

# Identification of an Osteogenic Protein-1 (Bone Morphogenetic Protein-7)-Responsive Element in the Promoter of the Rat Insulin-Like Growth Factor-Binding Protein-5 Gene\*

LEE-CHUAN C. YEH AND JOHN C. LEE

Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78229-3900

## ABSTRACT

Osteogenic protein-1 (OP-1), a member of the bone morphogenetic protein subfamily of the transforming growth factor- $\beta$  superfamily, induces new bone formation *in vivo* and regulates the expression of numerous growth factors. We previously showed that OP-1 down-regulates the transcription of the insulin-like growth factor-binding protein-5 (IGFBP-5) in primary cultures of fetal rat calvaria (FRC) cells. In the present study we identified, within the IGFBP-5 promoter, a 21-bp region that confers OP-1 responsiveness in FRC cells. Within this region lie three putative *cis*-acting regulatory elements, *viz.* a CAAT-like sequence, a CCAAT/enhancer-binding protein (C/EBP $\alpha$ )-like element, and a c-Myb or E-box-like motif. Mutations in the CAAT-like sequence reduced the promoter activity in both control and OP-1-treated cells, but did not abrogate the OP-1-induced down-regulation. Mutations in the C/EBP $\alpha$ -like element reduced the pro-

motor activity in both control and OP-1-treated cells without significantly affecting the extent of down-regulation. Mutations in the putative c-Myb or E-box-like motif reduced the promoter activity in both the OP-1-treated and control cells and completely abolished the inhibitory effect of OP-1 on the IGFBP-5 promoter activity. Gel mobility shift analyses further showed specific interaction between nuclear protein(s) in FRC cells and the 21-bp region. OP-1 down-regulates the nuclear regulatory protein interaction with the 21-bp region by reducing either the cellular concentration of the regulatory protein(s) or the affinity of the regulatory protein(s) for the OP-1 responsive element. In conclusion, we identified an OP-1 response region in the rat IGFBP-5 promoter and further showed that OP-1 down-regulates the nuclear protein interaction with the response element(s). (*Endocrinology* 141: 3278–3286, 2000)

**O**STEOGENIC PROTEIN-1 (OP-1/BMP-7) is a member of the bone morphogenetic protein (BMP) family that can be divided into several subgroups according to their amino acid sequence similarity (1–3). The BMPs are members of the transforming growth factor- $\beta$  superfamily (4, 5). OP-1 was originally identified by its activity to induce bone formation *in vivo* (6). Subsequently, OP-1 was implicated in numerous biological processes, mostly based on the observation that transgenic mice with OP-1 deficiency survive for only a short time after birth. These animals show poor eye and kidney development and abnormal skeletal formation (7, 8). *In vitro*, OP-1 induces osteoblastic cell differentiation and stimulates the synthesis of biochemical markers characteristic of osteoblastic cell differentiation (6, 9–14).

OP-1 also stimulates the synthesis of several growth factors, such as the insulin-like growth factors (IGFs), in cultured osteoblastic cells. IGF-I and IGF-II show mitogenic activity for bone cells and enhance osteoblasts differentiation (15–17). The biological activity of the IGFs can be affected by their binding proteins, the IGF-binding proteins (IGFBPs) (18). For example, IGFBP-4 inhibits bone formation, but IGFBP-5 can be either stimulatory or inhibitory of the IGF-I

activity on bone cell (18–21). Synthesis of IGFBP-5 by osteoblastic cells appears to be tightly controlled (22) and can be affected by numerous factors (20, 23–36). For example, IGF-I (20, 24, 36), IL-6 with its soluble receptor (31), PGE<sub>2</sub> (27, 28, 34, 35), PTH fragments (26), and retinoic acid (24, 36) stimulate IGFBP-5 messenger RNA (mRNA) expression in osteoblastic cells. In contrast, basic fibroblast growth factor, cortisol, dexamethasone, platelet-derived growth factor, and transforming growth factor- $\beta$  inhibit IGFBP-5 mRNA expression (20, 23, 30, 32, 37). In fetal rat calvaria (FRC) cells, OP-1 decreases the steady state IGFBP-5 mRNA level by reducing transcription of the IGFBP-5 gene. The stability of the IGFBP-5 mRNA is unaffected (25). We further show that exogenous human IGFBP-5 protein inhibits osteoblast differentiation induced by OP-1, as determined by the measurement of a differentiation biochemical marker, alkaline phosphatase activity (37). Taken together, these data suggest that IGFBP-5 plays a critical role in OP-1-induced bone cell differentiation.

To investigate the molecular basis for the OP-1-induced down-regulation of IGFBP-5 gene expression, a series of plasmid constructs that contain the rat IGFBP-5 promoter fused to a reporter gene were generated. These plasmids were used to identify OP-1-response elements in transient transfection experiments in primary cultures of FRC cells. We found that OP-1 down-regulates rat IGFBP-5 promoter activity via a 21-bp region. The region contains several sequences analogous to certain highly conserved transcriptional regulatory

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Address all correspondence and requests for reprints to: Lee-Chuan C. Yeh, Ph.D., Department of Biochemistry (Mail Code 7760), University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78229-3900. E-mail: carolyeh@biochem.uthscsa.edu.

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elements. Mutation studies showed that sequences within the E-box-like motif are involved in the OP-1-induced down-regulation of the rat IGFBP-5 promoter. Gel mobility shift analyses further showed that OP-1 decreases the interaction between the 21-bp region and a *trans*-acting factor(s).

