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Parathyroid Hormone (PTH 1–34) Regulation of Rat Osteocalcin Gene Transcription

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ABSTRACT

Osteocalcin (OC) is a bone-specific extracellular matrix protein expressed by mature osteoblasts during late stages of differentiation. Previous studies have shown that forskolin, an activator of adenylate cyclase, stimulated OC production. Because PTH has been shown to activate several intracellular signal transduction pathways including cAMP, inositol phosphate and intracellular calcium mobilization, we investigated whether PTH action on cAMP accumulation leads to OC promoter activation. The rat OC promoter (1095 bp) was cloned into the promoterless luciferase gene reporter vector. The transcriptional activity of the rat OC promoter was evaluated after transfection of SaOS-2, an osteosarcoma cell line, with the OC promoter followed by treatment with PTH. Maximal OC promoter activity was observed within 4–8 h after the addition of 10^{-8} M PTH, whereas very little induction was seen after 24 and 48 h of treatment. The induction of OC promoter activity by PTH was concentration dependent. PTH analogs (PTH 1–84, PTH 1–34, and PTH 1–31) that stimulate intracellular cAMP accumulation, induced OC promoter activity, whereas other PTH analogs (PTH 3–34, PTH 7–34, PTH 13–34, and PTH 53–84) that do not stimulate cAMP production had no effect on OC promoter activation. Furthermore, PTH activation of the OC promoter was significantly enhanced in the presence of 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor. Inactivation of cAMP-dependent protein kinase A activity by either a selective protein kinase A inhibitor, H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5 isoquinolinesulfonamide), or antisense oligonucleotide directed against the regulatory subunit of cAMP-dependent protein kinase A, led to a corresponding loss of OC promoter activation by PTH. 5′ deletion analysis of the OC promoter demonstrated that the promoter (1095 bp) exhibited the greatest response to PTH, whereas the −198 bp construct of the OC promoter, containing only one cAMP response element and OC box, was no longer responsive. The constructs with further deletions (−120, −92, and −74) retained PTH responsiveness, but to a lesser extent. In summary, our results indicate that PTH activation of the OC promoter is a rapid event and mediated by the cAMP-dependent protein kinase A pathway. Although the novel cAMP response region overlapping the OC box is required for activation, full activation may require several *cis*-acting cAMP response elements or other response elements. (*Endocrinology* **138:** 3085–3092, 1997)

OSTEOCALCIN (OC) is the most abundant noncollagenous protein of bone extracellular matrix. It is synthesized almost exclusively by mature osteoblasts during the onset of extracellular matrix mineralization. However, the precise function of OC in regulating osteoblast function, mineral deposition, and matrix organization is unknown. Several physiological mediators of osteoblast function have been shown to influence the expression of the OC gene. The OC gene has been cloned from rat, mouse, and human (1–3). Both rat and mouse OC genes exist as clusters of related genes (4), although only two of three mouse OC related genes are expressed in bone (2). The rat and human promoters (1.8 kb and 3.9 kb, respectively) appear to contain the necessary information to direct tissue specific expression of the OC gene (5, 6). Several *cis*-acting regions including the response elements for vitamin D_3, estrogen, glucocorticoids, and cAMP, as well as the binding regions for activator protein (AP)-1 and AP-2 that are putative sites of interactions with general and bone-specific factors have been identified within the OC promoter (7–10).

Bone formation is a critical feature during development and in remodeling associated with fracture and diseases of bone, such as osteoporosis and Paget's disease. PTH, a calcium-regulating hormone, enhances bone formation when administered intermittently to experimental animals and humans (11–15). Although the exact mechanism of action of PTH in stimulating bone formation is not known, it increases osteoprogenitor differentiation (16). PTH action on these cells is transduced through G protein-coupled PTH/PTH-related peptide receptors that contain seven transmembrane domains (17–19). PTH occupation of the receptor leads to the activation of adenylate cyclase and phospholipase C pathways that results in the accumulation of multiple signal transducers including cAMP, inositol phosphate, a transient increase in the intracellular calcium, and activation of protein kinase C (20–24). Further studies have established that distinct domains within PTH may be associated with distinct biological activities (25). In addition to its effects on signal transducing factors, PTH has also been shown to increase the steady-state levels of OC messenger RNA in a rat osteosarcoma cell line, ROS 17/2.8 cells (26). The increased transcriptional activity of the rat OC promoter in MC3T3 cells by forskolin (FSK) is mediated through a novel cAMP responsive region (27). Because PTH activates multiple signal transduction pathways, we investigated whether PTH activates the OC gene, and whether this involves the cAMP-dependent protein kinase A (PKA) pathway. Using transient transfection assays in which SaOS-2 cells were transfected with the rat OC promoter (1095 bp) fused with luciferase reporter gene, we show that PTH (1–34) increased the rat OC promoter activity. We also show that cAMP-dependent PKA activation is associated with OC expression. We also demonstrate that a minimal promoter near the OC box can be

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activated by PTH, but a longer promoter (1095 bp) is required for maximal activation.

