Fibroblast Growth Factor Receptor 3 Gene Transcription Is Suppressed by Cyclic Adenosine 3’,5’-Monophosphate

IDENTIFICATION OF A CHONDROCYTIC REGULATORY ELEMENT

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Signaling through fibroblast growth factor receptors (FGFRs) is critical for the development and patterning of the vertebrate skeleton. Gain-of-function alleles of \textit{fgfr2} and \textit{fgfr3} have been linked to several dominant skeletal disorders in humans, while null mutations in \textit{fgfr3} result in the overgrowth of long bones in a mouse model system. Interestingly, the expression pattern of \textit{fgfr3} in growth plate chondrocytes overlaps that of the parathyroid hormone (PTH)-related peptide (PTHrP) receptor, a signaling molecule that also regulates endochondral ossification. The coincident expression of these two receptors suggests that their signaling pathways may also interact. To gain insight into the regulatory mechanism(s) that govern the expression of the \textit{fgfr3} gene in chondrocytes, we have identified a cell-specific transcriptional regulatory element (CSRh) by measuring the activity of various promoter fragments in FGFR3-expressing (CFK2) and nonexpressing (RCJ) chondrocyte-like cell lines. Furthermore, we demonstrate that activation of PTH/PTHrP receptors, either by stimulation with PTH or through the introduction of activating mutations, represses CSRh-mediated transcriptional activity. Finally, the transcriptional repression of the CSRh element was mimicked by treatment with forskolin, 8-bromo-cAMP, and 3-isobutyl-1-methylxanthine or by overexpression of the catalytic subunit of protein kinase A. Together, these data suggest that protein kinase A activity is a critical factor that regulates \textit{fgfr3} gene expression in the proliferative or prehypertrophic compartment of the epiphyseal growth plate. Furthermore, these results provide a possible link between PTHrP signaling and \textit{fgfr3} gene expression during the process of endochondral ossification.

Elaboration of the vertebrate skeleton occurs via two overlapping, yet distinct developmental pathways. Intramembranous ossification, the primary pathway for flat bone development, relies upon the direct differentiation of condensed mesenchyme into osteoblasts. These osteoblasts then secrete various matrix components until they are encapsulated by calcified bone, thus linking the rate of intramembranous bone growth to the rate of osteoblast differentiation. Alternatively, endochondral ossification, the primary pathway for formation of the axial and appendicular skeleton, differs from intramembranous ossification in that condensed mesenchymal cells (chondrocytes) elaborate a complex cartilaginous template as they progress through a series of developmental stages at the epiphyseal growth plate. The epiphyseal growth plate is organized into distinct cellular compartments: the resting zone, which serves as a renewable source of chondrocytes; the proliferative zone, where rapid cell division results in stacked columns of chondrocytes; and the hypertrophic zone, where the cells terminally differentiate, hypertrophy, and secrete a specialized matrix. After the encapsulated hypertrophic chondrocytes mature, the associated extracellular matrix is rapidly invaded by blood vessels and bone-forming osteoblasts, which synthesize trabecular bone. Thus, the overall rate of longitudinal bone growth is regulated by the progression of chondrocytes through these distinct developmental stages within the epiphyseal growth plate.

\textit{In vitro} and \textit{in vivo} model systems have been utilized to study both the commitment and subsequent differentiation of chondrocytes (1–5). These studies demonstrate that many physiological effectors are capable of modulating chondrocyte differentiation \textit{in vitro} and that mutations or alterations in gene expression can result in skeletal defects \textit{in vivo}. Recent genetic evidence has linked eight different craniosynostosis and chondrodysplasia syndromes to mutations in three of the FGFRs\(^1\) (6, 7). These autosomal dominant mutations affect both intramembranous and endochondral ossification, suggesting that FGF signaling is an essential regulator of skeletal development.

Mutations in the \textit{fgfr3} gene have been linked to several skeletal dysplasias in humans, including achondroplasia (8, 9), thanatophoric dysplasia types I and II (10, 11), and hypochondrodysplasia (12). Achondrodysplasia is the most common genetic form of dwarfism in humans and results from a mutation in the transmembrane domain (G380R) of FGFR3, while thanato-

\(^1\) The abbreviations used are: FGFR, fibroblast growth factor receptor; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; PKA, protein kinase A; EMSA, electromophoretic mobility shift assay; PBS, phosphate-buffered saline; DTT, dithiothreitol; C/EBP, CAAT/enhancer-binding protein; BSV, Rous sarcoma virus; CREB, cAMP-response element binding protein; RORa1/2, retinoid-like orphan receptor \(a\) family 1/2; JMC, Jansen’s metaphyseal chondrodysplasia; CRE, cAMP-response element; FSK, forskolin; 8-Br-cAMP, 8-bromo-cyclic AMP; IBMX, 3-isobutyl-1-methylxanthine; CSRh, cell-specific transcriptional regulatory element; CRBP, CSRh-binding protein(s); S/THr, steroid/thyroid hormone; TRE, thyroid hormone response element; PIPES, piperoxane-\(N\)-\(N'\)-bis[2-ethanesulfonic acid]; nt, nucleotide(s).

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phoric dysplasia is the most common neonatal lethal skeletal dysplasia in humans and results from any of three independent point mutations in the *fgfr3* gene. Clinically, all of these mutations result in a characteristic disruption of growth plate architecture and disproportionate shortening of the proximal limbs. Biochemically, these mutations activate FGFR3 signaling (13, 14). In contrast, inactivation of FGFR3 signaling in mice results in an increase in the size of the hypertrophic zone, as well as a coincident increase in bone length (15, 16). Such complementary phenotypes suggest that FGFR3 signaling is an essential component of the hierarchy of regulatory mechanisms that regulate endochondral bone growth.