## Materials and Methods

### Materials

All reagents were of molecular biology grade. All buffers were prepared with diethylpyrocarbonate-treated water. Restriction endonucleases were purchased from New England Biolabs, Inc. (Beverly, MA). Recombinant human OP-1 was provided by Stryker Biotech (Hopkinton, MA) and was dissolved in 47.5% ethanol/0.01% trifluoroacetic acid. The NucleoBond plasmid kit was obtained from CLONTECH Laboratories, Inc. (Palo Alto, CA). Oligonucleotides were synthesized by the Center for Advanced DNA Technologies, University of Texas Health Science Center (San Antonio, TX). The luciferase assay system was purchased from Promega Corp. (Madison, WI). The Galacto-Light reporter gene assay for  $\beta$ -galactosidase and the Dual-Light reporter system for luciferase and  $\beta$ -galactosidase were obtained from Tropic (Bedford, MA). The BandShift kit was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

### Construction of rat IGFBP-5 promoter-luciferase genes

A 1-kb DNA fragment, comprising nucleotides from  $-888$  bp upstream to  $+114$  bp downstream from the transcription start site ( $+1$ ) of the rat IGFBP-5 gene was generated by PCR using genomic DNA isolated from rat liver. The sense primer is 5'-AGG ATC TGC CTG CCC TGT-3', and the antisense primer is 5'-ACC GAG GAG GGG GAT AAC-3'. The reaction was performed for 25 cycles at 94 C for 30 sec, 55 C for 30 sec, and 68 C for 60 sec, with a final extension at 68 C for 10 min. The PCR product was directly cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). The clone containing the sense (positive) orientation of the IGFBP-5 promoter was confirmed by restriction enzyme mapping and double stranded (ds) DNA sequencing. The IGFBP-5 promoter fragment was then subcloned into the *SacI* and *XhoI* sites of the pGL2-Basic vector (Promega Corp.) containing the promoterless luciferase reporter gene (Luc). The antisense fragment of the IGFBP-5 promoter was ligated into the *XhoI* and *HindIII* sites of the pGL2-Basic

vector. All plasmids were checked for purity on 1% agarose gels. Only the ultrapure DNA preparations were used for transfection studies.

### Generation of rat IGFBP-5 promoter mutants

Deletions in the 5'-end of the rat IGFBP-5 promoter were generated by digestion of the plasmid containing the 1-kb promoter fragment with unique restriction enzymes or by PCR (Fig. 1). Constructs with 5'-end beginning at positions  $-390$ ,  $-297$ ,  $-151$ ,  $-131$ ,  $-71$ , and  $+32$  were generated by digestion of the parent plasmid with *AccI*, *AlwNI*, *StuI*, *DraIII*, *SacI*, and *DraI*, respectively. Constructs with the 5'-end beginning at positions  $-50$  and  $-33$  were generated by PCR using sense primers 5'-TGG CAG CCA GGG GCC GTC-3' and 5'-CTA TTT AAA AGC GCC TGC-3', respectively. PCR conditions were 35 cycles at 94 C for 60 sec, 50 C for 60 sec, 72 C for 60 sec, and a final extension at 72 C for 10 min. The resultant DNA fragments were subcloned into the pGL2-Basic vector. The internal mutations within the 21-bp sequence of the promoter were generated by PCR with sequence-specific oligonucleotide primers (Fig. 3B) and wild-type DNA template ( $-71/+114$ ) as described above. Alternatively, the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used. The  $-390/+114$  mutant clones containing specific mutations in the CAAT, CCAAT/enhancer-binding protein (C/EBP $\alpha$ ), or E-box sequence were constructed by replacement of the wild-type sequence with the corresponding mutant sequence. The DNA products were sequenced in their entirety to ensure the absence of unintended mutations.

### FRC culture and transient transfection

Timed pregnant Sprague Dawley rats were obtained from Harlan (Indianapolis, IN) and were handled and killed following the procedures approved by the institutional animal care and use committee at University of Texas Health Science Center. Primary FRC cells were prepared as described previously (37). Confluent cells of the passage 3 were used for experimentation. Transfection studies were carried out as described previously (25). Briefly, FRC cells were grown in six-well plates to about 70% confluence in complete  $\alpha$ MEM plus serum and were transiently transfected with promoter constructs by the calcium phosphate-DNA coprecipitation method (38). Plasmid DNA ( $12 \mu\text{g}$ ) was used for each six-well plate. The medium was removed after 3 h of transfection, and the cells were treated with 15% glycerol/ $1 \times$  HEPES-buffered saline for 2 min at room temperature. Transfected FRC cells were

