

Prostaglandin E₂-Induced Up-Regulation of *c-fos* Messenger Ribonucleic Acid Is Primarily Mediated by 3',5'-Cyclic Adenosine Monophosphate in MC3T3-E₁ Osteoblasts*

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ABSTRACT

The mechanism by which the proto-oncogene, *c-fos*, is up-regulated in response to PGE₂ in the mouse osteoblastic (MC3T3-E₁) cell line was investigated using RT-PCR. *c-fos* messenger RNA up-regulation by dmPGE₂ is rapid, starting 10 min post stimulation, and transient. The specific protein kinase A (PKA) inhibitor, H89, inhibited *c-fos* induction. Moreover, down-regulation of protein kinase C (PKC) activity by chronic TPA treatment had no effect on the induction of *c-fos* by dmPGE₂. We conclude that up-regulation of *c-fos* by dmPGE₂ is primarily dependent on PKA in MC3T3-E₁ osteoblasts. In S49 lymphoma wild-type but not S49 *cyc*⁻ cells, which are deficient in cAMP

signaling, dmPGE₂ up-regulates *c-fos* and increases cell growth compared with unstimulated cells. Thus in S49 lymphoma cells, *c-fos* induction by PGE₂ is also dependent on cAMP signaling. The minimal *c-fos* promoter region required for dmPGE₂-induced expression was identified by transfecting *c-fos* promoter deletion constructs coupled to the chloramphenicol acetyltransferase (CAT) reporter gene into Vero cells. Transfection of a plasmid containing 99 bp *c-fos* proximal promoter was sufficient to direct *c-fos*/CAT expression following stimulation with dmPGE₂. Because induction of *c-fos* is mediated by cAMP, these data are consistent with activation of *c-fos* via the CRE/ATF cis element. (*Endocrinology* 141: 291–298, 2000)

AN IMPORTANT regulator of bone remodeling is the arachidonic acid metabolite, prostaglandin E₂ (PGE₂) (1–3). PGE₂ is synthesised by osteoblasts and has been shown to promote new bone formation in whole animals (4–6), and osteoblasts *in vivo* (7, 8).

Mechanical loading stimulates PGE₂ secretion in osteoblasts and osteoblastic cell lines to increase local bone formation (1, 8). However, in space flight under conditions of microgravity, where mechanical loading is reduced, the rate of new bone formation is decreased (9–12). This loss of bone in microgravity, or space osteoporosis, has been attributed to a reduction in osteoblastic function (9) although there are very little data to address this question. In cultured MC3T3-E₁ osteoblasts flown in space, steady-state synthesis of PGE₂ is reduced together with a decrease in glucose utilization and DNA synthesis (13). Therefore, PGE₂ may act as a general mechanical or gravitational sensing factor whereby under conditions of increased mechanical loading it is synthesized and released to stimulate bone growth, but under conditions of very little loading its synthesis is down-regulated and bone growth is reduced. Several signal transduction pathways are known to be perturbed in response to

microgravity in experiments performed in simulated microgravity (14, 15) and in experiments aboard sounding rockets (16, 17) and on recent space shuttle flights (18), including changes in growth factor-induced signal transduction (19) and protein kinase C levels (20, 21). Thus, in addition to direct effects on PGE₂ synthesis and release, indirect effects on signaling pathways upstream of PGE₂ may explain the decrease in PGE₂ levels observed in microgravity. However, the molecular mechanism of prostaglandin-induced bone growth regulation under normal conditions is not well understood.

At the molecular level, an increase in messenger RNA (mRNA) level for the proto-oncogene *c-fos* is associated with the PGE₂-induced increase in osteoblast cell growth (22). *c-fos* is one of a family of transcription factors that include *c-fos*, *fosB*, *fra-1*, *fra-2*. Recognition elements for the AP-1 complex are found in the promoter regions of several genes involved in the growth and mineralization of bone including osteocalcin, alkaline phosphatase, and type I collagen. Transgenic mice overexpressing *c-fos*, develop osteosarcomas early in development (23), and *c-fos* null mice transgenes although not lethal, develop severe osteopetrosis and have deficiencies in bone remodeling and altered hematopoiesis (24, 25). These studies indicate that regulation of *c-fos* gene expression is important for normal bone development (26–28).

The mechanism of PGE₂-induced up-regulation of *c-fos* has been investigated in several cell types. In Swiss 3T3 fibroblasts and glomerular mesangial cells, PGE₂ stimulates *c-fos* via a PKC-mediated mechanism (29, 30). However, in the osteoblast-like cell line, UMR 106–01, and a strain of Swiss 3T3 fibroblasts, *c-fos* mRNA accumulation appears to be dependent on cellular cAMP and PKA but not PKC (31, 32). The

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differential composition of cell surface prostaglandin receptor subtypes may explain these differences in response to PGE₂ between cell types.

In this report, we investigate how PGE₂ exerts its stimulatory effect on *c-fos* gene transcription in MC3T3-E₁ osteoblasts. We find that *c-fos* induction occurs primarily by activation of PKA and that elements required for *c-fos* activation by dmPGE₂ reside within the proximal 99 bp of the *c-fos* promoter.