Materials and Methods

Reagents

Rat PTH 1–34, human PTH 1–84, PTH 53–84, PTH 13–34, bovine PTH 3–34, and PTH 7–34 were obtained from Bachem Biochemicals (Torrance, CA). Human PTH 1–31 was obtained from Peninsula Labs (Bellmont, CA). For treatments, PTH was solubilized in a buffer containing 1 mM HCl, 0.15 M NaCl, and 1 mg/ml BSA. FSK and H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5 isoquinolinesulfonamide) were purchased from Calbiochem (San Diego, CA). 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Sigma (St. Louis, MO). FSK, H-89, and IBMX were solubilized in dimethylsulfoxide. FBS was obtained from Hyclone Laboratories (Logan, UT). DMEM/F-12 (3:1) medium, glutamine, penicillin, streptomycin, and lipofectin reagent were purchased from Gibco/BRL Life Technologies (Gaithersburg, MD). All other reagents were of the best grade commercially available.

Cell culture

Human osteoblastic cell line, SaOS-2, was maintained in DMEM/F-12 (3:1) medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. The cells have been previously shown to produce cAMP in response to PTH and FSK (28).

Construction of rat OC promoter-luciferase reporter vector

The sequence for the rat OC promoter was obtained from the published data (GenBank accession number M23637). The promoter was generated by PCR, using rat genomic DNA as a template. The resulting promoter (1095 bp) was cloned into the *Kpn*I/*Xho*I sites of the promoterless pGL-2 basic luciferase gene reporter vector (Promega Corp., Madison, WI) in the 5′ to 3′ orientation. Various deletions of the OC promoter were constructed by progressively shortening the 5′ region with fixed 3′ end by PCR using ExSite PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were subsequently sequenced to verify insert identity.

Transfection and luciferase assay

SaOS-2 cells grown to about 60% confluence were transfected with the OC promoter constructs, using the standard calcium phosphate coprecipitation technique, followed by a 15% glycerol shock for 2 min. After an overnight recovery, transfected cells were trypsinized, pooled, and seeded in 24-well plates in DMEM/F-12 medium + 10% FBS. After an overnight incubation, the medium was removed and fresh DMEM/F-12 medium + 1% BSA containing either vehicle, PTH, or FSK at the indicated concentrations was added. In the experiment using H-89, transfected cells were pretreated with 10^{-5} M H-89 in DMEM/F-12 + 0.1% BSA for 1 h. The inhibitor was also included during treatments with PTH or FSK. After treatment with PTH or FSK for the indicated time, cells were lysed in lysis buffer (100 mM KPO_4, pH 7.8, 1 mM dithiothreitol and 0.2% Triton X-100). Aliquots of lysate were assayed for luciferase activity in the presence of luciferin (0.2 mM) and luciferase assay buffer (15 mM tricine, pH 7.8, 15 mM $MgSO_4$, 5 mM dithiothreitol and 1.5 mM ATP) using a ML 3000 automated injection Luminometer (Dynatech Labs., Chantilly, VA). To control for transfection efficiency within 5′-deletion constructs of the OC promoter (Figs. 8 and 9), SaOS-2 cells were cotransfected with pSV-β-galactosidase control vector (Promega Corp.). β-Galactosidase activity was determined in the cell lysates using Galacto-Light Plus system (Tropix, Bedford, MA). Protein concentrations of lysates were measured with the Bradford method (Bio-Rad, Richmond, CA). Luciferase activity of lysates was normalized to the protein concentrations, and the data from several independent experiments are presented as the mean ± SD. Statistical significance between samples were calculated using ANOVA and the Turkey-Kramer HSD test employing JMP 3.1 software (SAS Institute, Cary, NC).

Antisense oligonucleotide treatment

Antisense oligonucleotide (5′-GCG-TGC-CTC-CTC-ACT-GGC-3′) and sense oligonucleotide (5′-GCC-AGT-GAG-GAG-GCA-CGC-3′) were derived from the complementary DNA sequences encoding amino terminus 8–13 codons of the human PKA regulatory subunit (29). SaOS-2 cells transfected with the OC promoter were further incubated with or without phosphorothioate oligonucleotides in the presence of lipofectin reagent (30) for 36 h, followed by treatment with PTH or FSK for 8 h. The luciferase activity and protein concentrations were quantitated as described.

Results

Time course of transcriptional activation of rat OC promoter by PTH in transfected SaOS-2 cells

The effect of PTH 1–34 on OC transcription was evaluated in SaOS-2 cells transfected with the rat OC promoter (1095 bp) fused to the luciferase reporter gene. The transfected cells were treated with PTH 1–34 (10^{-8} M) for various time intervals (1, 2, 4, 8, 24, and 48 h), and the OC promoter activity was evaluated by assaying for luciferase activity. As illustrated in Fig. 1, measurable activation of the OC promoter activity (significance at $P < 0.005$) was observed at 2 h after PTH addition, with maximal induction occurring between 4–8 h after the addition of PTH, whereas very little induction was seen after 24 h of treatment. For subsequent experiments, the OC promoter activation was determined after a 8 h treatment with PTH or FSK.

