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Induction of Rat Interstitial Collagenase (MMP-13) mRNA in a Development-Dependent Manner by Parathyroid Hormone in Osteoblastic Cells

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The purpose of this study was to determine whether the production of interstitial collagenase mRNA in response to parathyroid hormone (PTH) changes with osteoblast phenotypic development. To accomplish this, cells derived from fetal rat calvaria were examined. The calvarial osteoblasts, which proliferate when placed in culture, can be made to differentiate after confluence. Studies were performed on cells while they were proliferating, at confluence, and during the differentiation process. The cells were treated with PTH for various times, and interstitial collagenase mRNA was quantified by RNase protection assay. We concluded that the ability of PTH to induce interstitial collagenase mRNA in these cells increased with osteoblast phenotypic development. We also determined that the response could be mimicked by combining the effect of 8-bromo-cAMP and 12-*O*-tetradecanoyl-phorbol-13-acetate, stimulators of the protein kinase A and protein kinase C pathways, respectively, both known to be activated by PTH. The binding of nuclear factors to two regions previously reported to be important for PTH induction of the gene in UMR 106-01 cells was also examined. These data indicated that the binding of nuclear factors to oligonucleotides encompassing the TRE (-51) or the PEA3 (-80) elements changed with development of the osteoblast phenotype. The latter was also shown to be PTH responsive.

Key Words: Matrix metalloproteinase; cyclic adenosine monophosphate; protein kinase C; bone; osteoblasts; parathyroid hormone.

Introduction

Parathyroid hormone (PTH) is one of several systemic hormones and factors that regulates bone remodeling *(1)*. This process consists of a breakdown phase followed by a period of resynthesis and takes place to accommodate growth and healing of fracture *(2)*. Bone is also remodeled to maintain calcium homeostasis in the body, which is crucial for life functions *(3)*. To study the action of PTH during the bone remodeling process, our laboratory has been examining the production of interstitial collagenase (MMP-13) in rat osteoblastic cells. In the present study, we utilized rat calvaria–derived osteoblasts in culture to correlate osteoblastic differential stage with the ability of PTH to induce interstitial collagenase *(4)*.

Calvarial osteoblasts from 21-d-old fetal rats (ROB), when placed in culture, will progress through various stages of developmental maturation in which the expression of certain genes related to osteoblast phenotype change *(4)*. While proliferating, they are considered to be in a preosteoblastic stage, developing into mature osteoblasts when proliferation has stopped and matrix formation has begun; a marker for transition into the mature osteoblast is the production of alkaline phosphatase (AP) *(5)*. Osteocalcin and the beginning of hydroxyapatite crystal formation signals the time when cells transition into preosteocytes and finally into osteocytes. The latter are found surrounded by mineralized osteoid and are considered the terminal differentiation stage of this cell type. These phenotypic changes are induced in culture through incubation with β-glycerophosphate and ascorbic acid.

The mechanism by which PTH stimulates signal transduction in target cells is primarily through cyclic adenosine monophosphate (cAMP) *(6–8)*. It has been reported, however, that PTH also stimulates phosphatidylinositol 4,5-bisphosphate hydrolysis with production of inositol 1,4,5-triphosphate and diacylglycerol in osteoblastic cells *(9)*, which are second-messenger signals for the release of intracellular stores of calcium and activation of protein kinase C (PKC), respectively *(10,11)*. PTH has also been shown to increase transiently cytosolic calcium in transformed and

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normal osteoblastic cells through the release of calcium from intracellular stores and influx from the extracellular milieu *(12,13)*.

The rat MMP-13, a matrix metalloproteinase, is virtually identical to the mouse MMP-13 and is highly homologous to the human collagenase-3 *(14–16)*. Agents capable of affecting interstitial collagenase mRNA levels in rat calvaria–derived osteoblasts in culture include retinoic acid, platelet-derived growth factor BB, bone morphogenetic protein 2, and interleukins 1 and 2 *(17–20)*. Interstitial collagenase, secreted by the osteoblast in response to bone resorption stimulators, is thought to initiate the catabolic portion of the remodeling process through removal of the unmineralized osteoid layer, allowing osteoclast access to the mineralized matrix *(21)*. Previous data indicated that induction of interstitial collagenase by PTH in UMR 106-01 cells, a rat osteoblastic osteosarcoma-derived cell line, was in part transcriptional *(16,22)*; the UMR 106-01 cells are osteoblastic in phenotype-producing type I collagen and AP. We also demonstrated that the cAMP analog, 8-Br-cAMP, was able to mimic the transcriptional stimulation of the interstitial collagenase gene in UMR 106-01 cells *(22)*. In addition, our data indicated that this induction occurred through a TRE (–51; TPA-responsive element) in the 5' regulatory region of the interstitial collagenase gene. The cAMP-responsive element binding protein (CREB) was shown to bind to this region, with activation most likely occurring through PTH-mediated phosphorylation of CREB *(23)*. A PEA3 site (–80) in the 5' regulatory region of the gene was reported also to participate in the PTH response (Quinn, C. O., Rajakumar, R. A., and Agapova, O. A., unpublished data) *(24)*. A PTH-mediated time-dependent increase in binding of nuclear factors to this region was demonstrated. Components of this complex include Ets-1 and the CREB binding protein (CBP).

In the current study, we used RNase protection assay to examine the production of stable mRNA in response to PTH, 8-Br-cAMP, and the PKC stimulator, 12-*O*-tetradecanoylphorbol-13-acetate (PMA). The calvarial osteoblasts were examined in culture while still proliferating, at confluence and during the differentiation process. Our data indicate that the ability of PTH to induce interstitial collagenase mRNA increases with development of the osteoblastic phenotype in rat calvarial osteoblasts. The pattern of nuclear factors binding to the TRE and PEA3 regions of the interstitial collagenase gene was also shown to change with development of the osteoblast phenotype. In addition, factors binding to the latter responded to PTH in a development-dependent manner.