Materials and Methods

Materials

α MEM and DMEM were purchased from Fisher Scientific (Pittsburgh, PA). 16, 16-Dimethyl prostaglandin E₂ was from Cayman Chemical Co. (Ann Arbor, MI). 8-bromo-cAMP was obtained from Biomol (Plymouth Meeting, PA). 12-O-Tetradecanoylphorbol 13-acetate (TPA) and H-89 were from LC Laboratories (Woburn, MA). H-7 was from Calbiochem (La Jolla, CA). Indomethacin was from Sigma (St. Louis, MO). FCS was from HyClone Laboratories, Inc. (Logan, UT). Vero, S49 wild-type (wt) and S49 adenylate cyclase mutants (*cyc*⁻) cells, L-glutamine and HEPES buffer and Opti-MEM were obtained from the University of California Cell Culture Facility (San Francisco, CA). Moloney murine leukemia virus (MMLV), *Taq* DNA polymerase, Lipofectamine, and the green fluorescent protein vector, pGreen Lantern, were from Life Technologies, Inc. (Grand Island, NY). RNase inhibitor was from Roche Molecular Biochemicals (Indianapolis, IN). Oligonucleotides were ordered from Operon Technologies Inc. (Alameda, CA).

Cell culture

The MC3T3-E₁ cell line was clonally derived from embryonic mouse calvaria (33). Cells were plated and grown to confluence in α MEM containing 10% FCS. Cells were serum deprived for 16–18 h before the start of each experiment by incubation in media containing 1% FCS. We have determined that *c-fos* mRNA increase is maximal at a concentration of 4 μ g/ml (11 μ M) of dimethyl prostaglandin E₂ (dmPGE₂), a stable analog of PGE₂, in serum-deprived, confluent MC3T3-E₁ osteoblasts (Fig. 2). Confluent cultures of osteoblasts were treated for 30 min to 2 h with various agents as stated in the figure legends and then with 4 μ g/ml of dmPGE₂ or 500 μ M 8-bromo-cAMP for 30 min. Vero cells were cultured in α MEM containing 10% FCS as described for osteoblasts. S49 cells were grown in DMEM supplemented with 10% heat inactivated horse serum, antibiotic, 20 mM L-glutamine and 0.11 mg/ml sodium pyruvate. S49 cells were serum deprived for 16–18 h in 4% heat inactivated horse serum before each experiment. Cell counts were performed in a ZBI Coulter counter cell number is reported \pm sd.

RNA isolation, RT, and PCR

RNA from cultured MC3T3-E₁ osteoblasts was isolated using a modified guanidinium thiocyanate method based on the protocol previously described by Chomczynski and Sacchi (34). RNA was quantitated and 1.5 μ g was added to an RT reaction in 30 μ l containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 1 mM dNTPs, 1.67 μ M oligo d(T) primer, 6.67 U of MMLV, 0.67 U of RNase inhibitor. The RT reaction was incubated at room temperature for 10 min, then at 42 C for 45 min and 72 C for 10 min. The reaction was stopped by incubation at 99 C for 5 min. Five microliters of complementary DNA (cDNA) from the RT reaction was added directly to a 50 μ l PCR. The amplification conditions were as follows: 94 C/100 sec, 63 C/70 sec, 72 C/100 sec. *c-fos* was amplified for 30 cycles and CPH1 for 24 cycles. Oligonucleotide primers were designed to span at least one intron to detect any contaminating genomic DNA carried over from the RNA isolation step and to assume determination of RNA alone. *c-fos* primer sequences have been previously described (35) and CPH1 primer sequences were designed from GenBank sequences by M. H. F. as follows: cyclophilin, CPH1-F primer, 5'-CGT CTC CTT TGA GCT GTT TGC AGA C-3' and CPH1-R primer, 5'-CAT AAT CAT AAA CTT AAC TCT GCA ATC CAG C-3' for mouse, the product size is 622 bp. Primers for actin were developed by MHF from published GenBank sequences for β -actin. These primers detect β -

and *x-actin* in the mouse at 656 bp and 620 bp. The cycle parameter of the polymerase reactions of each gene was established so that the PCR product analysis was in linear amplification range, allowing the amount of PCR products to be accurately quantified and compared between experiments. As a part of this strategy, small variations between experiments were corrected by comparison to CPH1 PCR products derived from the same RT reaction. Hence, the level of *c-fos* expression is semi-quantitative relative to CPH1. The RNA content was held constant; linear RT-PCR was accomplished by varying the number of PCR cycles. RT conditions were established so that the RNA was not limiting and the PCR amplification reaction was stopped in the linear range and reaction products could be accurately quantified and compared. PCR bands were identified by size after electrophoresis on a 2% agarose gel, stained with ethidium bromide, and photographed with a Polaroid camera over a UV lightbox. For quantification, the bands of interest were scanned at 400 dpi with HP Scanjet IIcx scanner (Hewlett-Packard Co., Palo Alto, CA) and stored as Macintosh TIFF files. The peak areas and densities were determined using NIH Image 1.55 matching software (National Institutes of Health; Bethesda, MD).