Recent studies have demonstrated that PTH/PTHrP receptor signaling, like FGFR3 signaling, regulates the process of endochondral bone growth (17–20). Inactivation of either PTHR or the PTH/PTHrP receptor in mice results in the premature ossification of proliferating chondrocytes as well as a marked decrease in the size of the proliferative zone (17, 18), a phenotype resembling that seen with constitutive activation of FGFR3 signaling. The hypothesis that PTHR signaling regulates the proliferation of chondrocytes by modulating FGFR3 activity provides an explanation for the similarity in these phenotypes. Such control may involve transcriptional, translational, and/or post-translational mechanisms. To this end, we have examined whether PTH/PTHrP receptor signaling regulates the transcriptional activity of the *fgfr3* gene.

To examine the mechanisms governing expression of the *fgfr3* gene in chondrocytes, an “enhancer” element capable of recapitulating the chondrocytic expression pattern of *fgfr3* in *vitro* was identified and shown to be suppressed by PTH/PTHrP receptor signaling. Furthermore, activation of protein kinase A (PKA) signaling, either through the elevation of cAMP levels or through the overexpression of the catalytic subunit of PKA, also suppressed the transcriptional activity of the enhancer element and decreased the steady state levels of the endogenous *fgfr3* gene. These results suggest a model whereby the attenuation of PKA signaling, by effectors such as the PTH/PTHrP receptor, serves to regulate endochondral ossification by modulating the chondrocytic expression of *fgfr3*.

Electrophoretic mobility shift assays (EMSAs) were used to identify a single hexameric “orphan” receptor half-site in the enhancer element that interacts with nuclear protein(s). Elevation of cAMP levels failed to disrupt this DNA-protein complex; however, mutations in this motif that inhibit nuclear protein(s)/DNA interactions do dramatically impair chondrocytic transcriptional activity. Nevertheless, these same mutations did not prevent the cAMP-mediated suppression of transcriptional activity, thus suggesting that there are additional DNA/protein interactions within the enhancer element that are required for the cAMP-dependent regulation of *fgfr3* expression.

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction—* pCMV-luc, pCMV-β-gal (29), 30) were diluted to a final volume of 20 μl with distilled, deionized water. To that was added 250 μl of 0.272 m CaCl2 and 270 μl of 3 m sodium hydroxyl-2-aminoethanesulfonic acid (BES), 280 m NaCl, 1.5 m NaHPO4). The DNA mixture was then allowed to precipitate at room temperature for 15 min, and 80 μl of the precipitant was washed with distilled, deionized water.

**Expression Plasmids and Other DNA Reagents—** The rat FGFR3 RNase protection probe was generated by amplifying rat cDNA with the following primers: DO80, 5′-GGT ATG GGA AGT GTG G-3′; and DO113, 5′-GGT CCG ACA CAT TGG-3′. The resulting product was cloned into pGEM-T (Promega Corp.), and both the identity and orientation of the insert was confirmed through sequencing. The plasmid pTRI-β-Actin-125-Rat was purchased from Ambion Inc.

The expression plasmid for the catalytic subunit of protein kinase A (MT-Ca) was obtained from S. McKnight (23), while empty vector control (pEV142) was provided by R. Palmiter (24). Expression plasmids for wild-type (HKrk) (25) and mutated versions (HKrk-H223R (25) and HKrk-T410P (25)) of the human PTH/PTHrP receptor were provided by H. Jüppner.

Cell Culture and Transfection—**CFK2 (26) and RC3 (27) (clone 3.1.5.18) cell lines were obtained from J. Henderson and J. Aubin, respectively. Cell lines were maintained subconfluent in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% fetal calf serum (Sigma), 2 mML-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). All cells were transfected in triplicate using a modified calcium phosphate precipitate method (28). The day prior to transfection, cells were plated at a density of 8 × 10^4 cells/well in a 12-well plate (Corning). Four hours prior to transfection, cells were seeded with 0.8 ml of fresh media. Five micrograms of each DNA construct (double-banded on a CaCl2 gradient) and 0.5 μg of pS Covently-M (29, 30) were diluted to a final volume of 20 μl with distilled, deionized water. To that was added 250 μl of 0.272 m CaCl2 and 270 μl of 3 m sodium hydroxyl-2-aminoethanesulfonic acid (BES), 280 m NaCl, 1.5 m NaHPO4). The DNA mixture was then allowed to precipitate at room temperature for 15 min, and 80 μl of the precipitant was washed with distilled, deionized water.

**RNase Protection Analysis—** A 2000 μl of luciferase lysis buffer (25 m Tris-HCl (pH 7.8), 2 m dithiothreitol (DTT), 2 m trans-1,2-diaminocyclohexane-N,N,N,N-N-tetracetic acid , 10% glycerol, 1% Triton X-100). Insoluble materials were removed from all lysates by centrifugation at 14,000 rpm for 4 min, and 50–100 μl of the cleared lysate was used to analyze luciferase reporter expression as described previously (31). β-Galactosidase activity was determined with the Galacto-Light Plus system, as described by the manufacturer (Tropix, Inc.). Forskolin (Sigma) was dissolved in MeSO (Sigma) to generate a 1000× stock. The stock solution was diluted to 100× with Ham’s F-12 medium 24 h prior to addition to cells. Synthetic human PTH (1–34) (Sigma) was resuspended in 0.01 m acetic acid and stored at −80 °C. A 2000× stock of PTH was diluted 1:20 with Ham’s F-12 medium prior to the stimulation of PTH/PTHrP receptor-transfected cells.

