June 2001

Parathyroid Hormone Regulation of the CREMP2 Promoter in Osteoblastic Cells

Danielle E. Battisti

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PARATHYROID HORMONE REGULATION OF THE 
CREMP2 PROMOTER IN OSTEOBLASTIC CELLS

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A Thesis
Submitted in the Partial Fulfillment of the
Requirements for the Degree of
Master of Dental Science
at the
University of Connecticut
2001
Master of Dental Science Thesis

PARATHYROID HORMONE REGULATION OF THE CREMP2 PROMOTER IN OSTEOBLASTIC CELLS

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University of Connecticut
2001
ACKNOWLEDGEMENTS

It has been a privilege to do my post-graduate orthodontic training at the University of Connecticut Health Science Center over the past three years. To reach this final point and present my study, there are many people whom I need to thank. First of all, I would like to thank Dr. Ravindra Nanda for giving me the opportunity to fulfill my dream as an Orthodontist. Without him I would not be where I am today. He has been a great teacher and leader, but more importantly a dear friend. Secondly, I would like to thank Dr. Andrew Kuhlberg for always being there and his constant support. He has been an outstanding teacher, researcher, and a wonderful friend in so many ways that I could not do justice with only words. I would like to thank Dr. Jonny Feldman for his exceptional skills and knowledge. I truly appreciate all of the sound advice and direction he has given me. He inspires me to become a great teacher and astute clinician. I would like to thank all of the residents, specifically Kristen, Todd and Brad and my two classmates, Stanton and Zack, for making my time here nothing but fun. I would also like to thank my “big brothers”, Bobby and Derek, for taking me under their wings and teaching me the ropes.

I would like to thank everyone in Dr. Kream’s lab including Jin, Jenny, Fei, Ante, Henning, Penny, Denise and Dr. John Harrison who have made my time in the lab fun yet productive. A big heartfelt thanks goes to my guardian angel in the lab, Dr. Winston Huang. Winnie has had to teach me everything there is to know about doing basic science research from pipetting to writing my thesis. His patience, friendship and color coordinating schemes in power point are things that I will treasure forever. I consider
myself lucky to have worked under his supervision. Finally, my major advisor, Dr. Barbara Kream: I cannot thank her enough for allowing me to do research in her lab while accommodating my tight clinical program schedule. Her guidance and insight into research have helped me become a better scientist. I would like to express my most sincere appreciation to her.

I would like to thank my parents. Their unconditional love and support has gotten me through twenty-four years of schooling. My appreciation and love towards them is beyond what words can express. I would also like to thank my brother, Alan, and his family, my brother Brian, and his wife, Amy, my brother, Eric, and my sister, Claudine. They are not only my family, but they are my best friends. I cannot forget to thank my Uncle James and my Nana Anzalone for all their prayers.

Finally, I would like to thank my husband, Jonathan, and my precious son, Noah for giving me something to work towards. They fill my life with nothing but joy and love. I dedicate this glorious moment to them.
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The stage of osteoblast differentiation of stably transfected MC3T3-E1 cells with CREMP2-Luc238 may not determine the PTH response.
Abstract:

The inducible cAMP early repressor (ICER) is transcribed from an alternative intronic promoter (P2) of the CREM gene and functions as powerful repressor of cAMP induced transcription. ICER represses the activity of its own promoter, forming a negative autoregulatory loop. PTH induces ICER expression in osteoblastic cells and mouse calvariae. To determine the pathways used by PTH to regulate ICER expression, the activity of a construct containing the ICER 5’ regulatory region linked to a luciferase reporter (CREMP2-Luc238) was analyzed in stably transfected osteoblastic MC3T3-E1 cells. Cells were treated with pharmacological activators (forskolin, 8-bromo-cAMP, phorbol myristate acetate (PMA), PTH(3-34) and ionomycin) and a pharmacological inhibitor (H89), to investigate the signaling pathways that regulate PTH-dependent ICER promoter activity. To determine whether the state of osteoblast differentiation affects PTH induction of the ICER promoter, MC3T3-E1 cells were grown to confluence for 6-7 days, then supplemented with ascorbic acid and assayed for markers of osteoblastic differentiation at each time point by Northern blot analysis. The cells were treated with PTH and ICER promoter activity measured. CREMP2-Luc238 expression was induced by PTH(1-34), FSK, 8-bromo-cAMP, PGE2, but not PMA, ionomycin, or PTH(3-34). In addition a specific PKA inhibitor, H89, blocked PTH induction of CREMP2-Luc238 activity, suggesting that PTH induces CREMP2-Luc238 expression through the cAMP-PKA pathway. Moreover, our data show that the stages of osteoblast differentiation may not affect PTH induction of CREMP2-Luc238 expression. Therefore, ICER induction may represent a novel mechanism by which PTH regulates gene expression in
osteoblastic cells. Further studies in this area will enhance our understanding of hormonal regulation of gene transcription in osteoblasts.
**Background:**

