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Phosphate Regulates Osteopontin Gene Transcription

ABSTRACT

Extracellular inorganic phosphate (ePi) is a key regulator of cementoblast behavior, both *in vivo* and *in vitro*, and results in a marked increase in osteopontin expression *in vitro*. To examine the molecular mechanisms involved in ePi induction of osteopontin gene expression, we transfected a series of osteopontin promoter-luciferase constructs into OCCM-30 cementoblasts. Our results demonstrate that ePi can directly induce osteopontin gene transcription. The region responsive to ePi signaling was localized to a 53-bp region of the promoter between -1454 and -1401 that contains a glucocorticoid response element (GRE). Mutation of the GRE abolished the ePi response, suggesting that glucocorticoid receptor (GR) signaling is required for ePi-mediated transcription. In addition, treatment of cells with the GR antagonist RU-486 (Mifepristone) prevented promoter activation by ePi. The results presented support a model demonstrating that inorganic phosphate regulates OPN gene transcription in cementoblasts through a pathway that requires a functional GR. *Abbreviations*: BSP, bone sialoprotein; DMP1, dentin matrix protein 1; EMSA, Electrophoretic mobility shift assay; ePi, extracellular inorganic phosphate; GRE, glucocorticoid response element; GR, glucocorticoid receptor; OPN, osteopontin; Pi, inorganic phosphate; Q-PCR, quantitative PCR.

KEY WORDS: phosphate, osteopontin, gene transcription.

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INTRODUCTION

To improve therapies targeted at repairing or regenerating oral tissues, we must understand the molecular factors that control the development and remodeling of the hard tissues of the oral cavity. Tooth development occurs during embryogenesis and involves numerous different cell types, including odontoblasts that produce dentin, and cementoblasts (a specialized type of osteoblast) that produce cementum (Fong *et al.*, 2005; Foster *et al.*, 2007). Cementoblasts produce collagenous proteins, primarily collagen type I, and a large number of non-collagenous proteins, including osteopontin, that function to regulate the mineralization of hard tissues.

Osteopontin (OPN) is a glycosylated phosphoprotein that belongs to the SIBLING family of proteins. SIBLING (Small Integrin-Binding Ligand N-linked Glycoproteins) proteins are a family of glycoproteins that are encoded by a gene cluster on chromosomes 4 and 5 in humans and mice, respectively, and play important roles in mineralization (Fisher *et al.*, 2001; Fisher and Fedarko, 2003). OPN plays an important role in bone development as a negative regulator of hydroxyapatite crystal formation, a function supported by studies of OPN-deficient mice which exhibited increased mineral content and crystallinity compared with normal animals (Boskey *et al.*, 2002). OPN can also function as a cytokine during inflammation by binding to integrin receptors, stimulating IL-12 expression (Ashkar *et al.*, 2000). In vascular diseases such as atherosclerosis, blood vessels calcify and ectopically express mineralization markers. These markers include OPN, which inhibits mineralization, as well as other genes that promote mineralization, with the end result being rupture of the damaged vessels (reviewed in Giachelli, 2005).

Using a murine first molar model of tooth development, researchers have shown that OPN is expressed along the tooth root surface during the initial stages of cementogenesis and in the non-mineralized tissues of the periodontal ligament (MacNeil *et al.*, 1995; D'Errico *et al.*, 1997). However, the molecular mechanisms controlling the expression of OPN and other mineralization-associated genes are not well-understood. *In vitro* studies have shown that inorganic phosphate (Pi) is an important regulator of gene expression during bone mineralization. In Pi-treated cementoblasts, mRNA expression of SIBLING family members either increased (OPN, DMP-1) or decreased (BSP) (Foster *et al.*, 2006). Moreover, quantitative PCR and microarray studies have shown that ePi affects the mRNA expression of a wide variety of genes *in vitro,* including genes functioning in phosphate homeostasis (*e.g.*, ANK, TNAP, Pit-1) and cell signaling (*e.g.*, Wnt, BMP-4, and Egr1/2) (Foster *et al.*, 2006; Rutherford *et al.*, 2006). Pi also induces the expression of mineralization-associated genes such as OPN in vascular smooth-muscle cells (Jono *et al.*, 2000). In the present study, we hypothesized that Pi regulates OPN expression at the transcriptional level, and used a promoter-based approach to explore the mechanisms.