**RNA Protection Analysis—** Gene expression was determined by RNase protection analysis. Briefly, total cellular RNA was purified from a confluent 10-cm dish using the RNaseasy kit (Qiagen, Inc.). Samples were denatured at 95 °C for 5 min and then incubated at 50 °C for 8 h. Unhybridized probe was removed by adding 350 μl of RNase digestion buffer (10 mTris-HCl (pH 7.5), 300 m NaCl, 5 m NaPO4, 0.5 m NaOAc, 2.5 m glycerol, 1% Triton X-100). Insoluble materials were removed from all lysates by centrifugation at 14,000 rpm for 4 min, and 50–100 μl of the cleared lysate was used to analyze luciferase reporter expression as described previously (31). β-Galactosidase activity was determined with the Galacto-Light Plus system, as described by the manufacturer (Tropix, Inc.). Forskolin (Sigma) was dissolved in MeSO (Sigma) to generate a 1000× stock. The stock solution was diluted to 100× with Ham’s F-12 medium 24 h prior to addition to cells. Synthetic human PTH (1–34) (Sigma) was resuspended in 0.01 m acetic acid and stored at −80 °C. A 2000× stock of PTH was diluted 1:20 with Ham’s F-12 medium prior to the stimulation of PTH/PTHrP receptor-transfected cells.
EDTA, 14 μg of RNase A, 248 units of RNase T1) and incubating the reaction for 30 min at 30 °C. Duplex RNA hybrids were then purified with RNAzol B (Teltest, Inc.), dried under vacuum, and resuspended in 8 μl of RNA loading buffer (1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanole FF, 50% formamide). Samples were denatured for 5 min at 95 °C, chilled on ice, and resolved on a 5% denaturing polyacrylamide gel (Long Ranger; J. T. Baker). Gels were dried under vacuum and exposed to Kodak X-Omat film. When RNase protection assays were quantified, gels were analyzed with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) and quantified with ImageQuant software (version 3.3, Molecular Dynamics). The sizes for the protected fragments are as follows: rat FGFR3, 291 nt; rat C/EBP, 126 nt. The specific activity of the probe was estimated and ultimately diluted to 25,000 cpm/0.1 ml, which consisted of 0.1 pmol of probe (25,000 cpm), 3 μl of bovine serum albumin (Fisher), 1.25 μg of poly(dIdC) (Amersham Pharmacia Biotech), 4 μg nuclear extract, and buffer D. Duplex oligonucleotide competitors were preincubated with the nuclear extract for 5–10 min at 25 °C. The resulting complexes were resolved on a 4–20% gradient gel (Novex) prerun in 0.375× TBE (230 mM Tris borate (pH 8.3), 75 μM EDTA) at 100 V for 10 min as described (31). Gels were dried under vacuum and subsequently exposed to Kodak X-Omat film.

The sense strand of each oligonucleotide used for EMSAs are shown in Table I. To demonstrate the functionality of CAAT/enhancer-binding protein (C/EBP), thyroid hormone response element (TRE), and retinoid-like orphan receptor α family 1 (ROrA1) competitors, the duplex DNA binding sites were radiolabeled and used to probe CFK2 nuclear extracts. All competitors were able to form gel shift complexes (data not shown).

**FGFR Expression**—Expression of fgfrs was determined by reverse transcriptase-polymerase chain reaction as described previously (34).

### RESULTS

**Identification and Localization of Chondrocytic Regulatory Elements—**FGFR3 is an essential regulator of endochondral ossification and is expressed in a subset of chondrocytes found within the developing epiphyseal growth plate (15, 16, 35). The specific expression pattern of fgfr3 suggests that it has stage-specific effects on the process of chondrocyte maturation. To examine the regulation of fgfr3 gene expression, two chondrocyte cell lines, CFK2 and RCJ, were utilized. CFK2 cells and RCJ cells, both derived from fetal rat calvarial mesenchyme, express chondrocyte-specific genes and elaborate a cartilage-specific expression pattern of the endogenous fgfr3 gene, which consists of 0.1 pmol of probe (25,000 cpm), 3 μl of bovine serum albumin (Fisher), 1.25 μg of poly(dIdC) (Amersham Pharmacia Biotech), 4 μg nuclear extract, and buffer D. Duplex oligonucleotide competitors were preincubated with the nuclear extract for 5–10 min at 25 °C. The resulting complexes were resolved on a 4–20% gradient gel (Novex) prerun in 0.375× TBE (230 mM

