CCAAT/Enhancer-binding Protein δ Is a Critical Regulator of Insulin-like Growth Factor-I Gene Transcription in Osteoblasts*

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Insulin-like growth factor-I (IGF-I) plays a major role in promoting skeletal growth by stimulating bone cell replication and differentiation. Prostaglandin E₂ and other agents that induce cAMP production enhance IGF-I gene transcription in cultured rat osteoblasts through a DNA element termed HS3D, located in the proximal part of the major rat IGF-I promoter. We previously determined that CCAAT/enhancer-binding protein δ (C/EBPδ) is the key cAMP-stimulated regulator of IGF-I transcription in these cells and showed that it transactivates the rat IGF-I promoter through the HS3D site. We now have defined the physical-chemical properties and functional consequences of the interactions between C/EBPδ and HS3D. C/EBPδ, expressed in COS-7 cells or purified as a recombinant protein from Escherichia coli, bound to HS3D with an affinity at least equivalent to that of the albumin D-site, a known high affinity C/EBP binding sequence, and both DNA elements competed equally for C/EBPδ. C/EBPδ bound to HS3D as a dimer, with protein-DNA contact points located on guanine residues on both DNA strands within and just adjacent to the core C/EBP half-site, GCAAT, as determined by methylation interference footprinting. C/EBPδ also formed protein-protein dimers in the absence of interactions with its DNA binding site, as indicated by results of glutaraldehyde cross-linking studies. As established by competition gel-mobility shift experiments, the conserved HS3D sequence from rat, human, and chicken also bound C/EBPδ with similar affinity. We also found that prostaglandin E₂-induced expression of reporter genes containing human IGF-I promoter 1 or four tandem copies of the human HS3D element fused to a minimal promoter and show that these effects were enhanced by a co-transfected C/EBPδ expression plasmid. Taken together, our results provide evidence that C/EBPδ is a critical activator of IGF-I gene transcription in osteoblasts and potentially in other cell types and species.

Insulin-like growth factor-I (IGF-I), a conserved 70-residue secreted protein, plays a fundamental role in regulating somatic growth in mammals and other vertebrate species (1, 2). IGF-I is synthesized by many cells including osteoblasts (1, 2) and can act as a growth and differentiation factor within the skeleton as well as in other tissues (2–4). Production of IGF-I by skeletal cells is controlled by local and systemic agents, including hormones (5–10). Both parathyroid hormone and prostaglandin E₂ (PGE₂) stimulate IGF-I synthesis in cultured osteoblasts by enhancing IGF-I gene expression (11, 12) through mechanisms that are secondary to hormonal induction of cAMP accumulation (5, 7, 11). Previous studies have shown that PGE₂ stimulates IGF-I gene transcription in osteoblasts by activating promoter 1, the major IGF-I promoter in bone (12–14) and in most other tissues (15). We have found that induction of IGF-I transcription by PGE₂ is part of a primary hormonal response that does not require ongoing protein synthesis (16), but like other cAMP-activated pathways, does require the catalytic subunit of cAMP-dependent protein kinase (13). We recently mapped a functional cAMP response element to the 5’-untranslated region of rat IGF-I exon 1 within a previously footprinted site termed HS3D (16) and identified CCAAT/enhancer-binding protein δ (C/EBPδ) as the principal cAMP-activated transcription factor in osteoblasts that binds to and transactivates IGF-I promoter 1 through the HS3D site (17).

The C/EBP family comprises a diverse group of transcriptional regulators with actions on tissue development and regeneration, inflammation, and intermediary metabolism (18). These proteins are members of the basic leucine zipper family of transcription factors (18, 19) and share strong amino acid similarity in their COOH-domains, which contain motifs responsible for protein dimerization and DNA binding (18). The first C/EBP proteins to be characterized, C/EBPα and C/EBPβ (20–22), function as transcriptional activators and play major roles in adipocyte differentiation and in regulating gene expression in the liver and other tissues (18, 23–26). C/EBPδ also has been implicated in the control of adipogenesis and in mediating the acute phase response to inflammatory stimuli (18, 23, 24). Its potential role in controlling hormone-activated IGF-I synthesis in bone cells had not been described until our recent report (17).