Results

Induction of Interstitial Collagenase mRNA by PTH in a Development-Dependent Manner

To determine whether the ability of PTH to induce collagenase mRNA changed during development of the osteoblastic phenotype, fetal rat calvaria–derived osteoblasts were grown in culture. The cells were incubated in growth media until they became confluent, at which point they were allowed to undergo differentiation in media containing β-glycerophosphate and ascorbic acid, which promote development of the osteoblastic phenotype *(4)*. At various times during the differentiation process, the cells were treated with PTH (10^{-8} *M*, 4 h) and total RNA was recovered; treatment conditions were based on previous studies with the UMR 106-01 cells *(22)*. RNase protection assays demonstrated that the peak stimulation by PTH, calculated as fold induction over control, occurred on d 10 in culture (Fig. 1A–C); the cells became confluent on d 5 in culture. Day 10 in culture was also the time of maximum AP production, monitored through staining (data not shown), and when mineralization was approx 50% of maximum (Fig. 2). With further time in culture, collagenase mRNA produced in response to PTH decreased until levels measured were equivalent to those of the control.

8-Br-cAMP Mimics Development-Dependent PTH Induction of Interstitial Collagenase mRNA

The cAMP analog, 8-Br-cAMP, was shown to be capable of mimicking the transcriptional response to PTH of collagenase in UMR 106-01 cells *(22)*. Here, the cAMP response was examined in the calvarial osteoblasts to determine the effect of osteoblast development on this induction process. The cells were treated with 8-Br-cAMP (10^{-3} *M*, 6 h) on the days indicated (*see* Fig. 1). RNase protection assays demonstrated that the time course of production of collagenase mRNA in response to 8-Br-cAMP was similar to the PTH response (Fig. 1A, C).

Capability of PMA to Induce Interstitial Collagenase mRNA

Although the PTH response in UMR 106-01 cells is mediated mainly by cAMP, a contributory effect of the calcium/PKC has been reported *(6,8)*. To determine the effect of PKC activation on collagenase production during osteoblast development, calvarial osteoblasts were treated with PMA (2.5×10^{-6} *M*, 6 h) on the days indicated (*see* Fig. 3). RNase protection assay demonstrated that unlike the results seen with PTH and 8-Br-cAMP, the greatest induction of interstitial collagenase mRNA occurred while the cells were still proliferating, at confluence, and during the early days of differentiation corresponding to d 4, 5, and 7, respectively (Fig. 3).

Increase in Constitutive Levels of Interstitial Collagenase with Time in Culture

The data already presented, are reported as the fold increase over control for the day being examined. To determine whether the decreased PTH response seen with the development of the osteoblast phenotype after d 10 in culture was a true reflection of what was occurring, collagenase mRNA levels were calculated as fold increase over

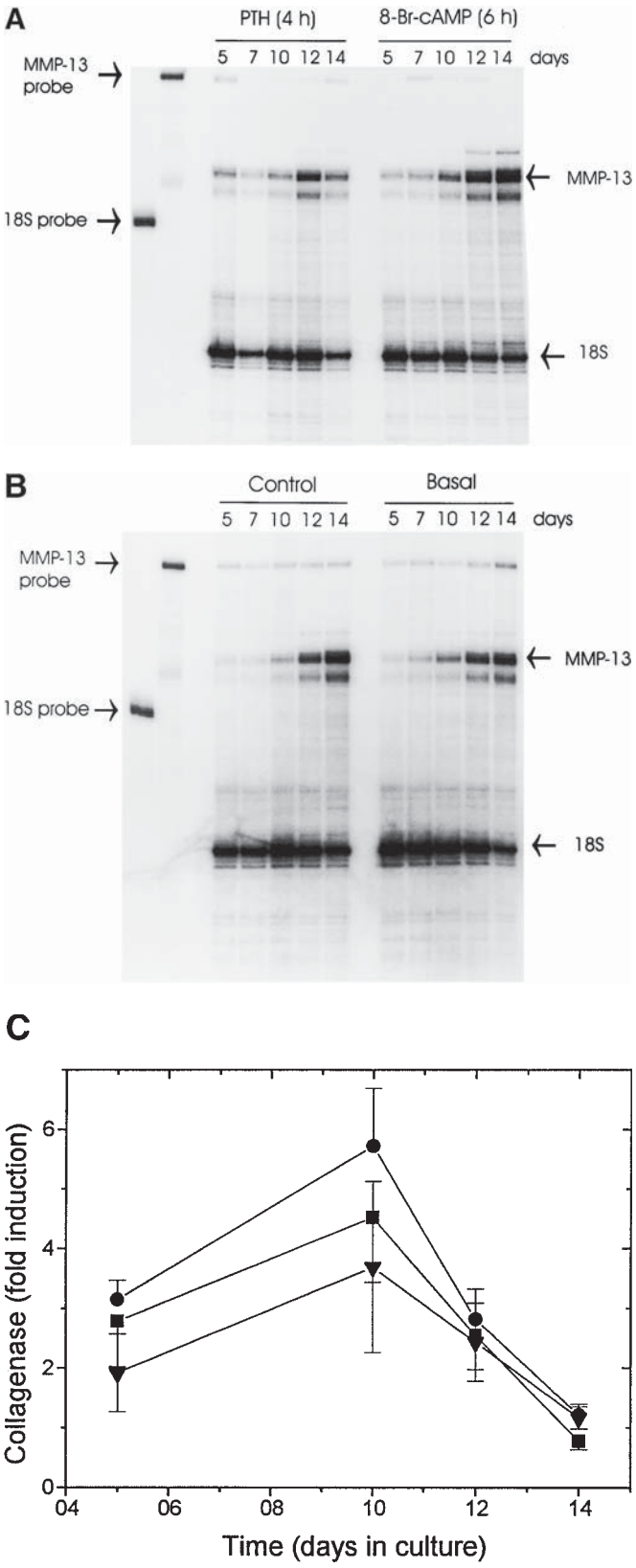


Fig. 1. Change in expression of interstitial collagenase mRNA correlated with days in culture. Osteoblasts derived from 21-d-old fetal rat calvaria were placed in culture. The cells were incubated in growth media until confluent (d 5), at which point they were switched to differentiation media. For treatment, the cells were preincubated in growth media containing 0.5% serum for 16 h and then treated with PTH (10^{-8} *M*, 4 h) or 8-Br-cAMP (10^{-3} *M*, 6 h) on the days indicated. Total RNA was isolated and RNase protection analysis performed on 20 μg of RNA. Collagenase levels were standardized to 18S ribosomal RNA. These are Phosphorimager scans of representative RNase protection gels of **(A)** PTH and 8-Br-cAMP, and **(B)** control and basal experiments. Basal levels were measured in cells that had not undergone any treatment, including preincubation. **(C)** A graphic representation of the mean ± SEM of three independent experiments expressed as the fold induction over control (underwent preincubation but were otherwise untreated). The means of the points were found to be significantly different for PTH (j) and 8-Br-cAMP (d) but not for basal (.) by analysis of vaiance (ANOVA) ($p < 0.05$ and $p > 0.05$, respectively).