### TABLE I

| Sense strand oligonucleotide sequences used for EMSAs |
|------------------------------------------------------|
| **CSRh** | 5′-CGCGGTACCTCAAAATTGACCTTATTGGAAAGGCTAAGC-3′ |
| **DM201** | 5′-CGCGGTACCTCAAAATTGACCTTATTGGAAAGGCTAAGC-3′ |
| **DM221** | 5′-CGCGGTACCTCAAAATTGACCTTATTGGAAAGGCTAAGC-3′ |
| **DM223** | 5′-CGCGGTACCTCAAAATTGACCTTATTGGAAAGGCTAAGC-3′ |
| **TRE** | 5′-GGTCTAGTCGTAGACGT-3′ |
| **CREB** | 5′-GGTACAGTCGACGTAGACGT-3′ |
| **RORE 1** | 5′-TCGACGTGATACGAAAGGTCTCGAC-3′ |
FIG. 1. Differential expression of FGF receptors and the fgfr3 promoter in chondrocytic cell lines. A, analysis of fgfr expression patterns in chondrocyte-like cell lines. First strand cDNA was synthesized from RNA obtained from both the CFK2 (lanes 1–3) and RCJ (lanes 4–6) cell lines. The kinase domains of fgfrs 1–4 were amplified simultaneously using a single oligonucleotide primer pair (34). The resulting mixture of polymerase chain reaction-amplified products was digested with PstI (lanes 1 and 4), PvuII (lanes 2 and 5), and EcoRI (lanes 3 and 6) to determine the expression of the fgfr1, fgfr3, fgfr2, and fgfr4 genes, respectively. Fragments were resolved on 2% agarose gels and visualized by silver staining. Predicted sizes for PstI digests (lanes 1 and 4) are as follows: fgfr1, 133 and 207 nt; fgfr3, 176 and 166 nt. Predicted sizes for PvuII digests (lanes 2 and 5) are as follows: fgfr2, 195 and 146 nt; fgfr2, 233 and 108 nt. Predicted sizes for EcoRI digests (lanes 3 and 6) are as follows: fgfr4, 217 and 124 nt. The arrowhead denotes the position of PstI-digested fgfr3 cDNA and is seen in RSV-derived cDNA (lane 1) but not in RCJ-derived cDNA (lane 2). The undigested polymerase chain reaction amplification product in lane 2 also indicates fgfr3 cDNA, which is not digestible with PvuII; this band is not present in lane 5, indicating the absence of fgfr3 expression in RCJ cells. M, 123-base pair molecular size standards (Life Technologies). B, comparison of p[−2951/−27]FR3-luc, p[−2311/−27]FR3-luc, and p[−1537/−27]FR3-luc transcriptional activities in CFK2 (shaded bars, fgfr3-expressing) and RCJ (open bars, fgfr3-nonexpressing) chondrocyte-like cell lines. All bar and column charts show data representative of at least three independent experiments. Constructs were transfected in triplicate, and both luciferase and β-galactosidase activities were determined as described under “Experimental Procedures.” Values are plotted as -fold induction over the empty vector (pGL2-Basic), and -fold induction was calculated by dividing the mean ± S.D. derived for each construct by the mean of the pGL2-Basic construct (pGL2-Basic = 1).

FIG. 2. CSR sequences confer selective transcriptional activity upon the RSV minimal promoter. Top, schematic representation of heterologous promoter construct. Bottom, sequences between −2311 and −1537 of the fgfr3 gene were cloned 5′ to the RSV minimal promoter and were tested for transcription-enhancing activity in CFK2 cells (shaded bars) and RCJ cells (open bars). Data are presented as in Fig. 1B; however, -fold induction was determined relative to the pRSV-luc heterologous promoter construct (pRSV-luc = 1).

elements capable of recapitulating the chondrocytic expression of the fgfr3 gene in vitro are located between nucleotides −2311 and −1537. Furthermore, the CSR sequences alone could confer selective transcriptional activity upon a heterologous minimal promoter and do not require additional fgfr3 promoter elements to selectively activate transcription in CFK2 cells.

To identify the chondrocyte-specific regulatory element(s) found within the −2311 to −1537 region, various pieces of the CSR sequences were multimerized into three-copy, head-to-tail arrays and placed 5′ relative to the RSV minimal promoter (Fig. 3A). The transcriptional activity of reporter constructs bearing the 5′-most 50 nucleotides of the CSR (pCSRa-luc, pCSRb-luc, pCSRe-luc, pCSRf-luc, pCSRh-luc) was dramatically enhanced in fgfr3-expressing CFK2 cells (Fig. 3A), while little or no enhancement of RSV-mediated transcriptional activity was observed in RCJ cells (data not shown). Therefore, the position of chondrocytic “enhancer” activity was further refined to nucleotides −2311 and −2263 of the fgfr3 gene. Analysis of these sequences, using the MatInspector program (36, 37), identified binding sites for several known transcription factors (Fig. 3B). Of interest were putative binding sites for the cAMP response element-binding protein (CREB), thyroid hormone receptor (T3R), and the RORα1/2, all components of signaling pathways that are known to affect chondrocyte development (2, 38–43).

FIG. 3. Localization of chondrocytic transcriptional regulatory element(s) in the region −2311 to −1537. A, the transcriptional activity of deletion constructs generated from the pCSR-luc reporter were assessed in CFK2 cells. A schematic diagram of the CSR of the fgfr3 promoter depicts the following constructs: CSRa, −2311/−1947; CSRB, −1968/−1534; CSRe, −2311/−2117; CSRd, −2139/−1947; CSRf, −2226/−2016; CSRg, −2311/−2167; CSRh, −2311/−2209; and CSRi, −2311/−2263. Note that enhanced transcriptional activity is observed with all constructs containing the 5′-most 50 nt. Data are presented as in Fig. 2 (pRSV-luc = 1). B, genomic sequence encompassing the −2311 to −2263 chondrocytic region of the fgfr3 gene. Shown both above and below the sequence are binding sites for known transcription factors: thyroid hormone response element (T3R), RORα1/2, CREB, and C/EBP. All sites show a greater than 90% similarity to known binding sites.
FIG. 4. Regulation of CSRh-mediated transcriptional activity by PTH/PTHrP receptors. A, repression of pCSRh-luc by co-expression of PTH/PTHrP receptors. pCSRh-luc was transfected into CFK2 cells along with pcDNA-I, HKrk (WT), HKrk-T410P (T410P), or HKrk-H223R (H223R) expression vectors (see “Experimental Procedures”). Thirty-six hours post-transfection, cells were starved in Ham’s F-12 medium containing 0.5% serum (12 h) prior to stimulation with or without 50 nM human PTH-(1–34) (12 h). Lysates were harvested, and data were plotted as percentage of control (pcDNA-I vector = 100%). B, regulation of pCRE-luc transcriptional activity by co-expression of PTH/PTHrP receptors. CFK2 cells were transfected with pCRE-luc, and one of the various expression vectors as described for A. Data are presented as fold induction (vector with or without PTH transfections = 1) and were calculated by dividing mean ± S.D. derived for each treatment by the mean of the mock transfection.