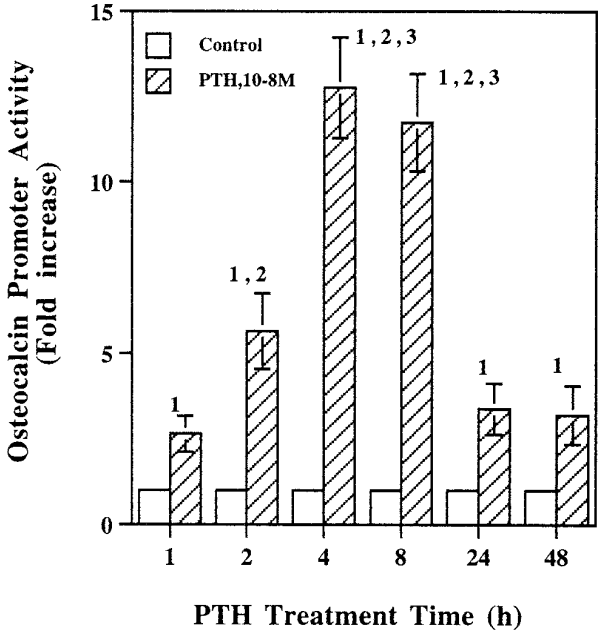

FIG. 1. Time course of PTH induction of OC promoter activity. SaOS-2 cells transfected with rat OC promoter-luciferase reporter vector (1095 OC Luc) were incubated with or without 10^{-8} M PTH for up to 48 h. At various time points, cells were washed with PBS and lysed in lysis buffer. Aliquots of lysate for each time point were used to determine luciferase activity and protein concentration. Cells treated with vehicle served as controls. Results are mean ± SD of three independent experiments (n = 9) and are expressed as fold increase of OC promoter activity in comparison with control. Luminescence light units per microgram protein at different time point upon activation are in the range of 0.37 (at 48 h) to 26.8 (at 4 h), whereas that of controls varied from 0.35–2.25. Fold increase was calculated using corresponding controls. 1, Significantly different from corresponding controls; 2, significantly different from 1-h treatment; 3, significantly different from 2-h treatment. All values were significant at $P < 0.005$.

Concentration-dependent activation of OC promoter

We next evaluated the effects of various concentrations of PTH 1–34 (10^{-11} M to 10^{-7} M) on the induction of the rat OC promoter activity. For comparison, FSK, a known stimulator of the OC promoter (27), was also tested at various concentrations (10^{-9} M to 10^{-5} M). As shown in Fig. 2, both PTH and FSK exhibited a concentration-dependent induction of OC promoter activity. PTH activation of OC promoter was detectable at 10^{-10} M, with maximal activation occurring at 10^{-9} M. FSK was effective at 10^{-6} M, with a sharp further increase at 10^{-5} M. At the latter concentration of FSK, the relative promoter activity was approximately 3-fold greater than that observed for the peak activity obtained with PTH. We also evaluated whether the effects of these two agents on the OC promoter activity was additive or synergistic by testing a saturable concentration of PTH (10^{-8} M) and the highest tolerable concentration of FSK (10^{-5} M). The results suggest that the effects are synergistic (Fig. 3). As shown in Fig. 3, the pGL-2 control vector that contains the luciferase gene driven by the SV 40 promoter was not significantly stimulated by either PTH or FSK or their combination.

Effect of PTH analogs on OC promoter activation

Because various domains of the PTH molecule have been demonstrated to exhibit distinct biological activities that are related to specific intracellular signals (20–25), we next evaluated whether the OC promoter activation is dependent on one or more of these pathways. Cells were treated with various analogs of PTH for 8 h and assayed for the OC promoter induction. As shown in Fig. 4, only those analogs capable of elevating intracellular cAMP levels (PTH 1–31, PTH 1–34, and PTH 1–84) elicited a concentration-dependent OC promoter response. Conversely, other analogs of PTH that do not elevate cAMP levels (PTH 3–34, PTH 7–34, PTH 13–34, and PTH 53–84) failed to stimulate OC promoter activation.

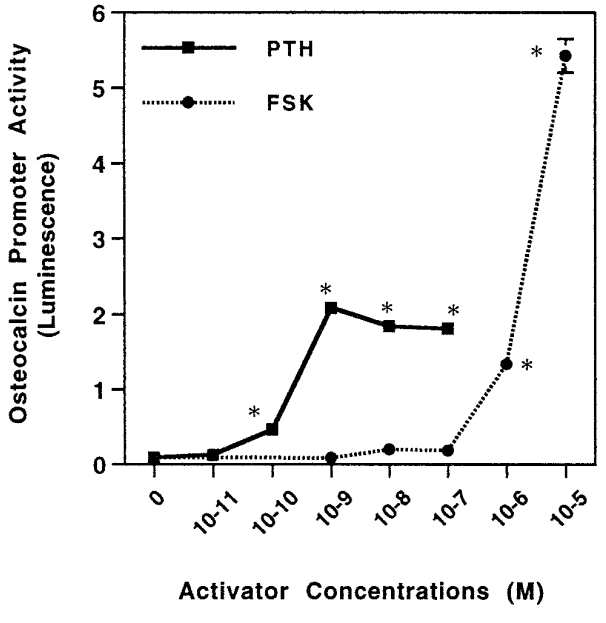

FIG. 2. Concentration-dependent activation of OC promoter activity by PTH and FSK. Following transfection of rat OC promoter-luciferase reporter vector into SaOS-2 cells, various concentrations of PTH (1–34) and FSK were added, and incubation was carried out for 8 h. At end of incubation, cells were harvested and assayed for luciferase activity and protein concentration. Results are mean ± SD from two independent experiments (n = 6) and are plotted as relative luminescence light units. *, Indicates statistical significance from controls at $P < 0.005$.