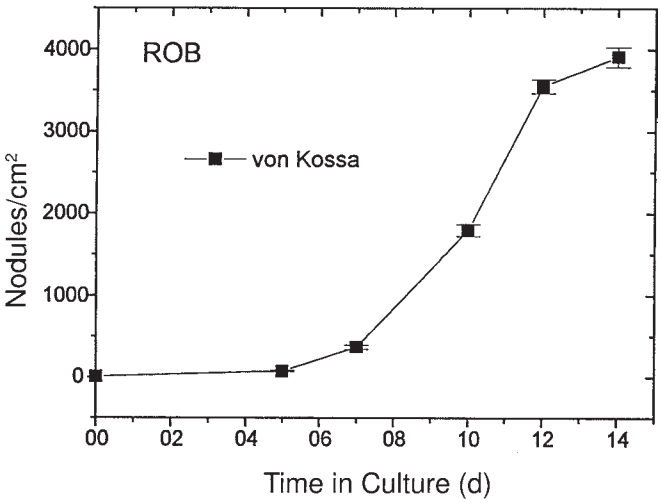


Fig. 2. Mineralized bone nodule formation by rat calvarial osteoblasts. Osteoblasts derived from 21-d-old fetal rat calvaria were placed in culture. Cultures were incubated in growth media until confluent (d 5), at which point they were switched to differentiation media. On the days indicated, the cells were von Kossa stained. Ten fields were quantified on each plate. This is a graphic representation of the mean ± SEM of eight independent experiments. The means were found to be significantly different by ANOVA ($p < 0.01$).

d 5 (confluence). It was apparent that the basal level (incubation maintained in 10% serum) was increasing rapidly with time, so that on d 14 it was 25-fold greater than on d 5 (Figs. 1B and 4). In addition, the control level (preincubation for 16 h in media containing 0.5% serum) was also increasing rapidly with time in culture. The amount of collagenase mRNA produced in response to PTH and 8-Br-cAMP also increased with time in culture, but to a much lesser extent (approx 10-fold) than under control and basal conditions (Figs. 1B and 4).

Mimicking of PTH Induction of Interstitial Collagenase mRNA by a Composite of Protein Kinase A and PKC Activation

The studies aforementioned examined the time course of expression of interstitial collagenase in response to the developing osteoblast phenotype. To establish the actual time

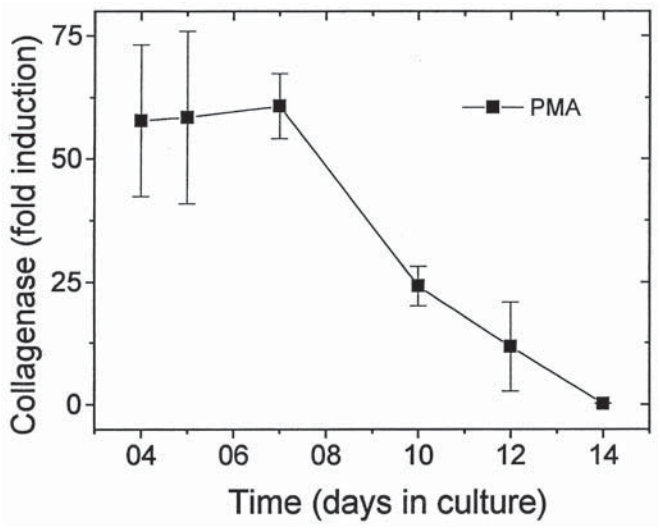


Fig. 3. Phorbol ester induction of interstitial collagenase mRNA correlated with days in culture. Osteoblasts derived from 21-d-old fetal rat calvaria were placed in culture. The cells were incubated in growth media until confluent (d 5), at which point they were switched to differentiation media. For treatment, the cells were preincubated in growth media containing 0.5% serum for 16 h and then treated with PMA (2.5×10^{-6} *M*, 6 h) on the days indicated. Total RNA was isolated and RNase protection analysis performed on 20 μg of RNA. Collagenase levels were standardized to 18S ribosomal RNA. This is a graphic representation of the mean ± SEM of three independent experiments expressed as the fold induction over control (underwent preincubation but were otherwise untreated). The means were found to be significantly different by ANOVA ($p < 0.05$).

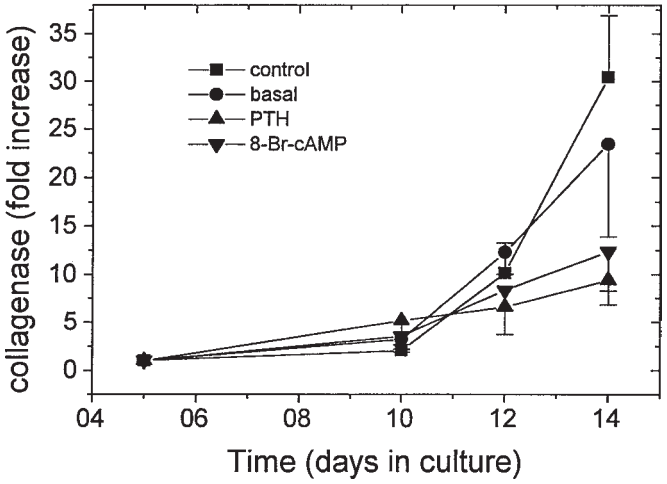


Fig. 4. Increase in interstitial collagenase mRNA with days in culture was serum independent in rat calvarial osteoblasts. Osteoblasts derived from 21-d-old fetal rat calvaria were placed in culture. Cultures were incubated in growth media until confluent (d 5), at which point they were switched to differentiation media. On the days indicated, the cells were preincubated in growth media containing 0.5% serum for 16 h and then treated with PTH (10^{-8} *M*, 4 h) or 8-Br-cAMP (10^{-3} *M*, 6 h) and total RNA harvested. Basal levels were measured in cells that had not undergone any treatment, including preincubation. Control cells were preincubated along with experimental cells but were not otherwise treated. RNase protection analysis were performed on 20 μg of RNA. Collagenase levels were standardized to 18S ribosomal RNA. This is a graphic representation of the mean ± SEM of three independent experiments expressed as the fold induction over the level at confluence (d 5 in culture). The means for the points on each curve were found to be significantly different by ANOVA ($p < 0.05$).