*Parathyroid hormone and bone*

Parathyroid hormone (PTH) is one of the most important regulators of serum calcium levels in humans. It normalizes a low serum calcium level by indirectly activating the formation and activity of osteoclastic cells to increase bone resorption, as well as causing many direct changes in the functions of osteoblastic cells [1]. The latter involves a switch in the phenotype of the osteoblast from one of bone formation to one of matrix degradation and active participation in the resorption process [2]. Cells of the osteoblast lineage, not osteoclasts, are the principal skeletal targets of PTH. PTH acts on osteoblasts, which then transmit a signal to the osteoclast that results in increased bone turnover. As a result, PTH-stimulated resorption and osteoclastogenesis require the presence of osteoblast cells [3]. PTH increases the expression of several mediators implicated in osteoclastogenesis and resorption, such as interleukin-11 (IL-11), interleukin-6 (IL-6) [4], prostoglandins [5], and the plasma membrane-bound osteoclast differentiation factor RANKL [6].

The effect of PTH on bone metabolism depends on the experimental model, dosage and mode of administration [7]. Continuous exposure of the skeleton to increased levels of PTH results in high bone turnover and bone loss. In marked contrast, intermittent administration of PTH increases net bone formation [8]. However, the exact mechanism for this anabolic effect is not clear. It is associated with activation of the lining cells on the surface of the bone and enhanced proliferation and differentiation of osteoprogenator cells in bone marrow [9]. It has also been suggested that there is an actual delay in osteoblast apoptosis, the fate of the majority of cells under normal
conditions. This would prolong the time spent in performing their matrix synthesizing function, as opposed to osteoclast precursor proliferation, which contributes to the anabolic effects in rats [10].

Thus, PTH has a diverse effect on osteoblastic cells. Studies of gene expression in osteoblasts are necessary to determine the mechanism by which PTH regulates both bone resorption and bone formation, biological processes absolutely necessary for orthodontic tooth movement.

PGE_2 and bone

Prostaglandins (PGs) are potent and complex regulators of bone metabolism. Prostaglandins, abundant in bone, are produced primarily by osteoblasts and derived from phospholipids in the membrane bilayer. They are released in response to direct cellular damage or by any non-destructive perturbation of the membrane of phospholipid cells, be it physical, chemical, hormonal or neurohormonal [11]. In orthodontics, the forces applied to move teeth through bone induce local inflammation, increasing vascular permeability and cellular infiltration causing lymphocytes, monocytes and macrophages to infiltrate into the inflammatory tissue where the prostaglandins are released. Prostaglandins, in turn, activate adenylate cyclase, which induces an increase in the intracellular levels of cAMP. This increase in cAMP stimulates the release of prostaglandins again. Prostaglandin levels appear to be regulated by a negative feedback mechanism with increased levels inhibiting adenylcyclase activity. They are reported to promote bone resorption, not only by increasing the number and size of the osteoclast but also by stimulating the activation of existing osteoclasts [12].
However, the effects of prostaglandins on bone metabolism are not that clear; they appear to be quite complex. Some studies show that prostaglandins enhance proliferation of osteoblastic cells [13] while others indicate the opposite [14]. Interestingly, Baylink et al. showed that the effects of prostaglandins on osteoblast proliferation are actually biphasic with stimulation at lower concentrations ($10^{-9}$ M) and inhibition at higher concentrations ($10^{-6}$ M).

The data on the effects of prostaglandins on the differentiation of osteoblasts are more conclusive. Prostaglandins have been shown to clearly enhance differentiation of osteoblastic progenitors into mature osteoblasts. However, they generally inhibit the function of mature osteoblasts preventing collagen production [11]. The effects of PGE$_2$ are similar to those of PTH. PGE$_2$ has been shown by many to stimulate bone resorption, decrease collagen synthesis and cause increased levels of cAMP.

cAMP-PKA signaling pathway

Individual cells of all types of organisms have evolved a mechanism by which they can sense and respond appropriately to environmental stimuli. The higher the level of the organism involved, the greater the diversity of signals they can respond to, including growth factors, hormones, nutrients and sensory input. These signals regulate cellular differentiation and proliferation. However, the receiving cell must have the appropriate receptor and signal transduction pathway to elicit a response [15].

When a ligand such as a growth factor or hormone binds to a receptor on the plasma membrane, a signal that binding has occurred is received inside the cell and transduced to other molecules by a second messenger such as cAMP, which is generated
by the membrane protein AC (adenylate cyclase). Ligand binding to a cell surface receptor can stimulate the activity of membrane associated AC converting ATP to cAMP. cAMP, in turn, binds cooperatively to two sites on the regulatory subunit of protein kinase A (PKA), causing dissociation of the inactive protein kinase A complex into active catalytic subunits and regulatory subunits. The catalytic subunits migrate into the nucleus where they phosphorylate and, thereby, activate transcription factors [16]. Transcription factors are proteins that control the rate of gene expression by integrating information from promoter sequences and signal transduction pathways.