FIG. 5. cAMP-mediated repression of FGFR3 gene expression. A, response of pCSRh-luc to modulation of cAMP levels. CFK2-transfected cells were starved for 12 h in 0.5% serum prior to treatment with 0.1% Me2SO (Mock), 10 μM FSK, 1 mM 8-Br-cAMP, or 0.5 mM IBMX. Cells were treated for 12 h, and then both luciferase and β-galactosidase activities were determined. Data are presented as percentage of control as in Fig. 4B. B, AMP/cAMP dose-response curves. CFK2 cells were transfected with pCSRH, starved in low serum conditions as described above, and then treated with varying concentrations of either AMP (●) or 8-Br-cAMP (○) for 12 h prior to harvest. C, cAMP-mediated repression of p(2311/27)FR3-luc-transfected CFK2 (shaded bars) and RCJ (open bars) cells were starved for 12 h in 0.5% serum prior to treatment with either 0.1% Me2SO (Mock) or 10 μM FSK. Cells were treated for 12 h prior to the determination of both luciferase and β-galactosidase activities. Data are presented as in Fig. 4A. D, FSK-induced repression of CFK2-specific endogenous rat fgfr3 gene expression. Cells were serum-starved for 12 h in 0.5% serum prior to treatment for 12 h with either 0.1% Me2SO (DMSO) or 10 μM forskolin. RNA was harvested, and the relative levels of rat FGFR3 mRNA and β-actin were determined simultaneously using RNASense protection. The rat FGFR3 probe protects a 291-nt fragment, and the β-actin probe protects a 158-nt fragment.

The repression mediated by both the wild-type and T410P mutant PTH/PTHrP receptors could be enhanced by the addition of 50 nM PTH, while treatment of the H223R-transfected cells with PTH failed to augment the observed transcriptional suppression (Fig. 4A, compare wild type, T410P, and H223R transfusions with and without PTH). Taken together, these data suggest that CFK2 cells express low levels of a PTHrP-like ligand and that signaling through the PTHrP receptor can regulate fgfr3 gene expression through the CSRh enhancer element.

To confirm that PTH/PTHrP receptors were functioning as expected in CFK2 cells, both wild-type and JMC-associated PTHrP receptors expression constructs were co-transfected with a cAMP-responsive (pCRE-luc) reporter gene. Relative to empty vector, co-expression of the wild-type PTH/PTHrP receptor resulted in a 2.2-fold induction of CRE-mediated transcriptional activity, while stimulation of wild-type receptor-expressing cells with 50 nM human PTH-(1–34) resulted in a 10-fold increase in transcriptional activity (Fig. 4B, compare wild-type receptor transfected cells with or without PTH with vector controls). Unlike the modest stimulation observed as a result of expression of the wild-type receptor, co-expression of either the T410P or H223R mutant PTH/PTHrP receptors resulted in a PTH-independent 5.6- or 6.3-fold increase in CRE-mediated transcriptional activity, respectively (Fig. 4B, compare T410P and H223R mutant receptor-transfected cells with vector transfection). Stimulation of the T410P mutant receptor with 50 nM PTH resulted in a significant increase in CRE-mediated transcriptional activity (Fig. 4B, compare T410P transfected cells with and without PTH), while treatment of the H223R-transfected cells with PTH failed to augment CRE-mediated transcriptional activity.

cAMP-mediated Repression of the Chondrocyte Regulatory Element and Endogenous fgfr3—To determine whether the PTH/PTHrP receptor-mediated transcriptional repression of the CSRh enhancer element depends upon cAMP-mediated signaling pathways, pCSRh-luc-transfected CFK2 cells were serum-starved for 12 h to lower background levels of cAMP. Transfected cells were then exposed to various pharmacological agents known to elevate the intracellular concentration of cAMP. Activation of adenylyl cyclase with 10 μM forskolin (FSK) resulted in a 90% decrease in the transcriptional activity of pCSRh-luc (Fig. 5A). Moreover, similar decreases in the CFK2-specific transcriptional activity of pCSRh-luc were observed when cAMP levels were elevated either directly with 8-bromo-cyclic adenosine 3’5’-monophosphate (8-Br-cAMP; a cell-permeable cAMP analog) or indirectly through the inhibition of cAMP phosphodiesterase activity with 3-isobutyl-1-methylxanthine (IBMX) (47) (Fig. 5A, 81 and 95%, respectively).

To examine the specificity of this transcriptional inhibition, the regulatory effects of AMP, the noncyclic precursor to cAMP, were compared with 8-Br-cAMP. The transcriptional activity of pCSRh-luc was unaffected by as much as 1 mM AMP, while a dose-dependent repression of CSRh-mediated transcriptional ac-
tivity was observed with 8-Br-cAMP (Fig. 5B). A 50% decrease (IC_{50}) in CSRh-mediated transcriptional activity resulted from treatment with 70 μM 8-Br-cAMP, while maximal inhibition (approximately 90%) was obtained with 300 μM 8-Br-cAMP. Together, these data demonstrate that the transcriptional potential of the CSRh enhancer element is regulated by cAMP.