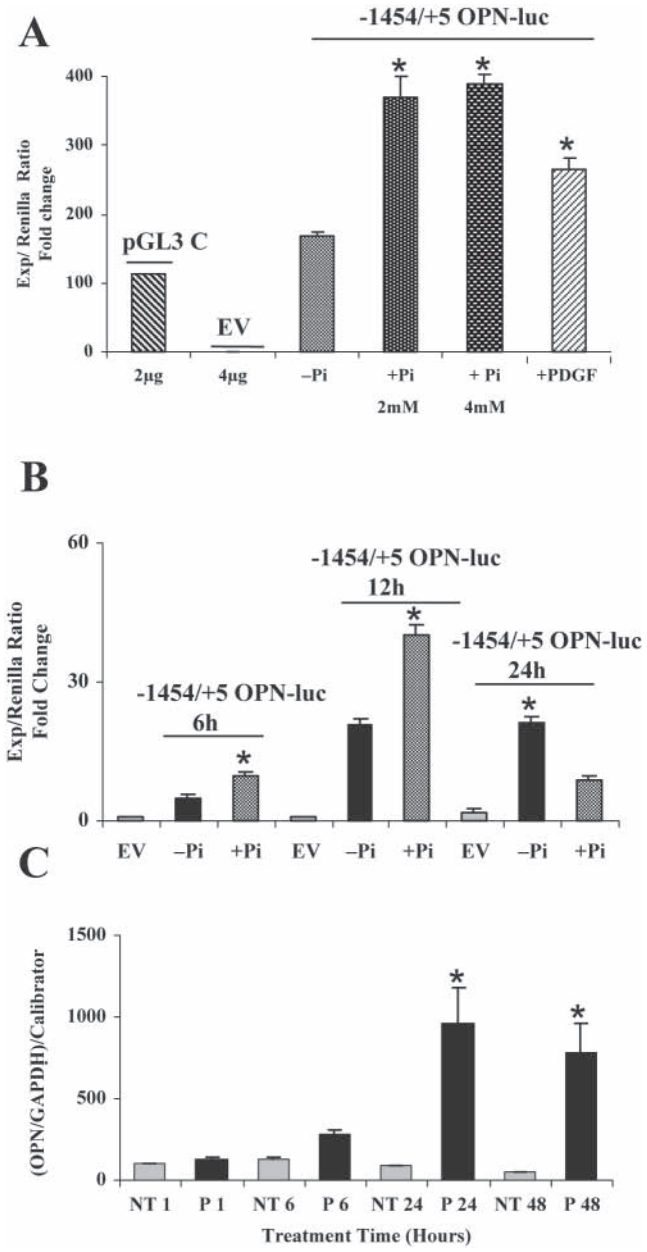

Figure 1. Phosphate activation of the OPN gene in cementoblasts. **(A)** The -1454/+ 5 OPN-luc construct was transfected into OCCM-30 cells, and cells were incubated with ePi for 12 hrs before being harvested. Results are shown normalized to the *Renilla* luciferase activity ± 1 SD. EV (empty vector) and pGL3 C represent cells transfected with pGL3-Basic and pGL3-Control, respectively. The mean Firefly/*Renilla* luciferase values (± SE) were: OPN –ePi/EV, 167.6 ± 5; OPN+ 2mM ePi/EV, 370 ± 29.5; and OPN+ ePi 4mM/EV, 388 ± 15.8. **(B)** Time-course of Pi activation of the OPN promoter. Cells were transfected with -1454/+ 5 OPN-luc and treated with 3 mM ePi (+ Pi) or media (-ePi) for 6, 12, and 24 hrs before being harvested. The mean Firefly/*Renilla* luciferase values of OPN promoter/EV (± SE) were: 6 hrs, 5.82 ± 0.6, 10.42 ± 1.2 (- Pi, + Pi); 12 hrs, 21.6 ± 1.6, 41.85 ± 1.7 (- Pi, + Pi); and 24 hrs, 22.22 ± 1.2, 9.6 ± 1.02 (- Pi, + Pi). **(C)** OPN mRNA expression measured by Q-PCR. Cells were treated with 5 mM ePi (P) or media only (NT) for between 1 and 48 hrs and then harvested for RNA analysis. Q-PCR was performed as described in MATERIALS & METHODS. Results are shown normalized to GAPDH. These experiments were performed in triplicate and represent 1 of the 5 experiments. *P ≤ 0.05.