The current experiments were designed to assess interactions between C/EBPδ and the HS3D DNA element of the major IGF-I promoter from both physical-chemical and functional perspectives. We find that C/EBPδ binds to the HS3D site from the rat IGF-I gene with an affinity equivalent to that of a known high affinity C/EBP element from the rat albumin promoter (21, 27) and that, like C/EBPα and C/EBPδ (21, 22, 28), it can form protein-protein dimers in the absence of DNA. C/EBPδ also binds to HS3D sites from the human and chicken IGF-I genes with high affinity and functions as a HS3D-de-
pLodent cAMP-inducible transcription factor for the major human C/EBP family. Together, taken together, our results provide evidence that C/EBPβ is a critical regulator of IGF-I gene transcription in osteoblasts and potentially in other cell types and species.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Primary osteoblast-enriched cell cultures were prepared from dissected and collagenase-digested parietal bones of 22-day-old Sprague-Dawley rat fetuses, as described previously (12, 29). Cells from the last 3 digestions were pooled and plated at 4800/cm2 in Dulbecco's modified Eagle's medium containing 20 mM HEPES, pH 7.2, 0.1 mM magnesium ascorbic acid, 100 units/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies, Inc.), and 10% fetal bovine serum (Sigma). COS-7 cells (ATCC CRL-1651) were incubated in antibiotic-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were plated at 1 × 10^6/ml. Transfection efficiency was stained with bisbenzimide (Promega Corp., Madison, WI) to normalize for transfection efficiency.

**Transfection**—Primary rat osteoblast cultures were de- 

**Nuclei were pelleted and resuspended in hypertonic buffer containing 20 mM HEPES, pH 7.4, 100 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 20% glycerol) containing the protease inhibitors listed above. Protein concentrations were determined using a modified Bradford assay (Bio-Rad).

**Western Blotting**—Western immuno blotting was performed after transfer of electrophoresed proteins to nitrocellulose membranes. Membranes were incubated in blocking buffer consisting of 5% nonfat dry milk and 20 mM bovine serum albumin in TBS-T (0.1% Triton X-100, pH 7.6, 137 mM NaCl, 0.03% Tween 20) for 1 h at 25 °C. Affinity-purified antibody prepared against full-length bacterially expressed C/EBPβ or C/EBPβ (diluted 1:500 in blocking buffer) was then added for 1 h at 25 °C. After washing the membranes in TBS-T, secondary antibody (rabbit anti- chicken IgG diluted 1:1000 in blocking buffer) was added for 1 h at 25 °C. Subsequent steps were performed as described previously (17).

**Preparation of Recombinant C/EBP Proteins**—Recombinant proteins were generated in bacteria as follows. Plasmids pET29a-C/EBPβ were transformed into the E. coli strain of E. coli. Bacterial cultures were grown to an A_600_nm of 0.6 in 50 ml of Circleg (Bio101, Vista, CA) containing 30 μg/ml kanamycin and 34 μg/ml chlor amphenicol and then were induced to express recombinant proteins by addition of isopropyl-1-thio-β-D-galactopyranoside (Sigma) to a final concentration of 1 mM for 3 h. Bacterial pellets were harvested, then re suspended into 5 ml of binding/wash buffer (20 mM Tris-HCl, pH 7.5, 1.5 mM NaCl, 1% Triton X-100) containing 6 μg/mL ribosomes were isolated from bacteria using the cycle of freezing and thawing followed by sonication. Bacterial debris was removed by centrifugation. The S-tagged proteins were purified using S-agarose (Novagen), according to the manufacturer's protocol. Pure proteins were eluted in binding/wash buffer supplemented with 2 mM guanidine thiocyanate and 2 mM urea, followed by dialysis against 20 mM HEPES, pH 7.9, 100 mM KCl, 2 mM EDTA, 20% glycerol. 0.5 mM phenylmethyl sulfonyl fluoride, 0.5 mM dithiothreitol for 2 h at 4 °C. The S-tag was cleaved using biotinylated thrombin as described by the supplier (Novagen). Recombinant proteins were aliquoted and stored at −80 °C until use.