c-fos promoter reporter gene constructs

To determine the minimal *c-fos* promoter required to direct PGE₂-inducible expression, a series of *c-fos* proximal promoter fragments were cloned upstream of the chloramphenicol transferase (CAT) and pGreen Lantern Green Fluorescent reporter plasmids (pGL). The four CAT/*c-fos* constructs, pFC99, pFC225, pFC700, pFC2000 (kindly provided by R. Roeder, Rockefeller University) contain 99 bp, 225 bp, 700 bp and 2,000 bp of the proximal *c-fos* promoter, respectively (36). pGL*c-fos*225 contains 225 bp of *c-fos* proximal promoter and were derived from pFC225. pFC225 was digested with *Xho*I and *Xba*I, the ends blunted and the resulting 225-bp fragment cloned into the *Sma*I site of the pGL. The 5' and 3' ends of pGL*c-fos*225 was sequenced to confirm identity and orientation.

Transient transfections

cDNAs were transfected into Vero cells using a standard Lipofectamine protocol. Briefly, cells were plated onto round 22 mm coverslips in 6-well multiwell plates and grown to 80% confluence in 5% FCS α MEM. 2 h before transfection the medium was removed and 0.5% FCS α MEM plus indomethacin was added. Three micrograms of plasmid cDNA was added to 10 μ l of Lipofectamine in 200 μ l of Opti-MEM (serum and antibiotic-free) and incubated at room temperature for 30 min. For the CAT reporter gene transfections, a plasmid containing the β -galactosidase gene (pSV- β -gal) was co-transfected with the CAT constructs to determine transfection efficiencies. Eight hundred microliters of Opti-MEM was added to the DNA/Lipofectamine mix and added to the cells, which had previously been rinsed with Opti-MEM. The transfection was allowed to proceed for 5 h at 37 C and stopped by replacing the medium with 0.5% FCS α MEM supplemented with the cyclooxygenase inhibitor indomethacin. At this time, 4 μ g/ml dmPGE₂ or 5 μ g/ml octanoic acid was added to the appropriate wells. After 30 h, cells were lysed and extracts prepared for standard CAT and β -galactosidase assays. Extracts for CAT assays were treated at 60 C for 10 min to inactivate endogenous acetylases. Chloramphenicol and its acetylated forms were separated by ascending TLC. The GFP fluorescence was examined after 24 h with a Carl Zeiss Axioscope (Oberkochen, Germany), using a FITC filter.

Results

Consistent with an earlier unpublished observation is the finding that dmPGE₂ significantly ($P < 0.0001$, Student's *t* test) increased the growth of serum-deprived MC3T3-E₁ cells 156% after 24 h when compared with untreated cells (control, $2.17 \times 10^4 \pm 0.13 \times 10^4$; dmPGE₂-treated, $5.56 \times 10^4 \pm 0.24 \times 10^4$ new cells). Associated with PGE₂-induced mitogenesis was an increase in *c-fos* gene induction within minutes of addition Fig. 1 (22, 37). To the maximize time and dose of the effect, we ran concentration dose response curves and time

FIG. 1. Time course for PGE₂-induced up-regulation of *c-fos* mRNA. Confluent osteoblast cultures were grown overnight in low serum media (1% FCS α MEM) and treated with dmPGE₂ for the times indicated (minutes). The RNA was isolated, subjected to RT and PCR as described in *Materials and Methods*.