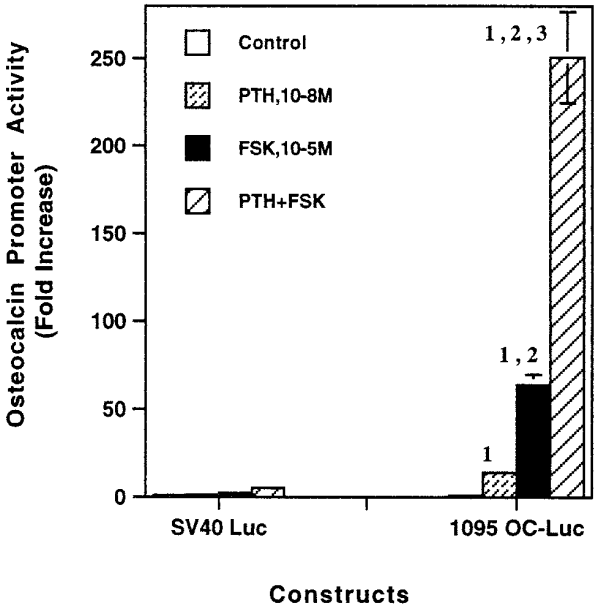

FIG. 3. Effect of PTH + FSK on OC promoter activity. SV40-luciferase vector (SV 40 Luc) and rat OC promoter-luciferase vector-transfected SaOS-2 cells were incubated for 8 h with 10^{-8} M PTH or 10^{-5} M FSK alone or in combination. At end of incubation, cells were harvested and assayed for luciferase activity and protein concentration. Results are mean ± SD of two independent experiments (n = 4) and are expressed as fold increase of OC promoter activity *vs.* control. Luminescence units per microgram protein are in the range of 0.2 (control) to 33.3 (PTH + FSK). 1, Significantly different from control; 2, significantly different from PTH; 3, significantly different from FSK or PTH. All values were significant at $P < 0.005$.

IBMX enhances PTH activation of OC promoter

To investigate further whether cAMP accumulation leads to enhanced transcriptional activation of the rat OC promoter, we next evaluated the effects of PTH on OC activation in the presence of IBMX, a phosphodiesterase inhibitor. This was tested at a constant concentration of PTH (10^{-8} M) and at two concentrations of IBMX (50 or 500 μM). IBMX demonstrated a concentration-dependent effect on OC activation (Fig. 5). IBMX alone at 50 μM was only minimally effective (1.8-fold induction), whereas at a higher concentration (500 μM), a strong activation was observed (35-fold induction). At either concentration of IBMX, PTH effect was much stronger. PTH (10^{-8} M) caused a 7-fold activation of OC promoter, and PTH + IBMX resulted in either a 33-fold (50 μM) or 75-fold (500 μM) induction of OC. Thus, conditions that enhanced cAMP accumulation (IBMX) also resulted in a corresponding increase in the OC promoter activation.

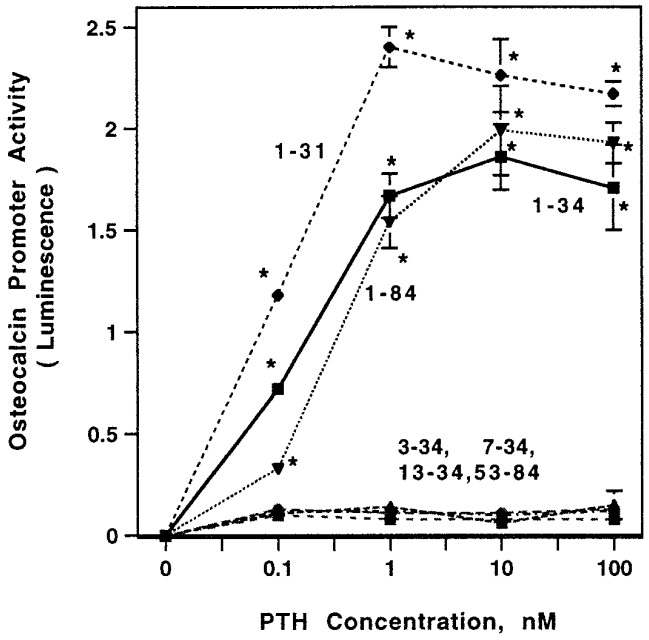

FIG. 4. Effect of PTH analogs on OC promoter activity. Transfected SaOS-2 cells were incubated with a series of 10^{-8} M PTH analogs for 8 h. Results are plotted as relative luminescence light units and are the average of triplicate values from a representative experiment. Values are mean ± SD. *, Significantly different from control ($P < 0.005$).