PTH mediates its effects on bone metabolism by binding to PTH-PTH-related protein (PTHrP) receptors present on the plasma membrane of osteoblasts. PTH binding to this receptor stimulates the adenylate cyclase and phospholipase C, thereby activating primarily the cAMP-PKA pathway, but also the protein kinase C (PKC) and calcium signaling pathways. Activation of these pathways leads to changes in gene expression by transcriptional and/or post- transcriptional mechanisms [17].

In eukaryotes, transcriptional regulation activated by the AC signaling pathway is mediated by the CREB (cAMP response element binding protein)/ATF (activating transcription factor) family of cAMP responsive transcription factors. cAMP regulation of gene expression in many types of cells involves the interaction of gene promoter sites with CREB [18]. CREM, the cAMP response element modulator, is a member of the CREB/ATF family. This family consists of many members which arise by alternative splicing and alternative promoter utilization and can act as transcriptional activators and repressors by binding to cAMP response elements (CRE) in the promoter region of genes. CRE is an eight base pair palindromic sequence (TGACGTCA). CREB/ATF
transcription factors contain basic domain/leucine zipper motifs and exhibit a great functional diversity by binding as heterodimers as well as homodimers to CREs [2, 5].

CREM is a multiexonic gene that by alternative splicing gives rise to both activators and repressors of the cAMP-dependent transcription [19]. The CREM gene encodes two glutamine-rich domains (Q1 and Q2) that are responsible for transcriptional activation, the phosphorylation box (P-box) or inducible kinase domain and two DNA binding domains (DBDI and II). The CREM antagonists α, β, and γ lack the two glutamine-rich domains. They bind to CREs but do not stimulate basal transcription, thus behaving functionally as repressors of cAMP-induced transcription. CREMr, another isoform, includes these domains and is a transcriptional activator. However, none of these isoforms are inducible by activation of the cAMP-signaling pathway.

**Inducible cAMP early repressor (ICER)**

An inducible CREM transcript encodes a novel isoform called inducible-cAMP early repressor (ICER) [19, 20]. ICER is a small protein of 120 amino acids with a predicted molecular weight of about 13.4 kDa. ICER lacks the Q and kinase inducible domains and is essentially only the DNA binding domain of CREM, consisting of the leucine zipper and basic region. Therefore, ICER functions as a powerful repressor of cAMP-induced transcription. ICER is generated by the use of an alternative intronic promoter, P2. In contrast to the promoter generating all the previously characterized isoforms (P1), which is GC rich and not inducible by cAMP, the P2 promoter has a normal A-T and G-C content and is strongly inducible by cAMP. The P2 promoter contains two pairs of closely spaced CREs in tandem separated by only three nucleotides.
This feature makes P2 unique among cAMP regulated promoters and is suggestive of cooperative interactions among factors binding to these sites [21]. ICER expression characterizes CREM as an early response gene. Following the induction of CREM by cAMP, there is a rapid rise in ICER that peaks after 2 hours of stimulation and does not require de novo protein synthesis. Significantly, the subsequent decline in ICER expression, occurring approximately after 5 hours, requires protein synthesis. ICER represses its own production via a negative autoregulatory mechanism that involves binding to its own promoter and blocking transcription[19]. ICER represents the first transcriptional repressor in the cAMP pathway whose function is regulated primarily by modulation of its intracellular levels and not by phosphorylation [16].

CRE-binding proteins play a pivotal role in the physiology of the pituitary gland, spermatogenesis, circadian rhythms and the molecular basis of memory. In the testis there exists a striking differential regulation of CREM expression according to the developmental stage. CREM mRNA is a highly abundant transcript in adult testis, whereas in prepubertal animals it is expressed at very low levels [16, 22]. In addition, CREM is expressed with a circadian rhythm in the pineal gland [16, 23]. Analysis reveals a dramatic day-night regulation, with peak expression occurring during the night. Experiments indicate that CRE-binding proteins are likely to play a role in the molecular processes leading to long-term memory [18]. ICER appears to be predominantly distributed in neuroendocrine tissues. This information suggests an important role for ICER in the cAMP regulation of neuroendocrine genes [19].
Significance to Orthodontics

Orthodontics involves the movement of teeth through bone. It is thought of as increased bone remodeling in response to the mechanical force applied to the teeth. Biologically, the process involves active bone remodeling, bone resorption and bone deposition. When a force is applied to a tooth the periodontal cells are compressed between the tooth root and alveolar bone. These cells secrete bone-resorbing cytokines, which stimulate osteoclast function and bone resorption [24]. The alveolar bone surrounding the tooth is remodeled in such a way that the surface under pressure is resorbed and the bone surface under tension is deposited. The role of bone metabolism, however, in controlling tooth movement has been considered secondary to the force applied. This is largely a result of the difficulty in altering bone metabolism systemically, in addition to the fact that the force applied is the most easily manipulated factor [25]. The remodeling of bone that occurs with the orthodontic force is at the local level in the bone microenvironment. However, remodeling that is regulated with hormones such as PTH is at the systemic level.