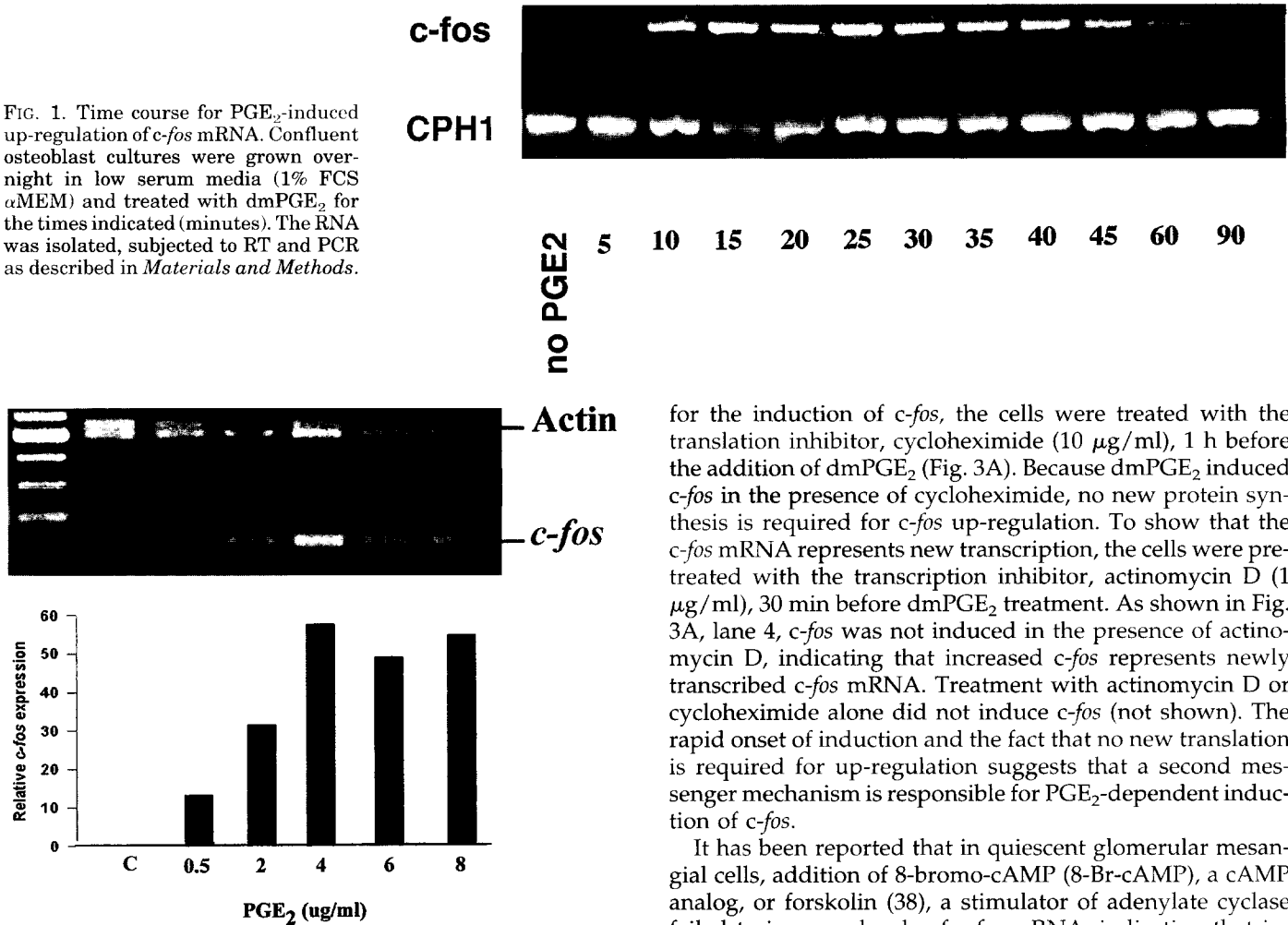


FIG. 2. *c-fos* response of osteoblasts to increasing amounts of dmPGE₂. Osteoblast cultures were down-regulated in 2% FCS α MEM media for 48 h before addition. dmPGE₂ was added directly to depleted media for 30 min before collection of RNA. RT-PCR was performed on both β -actin and *c-fos*. The β -actin primers produced dual bands, the lower being identified as α -actin, the upper band of β -actin was used as the housekeeping gene for quantification. The amount of *c-fos* induced by prostaglandin relative to β -actin is seen in the lower graph.

curves (Figs. 1 and 2) To investigate the timing of PGE₂-induced increase in *c-fos* mRNA, 4 μ g/ml of dmPGE₂ was added to serum-deprived confluent MC3T3-E₁ osteoblasts for various times and changes in mRNA assayed by RT-PCR (Fig. 1). An increase in *c-fos* mRNA levels was first detected 10 min after the addition of dmPGE₂ and was maximal after 25 min, where the increase was 15- to 20-fold above control levels (no added dmPGE₂). However, *c-fos* mRNA up-regulation was transient and decreased to near nonstimulated levels 90 min after dmPGE₂ stimulation. To determine if the *c-fos* response was relative to concentration of PGE₂, cells were incubated with varying doses of prostaglandin for 30 min. As seen in Fig. 2, the osteoblasts respond to as little as 0.5 μ g/ml of PGE₂ with maximum stimulation at 4 μ g/ml. This finding is consistent with our previous work that showed growth was maximal at 4 μ g/ml (37).

To determine whether new protein synthesis is required

for the induction of *c-fos*, the cells were treated with the translation inhibitor, cycloheximide (10 μ g/ml), 1 h before the addition of dmPGE₂ (Fig. 3A). Because dmPGE₂ induced *c-fos* in the presence of cycloheximide, no new protein synthesis is required for *c-fos* up-regulation. To show that the *c-fos* mRNA represents new transcription, the cells were pre-treated with the transcription inhibitor, actinomycin D (1 μ g/ml), 30 min before dmPGE₂ treatment. As shown in Fig. 3A, lane 4, *c-fos* was not induced in the presence of actinomycin D, indicating that increased *c-fos* represents newly transcribed *c-fos* mRNA. Treatment with actinomycin D or cycloheximide alone did not induce *c-fos* (not shown). The rapid onset of induction and the fact that no new translation is required for up-regulation suggests that a second messenger mechanism is responsible for PGE₂-dependent induction of *c-fos*.