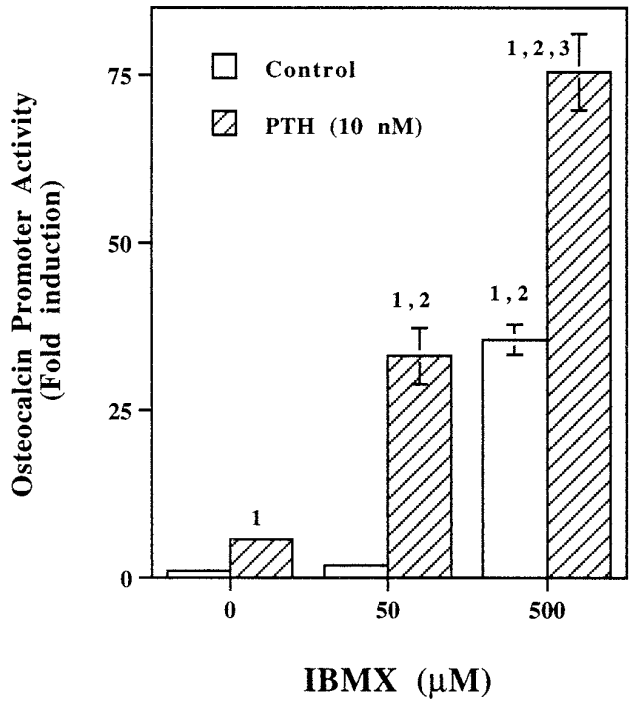

FIG. 5. Synergistic effects of PTH and IBMX on OC promoter activity. Transfected SaOS-2 cells were incubated with IBMX (50 μM or 500 μM), PTH (10^{-8} M), or in combination for 8 h. Results are average ± SD of two independent experiments (n = 6), and are expressed as fold increase of induced OC promoter activity (n = 6 ± SD) *vs.* control. Luminescence light units per microgram protein are in the range of 0.3 (control) to 25 (IBMX 500 μM + PTH). 1, Significantly different from corresponding controls; 2, significantly different from PTH alone; 3, significantly different from PTH + IBMX (50 μM) or IBMX (500 μM). All values were significant at $P < 0.005$.

cAMP-dependent PKA activation is required for OC promoter activation

To further demonstrate that PKA activation is a critical step in OC induction by PTH, we next asked whether direct inactivation of PKA would lead to a lack of OC activation. PKA inactivation was accomplished by either pretreating cells with H-89, a known inhibitor of PKA (31), or by pretreating cells with antisense oligonucleotides against PKA. In the first experiment, cells were pretreated with H-89 (10^{-5} M) for 1 h and then subsequently treated with PTH for 8 h in the presence of H-89 (10^{-5} M). The results show that H-89 treatment reduced the response to PTH (Fig. 6).

In the second experiment, the OC promoter activation was evaluated by cotransfecting cells with an antisense oligonucleotide to the regulatory subunit of human PKA (29). After transfection with the OC promoter, SaOS-2 cells were pretreated with 20 μM of either PKA antisense oligonucleotide (AS-PKA) or PKA sense oligonucleotide (S-PKA) for 36 h, followed by an additional 8-h treatment in the presence of either PTH or FSK. The result (Fig. 7A) showed that OC activation by PTH or FSK was blocked by AS-PKA but not by S-PKA. The inhibition of activation was further confirmed by treating cells with various concentrations of AS-PKA (Fig. 7B). From these observations, we conclude that cAMP-mediated PKA is the key mediator of the OC promoter activation.

5′-deletion analysis of PTH responsive region of rat OC promoter

The 5′-flanking region of the rat OC gene contains a series of *cis*-acting elements that regulate the transcription of the OC gene (7–10). These include AP-1, AP-2, vitamin D_3, glucocorticoid response element, and multiple cAMP response elements (CREs). Because FSK action on OC was mediated at least in part through the novel cAMP response region overlapping the OC box (27), we next evaluated whether this region is critical in the PTH activation of OC gene. Various 5′ deleted sequences of rat OC promoter were constructed and tested for response to either PTH or FSK. As shown in Fig. 8, the 1095-bp OC promoter showed the greatest response to PTH and FSK, whereas the −198 bp construct of the OC promoter (5′ in relation to transcription initiation site), containing only one CRE and the OC box, was no longer responsive to PTH and FSK. Constructs with further deletion, (−120, −92, and −74), regained PTH responsiveness, although the response was much less in comparison with the 1095-bp OC promoter. These observations are also true for FSK. The results suggest that 5′ deletion of the OC promoter (other than −198 construct) still allowed for PTH responsiveness, although the longer (1095 bp) OC promoter was required for full activity. To further validate that these constructs were activated via the cAMP pathway, cells transfected with various constructs were treated with various PTH analogs. The results (Fig. 9) confirmed that the promoters with further deleted sequences retained the requirement for cAMP as the mediator of activation.

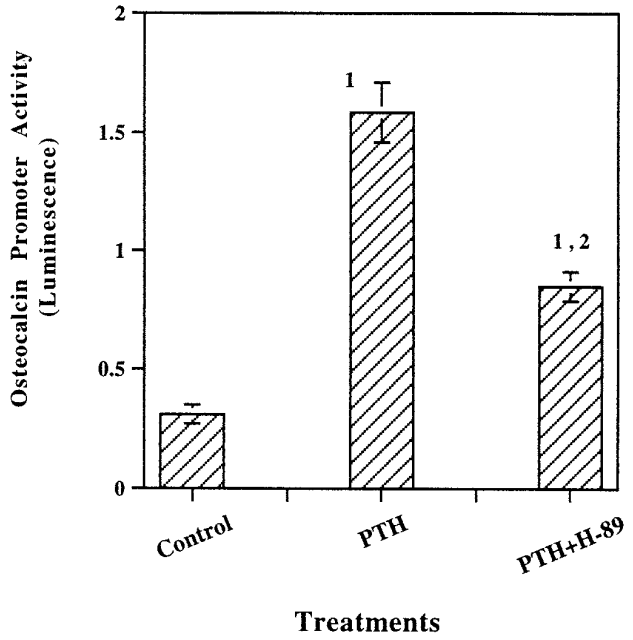

FIG. 6. Effect of PKA inhibitor on PTH induction of OC promoter activity. Transfected SaOS-2 cells were pretreated with PKA inhibitor, H-89 (10^{-5} M), for 1 h in 0.1% BSA medium followed by addition of 10^{-8} M PTH for 8 h. Results are average of two independent experiments (n = 6) and are expressed as relative luminescence units (n = 4 ± SD). 1, Significantly different from control; 2, significantly different from PTH ($P < 0.005$).