PTH regulates bone metabolism through an osteoblast-mediated mechanism. PTH increases osteoclastic bone resorption by inducing the production of local mediators in osteoblastic cells. Thus, since PTH is a potent stimulator of bone resorption, it has been proposed as a means of accelerating tooth movement in orthodontic treatment [24, 26]. Therefore, it is possible that administration of this bone-resorbing factor may increase bone-resorbing activity in compressed periodontal tissue. If PTH is to be considered as a treatment modality, it is important to understand the molecular pathways by which PTH stimulates bone resorption. The rapid and transient induction of ICER by
PTH may be a physiological mechanism by which it controls the transcription of genes involved in bone resorption.
Hypothesis:

PTH, signaling through the cAMP-PKA pathway, induces the expression of the CREMP2 promoter in MC3T3-E1 cells.

Objectives:

1. To examine regulation of the CREMP2 promoter by PTH in osteoblastic MC3T3-E1 cells and determine the signaling pathway(s) involved.

2. To determine whether the state of osteoblast differentiation of MC3T3-E1 cells alters the PTH response of the CREMP2 promoter.

Research Plan:

1. To examine regulation of the ICER promoter by PTH in osteoblastic MC3T3-E1 cells and determine the signaling pathway(s) involved.

We developed a model system by stably transfecting an ICER promoter-luciferase construct into osteoblastic MC3T3-E1 cells. Stably transfected cells were treated with PTH and luciferase activity measured. To investigate the signaling pathways involved in PTH’s induction of ICER transcription, cells were treated with: forskolin (FSK) and 8-bromo-cAMP (8Br-cAMP), which activate the cAMP pathway, phorbol myristate acetate (PMA) and PTH(3-34), which activate the protein kinase C pathway (PKC), and ionomycin, which increases the intracellular levels of Ca$^{2+}$. We also treated the cells with PTH and a pharmacological inhibitor of the cAMP pathway, H89, to investigate the signaling pathways involved.
2. To determine whether the state of osteoblast differentiation of MC3T3-E1 cells affects the PTH response on the ICER promoter.

MC3T3-E1 cells were grown to confluence under conditions described in experiment #1. At day 7, osteocalcin, bone sialoprotein, and type I collagen mRNA, markers of the osteoblastic differentiation, were measured. Then, ascorbic acid was added to differentiate the cells. At day 14, markers of osteoblast differentiation including osteocalcin, bone sialoprotein, and type I collagen mRNA were measured. At each time point, cells were treated with PTH, and ICER promoter activity measured.
Materials and Methods:

Materials

Synthetic bovine PTH(1-34), bovine PTH(3-34) amide, forskolin (FSK), phorbol myristate acetate (PMA), 8-bromocyclic AMP (8Br-cAMP), H89 and ionomycin were purchased from Sigma Chemical Company (St. Louis, MO). Lipofectamine reagent was purchased from GIBCO BRL (Gaithersburg, MD). PTH was prepared as a stock solution containing 1 mg/ml BSA containing 0.001 N HCl and diluted in culture medium at least 1000-fold. FSK, PMA and ionomycin were prepared as stock solutions in 100% ethanol and diluted in culture medium at least 1000-fold. 8Br-cAMP was dissolved directly in the culture medium to a final concentration of 1 mM. The ICER promoter-luciferase construct (CREMP2-Luc238) was kindly provided by Dr. Carlos Molina (UMDNJ-New Jersey Medical School and Graduate School of Biomedical Sciences, New Jersey). This construct contains the mouse CREM P2 promoter region from -238/+14 bp with 2 clusters of cAMP-responsive elements (CRE) cloned into the pGL3 luciferase reporter plasmid (Promega, Madison, WI) (Figure 1).
Figure 1 Drawing of the CREMP2-Luc238 construct.

A fragment of CREMP2 promoter from –238 to +14 bp, containing two clusters of CREs in tandem, was linked to a luciferase reporter.
**Cell Culture**

MC3T3-E1 cells stably transfected with CREMP2-Luc238 were plated at 5000 cells/cm² in 35- or 100-mm tissue culture wells, cultured in a humidified atmosphere of 5% CO₂ in air at 37° C and fed every 3 days with DMEM supplemented with 10% heat-inactivated fetal calf serum (HIFCS), 100 U/ml penicillin and 50 µg/ml streptomycin.

**Total RNA isolation and Northern blot analysis**

Total RNA was extracted by the guanidinium isothiocyanate method of Chomczynski and Sacchi [27]. MC3T3-E1 cells stably transfected with CREMP2-Luc238 from three 35-mm wells or one 100-mm plate were washed twice with cold PBS and lysed with a solution containing 4M guanidinium thiocyanate. RNA was extracted with H₂O-saturated phenol/chloroform-isoamyl (24:1); precipitated with an equal volume of isopropanol, in the presence of 0.2M sodium acetate, pH 4.0, at -20°C. The pelleted RNA was dissolved in guanidinium thiocyanate solution and precipitated with isopropanol. The final RNA pellet was washed twice with 80% ethanol, lyophilized, dissolved in diethylpyrocarbonate-treated water and quantitated by absorbance at 260 nm. Twenty micrograms of total RNA was fractionated by electrophoresis on a 1% agarose–6% formaldehyde gel and transferred to a GeneScreen Plus hybridization membrane. Membranes were prehybridized for 3 h, hybridized at 42°C or 56°C for 12-16 h with (³²P)dGTP labeled cDNA probes and exposed to x-ray film at -70°C.
Transfection