It has been reported that in quiescent glomerular mesangial cells, addition of 8-bromo-cAMP (8-Br-cAMP), a cAMP analog, or forskolin (38), a stimulator of adenylate cyclase failed to increase levels of *c-fos* mRNA, indicating that increased cAMP levels do not activate *c-fos* in these cells (30). We asked whether cAMP could increase *c-fos* in MC3T3-E₁ cells. When 500 μ M 8-Br-cAMP (Fig. 3B, lane 3; Table 1) or 20 μ M forskolin (Fig. 3C, lane 3; Table 1) was added to serum-deprived osteoblasts for 30 min, *c-fos* mRNA levels increased to a level comparable with mRNA levels following dmPGE₂ stimulation indicating that by raising cAMP levels, it is possible to induce *c-fos* in MC3T3-E₁ cells. Treatment with the potent PKC activator TPA for 30 min in the absence of dmPGE₂ induced *c-fos* 1.5-fold above the level of dmPGE₂ stimulation alone (Fig. 3B, lane 4; Table 1), indicating that activation of TPA-sensitive PKC can induce *c-fos* in MC3T3-E₁ cells.

Because agents that stimulate PKA and PKC induce *c-fos* in MC3T3-E₁ cells, we wanted to determine by which pathway(s) PGE₂ mediates stimulation of *c-fos* transcription. Short exposure to TPA stimulates PKC activity and overnight treatment down-regulates PKC activity (39), therefore we asked whether the dmPGE₂-mediated up-regulation of *c-fos* mRNA was acting through a PKC mechanism. In serum-depleted MC3T3-E₁ cells, 16 h incubation with TPA (1.6 μ M) did not abolish induction of *c-fos* by dmPGE₂ or 8-Br-cAMP (Fig. 3B, lanes 5 and 6; Table 1). In contrast, 16 h TPA treatment followed by TPA treatment for 30 min did prevent induction of *c-fos* (lane 7) This control confirms that PKC

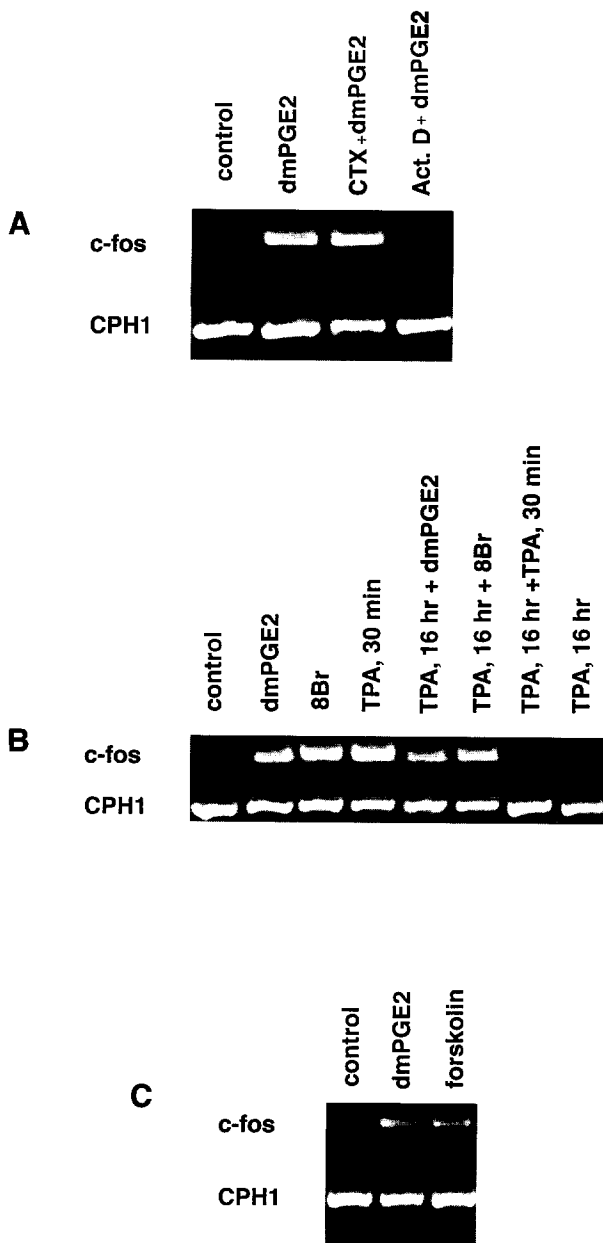


FIG. 3. Effect of various agents on dmPGE₂ and 8-Br-cAMP-induced *c-fos* mRNA levels. Confluent osteoblast cultures were grown overnight in low serum media (1% FCS α MEM). RNA was isolated, subject to RT and PCR as described. All dmPGE₂ (4 μ g/ml) and 8-Br-cAMP (500 μ M) treatments were for 30 min. A, Effect of cycloheximide and actinomycin D on dmPGE₂-induced *c-fos* mRNA levels. Cycloheximide (10 μ g/ml) and actinomycin D (1 μ g/ml) were added 2 h before dmPGE₂ treatment. B, Effect of TPA on dmPGE₂ and 8-Br-cAMP-induced *c-fos* mRNA levels. A total of 1.6 μ M TPA was added for the times indicated before dmPGE₂ or 8-Br-cAMP treatment. C, Effect of forskolin on *c-fos* mRNA levels. dmPGE₂ (4 μ g/ml) and forskolin (20 μ M) were added for 30 min.

activity is down-regulated by chronic TPA treatment. Because the induction of *c-fos* by dmPGE₂ or 8-Br-cAMP is unaltered when PKC is down-regulated, *c-fos* induction is not likely to be dependent upon activation of PKC. These data suggest that *c-fos* induction by dmPGE₂ is mediated by

cAMP and PKA but not by TPA-sensitive PKC in MC3T3-E₁ cells.