**Assay for Formation of C/EBP Homodimers**—Two μg of truncated recombinant C/EBPβ protein (C/EBPβ-ΔNcoI) was incubated with or without 0.01% glutaraldehyde for 10 min at 25 °C. Samples were separated by 5% polyacrylamide gel electrophoresis followed by Coomassie Blue staining.

**DNA-Protein Binding Studies**—Gel mobility shift experiments followed previously published methods (16, 17). Oligonucleotides and competitors are listed in Table I. Radiolabeled double-stranded DNA probes were synthesized by annealing complementary oligonucleotides followed by cleavage of single-stranded ends with CTCP, 5′-dATP, 5′-dGTP, 5′-dUTP, and 5′-dAMP (3′)-C (800 μM, Amersham Pharmacia Biotech) using the Klenow fragment of DNA polymerase I. Nuclear protein extracts or recombinant proteins were preincubated for 30 min on ice with 2 μg of poly(dI-dC) without or with unlabeled specific or nonspecific DNA competitors. The gel was dried, exposed to film, and the DNA-Protein binding was analyzed.

**TABLE I**

| Oligonucleotide used in gel mobility shift experiments | Top DNA strand | Reference |
|------------------------------------------------------|----------------|-----------|
| Rat HS3D 5′-TTTCAGGCGTACTGCCGACATGGAA (16) | Top | 1 |
| Human HS3D 5′-TCCAGGCGATGCGCGGATC (29) | Top | 16 |
| Albumin D-site 5′-GTTAGATTGTTGAGGGTAC (21) | Top | 30 |
| Oct-1 5′-TTTATAGAGGATCATCAGAACAGAAGTAC (39) | Top | 36 |
Expression of C/EBPβ in COS-7 cells. Antibodies to bacterially expressed C/EBPβ or C/EBPβ were raised in chickens and affinity-purified as described under "Experimental Procedures." Left panel, Western immunobots of bacterially expressed C/EBPβ (lanes 1 and 3) and C/EBPβ (lanes 2 and 4) demonstrate that each antisem recognizes the respective antigen. Right panel, Western immunobots of nuclear protein extracts from COS-7 cells transiently transfected with an expression plasmid for rat C/EBPβ (lanes 5 and 7) or for rat C/EBPβ (lanes 6 and 8) and probed with antibodies to each protein.

Quantitative DNA-protein binding studies were performed with a constant amount of protein (50 ng of bacterial recombinant C/EBPβ or 1.3 μg of COS-7 cell nuclear extract) and increasing concentrations of radiolabeled probe (0.5 to 100 nM). After electrophoresis, gels were dried, and the radioactivity in bands representing protein-bound DNA and free probe was measured by phosphoimager (Molecular Imager System, Bio-Rad). The dissociation constant (Kd) was calculated from these data as the negative reciprocal of the slope after results were graphed by Scatchard plot analysis.

HS3D DNA (nM)

Applied to 4–12 and 4–20% nondenaturating polyacrylamide gradient gels (Novex, San Diego, CA) or a 5% nondenaturating polyacrylamide gel. The dried gels were exposed to x-ray film at −80 °C with intensifying screens.