Discussion

Bone formation occurs during early development and repair. It is a carefully orchestrated process that requires multiple cellular interactions. As preosteoblasts differentiate into osteoblasts, they lay down new matrix, which subsequently becomes mineralized. This differentiation process is associated with the expression of specific matrix molecules. OC, a calcium binding protein, is present during late stage differentiation and in mineralized bone matrix. Although the exact role of OC in bone matrix mineralization is not known, it has been considered a marker for fully differentiated osteoblasts because of its unique expression pattern (32). Because PTH has an anabolic effect on bone formation by stimulating osteoblast differentiation, we investigated PTH-regulated rat OC promoter activation. The major findings of this study were 1) PTH caused a transcriptional activation of the OC promoter; 2) only those fragments of PTH that are known to induce cAMP accumulation were able to induce activation of the OC promoter; 3) inactivation of PKA either by a selective PKA inhibitor, H-89 or by antisense oligonucleotide to PKA resulted in the loss of OC activation by PTH; and 4) the novel cAMP response region overlapping the OC box was responsive to PTH, but full activation required the longer promoter (1095 bp). These results suggest that PTH activation of the OC promoter may involve cAMP-dependent PKA activation, and that the previously identified novel cAMP response region overlapping the OC box may at least in part play a role in mediating PTH effects.

The transcriptional activation of the rat OC promoter was evaluated by a transient transfection assay. A 1095-bp fragment of the OC promoter, which has been previously described to contain multiple *cis*-acting regions and also contains regions that direct the cell-specific expression of the OC gene, was fused with a luciferase reporter gene. This construct was transfected into SaOS-2 cells, and luciferase activity was quantitated as a measure of transcriptional activation of OC gene. The OC promoter activity obtained in response to FSK in SaOS-2 cells was much higher in our studies (15- to 30-fold or even greater) compared with other studies (3- to 5-fold) that used MC3T3 cells (27). This may be due to differences in species (human *vs.* mouse) or differences in the stage of differentiation. In experiments not reported here, we also evaluated OC transcription in ROS17/2.8 cells, a cell line that is generally considered to be more fully differentiated than SaOS-2 cells. In ROS17/2.8 cells, the basal transcriptional activity was much higher than in SaOS-2 cells, but the PTH activation was only about 1.5-fold (our unpublished observation). An interpretation of these studies is that basal activation is most likely to be dependent on the stage of differentiation of the cells, whereas PTH responsiveness may depend on whether or not a given cell exhibits PTH receptor. In addition, the duration of treatment with the activators was different in these studies. Our time course studies (Fig. 1) suggest that the maximal promoter activation occurred between 4–8 h, whereas only modest or no activity was detectable at 24–48 h. In a majority of the previously reported studies, the activation of OC promoter was evaluated only after 24–48 h of treatment with FSK or vitamin D_3, and this may account for differences in fold activation (27, 9, 33). The loss of activity at 24–48 h in the presence of PTH may be due to an unique activity of PTH. These *in vitro* effects of PTH may be analogous to the reported *in vivo* effects on bone formation, which is stimulated upon intermittent administration, whereas continuous administration resulted in resorption (34–36). It is thus possible that PTH may display time-dependent differential effects on OC activation *in vitro*.

PTH activates a variety of intracellular signal transduction pathways that lead to generation of second messengers including cAMP, inositol phosphate, and intracellular calcium (20–24). Our studies suggest that the OC promoter activation by PTH is mediated primarily by the cAMP-dependent PKA pathway for the following reasons. 1) Only those analogs of PTH that activate PKA (PTH 1–34, 1–84, and 1–31) were able to stimulate OC transcription. Although PTH 1–34 and PTH 1–84 have full spectrum of activity, PTH 1–31 activates only PKA with no demonstrable effects on protein kinase C (PKC) or phospholipase C (PLC) (37). In contrast, N-terminally truncated PTH analogs (PTH 3–34, 7–34, 13–34, and 53–84) were ineffective in activating the OC promoter. These analogs activate PKC, but not adenylate cyclase (38, 39). 2) IBMX, a phosphodiesterase inhibitor that enhances cAMP accumulation by reducing its degradation, enhances PTH effects. 3) Inactivation of PKA with H-89, a selective inhibitor of PKA, or an antisense oligonucleotide directed against the regulatory subunit of PKA, reduced PTH-stimulated OC activation. These results collectively establish that cAMP- dependent PKA activation is the critical mechanism of OC activation by PTH.