MATERIALS & METHODS

OPN Promoter Constructs

A 1459-bp fragment of the murine OPN promoter (Genbank accession number AY220127) from -1454 to +5 was amplified from murine genomic DNA with PCR primers (forward, 5′-AATTCTGCATG CCTCTTACACTG-3′; reverse, 5′- GCAAACCCAAGCAAGGAT-3′) and cloned into pGL3-Basic (Promega, Madison, WI, USA) to generate the construct -1454/+5 OPN-luc. Additional deletion constructs lacking distal promoter sequences (denoted -914/+5 OPN-luc, -724/+5 OPN-luc, and -540/+5 OPN-luc, Fig. 2A) were prepared by digestion of -1454/+5 OPN-luc with the restriction enzymes *Kpn* I plus either *Pst* I, *Nhe* I, or *Eco* RI. Additional constructs containing fragments of the upstream OPN promoter (-1454/-914 OPN-luc, -1454/-1134 OPN-luc, -1401/-1134 OPN-luc, and -1401/-1252 OPN-luc) were prepared by PCR and cloned into the pGL3-Promoter vector (Promega). The point mutations in the GRE 3′-half-site sequence were prepared by PCR with 1454/-1134 OPN-luc as the template (for details, see Appendix). We sequenced all constructs completely to confirm that the correct DNA sequence had been cloned.

Cell Culture and Transfections

Immortalized murine cementoblasts (OCCM-30 cells) were grown and maintained as described previously (Foster *et al*., 2006). Cells were treated with 2-5 mM Pi as described and harvested 12 hrs later, except where indicated. Details of the transfection procedures, RNA isolation, and quantitative PCR are provided in the Appendix.

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear protein extracts were prepared from OCCM-30 cells by means of a nuclear extraction kit (Panomics, Fremont, CA, USA), according to the manufacturer's protocol. Typically, 10^6 cells grown in several 100-mm dishes were used as the starting material for each experiment. Protein concentrations ranged from 2 to 4 µg/µL. EMSAs were performed with biotin-labeled double-stranded oligonucleotides as described in the Appendix.

RESULTS

The Murine Osteopontin Promoter is Active in Cementoblasts and is Up-regulated by Inorganic Phosphate

To study the transcriptional regulation of OPN by ePi, we prepared a construct that contains the OPN promoter sequence from -1454 to +5, relative to the RNA start site at +1 (Fig. 2A). The -1454/+5 OPN-luc construct was active in OCCM-30 cells (Fig. 1A). Luciferase activity was dependent on both input DNA concentration (data not shown) and time of transfection, as expected ($P \leq 0.05$; $n = 10$) (Fig. 1B). Phosphate up-regulated OPN promoter activity in a dose-dependent manner. In the presence of 3 mM ePi, OPN promoter activity increased by 63.0 ± 14 fold compared with the EV control ($P = 0.005$, $N = 5$ in triplicate), relative to the control OPN promoter activity seen without ePi treatment [30.2 ± 5.5/EV ($P = 0.005$), $N = 5$ in triplicate] (Fig. 2B). OPN promoter activity reached a maximum level within 12 hrs after ePi addition (Fig. 1B). This result is in contrast to the more sustained effect of ePi on OPN mRNA levels in OCCM-30 cells, which plateaued at 24 hrs after ePi addition (Fig. 1C) (Foster *et al*., 2006). These studies demonstrate that ePi can induce OPN gene transcription in

cementoblasts, which is reflected in an induction of OPN mRNA levels and, presumably, protein.

