Identification of the cAMP Response Element That Controls Transcriptional Activation of the Insulin-like Growth Factor-I Gene by Prostaglandin E₂ in Osteoblasts*

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Insulin-like growth factor-I (IGF-I), a multifunctional growth factor, plays a key role in skeletal growth and can enhance bone cell replication and differentiation. We previously showed that prostaglandin E₂ (PGE₂) and other agents that increase cAMP activated IGF-I gene transcription in primary rat osteoblast cultures through promoter 1 (P1), the major IGF-I promoter, and found that transcriptional induction was mediated by protein kinase A. We now have identified a short segment of P1 that is essential for full hormonal regulation and have characterized inducible DNA-protein interactions involving this site. Transient transfections of IGF-I P1 reporter genes into primary rat osteoblasts showed that the 328-base pair untranslated region of exon 1 was required for a full 5.3-fold response to PGE₂ mutation in a previously footprinted site, HS3D (base pairs +193 to +215), reduced induction by 75%. PGE₂ stimulated nuclear protein binding to HS3D. Binding, as determined by gel mobility shift assay, was not seen in nuclear extracts from untreated osteoblast cultures, was detected within 2 h of PGE₂ treatment, and was maximal by 4 h. This DNA-protein interaction was not observed in cytoplasmic extracts from PGE₂-treated cultures, indicating nuclear localization of the protein kinase A-activated factor(s). Activation of this factor was not blocked by cycloheximide (Chx), and Chx did not impair stimulation of IGF-I gene expression by PGE₂. In contrast, binding to a consensus CAMP response element (CRE; 5'-TGACGTCA-3') from the rat somatostatin gene was not modulated by PGE₂ or Chx. Competition gel mobility shift analysis using mutated DNA probes identified 5'-CGCAATCG-3' as the minimal sequence needed for inducible binding. All modified IGF-I P1 promoter-reporter genes with mutations within this CRE sequence also showed a diminished functional response to PGE₂. These results identify the CRE within the 5'-untranslated region of IGF-I exon 1 that is required for hormonal activation of IGF-I gene transcription by cAMP in osteoblasts.

The anabolic role of insulin-like growth factor-I (IGF-I) in skeletal growth is well documented (1–3), and until recent years it was believed that much if not all of the IGF-I in the skeleton arrived through the circulation following production by the liver. However, we now know that osteoblasts synthesize IGF-I and that its expression is stimulated by parathyroid hormone (PTH) and by locally produced prostaglandin E₂ (PGE₂) (4, 5). Both of these agents influence bone remodeling, both elevate intracellular levels of cAMP in osteoblasts, and each either directly or indirectly influences osteoblast and osteoclast activity, possibly in part through stimulatory effects on IGF-I expression (6–13). Consequently, locally produced IGF-I is likely to be important in regulating the activity of skeletal cells. Earlier we observed that IGF-I functions as a coupling factor through its ability to stimulate collagen synthesis following intermittent exposure to PTH (10). Therefore, IGF-I is now believed to serve as a key factor in skeletal growth, integrity, and bone remodeling. This central role of IGF-I in skeletal health has prompted us to examine the molecular events involved in cAMP-dependent regulation of IGF-I expression in bone cells. PGE₂ was chosen as the prototypical cAMP-inducing agent because 1) it is a natural product of arachidonic acid metabolism; 2) PGE₂ is made by osteoblasts in response to PTH, various growth factors, or lymphokines; and 3) it may function as a signal-transducing agent in mechanically strained skeletal tissue (14–19).