To investigate further, we examined the effect of protein kinase inhibitors on the dmPGE₂ induction of *c-fos*. Treatment with the nonspecific kinase inhibitor H-7 (40) (Fig. 4, lanes 6 and 7; Table 1) reduced the dmPGE₂ and 8-Br-cAMP-induced *c-fos* mRNA level to 21% and 33% of dmPGE₂-induced and 8-Br-cAMP-induced levels, respectively, indicating that *c-fos* up-regulation requires the activation of a protein kinase. Addition of the specific protein kinase A inhibitor, H-89 (30 μ M) (41), reduced dmPGE₂ and 8-Br-cAMP-induced *c-fos* mRNA to 24% and 37%, respectively, suggesting that PKA is required for *c-fos* up-regulation (Fig. 4, lanes 8 and 9, and Table 1).

To further understand the mechanism of up-regulation of *c-fos* by dmPGE₂, we examined the effect of dmPGE₂ on S49 lymphoma wild-type and *cyc*⁻ cells. S49 *cyc*⁻ cells are defi-

TABLE 1. Summary of effects of various agents on *c-fos* mRNA levels

Treatment	<i>c-fos</i> mRNA level (% of control)
A	
Control (No PGE ₂ or 8-Br-cAMP)	10.6 \pm 0.9
500 μ M 8-Br-cAMP	102.1 \pm 9.7
20 μ M forskolin	82.3 \pm 5.2
1.6 μ M TPA, 30 min	153.3 \pm 9.8
1.6 μ M TPA (ON) + dmPGE ₂	86.0 \pm 8.3
50 μ M H7 + dmPGE ₂	21.4 \pm 8.4
30 μ M H89 + dmPGE ₂	24.0 \pm 6.1
B	
50 μ M H7 + 8-Br-cAMP	33.3 \pm 4.9
30 μ M H89 + 8-Br-cAMP	37.0 \pm 11.5

MC3T3-E₁ osteoblasts were grown to confluence in α MEM containing 10% FCS and then grown for 16 h in medium containing 1% FCS before the experiment. Agents were added for 30–120 min before the addition of dmPGE₂ or 8-Br-cAMP. RNA was isolated and RT-PCR performed as described in *Materials and Methods*. Data are presented as a percentage of dmPGE₂ (A) or 8-Br-cAMP (B)-induced *c-fos* mRNA levels. dmPGE₂ (4 μ g/ml) and 8-Br-cAMP (500 μ M) treatment was for 30 min. Values represent the means and ses of four to seven experiments. ON, Overnight; TPA, 12-O-tetradecanoylphorbol 13-acetate.

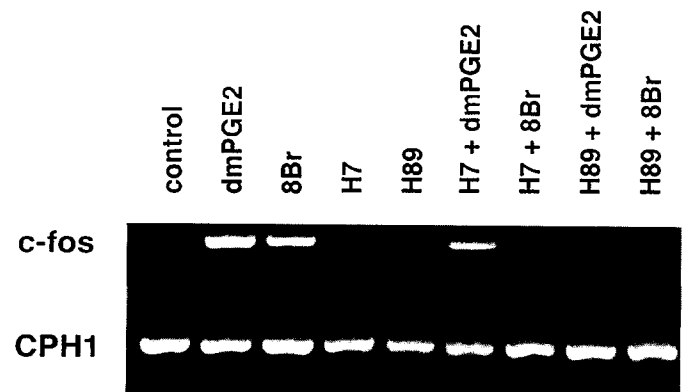


FIG. 4. Effect of kinase inhibitors on *c-fos* mRNA levels. Serum-deprived MC3T3-E₁ osteoblasts were grown, after RNA was isolated RT-PCR performed as described in *Materials and Methods*. All dmPGE₂ (4 μ g/ml) and 8-Br-cAMP (500 μ M) treatments were for 30 min. H-7 (50 μ M) was added for 30 min, and H89 (30 μ M) for 60 min before dmPGE₂ or 8-Br-cAMP treatment.