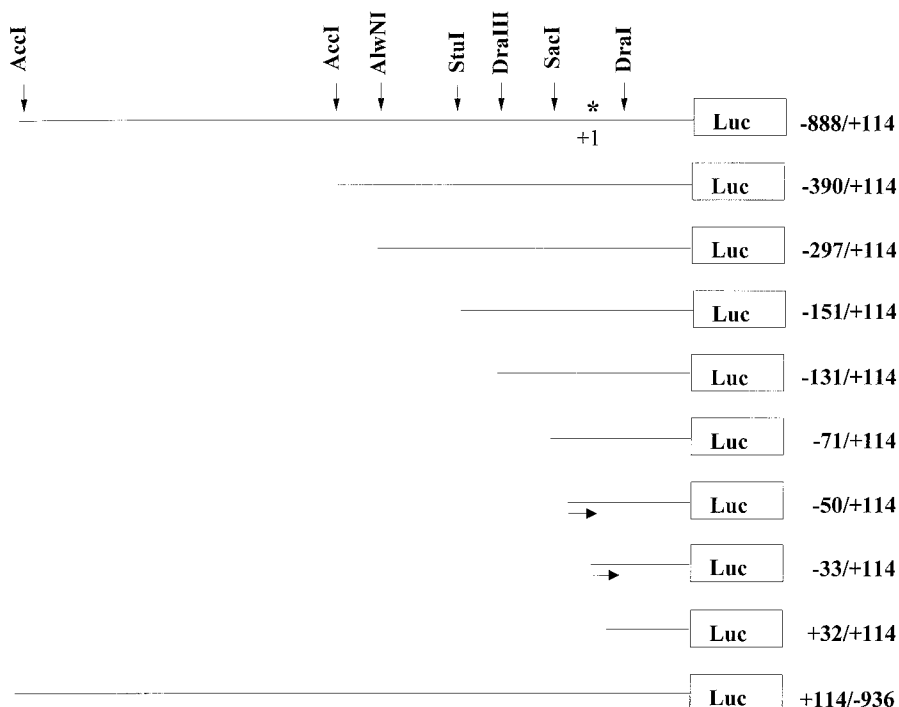


FIG. 1. DNA constructs containing different rat IGFBP-5 promoter regions. The transcription start site is  $+1$ . The 1-kb DNA fragment was subcloned into the pGL2-Basic vector containing the promoterless luciferase reporter gene (Luc). Six of the 5'-deletion fragments were generated by digestion of the parent (1-kb) DNA with unique restriction enzymes, and two ( $-50/+114$  and  $-33/+114$ ) were generated by PCR as indicated. The unique restriction sites are shown (perpendicular arrows). The positions of primers used for PCR are indicated as horizontal arrows.

incubated overnight in fresh complete  $\alpha$ MEM, followed by treatment with OP-1 (300 ng/ml) or vehicle in serum-free  $\alpha$ MEM for 24 h. The IGFBP-5 promoter activity was determined by measuring the luciferase reporter gene activity as previously described (25). Briefly, cells were lysed, and an aliquot (20  $\mu$ l) was taken to measure luciferase activity using a luciferase assay kit (Promega Corp.) and the OPTOCOMP I luminometer ILA911 (Tropix). Another aliquot (10  $\mu$ l) was used to determine transfection efficiency by measuring  $\beta$ -galactosidase activity using the Galacto-Light kit (Tropix). Luciferase activity was normalized to  $\beta$ -galactosidase activity. In some experiments the Dual-Light Reporter System was used. The system is a chemiluminescent reporter gene assay system for the combined luciferase and  $\beta$ -galactosidase, using as substrates luciferin and Galacton-Plus, respectively. The values obtained were comparable to those using the single enzyme system.

#### Gel mobility shift assay

Proteins for the gel mobility shift assays were extracted from control or OP-1-treated confluent FRC cells basically as previously described (39). Protein extracts were stored as aliquots at  $-80^{\circ}\text{C}$  until use. The protein concentration was determined using the Bradford method (40). The gel mobility shift experiments were carried out using the BandShift kit. Briefly, radiolabeled dsDNAs were produced by annealing complementary oligonucleotides (consisting of the sequence from  $-71$  to  $-51$  and its complementary sequence) followed by treatment with the T4 polynucleotide kinase in the presence of [ $\gamma$ - $^{32}\text{P}$ ]ATP. Varying concentrations of protein extracts were incubated with a fixed amount of radiolabeled DNA probe (5500 cpm) for 20 min at room temperature. Samples were analyzed on 5% nondenaturing polyacrylamide gels in  $1 \times \text{TBE}$ . Gels were dried, and the radioactive bands were detected and quantified using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) and ImageQuant software (Becton Dickinson and Co., Mountain View, CA). For the competition experiments, an excess amount (50- to 200-fold) of unlabeled DNA probe or unrelated *EBNA-1* or *Oct-1* DNA was included in the reaction.

#### Statistical analysis

Multiple means were compared by one-way ANOVA, followed by Student's *t* test for paired comparison with the control. The ANOVA and Student's *t* test programs in the PSI-Plot (Ploy Software International, Salt Lake City, UT) for personal computers were used for the analyses.

### Results

Previously we showed that OP-1 down-regulates IGFBP-5 transcription in FRC cells. To examine the potential mechanism(s) involved in the regulation of IGFBP-5 expression by OP-1, a 1-kb fragment of the rat IGFBP-5 promoter was generated by PCR using the rat genomic DNA as a template. The fragment containing the promoter sequence from  $-888$  to  $+114$  was subsequently fused upstream to a promoterless luciferase (Luc) reporter gene in the pGL2-Basic vector. Subsequently, several rat IGFBP-5 promoter-Luc reporter gene constructs with different 5'-deletions were generated by unique restriction endonuclease digestion or by PCR (Fig. 1). These DNA constructs were used in transient transfection studies of subconfluent FRC cells to define the OP-1-responsive element.