Although the rat IGF-I gene contains two promoters (promoter 1, P1, is 5’ to exon 1, and P2 is 5’ to exon 2 (20)), P1 functions selectively in cultures of primary fetal rat osteoblasts to control basal and PGE₂-stimulated IGF-I expression (21). Agents that elevate intracellular cAMP concentrations increase transcription of the IGF-I gene in these cells (21, 22). While the response to PTH and to PGE₂ in bone cells consists of stimulation of protein kinases A (PKA) and C and the mobilization of calcium (23–25), three pieces of evidence demonstrate the involvement of PKA in IGF-I gene activation. First, all hormones and chemical agents tested to date that significantly elevate cAMP levels in osteoblasts also increase IGF-I gene expression (26). Second, cotransfection of osteoblasts with an IGF-I P1-luciferase reporter gene and an expression plasmid for the catalytic subunit of PKA produces a constitutively high level of promoter activity that is not stimulated further by PGE₂ treatment (27). Finally, cotransfection of an IGF-I P1-luciferase reporter gene with an expression plasmid for a mu-

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tant regulatory subunit of PKA that is unresponsive to CAMP completely blocks the induction of IGF-I promoter function by PGE$_2$ (27).

In previous studies, we mapped a functional CAMP response element (CRE) to the 5′-untranslated region (UTR) of exon 1 within a segment (+196 to +328) that lacks consensus CRE or AP-2 binding sites, features often associated with conventional CAMP-responsive promoters (28–31). We now have identified a short region within this part of the 5′-UTR that is essential for full hormonal responsiveness and have characterized inducible DNA-protein interactions involving this site. Transient transfections of IGF-I P1-reporter genes showed that an intact pre-

CRE in IGF-I Promoter 1

EXPERIMENTAL PROCEDURES

Cell Cultures—Primary osteoblast-enriched cell cultures were prepared from the parietal bones of 22-day-old Sprague-Dawley rat fetuses (Charles River Laboratories, Raleigh, NC). Animals were housed and euthanized by methods approved by the Yale University Animal Care and Use Committee. Cranial sutures were eliminated during dissection, and the bones were digested with collagenase for five sequential 20-min intervals. The cell population released during the last three digestions and the bones were digested with collagenase for five sequential 20-min intervals. The cell population released during the last three digestions exhibited biochemical characteristics associated with differentiated osteoblasts, including PTH receptors, type I collagen synthesis, and alkaline phosphatase activity (20). All plasmids were propagated in strain DH5$_{\alpha}$ (20). All plasmids were propagated in strain DH5$_{\alpha}$ (20). All plasmids were propagated in strain DH5$_{\alpha}$ (20). All plasmids were propagated in strain DH5$_{\alpha}$ (20).

Plasmids—Rat IGF-I cDNA was kindly provided by Dr. Liam Murphy. Rat IGF-I promoter 1 constructs have been described previously (20). All plasmids were propagated in Escherichia coli strain DH5$_{\alpha}$ with ampicillin selection and were prepared using a Qiagen® Plasmid Kit (Qiagen Inc., Chatsworth, CA) and the manufacturer’s recommended protocol. Mutant constructs were produced by PCR, and the mutations were verified by DNA sequence analysis.

RNA Isolation and Analysis— Cultures of 9.6 cm$^2$ were solubilized in buffer consisting of 5 mM guanidine monothiocyanate, 25 mM trisodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol, followed by extraction with phenol/chloroform/isoamyl alcohol (75:25:1) in the presence of 0.2 mM sodium acetate (36). Total RNA was precipitated, ethanol-washed, dried, and resuspended in diethylpyrocarbonate treated water, and concentration and purity were determined by absorbance at 260 and 280 nm. Ten micrograms of RNA was denatured with 2.2 mM formaldehyde, 12.5 mM formamide at 65°C for 15 min and fractionated on a 1.5% agarose, 2.2 M formaldehyde gel. Co-electrophoresed RNA standards were included to assess integrity of RNA and to standardize sample loading. After blotting, RNA was cross-linked onto charged modified nylon (GeneScreen Plus™, DuPont, NEN). A restriction fragment containing the rat IGF-I cDNA clone was purified from an agarose gel and labeled with [α-32P]deoxythymidine triphosphate and [α-35S]thymidine triphosphate by random hexanucleotide primed second strand synthesis (37). Northern blots were hybridized with [32P]IGF-I cDNA, and the filters were washed under conditions of progressively increasing stringency. Finally, filters were rinsed with 0.2 × SSC (20 × SSC contains 3 mM NaCl, 0.3 mM trisodium citrate, pH 7.0) and 0.1% sodium dodecyl sulfate for 1 h at 55°C. The bound radioactive material was visualized by autoradiography using Amersham Hyperfilm® and a DuPont Cronex intensifying screen. Filters were eluted of specifically bound 32P-labeled cDNA by washing in deionized water for 5 min at 100°C before probing with 18 S antisense ribosomal RNA (Ambion, Austin, TX).