course of production of collagenase in response to PTH (10^{-8} *M*), 8-Br-cAMP (10^{-3} *M*), and PMA (2.5×10^{-6} *M*), cultures on the peak day of PTH induction (d 10) were examined. Cells were treated at the indicated times (*see* Fig. 5) and RNase protection assays were performed.

In response to PTH, interstitial collagenase mRNA levels increased slightly by 2 h, peaked at 9 h, and then returned to basal level by 16 h. The response to 8-Br-cAMP also peaked at 9 h; however, the rise was more gradual with less than half the maximum level evident at 6 h compared to the PTH response, which was approaching 90% of maximum by that time. By contrast, induction in response to PMA was more rapid, having peaked between 4 and 6 h with levels falling by 9 h. These data are presented as fold induction over control (untreated samples) in Table 1. To compare the time course of expression, the values were recalculated and graphed as a percentage of maximal response (Fig. 5).

Change in the Binding Pattern of Nuclear Factors to –36 to –58 Upstream Regulatory Region of Interstitial Collagenase Gene with Development of Osteoblast Phenotype Independent of PTH Action

Previously, we reported that PTH induction of collagenase in the UMR cells was through a TRE (–51) consensus binding sequence. It was determined that CREB was binding to this site and was most likely phosphorylated through PTH action *(23)*, thereby leading to activation of transcription. To determine whether the pattern of transcription factor binding to this region was affected by the development of the osteoblast phenotype, gel shift experiments were performed. Cells on d 5 and 10 in culture, the day on which the cells became confluent and the day of peak PTH inductive effect, respectively, were treated with PTH (10^{-8} *M*, 4 and 24 h) and then nuclear extracts were obtained. Protein binding to an oligonucleotide-containing sequence of the –36 to –58 region, which encompasses the TRE consensus binding sequence (–51), was examined. Two bands were evident when nuclear extracts from the d 5 cultures were used, neither of which were affected by PTH treatment (Fig. 6A). The upper band, however, decreased whereas the lower band increased in intensity with increased time in culture. This was particularly apparent in the time course with extracts from cells incubated under basal conditions (Fig. 6B). Incubation with antibodies against CREB and CBP produced supershifts of nuclear factors present in the d 5 basal extracts capable of binding to this region. By d 10 in culture, the supershift produced by the CREB antibody

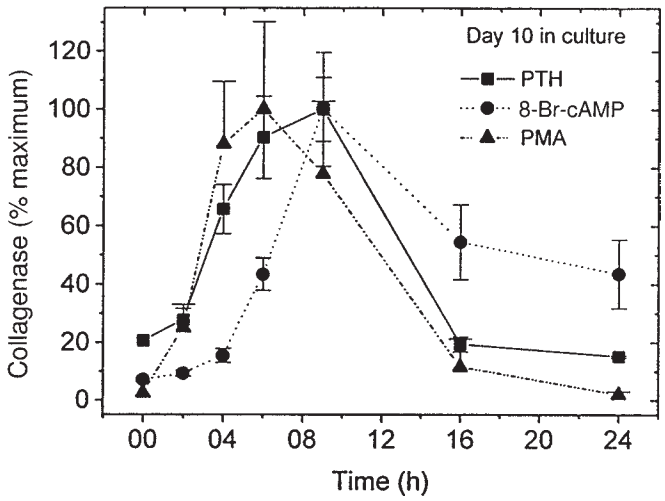


Fig. 5. Time course of expression of MMP-13 mRNA in rat calvarial osteoblasts at 10 days in culture. ROB cells were grown to confluence in growth media (5 d), at which point the media was switched to differentiation media. At d 9, cells were preincubated in media containing 0.5% serum for 16 h, and treatment with PTH (10^{-8} *M*), 8-Br-cAMP (10^{-3} *M*), or PMA (2.5×10^{-6} *M*) was for the indicated times on d 10 (maximum PTH induction time determined in Fig. 1). Total RNA was harvested and RNase protection assay performed. This is a graphic representation of the mean ± SEM for three to five independent experiments. Collagenase levels were standardized to 18S ribosomal RNA and expressed as a percentage of maximum. The means for the points on each curve were found to be significantly different by ANOVA ($p < 0.01$).

decreased, whereas the CBP supershift remained the same and an Ets-1 supershift was produced. Analysis of the d 10 nuclear extract from cells that had been treated with PTH indicated that antibodies against Ets-1, CBP, and CREB were all capable of producing supershifted bands (Fig. 6C).

Change in Transcription Factor Binding to –95 to –71 Region in Response to PTH in a Development-Dependent Manner

We demonstrated that in addition to the TRE (–51), a PEA3 binding site (–80) in the rat interstitial collagenase gene was also necessary for PTH induction of the gene (unpublished). Binding of nuclear factors to this region was shown to increase in a PTH time-dependent manner in UMR 106-01 cells *(24)*. Therefore, binding of nuclear factors from the calvarial osteoblasts was also examined. These data indicated that binding of nuclear proteins isolated from cells from d 10 cultures was dramatically affected by PTH treatment. A dramatic increase in binding was evident with extracts from cells after 4 h of PTH (10^{-8} *M*) treatment, which decreased in cells treated for 24 h (Fig. 7A). This increase in response to PTH treatment was not apparent with nuclear extracts from nuclear extracts from cells at confluence (5 d). An increase in binding of nuclear factors was also seen with increasing time in culture under basal conditions; cells maintained under basal conditions were not incubated in media containing 0.5% serum prior to the time of harvest (Fig. 7B).