Figure 2A shows the reporter gene activity of the various clones in FRC cells treated with OP-1 or vehicle. The promoter activity for the pGL2-Basic vector in transfected FRC cells treated with vehicle was very low and was not affected by OP-1. In control cells, the 1-kb promoter construct ( $-888/+114$ ) was about 110-fold more active than the parent pGL2-Basic (Fig. 2A). Sequential removal of the 5'-end sequence beginning from  $-888$  resulted in a stepwise reduction of the basal promoter activity. Deletion of the 500 nucleotides from

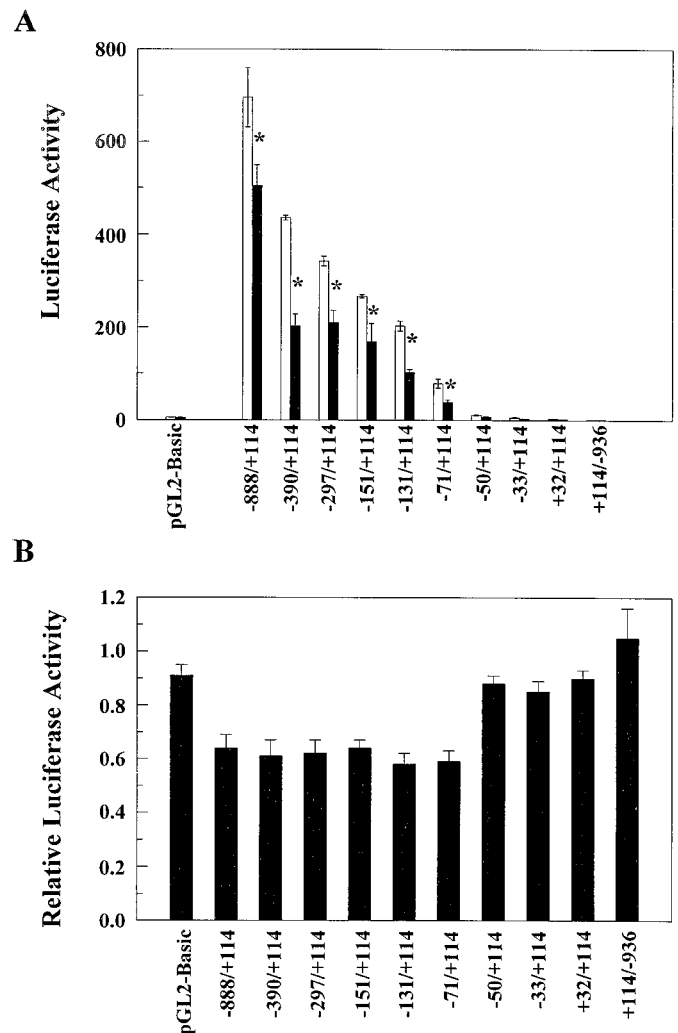


FIG. 2. Effects of OP-1 on the IGFBP-5 promoter activity in transiently transfected FRC cells. FRC cultures were transfected with the DNA constructs containing the different deletions of the IGFBP-5 promoter shown in Fig. 1. Cultures were treated with solvent (*open bars*) or OP-1 (*solid bars*; 300 ng/ml) for 24 h, followed by determination of reporter gene activity. A, Luciferase activity was normalized to  $\beta$ -galactosidase activity. Values represent the mean  $\pm$  SE of 6–10 independent determinations from 3 different FRC preparations. \*, The experimental value of the OP-1-treated sample is significantly different from the control ( $P < 0.05$ ). B, Ratios of the normalized luciferase activity in OP-1-treated culture/control culture as a function of the different promoter constructs.

the 5'-end (the  $-390/+114$  construct) led to an approximately 40% decline in the reporter activity compared with the  $-888/+114$  construct. Additional deletion of the promoter sequence to position  $-131$  led to a further 30% decline in promoter activity. The promoter containing the  $-71/+114$  sequence retained about 15% of the most active promoter (the  $-888/+114$  construct) activity and was about 13-fold more active than the pGL2-Basic. The promoter construct with only 50 nucleotides of the 5'-flanking sequence and the first 114 bp of exon 1 (the  $-50/+114$  construct) retained about 2–3% of the activity exhibited by the  $-888/+114$  construct and was about 2- to 3-fold more active than the parent pGL2-Basic.



(~64% of the basal promoter activity of the 1-kb construct). The promoter activity of each mutant was measured after transfection of FRC cells. Figure 4A shows the relative luciferase activities of the various shorter mutant promoter clones in FRC cells treated with OP-1 or vehicle. In agreement with the results shown in Fig. 2, OP-1 dramatically reduced (by ~47%) the activity of the IGFBP-5 promoter construct containing the wild-type sequence from -71 to +114. Mutations in the CAAT-like sequence reduced the promoter activity in both control and OP-1-treated cells. The extent of reduction in promoter activity induced by OP-1 was essentially unchanged (~40%). A single T to C mutation or a 4-base mutation within the C/EBP $\alpha$ -like element also reduced the IGFBP-5 promoter activity in both

control and OP-1-treated cells without affecting OP-1 responsiveness. Mutations in the E-box-like motif reduced promoter activity in both control and OP-1-treated cells and completely abolished the effect of OP-1 (Fig. 4A). Additionally, to substantiate these observations obtained with the shorter mutant promoter constructs, these analyses were conducted with the longer mutant constructs. Figure 4B shows the relative luciferase activities of the various longer mutant promoter clones. Similar to the results obtained with the shorter clones, the CAAT mutant promoter constructs showed reduced activity in both control and OP-1-treated cells, and the extent of the OP-1-induced reduction in promoter activity was similar to that of the wild-type (46% vs. 40%). Mutations in C/EBP $\alpha$  also reduced promoter activity in both control and OP-1-treated cells. The extent of the OP-1-induced reduction remained unchanged. Mutations in the putative E-box-like motif completely abolished down-regulation of promoter activity by OP-1. These observations suggest that the putative E-box-like motif (or at least the CAAC sequence within it) contains *cis*-acting elements responsible for the down-regulation of IGFBP-5 transcription by OP-1.