The significance of the synergistic effects of FSK on PTH (Fig. 3) is not clear. In these studies, we have used a saturable

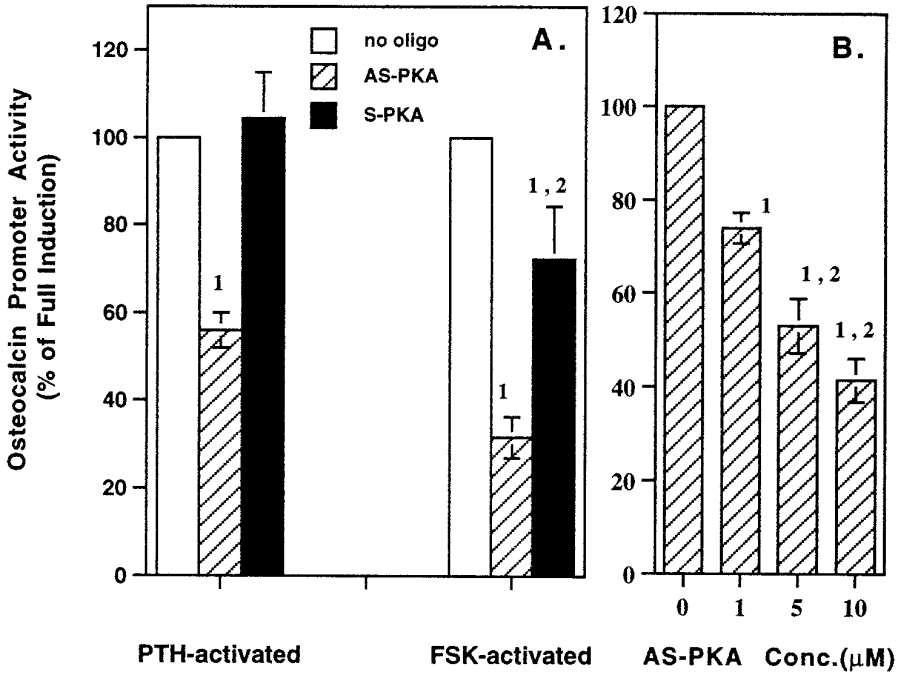

FIG. 7. Effect of PKA antisense oligonucleotide on PTH- or FSK-induced OC promoter activity. A, Transfected SaOS-2 cells were incubated in absence or presence of 20 μM PKA antisense (AS-PKA) or PKA sense (S-PKA) oligonucleotides for 36 h followed by an 8-h activation with PTH (10^{-8} M) or FSK (10^{-5} M). Results are average ± SD of two independent experiments (n = 6) and are plotted as percentage of OC promoter activity induced by PTH or FSK in absence of oligonucleotides. 1, Significantly different from no oligonucleotides control; 2, significantly different from AS-PKA treated ($P < 0.005$). B, Concentration-dependent inhibition of OC promoter activity by PKA antisense oligonucleotide. Transfected SaOS-2 cells were first treated with various concentrations of PKA antisense (AS-PKA) oligonucleotide in presence of 10 μg/ml of lipofectin for 36 h and then activated with PTH (10^{-8} M) for 8 h. Lysates were assayed for luciferase activity and protein concentration. Results are plotted as percentage (n = 3 ± SD) of rat OC promoter activity induced by PTH in absence of oligonucleotide. 1, Significantly different from no oligonucleotide control; 2, significantly different from 1 μM ($P < 0.005$).

concentration of PTH, but only a maximum tolerated concentration of FSK. The major mechanism of action of FSK is through adenylate cyclase pathway, but cAMP-independent mechanisms have also been recognized (40). Because PTH activates other pathways (PKC and PLC), it is conceivable that synergistic OC activation may be the result of other pathways that are not sufficiently active when treated with PTH or FSK alone. Similar potentiation of PTH activation has been recently observed for FSK in other systems (41, 42). In SaOS-2 cells, pretreatment with PTH resulted in a large increase in cAMP accumulation upon subsequent exposure to FSK (41, 42), which is consistent with the results presented here (Fig 3). Additionally, FSK treatment could result in higher PTH receptor numbers, resulting in greater response to PTH.

Previous studies using FSK and MC3T3 cells, have identified a novel cAMP response region overlapping the OC box of the rat OC promoter that was necessary and sufficient for FSK activation (27). We used similar approach to evaluate the effects of PTH. Starting with 1095 bp of the rat OC promoter, by 5′-deletion analysis, we found that the longer promoter (1095 bp) gave the greatest response to PTH and FSK. The −198-bp deletion construct containing only one CRE and the OC box, which was found to be active in MC3T3 cells (27), was not active in SaOS-2 cells (Figs. 8 and 9) and was not responsive to PTH or FSK. However, with further deletion of the rat OC promoter to −120, −92, and −74, response to PTH and FSK was regained, although to a lesser extent than the longer promoter (1095 bp). These deletion constructs (−120, −92, −74) displayed their dependence on cAMP production, because only those PTH analogs that stimulate cAMP production activated these constructs (Fig. 9). The reason for the loss and apparent reappearance of PTH-responsiveness is not understood. In contrast to the previous reports, it is clear that the longer promoter (1095 bp) is more strongly active than any other deletion constructs. This may indicate potential interactions between several *cis*-acting elements including vitamin D_3, glucocorticoid response elements, and AP-1 sites. In the rat promoter, there are at least three putative cAMP response elements, and all may be required for full activation. Another interpretation in the loss of activation of −198 construct and reappearance in the −120 construct might suggest a repressor element between −198 to −120 that might inhibit the response to PTH.