MC3T3-E1 cells were plated at 250,000 cells per 35 mm dish in DMEM containing 10% HIFCS, 100U/ml penicillin and 50 μg/ml streptomycin. When cells reached 60-80% confluence (overnight), transient transfection was performed using DOTAP reagent (Boehringer Mannheim, Indianapolis, IN). 1 μg/reaction of PGL3 and CREMP2-Luc238 constructs were diluted with HEPES and mixed with DOTAP reagent, which was also diluted with HEPES. Cells were incubated with the DOTAP-DNA mixture at 37°C for 5-6h. The DOTAP-DNA mixture was aspirated at the end of the incubation period, and fresh DMEM containing 10% HI-FCS, 100U/ml penicillin and 50 μg/ml streptomycin was added for 48 h prior to effector treatments.

Luciferase assay

The induction of CREMP2-Luc238 by the effectors was measured for luciferase activity. MC3T3-E1 cells stably transfected with CREMP2-Luc238 were grown to confluence in 35-mm wells as described above and treated with PTH and effectors for 4 h. In brief, the cells were washed twice with cold PBS and lysed in 200 μl of a lysis buffer containing 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N, N, N', N-tetraacetic acid, 10% glycerol, and 15% Triton X-100. The cell lysates were transferred to Eppendorf tubes and centrifuged for 30 sec. Ten microliters of each supernatant were assayed using a commercially available luciferase kit (Promega, Madison, WI), which was quantitated on a Berthold LB 9501/16 luminator (Wallac, Gaithersburg, MD). Luciferase activity was normalized to protein content, which was measured using the indicator bicinchoninic acid [28].
Statistics

Data were analyzed using a one-way ANOVA, and statistical differences between the groups were determined using the Student-Newman-Keuls *post hoc* test.
Results:

*Specific Aim 1*: To examine regulation of the CREMP2 promoter by PTH in osteoblastic MC3T3-E1 cells and determine the signaling pathway(s) involved.

Two populations of stably transfected MC3T3-E1 cells were obtained. Each population contained multiple individual clones of stably transfected cells.

To study the signaling pathways involved in the PTH induction of CREMP2 promoter activity, stably transfected MC3T3-E1 cells were treated for 4 h with the following signaling molecules: $10^{-8}$ M bovine PTH(1-34), $10^{-5}$ M forskolin (FSK), $10^{-6}$ M PGE$_2$, and 3 mM 8-bromo-cAMP (8Br-cAMP), which activate the cAMP pathway, and $10^{-6}$ M ionomycin, which increases intracellular levels of Ca$^{2+}$. In the following experiments, the fold-induction of each effector was calculated as the ratio of effector treatment vs. vehicle. The fold induction of the control group was set as 100% and the experimental groups were expressed as percentage of the control. All experiments were repeated at least twice with similar results.

In the first population, FSK and 8Br-cAMP significantly induced the CREMP2-Luc238 activity ($567\pm130\%$ and $346.5\pm19\%$, respectively, of the control group) (Figure 2). PTH(1-34) and PGE$_2$ induced CREMP2-Luc238 activity to $170.5\pm47.5\%$ and $150.5\pm31.5\%$, respectively, while ionomycin did not induce CREMP2-Luc238 (Figure 2). Population 2 showed similar results (Figure 3). In addition, Population 2 cells were also treated with $10^{-7}$ M PMA for 4h, which did not induce CREMP2-Luc238. (Figure 3).
These data suggested that CREMP2-Luc238 activity was induced mainly through the cAMP-PKA pathway.

To determine the involvement of the PKA pathway in the induction of the CREMP2-Luc238 by PTH, stably transfected MC3T3-E1 cells were pretreated with 40 μM H89, a pharmacological inhibitor of the cAMP-PKA pathway, for 1 h followed by either addition of vehicle or 10^{-8}M PTH(1-34) for 4 h. PTH caused a significant 1.65-fold induction of CREMP2-Luc238 (Figure 4). However, pretreatment with H89 blocked PTH induction to the level of the control suggesting that cAMP-PKA pathway is involved in PTH induction of CREMP2-Luc238 activity.