To examine the roles of nuclear proteins in the down-regulation of the IGFBP-5 promoter by OP-1, gel mobility shift assays were carried out using a radioactive oligonucleotide probe consisting of a 21-bp sequence (-71/-51) and proteins from control as well as OP-1-treated FRC cells. Figure 5A shows results of a representative gel shift assay. One major shifted band with a slower mobility in both control and OP-1-treated samples was observed. The intensity of the band was proportional to the protein concentration. However, at the same protein concentration, the band intensity was consistently lower in the OP-1-treated sample (by ~40–50%) than in the control sample (Fig. 5B). The decrease was readily observable 24 h after OP-1 treatment of FRC cells and was not changed further at 48 h. The radioactive probe in the shifted band was competed out by increasing concentrations of the nonradioactive, homologous oligonucleotide and was also competed out by the mutant oligonucleotide containing the E-box mutations (Fig. 6). The latter oligonucleotide was not as efficient in competing with the wild-type sequence. Unrelated oligonucleotides, *EBNA-1* and *Oct-1*, did not compete with the wild-type oligonucleotide (Fig. 6). The finding suggests that the nuclear protein-DNA interaction is specific.

To further examine the involvement of the individual conserved motif (CAAT, C/EBP $\alpha$ , and E-box) within the 21-bp sequence in the OP-1-induced down-regulation of IGFBP-5, the ability of mutant oligomers (Fig. 3B) consisting of mutations in each of the conserved element to bind nuclear protein(s) was examined. Gel mobility shift assays with radiolabeled wild-type and mutant oligonucleotide probes and proteins from control or OP-1-treated cells were conducted. In agreement with the results described above, OP-1 caused a decrease in the interaction between the nuclear protein(s) and the wild-type ds oligonucleotide probe (Fig. 7, lanes 1 and 2). The ds oligonucleotide probe containing mutations in the CAAT element only without changing the C/EBP $\alpha$  element and the E-box motif formed a DNA-protein complex. Although one would expect that the mutant DNA-protein complex might be smaller than the wild-type DNA-protein complex due to the failure of binding of the CAAT-box-

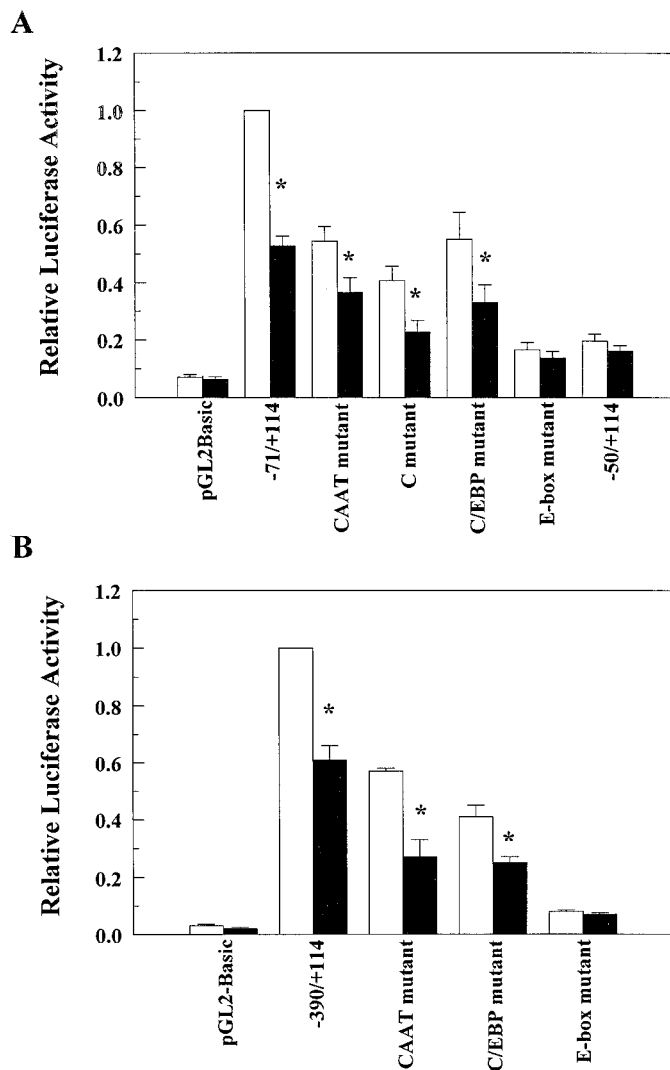


FIG. 4. Effects of OP-1 on the promoter activity of the different site-specific mutant IGFBP-5 promoter in transiently transfected FRC cells. Cultures were transfected with either the short (-71/+114) constructs (A) or the long (-390/+114) constructs (B) containing the different mutations shown in Fig. 3B. The transfected cells were treated with solvent (*open bars*) or OP-1 (*solid bars*; 300 ng/ml) for 24 h. Luciferase activity was normalized to  $\beta$ -galactosidase activity and compared with that in wild-type control as 1. Values represent the mean  $\pm$  SE of five to eight independent determinations from three different FRC preparations. \*, The experimental value of the OP-1-treated sample is significantly different from the control ( $P < 0.01$ ).

