM 17β-estradiol inhibited CAT activity up to 40%. CAT repression by 17β-estradiol was most prominent with constructs that also had the greatest basal activity in the 2% charcoal-stripped serum, i.e. the −326 and −174 constructs. Of additional interest is the fact that maximal promoter repression by 17β-estradiol averaged 40%, whereas reduction of the type I InsP$_3$ receptor mRNA is typically about 75% in similarly treated cells (24).

The lack of effect of 17α-estradiol and the observed antagonism of the 17β-estradiol response by tamoxifen and ICI 182,780 strongly support the contention that the transcriptional repression observed with 17β-estradiol is mediated by the estrogen receptor. However, the lack of consensus estrogen response elements in the type I InsP$_3$ receptor promoter sequence suggests a non-classical action of the estrogen receptor on gene expression (37, 38). Stein and Yang (34) observed similar estrogen receptor-dependent inhibition of IL-6 promoter activity in human U2-OS osteoblastic cells and presented evidence that the inhibition is not mediated through consensus estrogen response elements in the promoter but instead involves estrogen receptor binding to and repression of the stimulatory transcription factors NF-xB and C/EBPβ. Several observations reported here are consistent with a similar mechanism acting at the InsP$_3$ receptor promoter. First, consensus response elements for the estrogen receptor are not found in the isolated InsP$_3$ receptor promoter DNA. Second, transcription repression by 17β-estradiol was observed with the proximal promoter constructs, the same constructs that show maximal stimulation with serum. Furthermore, consensus binding sites for NF-xB, C/EBPβ, and serum response factor are found in the proximal promoter. Thus, the −350 bp and −174 bp promoter constructs had greatest activity in the presence of the 2% charcoal-stripped serum, and these were the constructs that showed the most significant repression with 17β-estradiol. The same parallel was observed in U2-OS osteoblastic cells between the regions of the IL-6 promoter necessary for transcription enhancement by either TPA or IL-1β and the ability of 17β-estradiol to repress transcription. In the case of the IL-6 promoter, experimental observations supported a mechanism by which the agonist-activated estrogen receptor was not required to be bound to the promoter to depress transcription, but rather the receptor sequestered and transrepressed the activator transcription factors, NF-xB and C/EBPβ. Studies are directed toward determining whether a similar mechanism is operative at the type I InsP$_3$ receptor promoter in osteoblasts and whether this estrogen-dependent down-regulation of the type I InsP$_3$ receptor plays a role in the blunted secretion of osteoclast activating cytokines from estrogen-treated osteoblasts.

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