To further confirm that H89 is a specific inhibitor of the cAMP-PKA pathway, stably transfected MC3T3-E1 cells were pretreated with 40 μM H89 for 1 h followed by either addition of vehicle or 10^{-5}M forskolin (FSK), for 4 h. FSK caused a significant induction of CREMP2-Luc238 to 371.5±63.5% of the control group (Figure 5). However, pretreatment with H89 blocked FSK induction to the level of the control. Population 2 showed similar results as Population 1 suggesting that cAMP-PKA pathway is involved in FSK induction of CREMP2-Luc238 activity.
To study the signaling pathways involved in the induction of CREMP2-Luc238 expression by PTH, stably transfected MC3T3-E1 cells were treated with PTH(1-34) or an N-terminal truncated PTH analog, PTH(3-34), which lacks the first two amino acids that are critical for activating the cAMP-PKA pathway [29, 30]. Since PTH(3-34) is in amide form, PTH-NH₂, was used for comparison. PTH(1-34) and PTH-NH₂ similarly induced CREMP2-Luc238 expression (170.5±47.5% and 165±14% of control respectively). In contrast, PTH(3-34) did not induce CREMP2-Luc238 (109±20% of control) (Figure 6). Population 2 showed similar results. These data suggest that PTH induces CREMP2-Luc238 expression primarily through the cAMP-PKA pathway.
cAMP activators induce CREMP2-Luc238 activity in stably transfected MC3T3-E1 cells.

MC3T3-E1 cells stably transfected with CREMP2-Luc 238 (population 1) were treated with 10 nM PTH, 10 μM FSK, 1 μM PGE2, 3 mM 8-bromo-cAMP, and 1 μM ionomycin for 4 h. Cell lysates were prepared and assayed for luciferase activity as described in Materials and Methods. Each value is a mean ± SEM of 2 to 5 separate experiments, each performed with triplicate determinations in each group. * Significant difference (p<.05) from control.
Figure 3  FSK and PGE₂ induce CREMP2-Luc238 activity in stably transfected MC3T3-E1 cells.

MC3T3-E1 cells stably transfected with CREMP2-Luc238 (population 2) were treated with 10 nM PTH, 10 μM FSK, 1 μM PGE₂, 100 nM PMA, and 1 μM ionomycin for 4 h. Cell lysates were prepared and assayed for luciferase activity as described in Materials and Methods. Each value is a mean ± SEM of 2 to 5 separate experiments, each performed with triplicate determinations in each group. * Significant difference (p<.05) from control.
A specific PKA inhibitor, H89, completely inhibits CREMP2-Luc238 activity in stably transfected MC3T3-E1 cells.

MC3T3-E1 cells stably transfected with CREMP2-Luc238 (population 1) were pre-treated with 40 μM H89 for 1 h and subsequently treated with $10^{-8}$M PTH(1-34) $10^{-5}$M for 4 h. Cell lysates were prepared and assayed for luciferase activity as described in Material and Methods. Each value is a mean ± SEM of an experiment with triplicate determinations in each group. * Significant difference (P<.01) from control; † Significant difference (P<.01) from PTH(1-34).
Luciferase/protein
Figure 5  A specific PKA inhibitor, H89, inhibits CREMP2-Luc238 activity in the second population of stably transfected MC3T3-E1 cells.

MC3T3-E1 cells stably transfected with CREMP2-Luc238 (population 1) were pre-treated with 40 μM H89 for 1 h and subsequently treated with 10^{-5}M forskolin (FSK) for 4 h. Cell lysates were prepared and assayed for luciferase activity as described in Materials and Methods. Each value is a mean ± SEM of 2 separate experiments with triplicate determinations in each group. \(^a\) Significant difference (P<.01) from control; \(^b\) Significant difference (P<.01) from FSK.
Percentage of Control (%)

- FSK
- H89
- H89 + FSK
Figure 6  PTH(3-34) does not induce CREMP2-Luc238 expression in stably transfected MC3T3-E1 cells.

MC3T3-E1 cells stably transfected with CREMP2-Luc238 (population 1) were treated with 10 nM PTH(1-34), 10 nM PTH(1-34) amide, and 10 nM PTH(3-34) for 4 h. Cell lysates were prepared and assayed for luciferase activity as described in Materials and Methods. Each value is a mean ± SEM of 2 separate experiments, each performed with triplicate determinations in each group.
Percentage of Control (%)

- PIH(1-34)
- PIH-NH₂
- PIH(3-34)
Since ascorbic acid did not seem to be necessary for differentiation of stably transfected MC3T3-E1 cells, a time course was performed without ascorbate to examine osteoblast mRNA markers in stably transfected MC3T3-E1 cells by Northern blot analysis and PTH induction of CREMP2-Luc238.

Northern blot analysis showed that Col1a1 mRNA was present on day 3 and remained constant up to day 14. BSP mRNA did not appear until day 7, peaked at day 10 and then remained constant. OC mRNA was not present on day 3, appeared on day 7 and was greatly increased on day 14 (Figures 10 and 11).

CREMP2-Luc238 expression was induced by PTH at all time points. The level of induction by PTH ranged from 2- to 4-fold throughout the entire experiment period (Figure 12). Note, however, that the baseline luciferase activity was constant until day 14, and then decreased at later time points. These data suggested that the stage of osteoblast differentiation may not determine the PTH response of stably transfected CREMP2-Luc238 cells.
Ascorbic acid is not required for the expression of osteoblastic differentiation markers in stably transfected MC3T3-E1 cells.