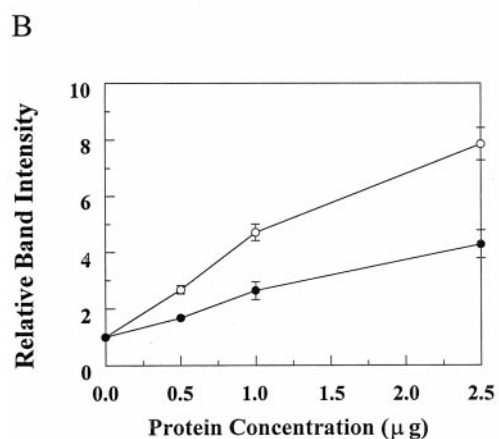
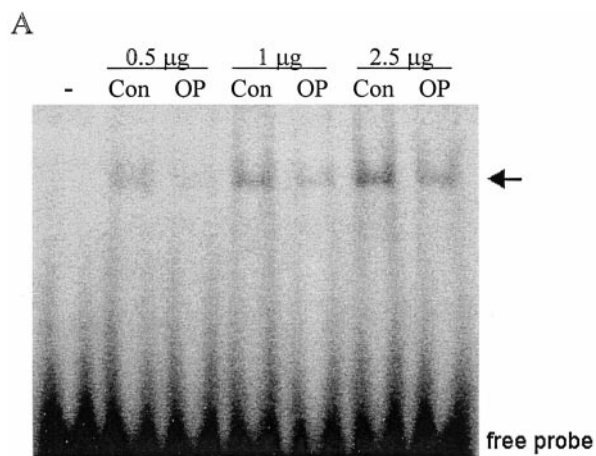


FIG. 5. DNA-protein interactions in the rat IGFBP-5 promoter as revealed by gel mobility shift assay. The  $^{32}\text{P}$ -labeled ds oligonucleotide probe spanning nucleotides  $-71$  to  $-51$  of the rat IGFBP-5 promoter was incubated with different concentrations ( $0$ – $2.5$   $\mu\text{g}$ ) of protein extracts from FRC cells that had been treated with solvent or OP-1 ( $300$  ng/ml) for  $24$  or  $48$  h. The reaction mixture was loaded onto a  $5\%$  nondenaturing polyacrylamide gel. DNA-protein complexes were detected by PhosphorImager. A, An image of a representative gel mobility shift experiment is shown. The position of the DNA-protein complex is indicated with an *arrow*, and that of the free oligonucleotide probe is also noted on the *right*. B, Quantitation of the relative band intensity shown in A compared with that without protein as  $1$ . Values are the mean  $\pm$  SE of four independent determinations from two different FRC cell preparations.

binding protein to the mutant complex, the apparent electrophoretic mobility of the mutant DNA-protein complex was not significantly dissimilar to that of the wild-type DNA-protein complex. A possible reason is that the gel system might not be sufficiently sensitive to detect small changes in the number of proteins bound to the complex. The band intensity of the complex was lower in the OP-1-treated sample than in the control (Fig. 7, lanes 3 and 4). Hence, the OP-1 induced decrease in protein binding to the wild-type  $21$ -bp sequence was probably not due to a change in the CAAT-binding protein(s). Similarly, the ds oligonucleotide probe containing mutations in the C/EBP $\alpha$  element only without changes in the CAAT element and E-box motif still displayed a decrease in band intensity in the OP-1 sample compared

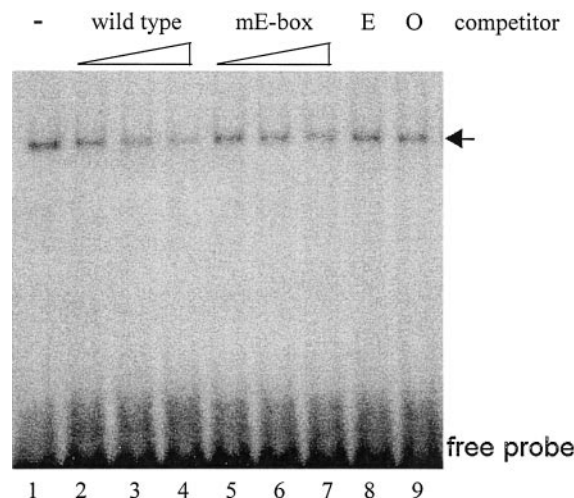


FIG. 6. Specificity of interactions between the rat IGFBP-5 promoter and nuclear proteins from FRC cells. Gel mobility shift experiments, described in Fig. 5, were conducted with radiolabeled oligonucleotide probes with wild-type sequence and protein extract from OP-1-treated cells in the presence of unlabeled homologous probes, wild-type oligonucleotide probes, unlabeled mutant E-box oligonucleotide probes, unlabeled *EBNA-1* probes, or unlabeled *Oct-1* oligonucleotide probes. Lane 1, No unlabeled competitor; lanes 2–4, unlabeled wild-type oligonucleotides in increasing concentrations ( $50$ -,  $100$ -, and  $200$ -fold molar excesses); lanes 5–7, unlabeled mutant E box oligonucleotide in increasing concentrations ( $50$ -,  $100$ -, and  $200$ -fold molar excesses); lane 8, unlabeled *EBNA-1* ( $200$ -fold molar excess); lane 9, unlabeled *Oct-1* ( $200$ -fold molar excess).

with the control (Fig. 7, lanes 5 and 6). The observation suggests that OP-1 did not change the C/EBP $\alpha$ -binding protein(s). However, the ds oligonucleotide containing mutations in the E-box motif only without changing the CAAT and C/EBP $\alpha$  elements no longer displayed the OP-1-induced decrease in band intensity (Fig. 7, lanes 7 and 8). The observation suggests that OP-1 altered either the E-box-binding protein(s) or a protein(s) that binds to the E-box-binding protein(s).