In addition, we examined the transcriptional response of both the full-length promoter construct (p(-2311/-27)FR3-luc) and the endogenous fgfr3 gene to increasing concentrations of cAMP. Treatment of serum-starved CFK2 cells with 10 μM forskolin specifically repressed CFK2-cell specific transcriptional activity of p(-2311/-27)FR3-luc while having little or no effect upon the basal transcriptional activity of p(-2311/-27)FR3-luc in RCJ cells (Fig. 5C). To examine the effects of cAMP on the expression of the endogenous gene, serum-starved CFK2 cells were stimulated for 12 h with either 0.1% Me_{2}SO or 10 μM forskolin, and the relative levels of fgfr3 mRNA were determined by RNase protection (Fig. 5D). Band intensities were quantified, and the decrease in fgfr3 mRNA levels was determined relative to an internal β-actin control. Treatment with Me_{2}SO failed to repress the steady state levels of fgfr3 mRNA, while treatment with 10 μM forskolin resulted in a 46% decrease in fgfr3 mRNA levels. A second experiment was performed following a 24-h induction with forskolin. At this time point, no change in fgfr3 mRNA levels was observed relative to β-actin. Together, the transcriptional response of the full-length promoter and of endogenous fgfr3 to forskolin support the hypothesis that fgfr3 can be regulated by the intracellular concentration of cAMP.

**Protein Kinase A Overexpression Represses CSRh-mediated Transcription—** cAMP levels can affect gene transcription by selectively activating PKA (48). Through its activation, PKA has been shown to augment the transcription of genes that contain a CRE(s) by selectively phosphorylating CREB at Ser-133 (49). Phosphorylated CREB is then capable of enhancing transcription by interacting with the CBP/p300 family of transcriptional co-activators (50, 51).

In the inactive form, PKA exists as a tetramer of two catalytic (Ca) and two regulatory subunits. Binding of cAMP by the regulatory subunits results in the dissociation of the tetramer complex and release of the active Ca subunit (52). To assess the ability of Ca overexpression to mimic the effects of PKA activation, the transcriptional response of pCRE-luc was examined after co-transfection with the Zn^{2+}-inducible metallothionein promoter-Ca (MT-Ca) expression vector (23). As expected, co-transfection of MT-Ca resulted in a 3-fold increase in the transcriptional activity of pCRE-luc, while the Zn^{2+}-dependent overexpression of Ca led to a 12-fold enhancement in CRE-mediated transcriptional activity (Fig. 6A, compare vector with and without Zn^{2+} with MT-Ca with and without Zn^{2+}). Therefore, overexpression of the catalytic domain of PKA mimics cAMP-mediated transcriptional activation of CRE-containing genes.

To determine if PTH/PTHrP receptor-mediated repression of the CSR sequences also depends on PKA activation, the MT-Ca expression vector was co-transfected with the pCSRh-luc reporter construct. Although treatment of the control vector-transfected cells with Zn^{2+} resulted in a 27% decrease in transcriptional activity (Fig. 6B, compare vector co-transfections with and without Zn^{2+}), an additional 50% decrease in CSRh-mediated transcriptional activity was observed when Co activity was induced with 80 μM Zn^{2+} (Fig. 6B, compare MT-Ca transfections with and without Zn^{2+} treatment). These data suggest that the repression of CSRh-mediated transcriptional activity by cAMP results from the activation of PKA signaling cascades.

**Nuclear Factor(s) Interact with 5' PuGGTCA-3' Core and Regulate the Transcriptional Potential of the CSRh Sequences—** Recruitment or stabilization of the transcriptional initiation complex by DNA-binding proteins and/or associated accessory factors is thought to be the underlying mechanism that regulates gene expression. To identify the DNA-protein interaction(s) that direct the observed chondrocytic transcriptional activity, the CSRh fragment was radiolabeled and used as a probe in EMSAs. Incubation of radiolabeled CSRh with extracts from CFK2 cells identified one major complex (Fig. 7A, lane 2); however, a complex with a similar mobility was also obtained in EMSAs with extracts from both the full-length promoter construct (pCSRF142 (vector) or MT-Ca expression plasmids. Transfected cells were then treated as described above; however, data are presented as percentage of control (untreated vector = 100%).

**Fig. 6. Catalytic subunit of protein kinase A (Ca-PKA) mimics transcriptional repression.** A, activation of pCRE-luc by overexpression of Ca. CFK2 cells were transfected with combinations of pCRE-luc and either pEV142 (vector) or MT-Ca expression plasmids. Transfected cells were then treated as described above; however, data are presented as percentage of control (untreated vector = 100%).

A number of different Zn^{2+} finger-containing transcription factors recognize DNA motifs similar to the 5' PuGGTCA-3' hexamer element found in the CSRh fragment (53). To determine if this motif is involved in the binding of the CSR-BP complex, nuclear extracts derived from CFK2 cells were preincubated with various unlabeled competitors. The CSR-BP-CSRh complex was completely disrupted by competition with a 10-fold molar excess of unlabeled probe (Fig. 7B, lanes 7–9), while a C/EBP consensus binding site failed to compete efficiently for the binding of CSR-BP even at a 100-fold molar excess (Fig. 7B, lanes 10–12). Significant competition for the binding of CSR-BP was also observed with TRE and RORα1 consensus binding sites (Fig. 7C, lanes 14–16 and 17–19, respectively), while, surprisingly, a known CREB binding site failed to compete effectively even at a 100-fold molar excess (Fig. 7C, lanes 20–22).

A number of different Zn^{2+} finger-containing transcription factors recognize DNA motifs similar to the 5'-PuGGTCA-3' hexamer element found in the CSRh fragment (53). To determine if this motif is involved in the binding of the CSR-BP complex, nuclear extracts derived from CFK2 cells were preincubated with several CSRh-like competitors (Fig. 8A). Significant competition for the binding of radiolabeled CSRh was observed when CFK2 cell extracts were preincubated with either DM201/2, a duplex DNA fragment corresponding to the sequence between -2311 and -2276 of the mouse fgfr3 gene, or DM221/2, a duplex DNA fragment containing a tetranucleotide substitution outside of the Zn^{2+} finger DNA binding motif (Fig. 8B, lanes 2–4 and 8–10, respectively). However, mutations that disrupt the 5'-PuGGTCA-3' motif (DM223/4) failed to compete for the formation of the CSR-BP-CSRh complex, even at a 100-fold molar excess (Fig. 8B, lanes 5–7). Identical results were obtained when DM201/2, DM223/4, and DM221/1
were radiolabeled and used as EMSA probes directly (data not shown). Incubation of CFK2 nuclear extracts with DM201/2 and DM221/2 resulted in the formation of a stable DNA-protein complex, while DM223/4 failed to form a resolvable DNA-protein complex (data not shown).