Gel supershift analyses were performed with nuclear extracts from cells either incubated under basal conditions or treated with PTH (10^{-8} *M*, 4 h). Cells were examined under basal conditions on d 5 and 10 in culture, whereas PTH treatment was examined only on d 10, the day of maximum response. Antibodies against Ets-1, CBP, and CREB were able to produce supershifts of bands with nuclear extracts from d 5 cells incubated under basal conditions (Fig. 7C). With the nuclear extracts from d 10 cells, the CREB antibody was no longer capable of producing a supershift, whereas the supershifted band with Ets-1 and CBP antibodies increased in intensity.

Discussion

Previous studies that we have conducted have been primarily in UMR 106-01 cells. To compare and extend these studies into nontransformed cells we chose to characterize interstitial collagenase induction by PTH in rat calvaria–derived osteoblastic cells. This is a well-established model system for examining osteoblastic cells while they are proliferating and during the differentiation process when they express osteoblast-specific proteins and markers in a developmental-dependent manner *(4)*. By examining these cells while they were proliferating and then differentiating, we determined that the pattern of PTH induction of interstitial collagenase changed with progression of the developmental phenotype.

Signal transduction by PTH in osteoblastic cells has been reported to be mainly through cAMP but with a contributory effect of the PKC/calcium pathways *(1,25)*. In the present study, we examined the induction of interstitial collagenase mRNA through agents that stimulate the protein kinase A (PKA) and PKC pathways in the rat calvarial osteoblasts. The surprising result was that PMA was capable of invoking a significant increase in the amount of collagenase mRNA produced, in contrast to the UMR 106-01 cells, in which very little effect was observed *(22)*. It was also interesting that the PKC response was much stronger in the proliferating cells and began to decrease with increasing mineralization. In comparing the PMA developmental pattern to that seen with 8-Br-cAMP, it was evident that response to the former was decreasing as that to the latter was increasing. The response at confluence was at a maximum for PMA, whereas the 8-Br-cAMP-induced mRNA production was only about half of maximum. Likewise, on the day of maximal response to 8-Br-cAMP, the PMA response was decreased to approximately half of maximum. Thus, PKC was most capable of stimulating interstitial collagenase mRNA production in the proliferating cells and in differentiating cells before mineralization was evident.

The time course of interstitial collagenase induction in response to PTH was performed on d 10 in culture, the day

Table 1
Collagenase mRNA (d 10 in culture)

Time (h)	PTH		8-Br-cAMP		PMA	
	Fold induction[a]	SEM	Fold induction[a]	SEM	Fold induction[a]	SEM
0	1	0	1	0	1	0
2	1.34	0.19	1.31	0.14	10.19	3.21
4	3.18	0.41	2.19	0.35	35.72	8.71
6	4.38	0.68	6.14	0.79	40.56	12.28
9	4.84	0.53	14.14	2.76	31.56	10.11
16	0.94	0.13	7.70	1.81	4.65	3.95
24	0.73	0.02	6.16	1.67	0.82	0.42

[a] Calculated as the mean for three to five independent experiments and expressed as the fold induction over control (underwent preincubation but were otherwise untreated).

of maximal stimulation by PTH. The data indicated that although the curve produced in the calvarial osteoblasts was similar to that seen with the UMR 106-01 cells, there was a definite difference. The induction was more gradual, peaking at approx 9 h with only about half the maximal amount evident at 4 h compared to peak levels at 4 h in the UMR 106-01 cells *(22)*. Another difference observed was that induced levels fell to control level by 16 h and remained there at 24 h, whereas in the UMR 106-01 cells, levels were still markedly elevated at 24 h (approx 30% of maximum) *(22)*.

The response to 8-Br-cAMP and PMA was also examined on d 10 in culture. The data indicated that unlike in the UMR 106-01 cells, the PKA pathway did not mimic the PTH response *(22)*. In the calvarial osteoblasts, there appeared to be a more even distribution between the two pathways. The response to 8-Br-cAMP was delayed compared to that of PTH. Although the time of peak expression was similar (9 h), the slope of the line to attain maximum expression for the PTH-generated curve was much steeper than that for 8-Br-cAMP. The early curve in the PMA induction was more reflective of the PTH curve during that time period, but unlike the 8-Br-cAMP response, mRNA induced by PMA was decreasing at the time of maximum PTH stimulation (9 h). Thus, we propose that in the calvarial osteoblasts, there was a greater contribution of the PKC pathway in the PTH stimulation of interstitial collagenase mRNA production than in the UMR 106-01 cells.

There was an increase in the basal level of interstitial collagenase expression with increasing time in culture. This increase in basal expression appeared to be serum independent because it was also apparent in cells incubated in media containing 0.5% serum (control samples). This dramatic increase in control levels over time masks the increase in response to PTH. Although the level of mRNA produced in response to PTH, when compared to the day of confluence, was increasing at an approximately linear rate, the control level increase was much more rapid. Therefore, we must conclude that interstitial collagenase mRNA induction by PTH was continuously increasing during the developmental progression of the calvaria-derived osteoblast in this system.

The increase in interstitial collagenase mRNA produced with progression of the osteoblast phenotype could be caused by the increasing accumulation of Ca^{2+}, which was previously reported to occur with time in these cultures *(4)*. This was particularly evident in the d 14 cultures in which the basal and control levels were increased 25- and 30-fold, respectively, over the d 5 cultures (confluence) in which mineralization had not yet started. In support of this explanation, it was previously reported that the addition of calcium phosphate crystals could stimulate collagenase production in articular condrocytes *(26)*. The cells are in a preosteocyte and osteocyte stage in their developmental sequence during this time period, and the production of collagenase within this setting may relate to maintenance of these cells in the bone environment in which these cells are found.

The gel shift data indicated that similar to what was reported for the UMR 106-01 cells, CREB was bound to the TRE (–51) *(23)*. We have also reported that binding to the PEA-3 site (–89) increased in a time-dependent manner in response to PTH in the UMR 106-01 cells *(24)*. In the case of the calvaria-derived osteoblasts, there was a time-dependent increase in binding to this site with progression through the development of the osteoblast phenotype. This increase in binding could be related to a previous report that Ets-1 mRNA production can be stimulated through Ca^{2+} *(27)*.