## Discussion

IGFBP-5 appears to play a significant role in bone cell growth. In turn, IGFBP-5 expression is affected by many growth factors. Previously, we showed that OP-1 down-regulates IGFBP-5 gene expression by reducing transcription without changing the stability of the IGFBP-5 mRNA ( $17$ ,  $25$ ). OP-1 reduced the steady state IGFBP-5 mRNA level by  $60$ – $80\%$ , as determined by Northern blot analysis ( $17$ ). Subsequent studies showed that OP-1 caused a  $43$ – $50\%$  reduction in the level of IGFBP-5 nuclear pre-mRNA transcription, as measured by ribonuclease protection assay with an intron 1-specific probe ( $25$ ). We also observed a  $40\%$  decrease in promoter activity in OP-1-treated FRC cells transfected with a human IGFBP-5 promoter construct ( $25$ ). Our current study revealed that OP-1 inhibited the rat IGFBP-5 promoter activity by about  $40$ – $50\%$  in FRC cells. Hence, there are good agreements among the *in vitro* and *in vivo* data obtained using different experimental approaches.

Our study also shows that the rat DNA fragment with  $151$  bp of the  $5'$ -flanking sequence and the first  $114$  bp of exon  $1$

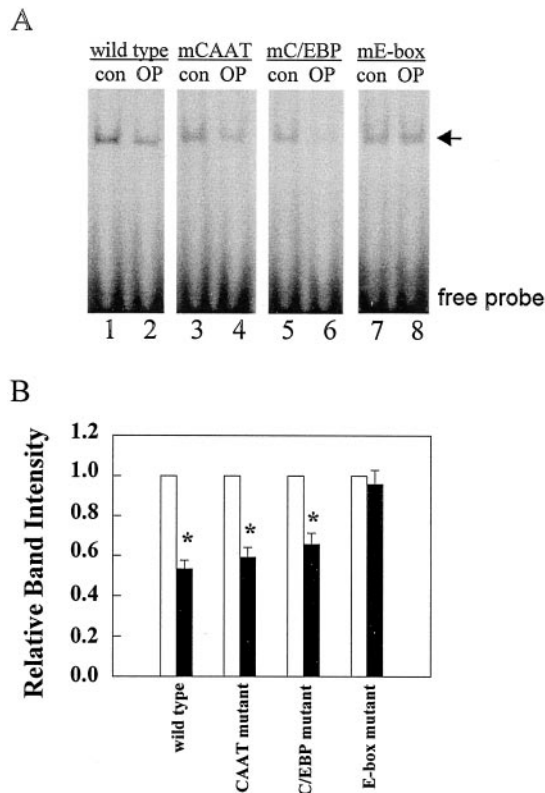


FIG. 7. Gel mobility shift assays for interactions between nuclear proteins and site-specific mutant IGFBP-5 promoters. The  $^{32}\text{P}$ -labeled ds oligonucleotide probes spanning nucleotides  $-71$  to  $-51$ , as shown in Fig. 3B, were incubated with  $2.5\ \mu\text{g}$  protein extracts from FRC cells that had been treated with solvent or OP-1 (300 ng/ml) for 24 h. The reaction mixtures were loaded onto a 5% nondenaturing polyacrylamide gel. DNA-protein complexes were detected by PhosphorImager. A, A phosphorimage of a representative gel mobility shift experiment is shown. Lanes 1 and 2, Radiolabeled probes containing the wild-type CAAT element, the C/EBP $\alpha$ -like element, and the E-box-like motif of the rat IGFBP-5 promoter; lanes 3 and 4, radiolabeled probes containing mutations in the CAAT element; lanes 5 and 6, probes containing mutations in the C/EBP $\alpha$ -like element; lanes 7 and 8, radiolabeled probes containing mutations in the E-box motif. The position of the DNA-protein complex is indicated with an *arrow*, and that of the free oligonucleotide probe is also noted on the *right*. B, Quantitation of the relative band intensity shown in the phosphorimage. Values are the mean  $\pm$  SE of three independent determinations from two different FRC cell preparations. \*, The experimental value of the OP-1-treated sample is significantly different from the control ( $P < 0.01$ ).

retained about 40% of the maximal promoter activity displayed by the  $-888/+114$  promoter sequence in fetal rat calvaria cells. The DNA fragment with only the  $-50/+114$  sequence retained about 2–3% of the maximal promoter activity. By comparison, Kou *et al.* (41) reported that the mouse DNA fragment with only 156 bp of the 5'-flanking sequence and the first 120 bp of exon 1 confers about 61% of the promoter activity in human Hep G2 cells. Maximal promoter activity was defined as that exhibited by the promoter containing the first 1 kb 5' upstream of the transcription start site of the mouse IGFBP-5 gene cloned 5' to the luciferase reporter gene. In addition, the mouse promoter fragment containing the  $-51/+120$  sequence shows about 23% of the maximal promoter activity. Only deletion to position  $-31$

leads to a marked decrease in promoter activity to about 6%. The reasons for this apparent difference between the two sets of data are not clear. One might speculate that the species difference in the promoter and the cell types used in the transfection studies might contribute to this dissimilarity. It is unlikely that the difference in activity lies in the two promoters (mouse *vs.* rat), because their base sequences are almost identical. It is possible that the difference in cell origin (human *vs.* rat and liver *vs.* calvaria) and the physiological state of the cells (transformed *vs.* nontransformed) used in these studies may contribute to the disparity. The published study was performed in a heterologous system with the mouse promoter in human hepatoma cells and the present study was performed in a homologous system with the rat promoter in rat calvaria cells.