Transfection Studies—IGF-I promoter 1–luciferase reporter plasmids (1.5 μg/9.6-cm$^2$ culture well) were co-transfected with a vector carrying the β-galactosidase gene under SV40 promoter control (1 μg/culture well; pSV-β-Galactosidase Control Vector, Promega Corp.) to normalize for transfection efficiency. Cultures at 50% confluence (48 h) were rinsed in serum-free medium and exposed to plasmids in the presence of Lipofectin™ (Life Technologies, Inc.) for 3 h. The solution was then replaced with growth medium containing 5% fetal bovine serum, and the cultures were grown to confluence (48 h). Confluent cultures were rinsed with serum-free medium and treated for 6 h with vehicle (ethanol diluted 1:1000 or greater), or PGE$_2$ (Sigma). At the end of the treatment interval, the medium was aspirated, cultures were rinsed with phosphate-buffered saline and then lysed in 100 μL of 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, 1% Triton X-100 (cell lysis buffer, Promega Corp.). Nuclei were pelleted at 12,000 × g for 5 min, and the supernatants were stored at −75°C until assay. Commercial kits were used to measure luciferase (Promega Corp.) and β-galactosidase (Tropix, Bedford, MA). Protein was determined by the Bradford assay (38).

Nuclear Protein Extracts—Confluent osteoblast cultures were serum-deprived for 20 h. The cultures then were rinsed with serum-free medium and exposed to vehicle or 1 μM PGE$_2$ for 1–4 h. Medium was aspirated, and cultures were rinsed twice with phosphate-buffered saline at 4°C; all subsequent steps were performed on ice or at 4°C. Cells were harvested with a cell scraper and gently pelleted, and the pellets were washed with PBS. Nuclear extracts were prepared by the method of Lee et al. with minor modifications (27, 39). Cells were lysed in hypotonic buffer (10 mM HEPES (pH 7.4), 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM dithiothreitol (with 3 μL phenylmethylsulfonyl fluoride), 10 mM sodium orthovanadate, 10 mM sodium fluoride, 0.4 μM microcin CT (Promega Corp.), and 1% Triton X-100). Nuclei were pelleted, and the supernatant (cytoplasmic extract) was collected and dialyzed as described below for nuclear extracts. Nuclei were resuspended in hypotonic buffer containing 0.4 mM NaCl, 0.2 mM Na$_2$EDTA, 25% glycerol, and the phosphate and protease inhibitors indicated above. Soluble proteins released by a 30-min incubation at 4°C were collected by centrifugation at 12,000 × g for 5 min, and the supernatant was dialyzed for 2 h against 2000 volumes of buffer (20 mM HEPES, pH 7.4, 0.1 mM KCl, 0.1 mM Na$_2$EDTA, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 20% glycerol), containing the protease inhibitors indicated above.