In summary, we have demonstrated that interstitial collagenase mRNA can be induced in the calvaria-derived osteoblasts in a development-dependent manner. The kinetics of induction appeared to be a composite of an initial PKC activity followed by a PKA response that we could mimic through PMA and 8-Br-cAMP, respectively. Although the time course of expression in these cells was similar to what was observed in the UMR 106-01 cells, major differences were observed. The time course

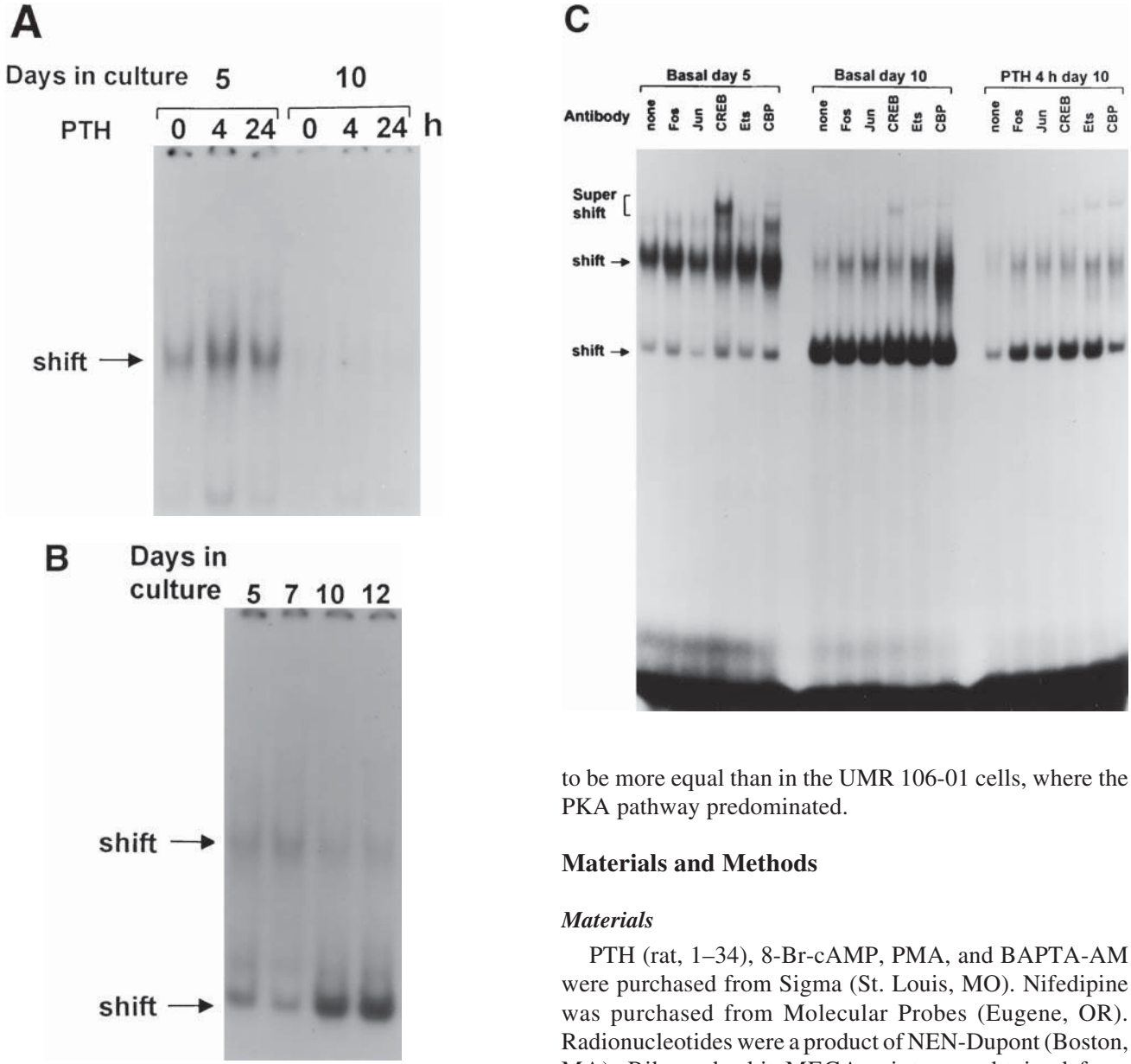


Fig. 6. Gel shift analysis of nuclear extracts binding to the –36 to –58 upstream regulatory region of the interstitial collagenase gene. Cells were harvested on the days indicated and nuclear extracts isolated. The equivalent of 10 μg of protein was incubated with a [^{32}P]-labeled oligonucleotide-containing sequence of the –36 to –58 region. Samples were run on a 4% polyacrylamide native gel. These are representative gels of three independent experiments. **(A)** PTH treatment: cells were preincubated in media containing 0.5% serum for 16 h and then treated with PTH (10^{-8} *M*, 4 and 24 h). **(B)** Basal expression: cells were grown in growth media until confluent (d 5), at which time they were switched to differentiation media. **(C)** Supershift assay: antibodies, as indicated, were added to the incubation mixture.

was delayed in the calvaria-derived osteoblasts, with a decreased slope in the ascending portion of the curve. The overall contribution of the PKC and PKA pathways appeared to be more equal than in the UMR 106-01 cells, where the PKA pathway predominated.