Methylation interference assays were performed by published methods (34, 35). Double-stranded DNA probes labeled at one end were synthesized as described above. Labeled probes were methylated by incubation with 0.2% dimethylsulfate in 50 mM sodium cacodylate and 1 mM EDTA at 25 °C for 4 min followed by 2 cycles of ethanol precipitation. Recombinant bacterial C/EBPβ protein (100 ng) was incubated with methylated labeled DNA for 30 min on ice, and the DNA-protein complex and free probe were separated by electrophoresis on a 5% polyacrylamide gel. The wet gels were exposed to x-ray film, and protein-bound and free probes were isolated and eluted. Eluted DNA was cleaved by 1M piperidine for 30 min at 95 °C followed by 3 cycles of precipitation. Samples were analyzed after electrophoresis on an 8% polyacrylamide, 5% urea gel and autoradiography for 16 h at −80 °C with an intensifying screen.

RESULTS

Our previous studies defined HS3D as an atypical cAMP response element located in the 5′-untranslated region of rat IGF-I exon 1 that mediated hormonally activated IGF-I gene transcription in primary rat osteoblasts (13, 16). We subsequently identified C/EBPβ as the cAMP-regulated transcription factor responsible for hormonally stimulated gene expression in these cells (17). The current experiments were designed to investigate the physical-chemical properties of the interac-
tions between C/EBPδ and the HS3D site and to determine whether C/EBPδ was involved as a mediator of cAMP-activated transcription in IGF-I genes from species other than rats.

**HS3D Is a High Affinity Binding Site for C/EBPδ**—Quantitative gel-mobility shift assays were used to determine the affinity of C/EBPδ for the HS3D DNA element following the methods outlined in “Experimental Procedures.” In the first series of experiments, nuclear extracts from COS-7 cells expressing C/EBPδ (Fig. 1) were used as the source of recombinant protein, and DNA-protein binding reactions were performed with a constant quantity of nuclear protein (1.3 μg) and a 200-fold concentration range of 32P-labeled double-stranded rat HS3D oligonucleotide (0.5–100 nM; Fig. 2A). Binding was saturable, with an EC50 of ~10 nM and a calculated Kd of 4.78 nM was very similar to the value obtained in parallel experiments using the previously described high affinity C/EBP binding site from the rat albumin promoter (Kd of 5.56 nM; Fig. 2B).

Analogous studies were performed using full-length recombinant C/EBPδ expressed and purified from *E. coli* (Fig. 3, lane 1). As seen with COS-7 nuclear protein extracts, binding of 32P-labeled double-stranded rat HS3D to bacterially derived C/EBPδ was saturable, with an EC50 of ~10 nM and a calculated Kd of 7.83 nM, approximately half that obtained using the albumin C/EBP site as the labeled DNA probe (Kd of 15.67 nM; Fig. 4).

Further evidence that HS3D functioned as a high-affinity binding site for C/EBPδ was obtained from a series of cross-competition gel-mobility shift experiments, using nuclear extracts from COS-7 cells expressing C/EBPδ. As seen in Fig. 5, unlabeled double-stranded HS3D and albumin D-site oligonucleotides competed identically with a 32P-labeled HS3D probe for binding to C/EBPδ and competed equivalently with 32P-labeled albumin D-site DNA. Based on these results and on the information shown in Figs. 2 and 4, we conclude that HS3D is a high affinity binding site for full-length C/EBPδ.

**C/EBPδ Binds to HS3D as a Dimer**—Previous studies had shown that other members of the C/EBP family, including C/EBPα and C/EBPβ, were able to bind to idealized palindromic recognition sites as dimers (21, 22, 28). We performed experiments to assess the stoichiometry of interactions between C/EBPδ and the nonpalindromic HS3D sequence. Bacterial fusion proteins were purified containing full-length and internally truncated rat C/EBPδ fused to an NH2-terminal S-tag (Fig. 3, lanes 1 and 3). The truncated recombinant protein, C/EBPδΔSacII, lacked amino acids 23 through 152 of C/EBPδ, which compose part of the transcriptional activation domain (18). Its absence would not be predicted to alter DNA-protein binding parameters. Both proteins could bind to the labeled HS3D probe, as indicated by results of gel-mobility shift experiments pictured in Fig. 6, lanes 2 and 4. As anticipated, DNA-protein complexes containing truncated C/EBPδ (T:T) exhibited faster mobility on native gel electrophoresis.