Gel Mobility Shift Assay—Gel mobility shift experiments followed previously published methods (40–42). Briefly, radiolabeled double-stranded probes were prepared by annealing complementary digoxigenin-labeled oligonucleotides, followed by fill-in of single-stranded overlaps with dCTP, dGTP, dATP, and [α-32P]dATP, using the Klenow fragment of DNA polymerase I. Twenty micromolars of nuclear extract or 50 μg of cytoplasmic proteins were preincubated for 20 min on ice with 2 μg of poly(dI-dC) with or without unlabeled specific or nonspecific competitor DNAs in 60 mM KCl, 25 mM HEPES, pH 7.6, 7.5% glycerol, 0.1 mM EDTA, 5 mM dithiothreitol, and 0.025% bovine serum albumin. After the addition of 5 × 10$^{-9}$ M of DNA probe (0.1–2 ng) for 30 min on ice, samples were applied to a 4–20% non-denaturing polyacrylamide gradient gel (Novex, San Diego, CA) that had been pre-electrophoresed for 30 min at 12.5 V/cm at 25°C in 45 mM Tris, 45 mM boric acid, 1 mM EDTA. Electrophoresis proceeded for 2.5 h under identical conditions. The dried gels were exposed to X-ray film at −80°C with intensifying screens.

2 T. McCarthy and M. Centrella, unpublished results.
The nucleotide sequences of the sense strand of oligonucleotides used in the gel mobility shift assays were as follows: HS3D, 5'-TCCAGAGCAGATAGACCTGCGCAATCGAAA-3'; Oct 1, 5'-TTTATGAGATCATGCAAATGGACGTACGAAA-3'; rat somatostatin CRE, 5'-TCCCTCAGCAGCTCAGAAGACAGAGTTTAA-3'.

Statistical Analysis—When statistical analysis was conducted, data were assessed by one-way analysis of variance, using Kruskal-Wallis or Bonferroni methods for post hoc analysis.

RESULTS

Our previous studies showed that transcription of the major promoter of the rat IGF-I gene, promoter 1 (20, 43-45), was activated by treatment of osteoblast cultures with PGE$_2$ (21), and transient transfection experiments indicated that a portion of the 5'-UTR of exon 1 was required for full hormonal responsiveness (27). Additional studies demonstrated that PGE$_2$-stimulated IGF-I gene expression by a classical cAMP-mediated pathway that required activation of PKA, since IGF-I promoter function could be induced by cotransfection with the catalytic subunit of PKA, and could be blocked by a dominant negative mutant regulatory subunit (27). A single DNase I footprint site was identified within this hormonally responsive segment of exon 1 at a site termed HS3D (27). The current experiments were designed to test the hypothesis that HS3D mediated PGE$_2$-activated IGF-I gene transcription.

As seen in Fig. 1, and in agreement with previous results (27), a 6-h incubation of osteoblast cultures with 1 $\mu$M PGE$_2$ stimulated a 5.3-fold increase in luciferase activity regulated by a transiently transfected recombinant plasmid containing a 2039-nucleotide IGF-I promoter 1 fragment that included the entire 328-nucleotide 5'-UTR of exon 1 (IGF1711b/Luc). As previously reported (27), deletion of exon 1 to position +195 (IGF1711c/Luc) caused a substantial drop in hormonal activation of luciferase. We then examined specific substitution mutations within HS3D (HS3Dmut(AAA), HS3D LSM1, and HS3D LSM2; the nucleotide substitutions in these mutant constructs indicated in Fig. 1 are underlined and in boldface type). Each mutant construct exhibited a diminished response to PGE$_2$ treatment, leading to a decline in inducible luciferase activity that was equivalent to the level observed with plasmid IGF1711c/Luc (Fig. 1). By contrast, nucleotide substitutions in another DNase I footprint site, HS3C (+147 to +169), which was detected with rat liver nuclear extracts but not osteoblast extracts and was not observed with cytoplasmic proteins (Fig. 1), had little effect on regulation of IGF-I promoter function by PGE$_2$.