Materials and Methods

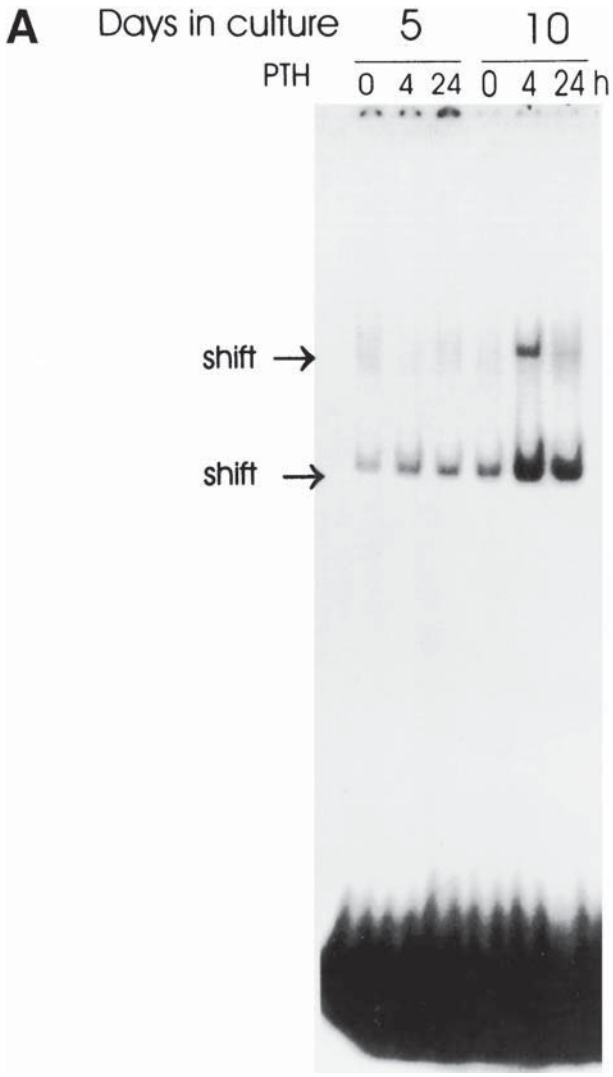
Materials

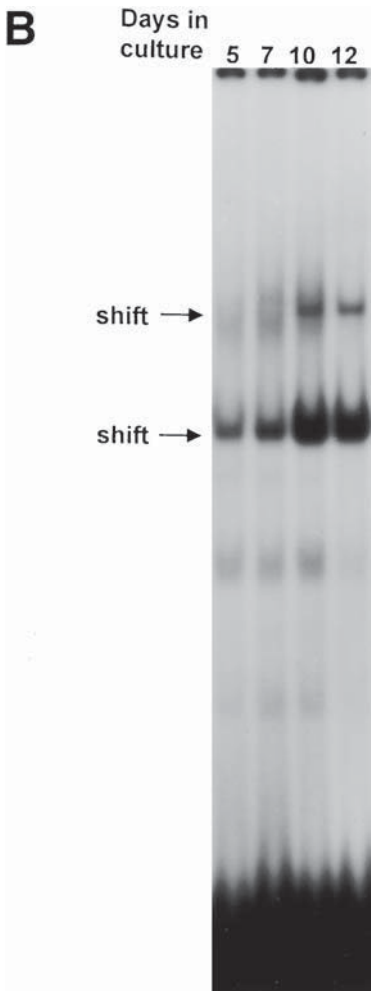
PTH (rat, 1–34), 8-Br-cAMP, PMA, and BAPTA-AM were purchased from Sigma (St. Louis, MO). Nifedipine was purchased from Molecular Probes (Eugene, OR). Radionucleotides were a product of NEN-Dupont (Boston, MA). Riboprobe kit MEGAscript was obtained from Ambion (Austin, TX), and collagenase P was purchased from Boehringer Mannheim (Indianapolis, IN).

Tissue culture media and reagents were purchased from Washington University Tissue Culture Center (St. Louis, MO). Fetal bovine serum (FBS) was a product of JRH Biosciences (Lenexa, KS). Timed pregnant rats were obtained from Taconic (Germantown, NY).

Cell Culture

Primary calvarial osteoblasts from 21-d-old fetal rats were prepared as previously described *(5)*. This protocol was approved by the Committee on Animal Care and Use at Saint Louis University in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. Calvaria were removed and stripped of their endosteum and periosteum and subjected to three successive digests of 20, 40, and 90 min





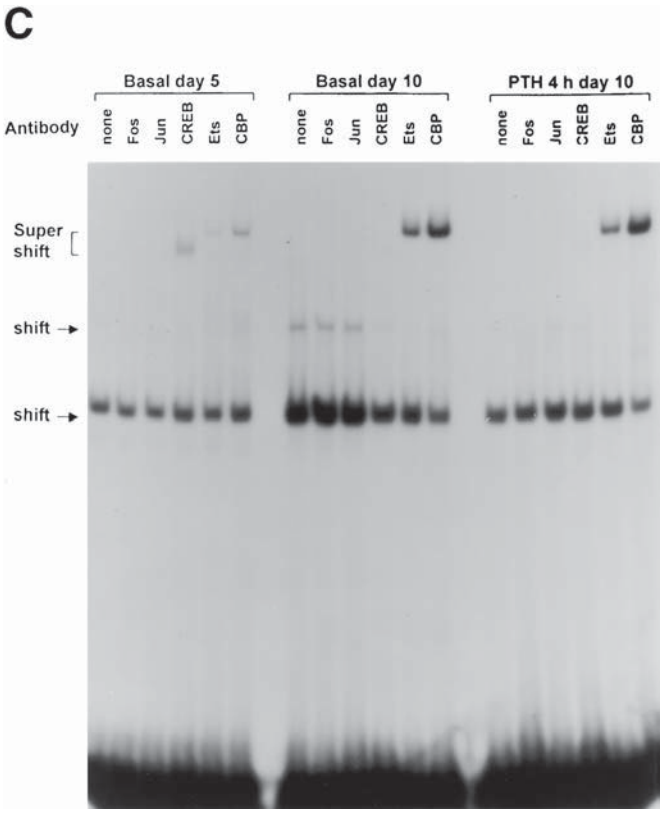


Fig. 7. Gel shift analysis of nuclear extracts binding to the –95 to –71 upstream regulatory region of the interstitial collagenase gene. Cells were harvested on the days indicated and nuclear extracts isolated. The equivalent of 10 μg of protein was incubated with a [^{32}P]-labeled oligonucleotide-containing sequence of the –95 to –71 region. Samples were run on a 4% polyacrylamide native gel. These are representative gels of three independent experiments. **(A)** PTH treatment: cells were incubated in media containing 0.5% serum for 16 h. Treatment with PTH (10^{-8} *M*, 4 and 24 h) was in the same media. **(B)** Basal expression: cells were grown in growth media until confluence (d 5), at which time they were switched to differentiation media. **(C)** Supershift assay: antibodies, as indicated, were added to the incubation mixture.

with trypsin (2.5 mg/mL) plus collagenase P (2.0 mg/mL) at 37°C. The first digest was discarded. Cells from the second and third digests were combined and plated at a density of 5×10^5 cells per 100-mm dish. Cells were grown in Eagle's MEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FBS. When the cells were confluent (5 d), the media was changed to BGJ_b (Fitton-Jackson modification) supplemented with 10% FBS, 50 μg/mL of ascorbic acid, and 10 m*M* β-glycerophosphate.