**Fig. 3.** Expression and purification from *E. coli* of fusion proteins containing full-length and truncated C/EBPδ. Photograph of purified fusion proteins containing an NH2-terminal S-tag derived from bacteria transformed with expression plasmids pET29a-C/EBPδ (lane 1), pET29a-C/EBPδΔSacI (lane 2), and pET29a-C/EBPδΔSacII (lane 3). Purification on S-agarose was performed as described under “Experimental Procedures.” Approximately 250 ng of protein has been applied to each lane of this 10% SDS-PAGE gel and photographed after staining with Coomassie Blue.

**Fig. 4.** HS3D is a high affinity binding site for bacterially expressed C/EBPδ. Quantitative gel-mobility shift experiments were performed with 32P-labeled rat HS3D (A) or rat albumin D (Alb D) site double-stranded oligonucleotides (B), and full-length C/EBPδ was expressed and purified from *E. coli*, as described under “Experimental Procedures.” DNA binding was quantified by phosphoimager, and results of three experiments were plotted as shown. Binding curves are illustrated in the left panels, and Scatchard plots are illustrated in the right panels (B/F, protein-bound CPM/ unbound CPM).

**TABLE**

| Protein | Binding Constants | nM |
|---------|-------------------|----|
| C/EBPδ | Kd | 7.83 |
| C/EBPδΔSacII | Kd | 15.67 |

**A. HS3D**

**B. Albumin D-site**
than did complexes with full-length protein (Fig. 6, W:W, compare lanes 4 and 2). When both C/EBPα isoforms were mixed together before the addition of the labeled oligonucleotide probe, an additional DNA-protein band of intermediate mobility (W:T) was detected after gel electrophoresis and autoradiography (lane 3), indicating formation of a relatively stable heterooligomeric complex containing full-length and truncated C/EBPα species. Thus, C/EBPα interacted with HS3D as a dimer.

C/EBPα and C/EBPβ have been shown to form protein-protein dimers in solution even in the absence of DNA (21, 22, 28). To assess the potential for C/EBPδ to self-associate, protein cross-linking studies were performed with the purified truncated recombinant protein, C/EBPδ-DΔNcoI (Fig. 3, lane 2), in the absence or presence of low concentrations of glutaraldehyde (0.01%) for 10 min at 25 °C. As shown in Fig. 7, only the monomeric protein of ~32 kDa was visualized after gel electrophoresis in the absence of cross-linker, whereas a larger band of ~65 kDa additionally was observed after incubation with glutaraldehyde. Similar results were seen with full-length C/EBPδ. Thus, like C/EBPα and C/EBPβ, C/EBPδ is able to form protein-protein dimers, which can be assembled into oligomeric DNA-protein complexes in the presence of a high affinity element like HS3D.

Identifying DNA-Protein Contact Sites—We initially characterized the HS3D element by in vitro DNaseI footprinting with rat liver nuclear protein extracts (33) and subsequently identified a qualitatively similar hormone-inducible DNA-protein interaction using rat osteoblast proteins (13). To determine whether recombinant C/EBPδ could recognize the same segment of DNA as osteoblast-derived nuclear proteins, in vitro DNaseI footprinting was performed with end-labeled double-stranded DNA probes derived from rat IGF-I promoter 1 and graded concentrations of C/EBPδ expressed and purified from bacteria. As seen in Fig. 8, recombinant C/EBPδ protected the HS3D site (nucleotides 200–214 on both strands) from nuclease digestion, effectively recapitulating what was observed previously with rat osteoblast nuclear proteins (13).