To determine whether the loss of protein binding activity observed with the DM223/4 mutations correlated with a loss in CSRh-mediated transcriptional activity, mutations in the Zn\(^{2+}\) finger DNA binding motif (Fig. 8A, DM223/4) were introduced into pCSRh-luc (pmCSRh-luc), and the resulting transcriptional activity was examined in CFK2 cells. Disruption of the 5′-PuGGTCA-3′ motif resulted in a 75% decrease in CSRh-mediated transcriptional activity (Fig. 8C). These data suggest that the 5′-PuGGTCA-3′ sequence motif regulates the transcriptional activity of the CSRh fragment by facilitating the formation of a CSR-BP-CSRh transcriptional regulatory complex.

To determine if cAMP levels affect the transcriptional activity of the CSRh element by regulating the formation of the CSR-BP-CSRh complex, modified nuclear extracts were prepared from cells treated with 0.1% Me\(_2\)SO, 10 \(\mu\)M forskolin, or 0.5 mM IBMX. Although treatment with either forskolin or IBMX resulted in a 90–95% decrease in transcriptional activity (Fig. 5A), the CSRh binding activity found in CFK2 cells was not affected by elevations in the concentration of cAMP (Fig. 8D).

The 5′-PuGGTCA-3′ Motif Is Not Sufficient for cAMP-mediated Suppression of the CSRh Element—To determine whether the Zn\(^{2+}\) finger binding motif is both necessary and sufficient for the cAMP-mediated suppression of the CSRh enhancer element, the transcriptional response to forskolin of the full-length promoter containing the Zn\(^{2+}\) finger mutation (pm(-2311/-27)FR3-luc) was examined. Like pm(-2311/-27)FR3-luc, the transcriptional activity of pm(-2311/-27)FR3-luc was suppressed by treatment with forskolin, whereas the activity of the promoter lacking the CSR element (p(-1537/-27)FR3-luc) was largely unaffected (Fig. 9). Despite the marked decrease in basal activity, pmCSRh-luc also remained responsive to cAMP (Fig. 9). Together, these results suggest that another CSRh binding activity (or activities) is required for cAMP-mediated suppression of the CSRh enhancer element.

**DISCUSSION**

Recently, both gain-of-function and loss-of-function mutations in fgfr3 have revealed unique roles for this receptor dur-
ing development (6, 15, 16, 54). Loss-of-function alleles of fgfr3 lead to an increase in the size of the hypertrophic zone and the subsequent overgrowth of long bones (15, 16), while gain-of-function mutations in fgfr3 have been genetically linked to autosomal dominant disorders where both the size and architecture of the epiphyseal growth plate are altered (8–11). Together, these observations demonstrate that FGFR3-mediated signaling is an essential regulator of endochondral ossification.

Previous studies that initially characterized the fgfr3 promoter identified sequences between −220 and +612 of fgfr3 that were capable of promoting efficient transcription in vitro, as well as supporting tissue-specific expression in vivo (21). Nevertheless, these minimal elements failed to recapitulate the entire tissue-specific expression pattern of fgfr3 and suggested the existence of other “enhancer” elements. A chondrocytic “enhancer” capable of recapitulating the in vitro expression pattern of the endogenous fgfr3 gene was identified by examining the transcriptional activity of a number of different reporter constructs in fgfr3-expressing (CFK2) and nonexpressing (RCJ) cell lines. The sequences found between −2311 and −1537 (CSR) of fgfr3 enhanced the transcriptional activity of the fgfr3 promoter in CFK2 cells, yet did not affect basal transcriptional activity in RCJ cells. The inability of the CSR enhancer to stimulate transcriptional activity in RCJ cells demonstrated that this element is not required for fgfr3 basal promoter activity and that it functions in a cell-specific manner. It should also be noted that sequences located 5′ to the CSR suppressed CFK2-specific transcriptional activity; however, the nature of this repression remains to be identified and characterized.

In many cases, transcription factor binding sites are modular and can confer transcriptional activity upon heterologous promoters. Using the RSV minimal promoter, sequences between −2311 and −1537 of fgfr3 were found to selectively enhance the RSV-mediated transcriptional activity in CFK2 cells, thus demonstrating that these sequences are both necessary and sufficient for chondrocytic cell-specific regulation in vitro. Additional deletion constructs were used to map the chondrocytic “enhancer” activity to sequences between −2311 and −2263 (referred to as CSRh). Analysis of this sequence identified overlapping binding sites for two steroid/thyroid hormone receptor superfamily members and a CREB-like binding site.

Like FGFR3, both gain-of-function and loss-of-function alleles of the PTH/PTHrP receptor have been linked to defects in endochondral bone growth (17, 25, 55). These results demonstrate that PTHrP signaling, like FGF signaling, regulates endochondral ossification. In situ hybridization studies have demonstrated that the expression pattern of the PTH/PTHrP receptor and fgfr3 overlap in the epiphyseal growth plate and suggest that PTHrP signaling may modulate the chondrocytic expression of fgfr3 in vivo.