cient in cAMP-mediated intracellular signaling because they lack the G α subunit of the $\alpha\beta\gamma$ heterotrimeric complex that binds to G protein-coupled surface receptors. A total of 5.6×10^5 S49 wild-type and *cyc*⁻ cells were plated and grown for 16 h in low serum medium, then counted and either dmPGE₂ or vehicle (ethanol) was added. After 24 h, the cells were counted again and the increase in cell number determined. Treatment with dmPGE₂ for 24 h caused a 31% increase ($3.49 \times 10^5 \pm 0.16 \times 10^5$ new cells) ($P < 0.005$, Student's *t* test) in cell number compared with untreated control cells ($2.67 \times 10^5 \pm 0.12 \times 10^5$ new cells). In S49 *cyc*⁻ cells, no significant change in growth was detected (dmPGE₂-treated, $1.66 \times 10^5 \pm 0.03 \times 10^5$ new cells; control, $1.94 \times 10^5 \pm 0.25 \times 10^5$ new cells). Because a significant increase in cell number was detected in wild-type cells, the data suggest that dmPGE₂-induced growth in S49 cells is mediated via a cAMP-dependent mechanism. To further examine this mechanism, we asked whether dmPGE₂ could induce expression of *c-fos* in S49 wild-type and *cyc*⁻ mutant cells. Addition of dmPGE₂ for 30 min increased *c-fos* mRNA levels in wild-type but not *cyc*⁻ S49 cells (Fig. 5). To ensure that other components of the cAMP signaling pathway are present, *cyc*⁻ cells were treated with forskolin. Forskolin induced *c-fos* in both wt and to a lesser extent in *cyc*⁻ cells, indicating that the cAMP-signaling pathway downstream from the G α subunit is largely intact. Differences in response of the two cell types to forskolin have been noted previously and include a delay in activation of cAMP accumulation in wild-type but not *cyc*⁻ cells, and a lack of desensitization of adenylate cyclase in wild-type cells (42). These differences in response to forskolin may have contributed to a reduced activation of *c-fos* by forskolin in *cyc*⁻ cells. The data suggest that, in S49 cells, dmPGE₂ induces the *c-fos* gene by raising cAMP levels via activation of a G protein-coupled receptor of the subtype that activates adenylate cyclase.

Because we have evidence that cAMP plays a major role in *c-fos* induction by dmPGE₂ in MC3T3-E1 osteoblasts and S49 cells, we asked whether the *c-fos* promoter region con-

taining the cAMP response element (CRE) mediated PGE₂-specific expression. Because MC3T3-E₁ cells start to enter apoptosis when left in NSAID for extended periods, we tested the constructs in Vero cells that maintain well in NSAID. Four different length *c-fos* promoter constructs that contain 99 bp, 225 bp, 700 bp, and 2,000 bp of the *c-fos* promoter were transfected into Vero cells (Fig. 6B). In cells transfected with two of the *c-fos*-directed constructs, pFC225 and pFC99, PGE₂ specifically up-regulated *c-fos*-directed CAT expression approximately 6- to 10-fold (lanes 8 and 11). CAT expression directed by pFC700 and pFC2000 was independent of dmPGE₂ treatment (lanes 1-5). This is not unexpected because several other cis-acting elements are present in the region between base pairs 225 and 700 upstream of the CAP site, including the serum response element, which can stimulate *c-fos* expression (6A). The structurally related fatty acid, octanoic acid, failed to stimulate CAT expression in cells transfected with pFC225 and pFC99, indicating that the increase in transcription is due to the action of dmPGE₂ and not to a closely related compound.

To confirm the CAT expression finding that the element directing PGE₂-induced expression resides within the proximal *c-fos* promoter, the proximal 225 bp promoter region was cloned upstream of the Green Fluorescent protein reporter gene to generate the construct pGLc*fos*225. Vero cells were transiently transfected with pGLc*fos*225, treated with 4 μ g/ml dmPGE₂ and examined under a fluorescent microscope for GFP fluorescence (data not shown). In transfected cells that were not treated with dmPGE₂, no GFP-fluorescence was detected; however, following the addition of dmPGE₂, a fluorescent signal was present in many cells. Thus in cells that express the pGL225*c-fos* construct, which excludes the SRE, *c-fos* can be induced by dmPGE₂. Taken together the CAT and GFP expression studies suggest that in MC3T3-E₁ osteoblasts the cis-element responsible for *c-fos* activation by dmPGE₂ is present in the proximal *c-fos* promoter and most likely within the 99 bp immediately preceding the transcription start site.

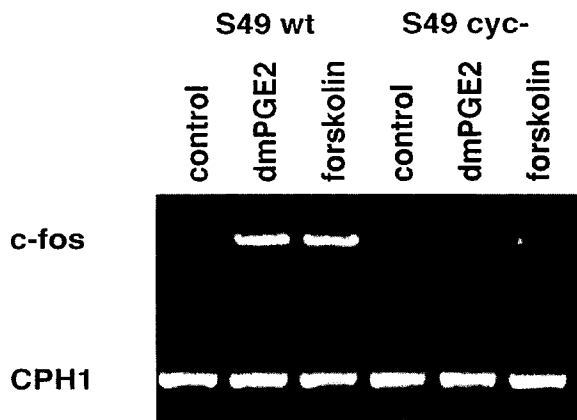


FIG. 5. Effect of dmPGE₂ on *c-fos* mRNA levels in S49 wild-type and *cyc*⁻ lymphoma cells. S49 cells were seeded at a density of 600,000 cells/ml and incubated in low serum for 16 h. Following each treatment the cells were briefly centrifuged to pellet cells, and the RNA isolated immediately and subjected to RT and PCR as described in *Materials and Methods*. dmPGE₂ (4 μ g/ml) and forskolin (20 μ M) treatment was for 30 min.