To delineate the promoter region responsive to ePi regulation, we prepared several deletion constructs, removing distal sequences from the -1454/+5 OPN-luc construct (Fig. 2A). The results of these transfections showed that all of the deletion constructs lacking the distal region between -1454 and -914 did not respond to ePi addition, suggesting that this upstream sequence contained the Pi-responsive region (Figs. 2B, 2C). This was confirmed with additional OPN-luc constructs. Hence, 2 constructs, containing regions of the OPN promoter from -1454 to -914 and -1454 to -1134, showed a robust response to ePi treatment (Fig. 3A). The other 2 constructs (-1401/-1134 OPN-luc and -1401/-1252 OPN-luc) did not respond to ePi addition, suggesting that the essential Pi-responsive region was located within the 53-bp region between -1454 and -1401 (Fig. 2A).

A GRE in the OPN Promoter is Required for Phosphate Responsiveness

An inspection of the minimal 53-bp ePi-responsive region demonstrated the presence of a GRE located between -1417 and -1403. The putative OPN GRE (5′ ATAAGCCTGTGTTCT 3′, with the 2 half-sites underlined) shows 100% identity in the critical 3′ half-site of the GRE that is conserved in a large number of glucocorticoid-responsive genes (Schoneveld *et al.*, 2004) (Fig. 2A). To confirm the involvement of the GRE in the Pi response, we created 2 point mutations, changing the TGTTCT 3′-half-site sequence to ACTTCT and ACTTAC. These mutant GRE constructs, when transfected into OCCM-30 cells, no longer responded to ePi (Fig. 3B). Our results demonstrate that the GRE site is required for ePi induction of OPN gene transcription.