We next performed in vitro dimethylsulfate footprinting to define some of the nucleotides directly involved in protein-DNA binding. As depicted in Fig. 9, two guanosine residues on the upper DNA strand and three on the lower strand that collec...
tively span 8 bp were required for binding by recombinant full-length C/EBPδ. Methylation of these residues inhibited binding, resulting in the accumulation of modified DNA in the unbound fraction and its subsequent cleavage by dimethylsulfate and piperidine. These results are in good agreement with our previous analysis of the HS3D site by site-directed mutagenesis (16, 17).

The HS3D Site Is Structurally and Functionally Conserved in IGF-I Promoters from Different Species—Table II depicts an alignment of the HS3D region of the rat IGF-I gene with analogous portions of the human, chicken, and chum salmon genes (36–38). The 25-bp segment shown is highly conserved, with 1 nucleotide substitution, a G to C transversion, and 1 deletion in the human and chicken DNAs compared with rat, and 2 substitutions and 1 deletion in salmon. The human and chicken sequences are identical, whereas salmon HS3D differs by only a single nucleotide. Cross-competition gel-mobility shift studies were performed to determine the relative affinity of the human/chicken HS3D region for C/EBPδ expressed in COS-7 cells. As seen in Fig. 10, both rat and human/chicken 32P-labeled double-stranded probes gave rise to DNA-protein complexes of identical mobility, and both unlabeled HS3D oligonucleotides competed equivalently for binding to C/EBPδ with either 32P-labeled DNA sequence. Thus the human and chicken HS3D sequences behave as high-affinity C/EBPδ binding sites.

Functional analyses of the potential role of HS3D in mediating hormonal regulation of human IGF-I gene transcription were performed with a chimeric human IGF-I promoter 1-luciferase fusion plasmid. Transient transfection experiments using rat primary osteoblast cultures showed that a fragment of human promoter 1 from −1630 to +322 with respect to the most 5′ transcription start site (36) mediated a 6-fold increase in reporter gene activity after incubation of cells with 1 μM PGE2 for 6 h (Fig. 11A). Co-transfection of the same plasmid with an expression vector for C/EBPδ led to a 5-fold rise in luciferase activity under basal conditions when compared with co-transfections with the empty expression plasmid and stimulated a further 3-fold increase in IGF-I gene activation after treatment with PGE2 (Fig. 11A).

To establish a specific role for the human/chicken HS3D site
in mediating PGE2-activated and C/EBPβ-regulated transcription, a reporter gene containing 4 tandem copies of the 19-bp natural human/chicken HS3D region cloned 5' to a minimal RSV promoter was transfected into rat osteoblasts. As seen in Fig. 11B, treatment with 1 μM PGE2 stimulated a 4-fold increase in luciferase activity but had no significant effect on a reporter plasmid containing the minimal RSV promoter alone. Co-transfection with an expression plasmid for C/EBPβ led to a 17-fold rise in luciferase activity under basal conditions as compared with co-transfections with the empty expression vector, and stimulated an additional 2-fold increase in promoter activity after incubation with PGE2 (Fig. 11B).

**DISCUSSION**

The studies described in this report define the physical-chemical properties and functional consequences of interactions between HS3D, the DNA element in IGF-I promoter 1 that mediates stimulation of IGF-I gene transcription by cAMP or PGE2 in osteoblasts (13, 16), and C/EBPβ, the key transcription factor responsible for cAMP-activated IGF-I expression in these cells (17). We show that C/EBPβ, expressed in COS-7 cells or purified as a recombinant protein from E. coli, bound to HS3D with an affinity at least equivalent to that of the albumin D-site, a known high affinity C/EBP binding sequence (21, 27), and that both DNA elements competed equally for C/EBPβ. C/EBPβ bound to HS3D as a dimer, with protein-DNA contact points located on guanine residues on both DNA strands within and just adjacent to the core C/EBP half-site, GCAAT. C/EBPβ also formed protein-protein dimers in the absence of interactions with its DNA binding site, as indicated by results of glutaraldehyde cross-linking experiments. In conjunction with functional studies, demonstrating cAMP-inducible transactivation by C/EBPβ of human IGF-I promoter 1 and of a reporter gene with four tandem copies of the conserved human/chicken HS3D site, our results provide evidence that C/EBPβ is a critical activator of IGF-I gene transcription in rodent osteoblasts and, potentially, in other cell types and species.