Both PTH and PTHrP, through their interaction with a common G protein-coupled receptor (PTH/PTHrP receptor), activate cAMP and Ca^{2+} second messenger signaling pathways by stimulating adenylate cyclase and/or phospholipase C activity, respectively (46). To determine if PTH/PTHrP receptor signaling could affect the transcriptional activity of the CSRh enhancer element, both wild-type and the constitutively active JMC isoforms of the human PTH/PTHrP receptor were co-transfected with pCSRh-luc. Although ligand-independent suppression of the CSRh enhancer was observed with all receptor isoforms, only cells expressing either the wild-type or the T410P mutant PTH/PTHrP receptors responded to PTH stimulation by further suppressing the transcriptional activity of the CSR element.

Biochemical characterization of the JMC mutations has demonstrated that both the T410P and H223R mutations induce the ligand-independent production of cAMP (55); however, PTH/PTHrP receptors harboring the H223R mutation fail to stimulate inositol phosphate turnover in response to ligand binding (25). Therefore, these results, as well as preliminary studies looking at the role of intracellular Ca^{2+}, suggest that maximal repression of the CSRh enhancer element requires both the cAMP- and Ca^{2+}-dependent signaling pathways. Such synergistic “cross-talk” between cAMP and Ca^{2+} second messenger signaling cascades may result from a positive feedback loop involving the activation of Ca^{2+}/calmodulin-stimulated adenylate cyclases (56). Additional studies will be required to elucidate the transcriptional role of Ca^{2+}.

Forskolin, a naturally occurring diterpene, elevates intracellular cAMP levels by directly activating adenylate cyclase. Unlike activation of the well-characterized CREB signaling pathway, forskolin suppressed the CFK2-specific transcriptional activity of the p(−2311/−27)FR3-luc and pCSRh-luc reporter constructs, as well as expression of the endogenous fgfr3 gene. These observations, as well as the dose-dependent repression of pCSRh-luc activity by 8-Br-cAMP, demonstrated that the chondrocyte-specific transcriptional activity of the CSRh enhancer element can be down-regulated by cAMP.

Enhancer elements influence transcription by facilitating and/or stabilizing the assembly of the transcriptional initiation complex. Therefore, EMSAs were used to identify potential protein/CSRh DNA interactions. These experiments identified one DNA-protein complex in CFK2 nuclear extracts (CSR-BP/CSRh). Proteins in the CSR-BP/CSRh complex were recognized by steroid/thyroid hormone (S/THR) binding site (T3R, RORα1, and rOCRE (data not shown)) competitors but did not interact with known cAMP-responsive consensus binding sites (C/EBP and CREB), even at a 100-fold molar excess. The interaction of steroid/thyroid hormone transcription factors with the DNA helix relies upon the conserved 5′-PuGGTCA-3′ hexamer motif (53). Although most of the S/THRs bind as homo- or heterodimers to variably spaced direct or indirect repeats of this motif (57), several of the orphan S/THR have been shown to bind as monomers to single half-sites (58, 59). Previously, S/THR have been implicated in the cAMP-dependent transcriptional regulation of various cytochrome P450 (steroid hydroxylase) genes (60). Characterization of the transcriptional regulation of the P450c17 and P450c21 genes identified cAMP-responsive half-site binding that differ from the classic CRE-motif (61). These sites resemble the classic 5′-PuGGTTCA-3′ hexamer orphan receptor half-site and bind various members of the S/THR superfamily (SF-1 and COUP-TF (62–64) and NGFI-B (65), respectively). Together, these results suggest that the factor(s) binding to the CSR enhancer element belong to the S/THR superfamily and are not related to the CREB/CREM/ATF family of basic leucine zipper transcription factors.

Because the CSRh sequence appears only to contain a single half-site, the effects of elevated cAMP levels and disruption of the 5′-PuGGTTCA-3′ sequence motif on CSR-BP/CSRh complex formation were examined. First, treatment of cells with forskolin does not appear to alter the formation of the CSR-BP/CSRh complex, suggesting that additional elements not identified by EMSAs interact with the CSRh sequence. Second, such factors are required to modulate the cAMP response, and the action of these elements is independent of CSR-BP/CSRh complex formation. Third, a mutation that altered three of the six consensus nucleotides inhibited formation of the CSR-BP/CSRh complex and significantly reduced the CSRh-mediated transcriptional activation of pCSRh-luc; however, it does

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2 D. G. McEwen and D. M. Ornitz, unpublished observations.
not abolish the CAMP-induced suppression of the CSRh enhancer element or the full-length promoter. Together, these results suggest that the orphan receptor half-site is required for maximal CFK2-specific transcriptional activity, but other undefined DNA/protein interactions are required for CAMP responsiveness. Additional experiments will be required to determine which portion of the CSRh sequence is important for mediating the CAMP response and the proteins that mediate the response.

The data presented in this study suggest that signaling cascades in the epiphysial growth plate capable of modulating the amount of CAMP and the activity of PKA may affect chondrocyte growth and differentiation by regulating fgfr3 gene expression in proliferating chondrocytes. Recently, interactions between the signaling pathways involving Indian hedgehog and PTHrP have been reported in the growth plate (17, 45). Coordination of these signals appears to be necessary to maintain the orderly transit of chondrocytes through the growth plate. Animals deficient in either PTHrP or the PTHrP receptor have decreased chondrocyte proliferation (17, 18). Ligand-in-dependent activating mutations in FGFR3 (G380R, R248C, or K650E) (13, 14) also decreases chondrocyte proliferation (44, 66). Therefore, we hypothesize that one pathway by which PTHrP can stimulate chondrocyte proliferation may involve down-regulation of fgfr3 expression. This would link the Indian hedgehog/PTHrP signaling pathway to the FGFR3 signaling pathway in the epiphysial growth plate. Interestingly, recent in vivo studies have demonstrated that FGFR3 signaling can repress Indian hedgehog activity in the growth plate (44). This completes a potential feedback loop that may coordinate endochondral bone growth.

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