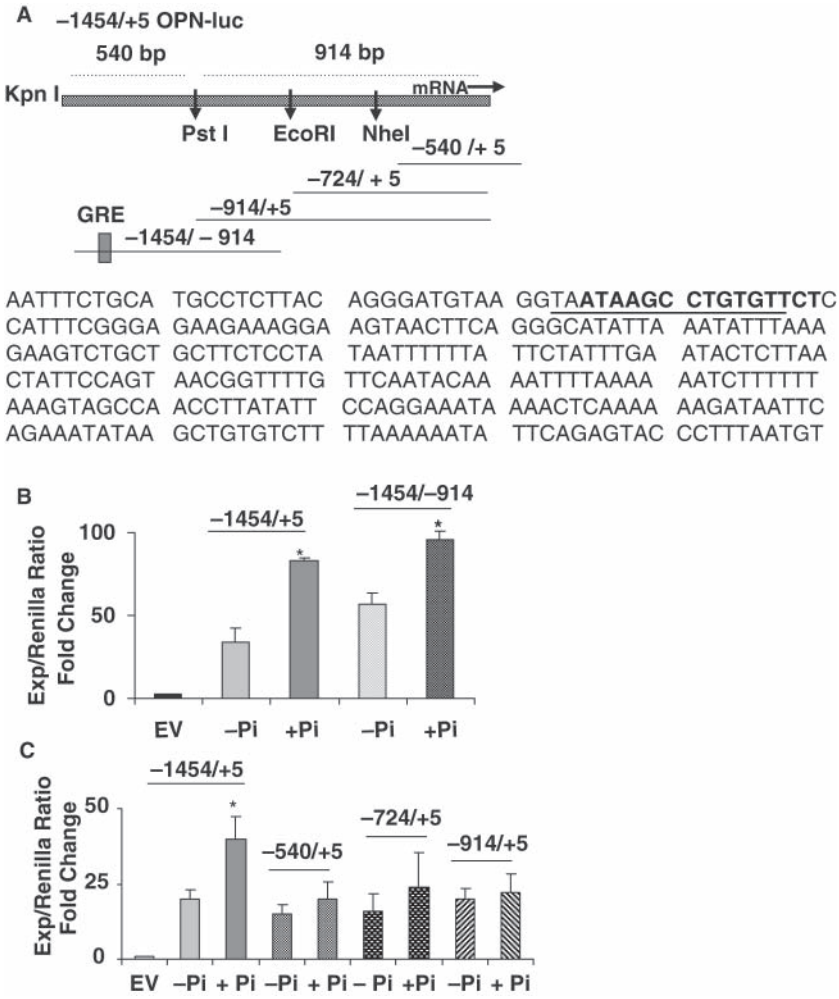

Figure 2. Deletion mapping to identify the phosphate-responsive region. **(A)** Diagram of the OPN constructs generated by restriction enzyme digestion or PCR. The distal 300 bp of the mouse OPN promoter sequence contained in the -1454/+5 OPN-luc construct is shown. The GRE motif is underlined in bold. **(B,C)** Luciferase activity of the OPN promoter constructs in the absence (- Pi) and presence (+ Pi) of 3 mM ePi. **(B)** Mean Firefly/*Renilla* values ± SE for the OPN constructs/EV (left to right) were: 33.66 ± 8.8, 82.8 ± 7.4, 57.0 ± 6.2, and 95.4 ± 10.9. **(C)** Mean Firefly/*Renilla* values ± SE for the OPN constructs / EV (left to right) were: 20 ± 2.9, 40 ± 7.5, 15 ± 3.2, 20 ± 5.8, 16 ± 5.9, 24 ± 11.4, 20 ± 3.4, and 22 ± 6.4, respectively. EV represents a control transfection with pGL3-Basic. All results are shown normalized to *Renilla* luciferase activity. These experiments represent the mean of triplicates and represent 1 of the 4 experiments. *$P \leq 0.05$.

Effects of Glucocorticoid Agonist and Antagonists on Phosphate Activation of OPN Transcription

Previous studies have suggested that OPN might be a glucocorticoid-inducible gene (Hakki *et al.*, 2005), while analyses of other data showed no effect of dexamethasone on OPN expression (Ogata *et al.*, 1995; Mikami *et al.*, 2007). For direct examination of whether OPN is transcriptionally regulated by glucocorticoids, and for study of the possible synergism between the glucorticoid response and Pi, cells were transfected with the -1454/+5 OPN-luc construct and then treated with either dexamethasone, or dexamethasone and ePi. The results showed that dexamethasone caused a small increase in promoter activity, but it was not statistically significant (Fig. 4A). Treatment of cells with both dexamethasone and ePi did not increase luciferase activity above what was seen with ePi alone, demonstrating that there is no synergism between ePi and glucocorticoids. In contrast, the control GRE-luc plasmid was strongly induced by dexamethasone treatment, while ePi alone had no effect on GRE-luc activity (Fig. 4B).

We also tested the effect of a glucorticoid receptor antagonist, RU-486 (Mifepristone), on the Pi induction of OPN transcription. The results demonstrate that RU-486 prevented the normal increase in luciferase activity observed with ePi treatment (Fig. 4C). This effect was dose-dependent, with 1 μM RU-486 completely abolishing promoter activation. Interestingly, the antagonist did not suppress OPN promoter activity below the control