In the present study we identified a 21-bp promoter element that mediates the down-regulation of IGFBP-5 by OP-1. The element is located between  $-71$  and  $-51$  in the rat IGFBP-5 promoter and is the first reported negative OP-1-responsive element that causes repression of the IGFBP-5 gene. The 21-bp OP-1-responsive element contains a CAAT-like sequence, a C/EBP $\alpha$ -like element, and a c-Myb-like or E-box-like motif. Mutations of the CAAT-like sequence and the C/EBP $\alpha$ -like elements reduced the overall transcription activity of the promoter, but did not eliminate the OP-1 response specifically. Thus, it is unlikely that this *cis*-element plays an important role in the OP-1-induced down-regulation of IGFBP-5 gene expression. Mutation studies further allowed identification of the putative E-box or c-Myb motif as part of the OP-1-responsive element.

In addition, our results on gel mobility shift assays with the 21-bp sequence and nuclear extract from control and OP-1-treated cells suggest that the OP-1 reduces either the concentration or the affinity of the nuclear protein(s) that interacted with this promoter region in FRC cells, resulting in a decrease in transcription. Such an interpretation is supported by our prior observation that OP-1 down-regulates IGFBP-5 transcription (17). However, the present data could not distinguish the two possibilities. Furthermore, the data on gel mobility shift assays using oligonucleotides with mutations in each of the three conserved elements indicated that an E-box-binding protein(s) or an ancillary protein(s) that binds to the E-box-binding protein(s) may play an important role, through the IGF-IGFBP-axis, in mediating at least in part the effects of OP-1 in inducing bone cell differentiation. Alterations in the ancillary protein may result in a change in the DNA binding affinity of the E-box-binding protein through protein-protein interaction. Although the molecular basis for the decrease in binding remains to be defined, phosphorylation/dephosphorylation of the *trans*-acting factor may be involved. Such a posttranslational modification, in turn, can alter the binding affinity of a regulatory protein.

These conserved elements in the IGFBP-5 proximal promoter are probably involved in the regulation of IGFBP-5 transcription by various agents. For example, previous reports showed that cortisol inhibits IGFBP-5 gene expression in osteoblasts (30). Deletion studies of the IGFBP-5 promoter further showed that the region responsive to cortisol lies between  $-70$  to  $+22$  bp of the promoter, and that the putative E-box or c-Myb motif is required for basal transcription and

cortisol-mediated transcriptional repression. Although this DNA region appears to be responsive to both OP-1 and cortisol, whether an identical base sequence is responsive to both agents is not clear at present. However, the actions of OP-1 and cortisol on bone cells are different; thus, it is unlikely that the two agents use an identical regulatory mechanism(s) for the down-regulation of the IGFBP-5 promoter. It is conceivable that different *trans*-acting factors might be involved. Knowledge of the mechanism(s) of transcriptional down-regulation by OP-1 and cortisol should be informative.

A minimal DNA sequence, spanning  $-69$  to  $-35$  bp, for PGE<sub>2</sub> stimulation of IGFBP-5 promoter activity has been reported recently (28). The sequence contains E box, C/EBP, nuclear factor-1, and activating protein-2 (AP-2) like elements. Mutation of the E box-like element reduced the basal promoter activity by 50% and eliminated the stimulatory effect of PGE<sub>2</sub>. Mutations in the C/EBP or nuclear factor-1 like elements reduced the basal promoter activity without affecting the PGE<sub>2</sub> effects. An earlier report revealed two PGE<sub>2</sub>-responsive regions, located between  $-2695$  to  $-1470$  and  $-989$  to  $-332$  bp (35).

Progesterone also stimulates IGFBP-5 gene transcription. Recently, a progesterone-responsive region, spanning from  $-162$  to  $-124$  bp in the human IGFBP-5 has been identified. The region contains two tandem CACCC box sequences, and mutation of the proximal CACCC box eliminated PG activation (27).

The base sequence of the negative OP-1-responsive element that participates in the OP-1-induced down-regulation of the IGFBP-5 promoter reported here differs from that of the positive OP-1-responsive elements reported previously (45, 46). For example, OP-1 stimulates mouse collagen type X promoter activity involving a 33-bp region consisting of nucleotides from  $-310$  to  $-278$  (45). The sequence is TTAAAAATAAAAAGGGTGAATCATCATTCCATC. The sequence contains a myocyte-specific enhancer binding factor (MEF)-2-like (TTAAAAATAAAA) sequence and an AP-1-like (TGAATCATCA) sequence. Both elements are necessary for the OP-1 effect. In rat calvaria-derived chondrogenic cells, OP-1 induced nuclear protein interaction with the MEF-2-like sequence, but not with the AP-1-like sequence. Additionally, a 316-bp BMP-responsive region in the chicken collagen type X promoter has been reported (46). The region was more responsive to BMP-4 than to BMP-2 and -7. Although the BMP-responsive element contains numerous putative regulatory protein-binding sites (such as Inf- $\alpha$ , NF-E1, Ets-1/PEA-3, HNF-5, CBF, AP-2, MBF-1, GATA-1, and c-Mos), it does not contain the MEF-2-like sequence or the AP-1-like sequence reported for the OP-1-responsive element in the mouse collagen type X promoter. The region also does not contain the E-box/c-Myb-like sequence reported here for the negative OP-1-responsive element in the rat IGFBP-5 promoter.

In summary, the present study demonstrates that the gene element responsible for the down-regulation of IGFBP-5 by OP-1 is located between  $-71$  and  $-51$  in the rat IGFBP-5 promoter. The E-box/c-Myb-like motif is necessary for the OP-1 effect. The results also show the presence of a nuclear protein(s) in FRC cells that binds to the DNA region. The observed decrease in the concentration or activity of this protein(s) in OP-1-treated FRC cells further suggests that a

change in the nuclear protein might be the mechanism for the down-regulation of IGFBP-5 in OP-1-treated FRC cells.

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