We next employed gel mobility shift studies to examine the interaction of a $^{32}$P-labeled double-stranded HS3D DNA probe with proteins from osteoblast cultures. Figs. 2 and 3 show time course experiments. PGE$_2$ treatment led to inducible binding of nuclear proteins to the HS3D probe. No binding was seen in extracts from vehicle-treated cells. A strong gel shift that migrated as a closely spaced doublet on a 4–20% gradient gel was observed after 2–4 h of incubation with PGE$_2$ and occasionally could be detected within 1 h of PGE$_2$ treatment. The DNA-protein interaction stimulated by PGE$_2$ was confined to nuclear extracts and was not observed with cytoplasmic proteins (Fig. 3), indicating that a PGE$_2$-activated signaling pathway did not stimulate nuclear translocation of a previously activated cyto-
for purines and pyrimidines for pyrimidines in consecutive blocks of four bases (Fig. 5A). Results using these competitor DNAs are presented in Fig. 5 and show that all but two mutants (M6 and M7) inhibited binding of the [32P]HS3D probe when tested at a 200-fold molar excess. In addition, a double-stranded oligonucleotide containing the AAA mutation that lacked inducible promoter function (Fig. 1) also did not compete for binding to the [32P]HS3D probe and neither a labeled AAA nor M6 oligomer was able to bind osteoblast nuclear proteins (data not shown). Taken together, these data define the minimal binding sequence as 5'-CGCAATCG-3', located at nucleotides +202 to +209 of exon 1 (20, 27), although additional flanking DNA sequences could potentially modulate DNA-protein interactions at this site. Since all IGF-I P1-luciferase reporter genes with mutations within this eight-nucleotide DNA binding core sequence showed a diminished response to PGE_2 (Fig. 1), these results additionally demonstrate the functional importance of this inducible DNA-protein interaction.

Our previous studies showed that induction of IGF-I gene expression by PTH in osteoblast cultures was not blocked by the protein synthesis inhibitor, cycloheximide (Chx) (4). As shown in Fig. 6A, PGE_2 treatment also stimulated accumulation of IGF-I mRNA, even in the presence of 2 μM Chx, a concentration that inhibited >90% of ongoing protein synthesis, as measured by incorporation of [3H]proline (~Chx, 7.2 ± 0.1 x 10⁻³ cpm versus +Chx, 0.71 ± 0.05 x 10⁻³ cpm). However, at 24 h Chx caused a small increase in basal steady-state IGF-I transcripts and modestly reduced the stimulatory effect of PGE_2. Pretreatment of osteoblast cultures with Chx also did not prevent the PGE_2-induced gel shift of the [32P]-labeled HS3D probe (Fig. 6B). These results indicate that PGE_2-regulated DNA-protein interactions at HS3D and PGE_2-stimulated IGF-I gene transcription represent primary hormonal responses that do not require ongoing protein synthesis and suggest that PGE_2 treatment modifies a preexisting DNA-binding protein(s), leading to an increase in its affinity for HS3D.
Our discovery that the calcitropic hormone PTH stimulates IGF-I gene expression in primary fetal rat osteoblasts and that IGF-I serves as a coupling factor for bone remodeling (4, 10) has motivated an investigation into the mechanism(s) regulating IGF-I gene activation in these cells. Initially, a PKA-dependent pathway was implicated, since all agents tested that significantly elevated cAMP levels in osteoblasts, including PGE_2, also diminished responsiveness to cAMP. These results define the minimal binding site as 5'-CGCAATCG-3'. Arrows point to the DNA-protein complexes. Lane 18 shows results of a competition experiment with an unlabeled consensus CRE oligonucleotide used at a 200-fold molar excess. Autoradiographic exposure was for 14 h at -80 °C with intensifying screens. The experiments were performed twice with identical results.

**DISCUSSION**

We now have identified the DNA sequence required for stimulation of IGF-I promoter function by cAMP (PGE_2). Point mutations in the previously footprinted HS3D site in the context of full-length promoter 1 diminished PGE_2-induced luciferase expression to a level identical to that found with deletion of nucleotides +196 to +328 of the 5'-UTR of exon 1. Moreover, mutation of another site, HS3C (+147 to +169), which is not footprinted by osteoblast nuclear proteins (27), had no effect on PGE_2-regulated promoter function. PGE_2 treatment also induced the binding of osteoblast nuclear proteins to HS3D, and neither this hormonal response nor activation of IGF-I gene expression by PGE_2 was blocked by the protein synthesis inhibitor, cycloheximide. Taken together, these results indicate that PGE_2-activated binding of osteoblast nuclear proteins to HS3D initiates a primary signaling pathway that stimulates IGF-I gene transcription through promoter 1.