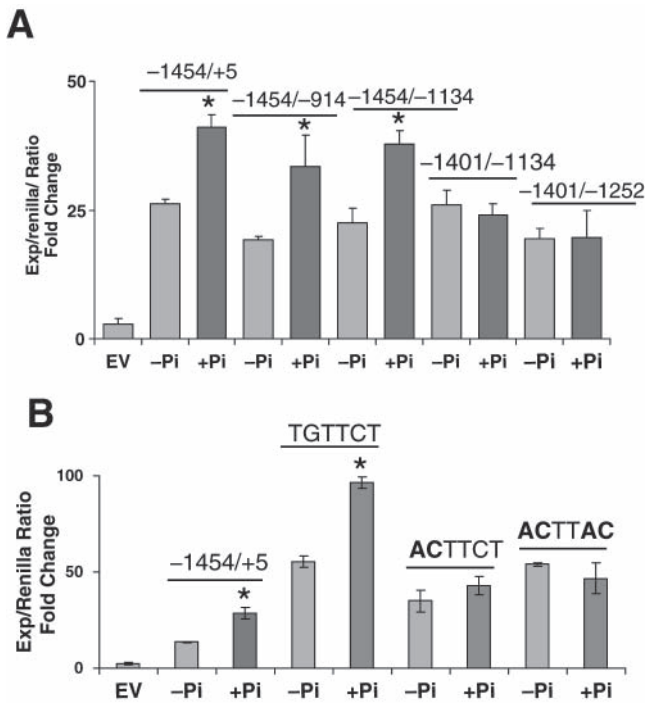

Figure 3. Phosphate induction of the OPN promoter requires a Glucocorticoid Receptor Element. OPN-luc promoter constructs were prepared by PCR and ligated into a PGL3-Promoter vector. Constructs were transfected into OCCM-30 cells as described in MATERIALS & METHODS. Cells were treated with 3 mM Pi and analyzed for luciferase activity as described in Figs. 1 and 2. **(A)** Mean Firefly/*Renilla* values ± SE for the OPN constructs /EV (left to right) were: 26.15 ± 1, 40.94 ± 2.5, 19.23 ± 0.7, 33.4 ± 6.1, 22.42 ± 2.95, 37.9 ± 2.5, 26 ± 2.9, 24 ± 2.1, 19.4 ± 1.2, and 19.54 ± 5.2, respectively. **(B)** Mutagenesis of the OPN GRE prevents Pi induction of promoter activity. Cells were transfected with the indicated mutant constructs that contained 2 or 4 base substitutions in the 3′ half-site of the GRE, and then tested for Pi responsiveness. The control construct (denoted TGTTCT) was -1454/-1134 OPN-luc. Mean Firefly/*Renilla* values ± SE for the OPN constructs /EV (left to right) were: 13.5 ± 3, 28.66 ± 2.8, 55.3 ± 3, 97 ± 5.8, 34.85 ± 4, 42.86 ± 1, 54 ± 7.9, and 46.7 ± 1.5, respectively. Note that the 2 mutant constructs with altered 3′ half-sites are no longer responsive to ePi addition. These experiments were performed in triplicate and represent 1 of 3 experiments. *$P \leq 0.05$.

(basal) activity. As expected, the dexamethasone-inducible activity of GRE-luc was fully suppressed by RU-486, which demonstrates that this compound can antagonize GR function in cementoblasts (Fig. 4D). Overall, these findings demonstrate that a functional GR is required for Pi activation of OPN transcription in OCCM-30 cells; however, GR is apparently not necessary for the basal (uninduced) transcription of the OPN gene.

EMSAs Demonstrate Protein Binding to the OPN GRE Sequence

To examine nuclear protein binding to the OPN GRE sequence, we performed EMSAs. The 27-bp OPN probe bound a protein present in OCCM-30 nuclear extracts (Appendix Fig. A). Experiments with GRE-luc (Figs. 4B, 4D) and EMSAs carried out with a canonical GRE probe (Appendix Fig. B) confirmed that OCCM-30 cells contained GR. To determine if the OPN gel shift was dependent on the GRE sequence, we carried out EMSAs using OPN probes containing several GRE mutations. Mutant probes 1 and 2, containing nucleotide substitutions in the 3′ half-site, exhibited reduced protein binding, while mutant probe 3, containing 2 additional base changes in the 5′ half-site, showed no gel shift (Appendix Fig. 1A). These results suggest that GR or a related protein can bind to the OPN GRE sequence. Attempts to confirm the observed gel shift by supershift analysis with GR antibody were unsuccessful. *In silico* analysis of the 27-bp OPN sequence used for EMSAs with the program MatInspector (Genomatix Software, Ann Arbor, MI, USA) did not identify any transcription-factor-binding sites besides GR. Therefore, unlike some GREs (Ogata *et al.*, 1995; So *et al.*, 2007), the OPN GRE does not appear to be a composite transcriptional enhancer.

DISCUSSION

Osteopontin (OPN), a member of the SIBLING family of extracellular matrix proteins, plays an important role in regulating mineralization and modulating osteoclast-mediated resorption of bone. Previous studies by our laboratory (Foster *et al.*, 2006; Rutherford *et al.*, 2006) and others (Beck and Knecht, 2003; Conrads *et al.*, 2005) have demonstrated that Pi regulates the expression of many genes in cementoblasts or osteoblasts, including OPN and other SIBLING proteins, as well as signaling molecules and transcription factors. In this study, we sought to determine if Pi affects OPN transcription directly or operates through a post-transcriptional mechanism. Our results demonstrate that Pi affects OPN expression directly by inducing OPN transcription, and further, that this response requires GR.