The role of osteocalcin in bone differentiation and matrix formation is not known. Although the association of the protein expression during late stage mineralization is well documented, what function if any, the protein may play is only speculative. A recent study of osteocalcin-deficient mice has demonstrated an increased bone mineral density in these animals, with no apparent alterations in mineralization or rate of resorption (43). This led to a provocative hypothesis that osteocalcin may be an inhibitor of bone formation. It is possible that OC expression may be the termination signal for the anabolic response. Although OC expression in cultures is not manifested until at least after 2–3 weeks *in vitro*,

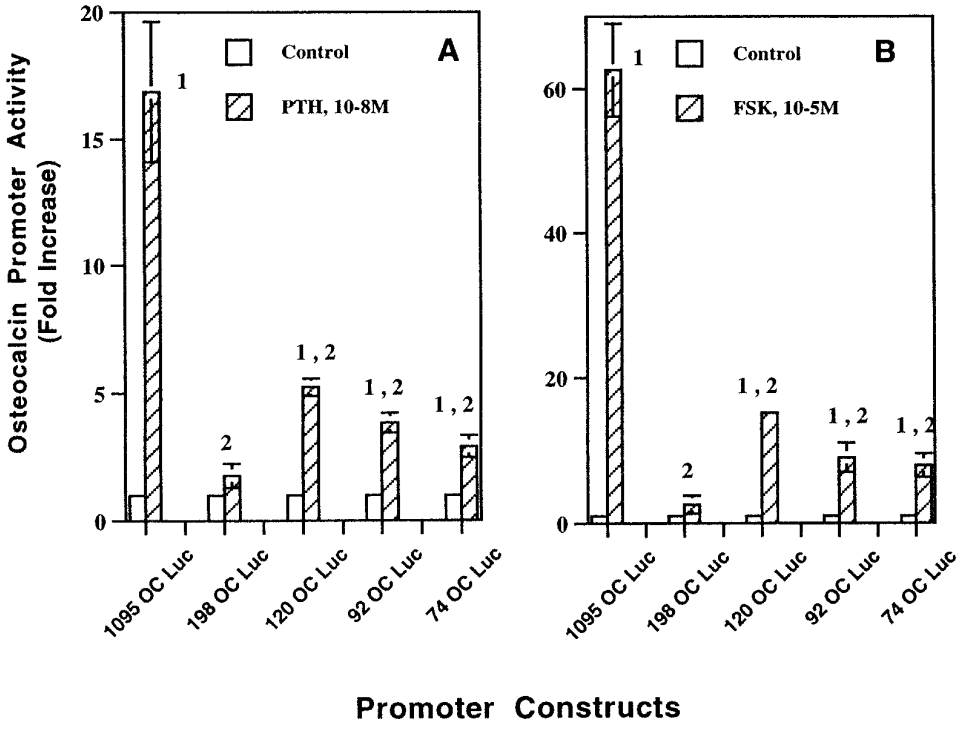

FIG. 8. 5′-deletion analysis of OC promoter response to PTH or FSK. Various OC promoter constructs (named numerically according to position of deletion relative to transcription initiation site) directing expression of an luciferase reporter gene were cotransfected with pSV-β-galactosidase vector into SaOS-2 cells, treated with 10^{-8} M PTH (A) or 10^{-5} M FSK (B) for 8 h, then harvested and assayed for luciferase activity, galactosidase activity, and protein concentration. Transfection efficiency between various 5′-deletion constructs were monitored. Results are average of two independent experiments (n = 4) ± SD and are expressed as fold increase of OC promoter activity *vs.* their control. 1, Significantly different from control; 2, significantly different from PTH or FSK treated −1095 OC promoter. All values are significant at $P < 0.005$.

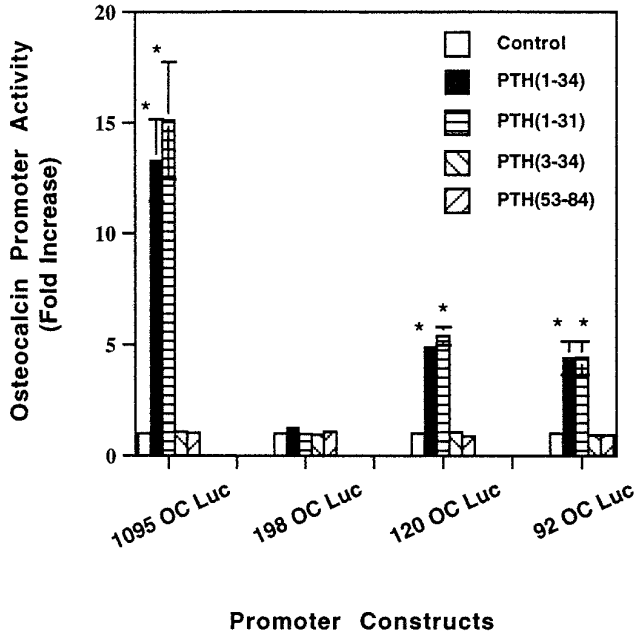

FIG. 9. Effect of PTH analogs on 5′-deleted OC promoter constructs. SaOS-2 cells transfected with various 5′-deleted OC promoter constructs and treated with a series of 10^{-8} M PTH analogs, including PTH 1–34, PTH 1–31, PTH 3–34, and PTH 53–84 for 8 h. At end of incubation, cells were harvested and assayed for luciferase activity and protein concentration. Results are average of triplicate ± SD and are plotted as fold increase (n = 3 ± SD) of OC promoter activity *vs.* control. *, Significantly different from respective controls ($P < 0.005$).

in vivo studies have suggested that PTH can induce serum osteocalcin levels within 3 days of treatment (Chandrasekhar, Harvey, and Hock, unpublished observation). This would imply that PTH effects on OC may be a relatively early event and may influence osteoprogenitor differentiation and function both directly and indirectly. The consequence of such induction is not clear. When the cells become fully mature, OC may signal the beginning of terminal differentiation.

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