The chemical properties of C/EBPβ assessed here resemble those of the related factors, C/EBPα and C/EBPβ. With the addition of our new studies, it is now clear that all three proteins can rapidly form dimers in dilute solution without a requirement for the presence of the specific DNA binding site (Refs. 21, 22, and 28; and this report). Dimerization is mediated by the COOH-terminal leucine zipper, which consists of a heptad of leucine repeats within a relatively preserved 35 amino acid core (18). This region and the adjacent basic DNA binding domain are the most conserved portions of C/EBPs, having ~60% identity among C/EBPα, β, and δ (18). Dimerization appears to be a prerequisite for DNA binding, since in previous studies with C/EBPα, mutation of any of the leucine residues blocked recognition of a high affinity C/EBP element (28).

Prior results using the COOH-terminal portion of C/EBPα in dimethylsulfate footprinting experiments with an idealized dyad-symmetrical high affinity C/EBP site had identified four nucleotide contact points and several other sites of enhanced DNA cleavage (34). Our observations with full-length C/EBPβ and HS3D DNA are very similar. We detected the same four protected nucleotides and mapped an additional protection to a more 5' guanine on the lower DNA strand (Fig. 9). The slight differences between results may be explained potentially by variation in the DNA binding sites, although the 8-bp central region is identical, or by the different proteins used, a COOH-terminal fragment of C/EBPα previously (34) versus full-length C/EBPβ here.

Our previous results defined HS3D as a functionally important component in the major IGF-I promoter from the rat (13, 16). The current studies demonstrate that human IGF-I promoter 1 also is activated by PGE2 and that a multimerized HS3D element from human or chicken IGF-I promoter functions as a PGE2-induced and C/EBPβ-regulated hormone response element. Based on these observations and on the similarity of HS3D sites in IGF-I genes from human, rat, chicken, and salmon (Table II), we tentatively predict that regulation of IGF-I transcription via C/EBPβ also is conserved and postulate that C/EBPβ may be a critical intermediate in the hormonal control of IGF-I synthesis in osteoblasts in several species. Detailed analysis of recently generated C/EBPβ-deficient mice (24) should provide additional insights into the role of this transcription factor in regulating production of IGF-I in bone and other tissues.

Other C/EBP isoforms also may play roles in regulating IGF-I gene expression. We had shown previously that C/EBPβ
could bind to the HS3D site and, in co-transfection experiments, could transactivate a rat IGF-I promoter 1–luciferase reporter gene (17). Because both C/EBPα and C/EBPβ are expressed in liver, fat, and other tissues (18, 20–22) where IGF-I mRNA also is synthesized (2, 15), it is reasonable to expect that these proteins also may modulate IGF-I gene transcription under different physiological conditions.

We previously found that C/EBPδ was activated and IGF-I transcription was stimulated in primary rat osteoblasts by a cyclic AMP-dependent protein kinase-dependent pathway that did not require ongoing protein synthesis (16). Preliminary experiments have shown that C/EBPδ can be translocated from the cytoplasm to the nucleus of osteoblasts after PGE₂ treatment, even when protein synthesis is blocked.² Goals for the future will be to characterize the pathways responsible for induction of C/EBPδ activity in these cells and to determine the pathways through which C/EBPδ stimulates IGF-I gene transcription.

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² Y. Umayahara, J. Billiard, C. Ji, M. Centrella, T. L. McCarthy, and Peter Rotwein, unpublished observations.
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