Osteoblastic MC3T3-E1 cells (population 1) were plated at 5000 cells/cm². On day 7, the medium was changed and cells were cultured in medium with or without ascorbic acid (50 µg/ml) for another 7 days. Total RNA isolation was performed on day 7 and 14 subjected to Northern blot analysis.
Figure 8  Ascorbic acid is not required for the expression of osteoblastic differentiation markers in stably transfected MC3T3-E1 cells.

Osteoblastic MC3T3-E1 cells (population 1) were plated at 5000 cells/cm². On day 7, the medium was changed and cells were cultured in medium with or without ascorbic acid (50 μg/ml) for another 7 days. RNA was extracted on day 7 and 14 and subjected to Northern blot analysis.
The stage of differentiation of stably transfected MC3T3-E1 cells with CREMP2-Luc238 does not affect the PTH response.

A parallel set of stably transfected MC3T3-E1 cells was cultured as described in Figures 7 and 8. Cells were treated with either 10 nM PTH(1-34) or vehicle for 4 h. Luciferase was assayed on days 7 and 14. Each value is a mean ± SEM of 2 separate experiments, each performed with triplicate determinations in each group. *Significant difference (p<.05) from control.
Figure 10  Stably transfected MC3T3-E1 cells expressed osteoblastic mRNA markers on day 14 in the absence of ascorbic acid.

A time course was performed without ascorbic acid to determine the expression of osteoblastic markers in stably transfected MC3T3-E1 cells by Northern blot analysis.
Figure 11  Stably transfected MC3T3-E1 cells expressed osteoblastic differentiation markers on day 14 in the absence of ascorbic acid.

A time course was performed without ascorbic acid to determine the expression of osteoblastic markers in stably transfected MC3T3-E1 cells by Northern blot analysis.
Figure 12  The stage of osteoblast differentiation may not determine the PTH response of stably transfected CREMP2-Luc238 cells.

A parallel set of stably transfected MC3T3-E1 cells was cultured as those described in Figures 10 and 11. Cells were treated with either 10 nM PTH(1-34) or vehicle for 4 h and luciferase activity was assayed on day 3, 7, 10 and 14. Each value is a mean ± SEM of 2 separate experiments, each performed with triplicate determinations in each group. *Significant difference (p<.05) from control.
Discussion:

The goals of these experiments were to 1) examine the regulation of CREMP2-Luc238 promoter by PTH in osteoblastic MC3T3-E1 cells and determine the signaling pathway(s) involved and 2) determine whether the state of osteoblast differentiation of MC3T3-E1 cells alters the PTH response of the CREMP2-Luc238 promoter. Our data demonstrate that PTH regulates the CREMP2-Luc238 promoter through the cAMP-PKA pathway.

PTH exerts its biological effects by binding to the PTH/PTHrP receptor that is found predominantly, but not exclusively, in cells of the osteoblast lineage [31]. Upon PTH binding, the PTH/PTHrP receptor, a G protein-coupled transmembrane receptor, activates many signaling pathways causing an increase in intracellular messengers such as cAMP [32] and diacylglycerol [33], which activate the protein kinase A (PKA) and protein kinase C (PKC) signaling pathways, respectively. A third second messenger, inositol 1,4,5-triphosphate [34], is also activated leading to an increase in intracellular free calcium levels, which can also activate protein kinase C. However, cAMP-PKA is probably the predominant pathway mediating the PTH-regulated gene expression in osteoblastic cells [2, 17, 35, 36].

Since PTH can activate multiple signaling pathways, we used pharmacological activators and inhibitors of several signaling pathways by which PTH regulates CREMP2-Luc238 expression. Agents that activate the cAMP-PKA pathway such as FSK, 8-bromo-cAMP and PGE₂ increased CREMP2-Luc238 expression. PMA, which activates the PKC pathway, and ionomycin, which works through the inositol 1,4,5-
triphosphate system to increase the intracellular levels of calcium, also activating the PKC pathway, did not increase CREMP2-Luc238 expression. H89, a PKA specific inhibitor, significantly reduced FSK- and PTH-induced CREMP2-Luc238 activity. FSK induced PTH(3-34), an analog that lacks the two N-terminal amino acids of PTH and signals through the PKC pathway and intracellular calcium, but does not activate the cAMP-PKA pathway [29, 30] was unable to induce CREMP2-Luc238 expression. Collectively, the data suggest that PTH regulates CREMP2-Luc238 expression in osteoblastic MC3T3-E1 cells by the cAMP-PKA pathway. It is important to recognize that utilization of other pathways has been suggested for PTH-induced osteoclast-like cell formation, bone resorption, and PTH-mediated changes in osteoblastic proliferation [17].