**Fig. 5. Identification of the minimal DNA sequence mediating PGE_2-induced nuclear protein binding to the HS3D site.** A series of mutant oligonucleotides (mutations underlined) shown in panel A were used in the competition gel mobility shift assay at a 200-fold molar excess with nuclear protein extracts isolated from osteoblast-enriched cultures following a 4-h treatment with 1 μM PGE_2. Panel B illustrates representative autoradiographs of competition gel mobility shift assays using unlabeled wild-type (WT) HS3D and mutant 1 through mutant 8 (M1–M8) HS3D oligonucleotides. These results define the minimal binding site as 5'-CGCAATCG-3'. Arrows point to the DNA-protein complexes. Lane 18 shows results of a competition experiment with an unlabeled consensus CRE oligonucleotide used at a 200-fold molar excess. Autoradiographic exposure was for 14 h at -80 °C with intensifying screens. The experiments were performed twice with identical results.

**Fig. 6. Ongoing protein synthesis is not needed for activation of IGF-I gene expression by PGE_2, or inducible binding to HS3D.** Panel A, Northern blot of IGF-I mRNA using total RNA isolated from osteoblast-enriched cultures treated with ethanol vehicle (C) or 1 μM PGE_2 (P) for 6 or 24 h, in the absence (-) or presence (+) of 2 μM Chx. Total RNA (10 μg) was run on a 1.5% agarose, 2 M formaldehyde gel, blotted onto charge-modified nylon, and probed with 32P-labeled IGF-I cDNA, or 32P-18 S antisense rRNA (as described under "Experimental Procedures"). Panel B, gel mobility shift experiments were performed as described under "Experimental Procedures" with nuclear extract from osteoblast-enriched cultures treated for 4 h with 1 μM PGE_2 in the absence or presence of 2 μM Chx. Nuclear protein binding to 32P-labeled HS3D probe is shown in lanes 1–4. Nuclear protein binding to 32P-labeled rat somatostatin CRE probe is shown in lanes 5–8. In parallel cultures, Chx caused a >90% inhibition of protein synthesis. Autoradiographic exposures were for 36 h (lanes 1–4) or 16 h (lanes 5–8) at -80 °C with intensifying screens. The experiments were repeated twice with identical results.
flanking DNA are highly conserved in IGF-I genes from seven different vertebrate species (including human, pig, chicken, Xenopus, coho salmon, and rainbow trout) (47–52), further indicating the possible regulatory importance of the HS3D region of exon 1. While primary human osteoblasts also respond to cAMP with enhanced IGF-I gene expression (53), it remains to be shown if cAMP stimulates IGF-I expression or whether hormone treatment induces nuclear protein binding to HS3D in other species.

Since the initial characterization of the CRE and the CREB/ATF family of transcriptional regulatory proteins, an array of nucleotide sequences have been implicated in modulating gene expression through cAMP-activated signaling pathways (29, 54–57). The consensus CRE (5'-TGACGTCA-3') was recognized following comparison of DNA sequences from several cAMP-inducible genes (54). However, a growing list of diverse nucleotide sequences have been identified as putative CREs for various genes in a variety of cellular contexts. Greater than 60 CRE entries are present in the Wisconsin Sequence Analysis Package (GCG DNA sequence data base program), and a nucleotide sequence comparison indicates similarities between the rat IGF-I CRE we now describe and the rat osteocalcin CRE. Experiments are now in progress to determine if cAMP stimulates IGF-I expression or osteocalcin gene transcription in osteoblasts.

In summary, we have identified a mechanism through which cAMP stimulates IGF-I gene transcription in osteoblasts and have defined the nucleotide sequences responsible for mediating hormonally activated transcription through inducible nuclear protein binding. Further efforts will focus on characterizing the proteins that bind to this site and discerning the mechanisms of their regulation and action.

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