It is well-established that glucocorticoids play numerous key roles in bone development. Clinically, the use of glucocorticoids to treat inflammatory diseases often leads to osteoporosis, due to the inhibition of osteoclast-mediated bone resorption and arrested bone formation (Kim *et al.*, 2006). In osteoblasts and cementoblasts, glucocorticoids regulate the expression of many genes, including OPN, BSP, osteocalcin, and DMP-1 (Hakki *et al.*, 2005; Leclerc *et al.*, 2005; Ogata *et al.*, 1995; Mikami *et al.*, 2008). The mechanisms are not well-understood; however, recent evidence suggests that glucocorticoids function in part by regulating the phosphorylation state and activity of the transcription factor Runx2/Cbfa1 (Phillips *et al.*, 2006). The down-regulation of osteocalcin transcription by dexamethasone has been shown to involve inhibition of the activity of an Egr2/Krox20 enhancer located upstream of a Runx2 site (Leclerc *et al.*, 2005).

In this report, we demonstrated by EMSA that a nuclear protein was able to bind to the OPN GRE. However, we were unable to verify, *via* supershift experiments with GR antibodies, that this DNA-binding protein was GR. Dexamethasone caused a slight increase in OPN gene transcription, though the increase was not significant. This finding is in accordance with

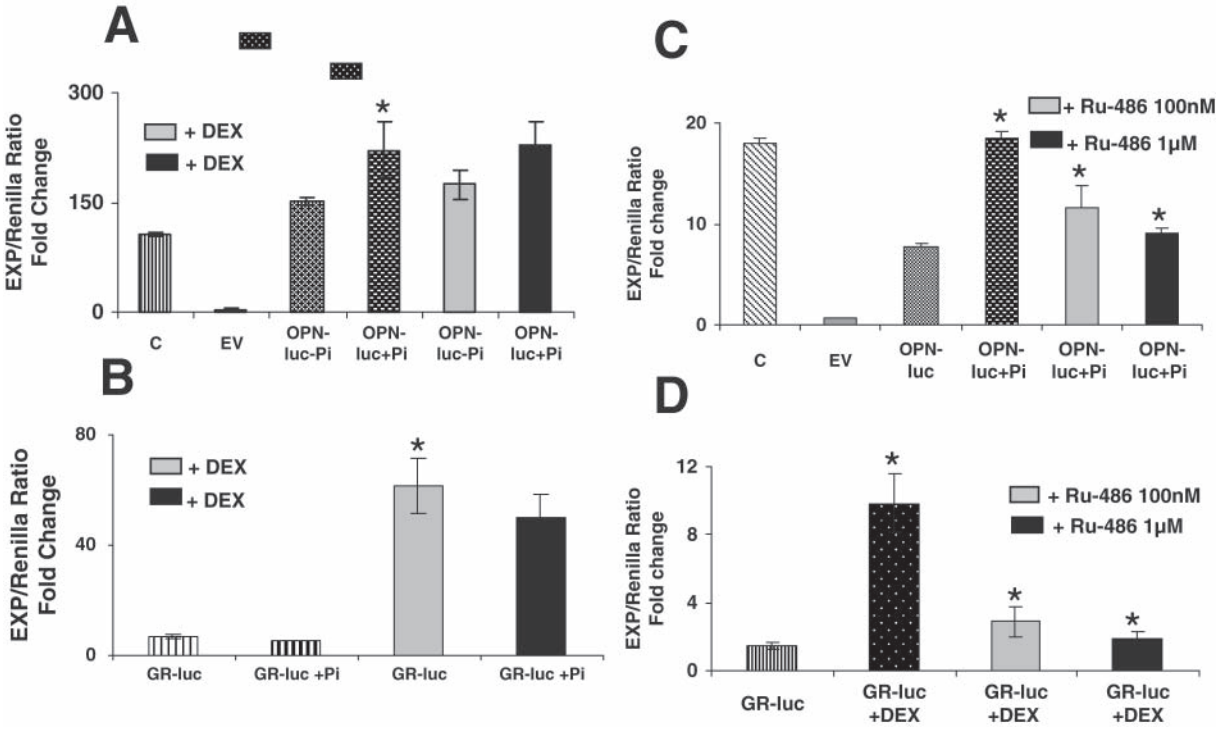

Figure 4. Effects of dexamethasone and RU-486 on OPN promoter activity. OCCM-30 cells were transfected with the indicated plasmids (4 μg/60 mm dish) and then incubated with Pi (ePi), dexamethasone, and/or RU-486, and luciferase assays were performed as described in MATERIALS & METHODS. Firefly luciferase activity is shown normalized to *Renilla* luciferase. **(A)** OPN promoter activity in the presence of dexamethasone and Pi. Cells were transfected with; pGL3-Control (C), empty vector (EV), or -1454/+ 5 OPN-luc (OPN-luc), followed by treatment with 3 mM Pi and/or 1 μM dexamethasone (DEX) as indicated. Mean Firefly/*Renilla* values ± SE for the OPN constructs/EV (left to right) were: 152 ± 5.2, 221 ± 38.7, 221 ± 38.7, and 228 ± 32.8. **(B)** GRE-luc promoter activity in the presence of dexamethasone and Pi. Cells were transfected with GR-luc and then treated with Pi, dexamethasone, or dexamethasone and Pi. Mean Firefly/*Renilla* values ± SE for the GR construct/EV (left to right) were: 7.21 ± 0.75, 5.6 ± 0.13, 61.43 ± 10.08, and 49.9 ± 8.55, respectively. **(C)** OPN promoter activity in the presence of 3mM Pi and RU-486 (100 nM-1 μM). Cells were transfected with pGL3-Control (C), empty vector (EV), or -1454/+ 5 OPN-luc (OPN-luc) and then treated with Pi, or Pi and RU-486 as indicated. Mean Firefly/*Renilla* values ± SE for the OPN constructs/EV (left to