Discussion

To study the effect of PGE₂ on signal transduction, we first determined the optimal time of exposure and concentration required. PGE₂ induction of *c-fos* was transient, with maximal signal occurring from 25-30 min. Induction of *c-fos* by PGE₂ was dose dependent with optimal concentration being 4 μ g/ml. This concentration is essentially the same as is needed for optimal stimulation of cell growth (22, 37).

To examine the signal transduction pathways involved in the up-regulation of *c-fos* by PGE₂, mRNA levels for *c-fos* stimulated with dmPGE₂ were examined using protein kinase inhibitors and activators. We found that the effect of dmPGE₂ is similar to that of 8-Br-cAMP where the up-regulation of *c-fos* is sharply reduced by H7 and H89 and is unaffected when PKC is down-regulated. Furthermore, PGE₂ failed to activate *c-fos* in S49 *cyc*⁻ mutant cells, which lack a component of the cAMP signaling pathway, but not in S49 wild-type cells. These data clearly demonstrate a major role for a cAMP-mediated mechanism in the PGE₂-induced up-regulation of *c-fos* in MC3T3-E₁ osteoblasts and S49 lym-

cAMP-dependent pathways, the cAMP-dependent pathway is primarily responsible for PGE₂ up-regulation of *c-fos* mRNA. Although we did not characterize the PGE₂ receptors present in MC3T3-E₁ osteoblasts in this study, our results are consistent with PGE₂ acting primarily through EP2 or EP4 receptors to up-regulate *c-fos* mRNA.

Two recent studies examined the effect of PTH on mitogenesis, second messenger signaling, and gene expression in UMR 106-01 osteoblast-like cells (31, 32). Treatment with forskolin, 8-Br-cAMP, or TPA up-regulated *c-fos* mRNA. However, treatment with PTH and PGE₂ stimulated a rise in UMR-106-01 cellular cAMP levels but no increase in growth. Chronic treatment with TPA failed to abolish PTH-induced up-regulation of *c-fos*. Taken together, the above experiments show that PTH can transiently up-regulate *c-fos* via a cAMP-dependent mechanism with little or no contribution from a TPA-sensitive PKC pathway. The effect of PTH on osteoblasts seems to be analogous to the effect of PGE₂ in MC3T3-E₁ cells, and both may act to induce *c-fos* via the same mechanism, namely by raising cAMP levels. The reason for the difference in growth response to PGE₂ between MC3T3-E₁ osteoblasts and UMR 106-01 osteoblast-like cells is unknown but may be due to the fact that UMR 106-01 cells are not fully differentiated osteoblasts and lack key markers that are typically found in true osteoblasts. For example, they do not synthesize osteocalcin and have variable levels of other bone matrix proteins (51, 52).

Fine mapping studies have identified several cis-acting domains that contribute to the basal promoter activity of the human *c-fos* gene. One, the serum response element (SRE) located -317 to -298 bp upstream of the start of transcription in the *c-fos* promoter plays a key role in transcriptional induction through the binding of several proteins (53-55). Mutations in the SRE binding site abolished transcriptional induction by serum, TPA, and growth factors. The cAMP response element (CRE) located 60 bp upstream of the CAP site binds the cAMP regulatory element binding protein (CREB/ATF). The CREB/ATF site overlaps the recognition sequence of the MTLF/USF transcription factor element in a GC-rich region (56). Mutational analysis and transient transfections have demonstrated that each of these domains to some extent contribute to the basal *c-fos* activity. We have shown that dmPGE₂-inducible *c-fos* activity requires at least the first 99 bp of the proximal promoter. Our transfection data, combined with protein kinase activator and inhibitor data, are consistent with the model that dmPGE₂ transiently raises cAMP levels and probably activates CREB to stimulate *c-fos* transcription via the ATF/CRE located at -60. However, our data do not exclude the possibility that dmPGE₂ binds directly to the *c-fos* promoter at an alternative site independent of PKA activation within the identified 99-bp region. Further studies, for example, site-directed mutagenesis of the identified region, should address this issue.

The relationship between mechanical loading and PGE₂ synthesis is intriguing. Upon mechanical stimulation, PGE₂ is released and local bone formation is increased in osteoblasts and osteoblastic cell lines (1). Conversely, under conditions of microgravity, where mechanical stress is very low,

PGE₂ synthesis is decreased and osteoblastic function is reduced (13). This correlation suggests that the rate of PGE₂ synthesis is sensitive to changes in mechanical loading. One possibility is that deformation of the cell membrane by mechanical loading alters the rate of arachidonic acid release from the membrane, thereby increasing levels of its metabolites including PGE₂. PGE₂ then would act on the cell to increase osteoblast *c-fos* gene expression within minutes and growth within 16-24 h.

In conclusion, we have shown that cAMP mediates, at least in part, the up-regulation of *c-fos*, a gene associated with the growth of bone cells. These findings may lead to therapies that counteract the bone loss experienced during space flight, a problem that needs to be overcome if the goal of long-term space flight is to be achieved.

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