As stated previously, PTH exerts its biological effects by binding to the PTH/PTHrP receptor. The PTH/PTHrP receptor is a member of a family of G protein-linked, 7 transmembrane domain receptors and binds with equal affinity to PTH and PTHrP. Although it is generally accepted that cells of the osteoblast lineage are the primary targets for PTH action in bone, it is not clear at what stage the osteoblastic cell begins to express surface receptors for PTH [37]. Rouleau et al indicated that an undifferentiated stromal cell had the greatest receptor binding sites, but others suggest that the mature osteoblast is more responsive to PTH [37]. McCauley and colleagues found that the normal differentiation of MC3T3-E1 preosteoblastic cells was accompanied by a progressive increase in the steady-state mRNA levels for the PTH/PTHrP receptor. The PTH/PTHrP receptor was low during cell proliferation and increased once cells began to differentiate. Thus, one would conclude that a more mature
osteoblast would be more responsive to PTH than a less differentiated osteoblastic cell. However, our results do not concur with this finding. PTH induced CREMP2-Luc238 expression by an average of 3-fold on day 7. Further culture and differentiation of the cells did not affect the CREMP2-Luc238 response to PTH stimulation. Similar results were found when the time course was performed without ascorbate. In this experiment CREMP2-Luc238 expression was induced 3-fold by PTH on day 3 and remained at that level throughout the 14 day experimental period. These data suggest that the stage of osteoblast differentiation may not determine the PTH response of stably transfected CREMP2-Luc238 cells.

Several studies report that ascorbic acid (AA) is necessary for the in vitro differentiation of a variety of cell types, including adipocytes, myoblasts, chondrocytes, odontoblasts, and osteoblasts. It is thought that the addition of AA stimulates the initial deposition of collagenous extracellular matrix, followed by the induction of specific genes associated with the osteoblast phenotype such as the bone/liver/kidney isozyme of alkaline phosphatase and osteocalcin.[38]. However, our study did not show that AA was necessary for the differentiation of the stably transfected osteoblastic MC3T3-E1 cells. As stated previously, we found by Northern blot analysis that osteoblast mRNAs were expressed on day 7, and the expression level increased on day 14 in the presence or absence of AA. However, an important factor seems to be the plating density. Preliminary results showed that on day 7 in the presence of AA and a plating density of 5,000 cells/cm² differentiation was enhanced. Furthermore, a plating density of 15,000
cells/cm² showed greater differentiation than the 5,000 cells/cm², suggesting that plating density plays a role in osteoblastic cell differentiation (unpublished observation).

In addition, when a time course was performed without AA, Northern blot analysis revealed that Col1a1 mRNA was present on day 3 and remained unchanged up to day 14. BSP mRNA did not appear until day 7, peaked at day 10 and decreased slightly by day 14. Osteocalcin mRNA was not present on day 3, appeared on day 7 and was highly expressed on day 14. We can conclude from these data that the length of culture plays a role in differentiation. In conclusion, our data show that AA is not necessary for the differentiation of CREMP2-Luc238 stably transfected cells, but the plating density and length of culture play a role.

As mentioned previously, PGE₂ binds to cell surface receptors and activates the cAMP-PKA pathway [39]. PGE₂ induces ICER mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR) in MC3T3-E1 cells and neonatal mouse calvaria [40] as well as playing a role in bone metabolism [11]. The cycle of bone turnover is divided into periods of activation, resorption and formation. It was reported for the first time by Klein and Raisz in 1970 that prostaglandins promote bone resorption by acting on osteoclasts. Such insight into the bone remodeling cycle is very important in orthodontic therapy. Over the years, orthodontics has evolved with advancing technology that has produced appliances and new materials that have resulted in improved results and reduced treatment time. Successful bone remodeling would produce less histological damage and pain, enhanced tooth movement, shorter treatment
periods, and more stable results. However, there may be a biological threshold beyond
which bone remodeling and, therefore, tooth movement cannot occur any more quickly.

Studies of factors, such as prostaglandins, which decrease the formation period of
the bone remodeling cycle on the pressure side during tooth movement and use activation
and resorption periods for efficient bone resorption, are considered to be of particular
significance to contemporary orthodontics [12]. Yamasaki et al [41, 42] have shown that
the rate of orthodontic tooth movement increases with local injection of PGE₂ in monkeys
and local injections of PGE₁ in humans. Kawata and Yamashita reported that pasting
PGF₂α on the oral mucosa of patients resulted in accelerated tooth movement and
alleviated pain during the process [12]. Chao et al reported that PGE₂ increases the total
number of osteoclasts and the osteoclastic-resorptive activity during orthodontic tooth
movement [11]. In addition, prostaglandin antagonists have been shown to decrease the
rate of tooth movement by inhibiting the increase of osteoclasts on the resorptive surface
[43] and the differentiation of osteoblasts on the bone-forming surface [44].
Consequently, the future of orthodontics may lie in improving the rate of bone
remodeling. PGE₂, in spite of its short-term local effects [11], may be useful in future
clinical orthodontic treatment by its local administration combined with tooth movement.
The combination could possibly lead to increased rates of bone remodeling, tooth
movement, and subsequently, treatment time. The reduction in treatment time may
potentially minimize the possible side effects of orthodontic treatment such as enamel
decalcifications, periodontal disease, and root resorption. Along the cutting edge of
technology, a more complete understanding of how prostaglandins regulate bone
formation and resorption may lead to the development of new therapies.
Bibliography:


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