right) were: 7.7 ± 0.4, 18.46 ± 0.7, 11.57 ± 2.2, and 9.06 ± 0.5, respectively. **(D)** GRE-luc promoter activity in the presence of dexamethasone and RU-486 (100 nM and 1 μM). Cells were transfected with GR-luc and then treated with dexamethasone, or dexamethasone and RU-486 (100 nM and 1 μM). Mean Firefly/*Renilla* values ± SE for the GR constructs/EV (left to right) were: 1.47± 0.19, 9.84 ± 0.9, 2.9 ± 0.9, and 1.84 ± 0.5, respectively. *P ≤ 0.05.

previous studies in rat osteoblastic cell lines which demonstrated that dexamethasone caused only a slight increase in OPN gene transcription, measured by nuclear run-on assay (Ogata *et al.*, 1995). In contrast, BSP is up-regulated by glucocorticoids, and osteocalcin decreases in expression. Therefore, while the OPN GRE can be classified as a GRE based on sequence considerations, it does not function as a classic glucocorticoid-inducible motif. Rather, we propose that it functions in a Pi signaling pathway, probably in association with other transcription factors or co-activators (Ogata *et al.*, 1995; So *et al*., 2007). The human OPN promoter also contains a potential GRE (5′ TTAATAaccTGTTCT 3′) at nucleotide positions -946 to -932. A potential GRE is present in the OPN promoter of other species, including dog, cow, gorilla, and macaque. Further studies will be needed to determine if this evolutionarily conserved GRE-like motif functions in other species to regulate Pi-mediated expression.

Phosphate is a potent regulator of bone mineralization; however, little is known about the molecular mechanisms involved (reviewed in Beck, 2003; Foster *et al.,* 2007). In this study, we have shown that GR signaling is required for Pi-regulated transcription. It was previously reported that Pi regulation of OPN expression requires the activity of ERK1/2 and protein kinase C, as well as the proteosomal/ubiquination pathway (Beck and Knecht, 2003). The well-established role of the proteasome pathway in the regulation of nuclear receptors, including GR (Dennis and O'Malley, 2005), is consistent with a model in which GR turnover is an important component of Pi signaling and the induction of gene expression.

In conclusion, we have characterized a region of the murine OPN promoter that is required for Pi-mediated transcription. This region contains a GRE which is required for the Pi response. Further, the Pi-induced transcription was inhibited with a GR antagonist, implying that GR plays a direct role in the Pi signaling pathway. This work should facilitate further studies on OPN and other Pi-inducible genes to determine the precise role of GR signaling in regulating gene expression during the mineralization of bone.

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