Media was changed every 2 d. Cells were preincubated (16 h) and treated in growth media containing 0.5% FBS at the indicated times. Treatment with PTH (10^{-8} *M*), 8-Br-cAMP (10^{-3} *M*), and PMA (2.5×10^{-6} *M*) was in growth media containing 0.5% FBS. Cells were also stained for AP and by von Kossa's method for mineralized bone nodules (Sigma).

RNA Isolation

Total cellular RNA was isolated as previously described *(28)*. Cells were rinsed twice in saline and then scraped into saline. Cells were recovered by centrifugation and resuspended in TSM (10 m*M* Tris, pH 7.4; 150 m*M* NaCl; 2 m*M* $MgCl_2$) + 0.5% IGEPAL CA-630. After incubation on ice (2 to 3 min), cellular debris was pelleted. An equal volume of TSE+S (10 m*M* Tris, pH 7.4; 150 m*M* NaCl; 5 m*M* EDTA, pH 7.4; 0.2% sodium dodecyl sulfate [SDS]) was mixed with the supernatant. The samples were extracted twice with 50% phenol + 50% $CHCl_3$:isoamyl alcohol (24:1) and once with $CHCl_3$:isoamyl alcohol (24:1). The supernatant was adjusted to 0.3 *M* sodium acetate, pH 5.2, and 2 vol of ethanol was added and precipitated overnight at –20°C. RNA was quantitated by measuring absorbance at 260 nm, and purity was assessed by calculating the absorbance ratio at 260:280 nm.

RNase Protection Assay

Radioactively labeled antisense transcripts were produced utilizing the appropriate RNA polymerase (SP6, T7, or T3) and α-[^{32}P]UTP according to the manufacturer's specifications (Ambion). The resulting transcripts were treated with RNase-free DNase (1 U/μL), 37°C for 30 min, extracted with an equal volume of phenol and $CHCl_3$:isoamyl alcohol (1:1). Ammonium acetate was added to 4 *M*, and samples were precipitated with 2 vol of ethanol and recovered by centrifugation (5 min at room temperature). Probes (1×10^6 cpm) were annealed to 20 μg of total RNA (85°C for 5 min; 54°C overnight) in hybridization buffer (40 m*M* PIPES, pH 6.8; 0.4 *M* NaCl; 1 m*M* EDTA; 80% formamide). The annealed sample was diluted 1:10 in RNase buffer (0.3 *M* NaCl; 10 m*M* Tris, pH 7.5; 5 m*M* EDTA). Digestion with RNase T1 (200 U) and RNase A (10 μg) was at 30°C for 1 h. The reaction was stopped by adding SDS to 0.6% and proteinase K to 0.15 μg/μL. Digested samples were extracted with an equal volume of phenol:chloroform/isoamyl alcohol (1:1), precipitated by adding ethanol (2 vol), and recovered by spinning for 15 min at room temperature. Samples were heated (90°C for 5 min) and run on 8% polyacrylamide sequencing gels *(29)*. Protected fragments were visualized and quantified utilizing a Molecular Dynamics Phosphorimager and ImageQuant program.

Preparation of Nuclear Extract

Nuclear extracts were prepared as previously described *(30)*. Cells were scraped into media and pelleted by centrifugation. The cells were rinsed in cold PBS, resuspended in approximately four times the volume of buffer I (20 m*M* Tris-HCl, pH 7.8; 5 m*M* $MgCl_2$; 0.5 m*M* dithiothreitol [DTT]; 0.3 *M* sucrose; 1 m*M* phenylmethylsulfonyl fluoride [PMSF]; 0.2 m*M* EGTA; 5 m*M* β-glycerol phosphate; and 1 ng/mL of each aprotinin, antipain, leupeptin, and pepstatin A), and incubated on ice for 1 min. This mix was then adjusted to between 0.5 and 0.6% NP-40, vortexed for 5 s, and centrifuged at 2000*g* for 5 min at 4°C to pellet the nuclei. The nuclei were resuspended in an equal volume of buffer II (10 m*M* Tris-HCl, pH 7.8; 5 m*M* $MgCl_2$; 350 m*M* NaCl; 0.2 m*M* EGTA; 0.5 m*M* DTT; 0.5 m*M* PMSF; 10 m*M* β-glycerol phosphate; 25% glycerol; and 1 ng/mL each of aprotinin, antipain, leupeptin, and pepstatin A), incubated on ice for 15 min, and centrifuged at maximum speed to pellet the nuclei. The supernatant was recovered and the protein concentration determined by Bio-Rad assay. Samples were stored in aliquots at –80°C.

Mobility Shift Assay

Gel shift assays were carried out as previously described *(31)* with modifications. Briefly, oligonucleotides were end labeled with [^{32}P] γATP and then annealed with a fivefold excess of cold complementary oligonucleotide. The end-labeled oligonucleotide was gel purified on an 8% polyacrylamide gel. The purified oligonucleotide (20,000 cpm) and nuclear extract (10 μg of protein) were incubated for 15 min at room temperature in 20 μL of buffer (10 m*M* Tris, pH 7.5; 50 m*M* NaCl; 1 m*M* DTT; 1 m*M* EDTA; and 5% glycerol) in the presence of polydeoxyinosine-deoxycytidylic acid or calf thymus DNA as competitor. The shifted bands were separated on a 4% polyacrylamide gel (28:1, acrylamide-bis), dried, and exposed for autoradiography.

Supershifts were carried out as described for mobility shift assays except specific antibodies (1 μL) were added. The incubations were carried out at 4°C overnight.

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