Prostaglandin E$_2$-Induced Up-Regulation of c-fos Messenger Ribonucleic Acid Is Primarily Mediated by 3',5'-Cyclic Adenosine Monophosphate in MC3T3-E$_1$ Osteoblasts*

JAMIE FITZGERALD†, THOMAS J. DIETZ, AND MILLIE HUGHES-FULFORD

From the Laboratory of Cell Growth, University of California, Department of Medicine and Department of Medicine, Veterans Affairs Medical Center, San Francisco, California 94121

ABSTRACT

The mechanism by which the proto-oncogene, c-fos, is up-regulated in response to PGE$_2$ in the mouse osteoblastic (MC3T3-E$_1$) cell line was investigated using RT-PCR. c-fos messenger RNA up-regulation by dmPGE$_2$ 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 dmPGE$_2$. We conclude that up-regulation of c-fos by dmPGE$_2$ is primarily dependent on PKA in MC3T3-E$_1$ osteoblasts. In S49 lymphoma wild-type but not S49 cyc- cells, which are deficient in cAMP priming with dmPGE$_2$ is rapid, starting 10 min post stimulation, and transient.

 fos

JAMIE FITZGERALD†, THOMAS J. DIETZ, AND MILLIE HUGHES-FULFORD

From the Laboratory of Cell Growth, University of California, Department of Medicine and Department of Medicine, Veterans Affairs Medical Center, San Francisco, California 94121

ABSTRACT

The mechanism by which the proto-oncogene, c-fos, is up-regulated in response to PGE$_2$ in the mouse osteoblastic (MC3T3-E$_1$) cell line was investigated using RT-PCR. c-fos messenger RNA up-regulation by dmPGE$_2$ 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 dmPGE$_2$. We conclude that up-regulation of c-fos by dmPGE$_2$ is primarily dependent on PKA in MC3T3-E$_1$ osteoblasts. In S49 lymphoma wild-type but not S49 cyc- cells, which are deficient in cAMP signaling, dmPGE$_2$ up-regulates c-fos and increases cell growth compared with unstimulated cells. Thus in S49 lymphoma cells, c-fos induction by PGE$_2$ is also dependent on cAMP signaling. The minimal c-fos promoter region required for dmPGE$_2$-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 dmPGE$_2$. Because induction of c-fos is mediated by CRE, 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 E$_2$ (PGE$_2$) (1–3). PGE$_2$ is synthesized by osteoblasts and has been shown to promote new bone formation in whole animals (4–6), and osteoblasts in vitro (7, 8).

Mechanical loading stimulates PGE$_2$ 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 decreases in PGE$_2$ 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 PGE$_2$-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 transgenese 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 PGE$_2$-induced up-regulation of c-fos has been investigated in several cell types. In Swiss 3T3 fibroblasts and glomerular mesangial cells, PGE$_2$ 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
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**

αMEM and DMEM were purchased from Fisher Scientific (Pittsburgh, PA). 16, 16-Dimethyl prostaglandin E₂ was from Cayman Chemical Co. (Ann Arbor, MI). 8-bromo-c·AMP was obtained from Biotium (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). COS was from HyClone Laboratories, Inc. (Logan, UT). Vero, S49 wild-type (wt) and S49 adenylate cyclase mutants (cyc-) cells, were obtained from the University of California Cell Culture Facility (San Francisco, CA). Moloney murine leukemia virus (MMLV), Tag 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 αMEM 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 μg/ml (11 μM) 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 μg/ml of dmPGE₂ or 500 μM 8-bromo-c·AMP for 30 min. Vero cells were cultured in αMEM 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 HEPES buffer and Opti-MEM were obtained from the University of California Cell Culture Facility (San Francisco, CA). Moloney murine leukemia virus (MMLV), Tag 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).

**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 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).

**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 Florescent 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). pGLc·fos225 contains 225 bp of c·fos proximal promoter and were derived from pFC225. pFC225 was digested with XhoI and XbaI, the blunted and the resulting 225-bp fragment cloned into the Smal site of the pGL. The 5’ and 3’ ends of pGLc·fos225 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 αMEM. 2 h before transfection the medium was removed and 0.5% FCS αMEM plus indomethacin was added. Three micrograms of plasmid cDNA was added to 10 μl of Lipofectamine in 200 μl 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 αMEM supplemented with the cycloxygenase inhibitor indomethacin. At this time, 4 μg/ml dmPGE₂ or 5 μg/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-E₃ cells 156% after 24 h when compared with untreated cells (control, 2.17 × 10² ± 0.13 × 10²; dmPGE₂-treated, 5.56 × 10² ± 0.24 × 10² 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
The lower being identified as c-0.5 min. As seen in Fig. 2, the osteoblasts respond to as little as were incubated with varying doses of prostaglandin for 30 media for 48 h before addition. dmPGE 2 was added directly to deformed on both 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 x-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.

cAMP MEDIATES PGE2 INDUCTION OF c-fos IN OSTEOBLASTS

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 αMEM) 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 αMEM 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 x-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.

c-fos is seen in the lower graph.

Curves (Figs. 1 and 2) To investigate the timing of PGE2-induced increase in c-fos mRNA, 4 μg/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 μg/ml of PGE2 with maximum stimulation at 4 μg/ml. This finding is consistent with our previous work that showed growth was maximal at 4 μg/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 μg/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 μg/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 transcription 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 adenylyl 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 μM 8-Br-cAMP (Fig. 3B, lane 3; Table 1) or 20 μM 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 μM) 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...
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 μM) (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 deficient in PKC. DM3T3-E1 osteoblasts were grown to confluence in αMEM 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 μg/ml) and 8-Br-cAMP (500 μM) treatment was for 30 min. Values represent the means and SEs of four to seven experiments. ON, Overnight; TPA, 12-O-tetradecanoylphorbol 13-acetate.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>c-fos mRNA level (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Control (No PGE2 or 8-Br-cAMP)</td>
<td>10.6 ± 0.9</td>
</tr>
<tr>
<td>500 μM 8-Br-cAMP</td>
<td>102.1 ± 9.7</td>
</tr>
<tr>
<td>20 μM forskolin</td>
<td>82.3 ± 5.2</td>
</tr>
<tr>
<td>1.6 μM TPA, 30 min</td>
<td>153.3 ± 9.8</td>
</tr>
<tr>
<td>1.6 μM TPA (ON) + dmPGE2</td>
<td>86.0 ± 8.3</td>
</tr>
<tr>
<td>50 μM H7 + dmPGE2</td>
<td>21.4 ± 5.4</td>
</tr>
<tr>
<td>30 μM H89 + dmPGE2</td>
<td>24.0 ± 6.1</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>50 μM H7 + 8-Br-cAMP</td>
<td>33.3 ± 4.9</td>
</tr>
<tr>
<td>30 μM H89 + 8-Br-cAMP</td>
<td>37.0 ± 11.5</td>
</tr>
</tbody>
</table>

MC3T3-E1 osteoblasts were grown to confluence in αMEM 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 μg/ml) and 8-Br-cAMP (500 μM) 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. 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 αMEM). RNA was isolated, subject to RT and PCR as described. All dmPGE2 (4 μg/ml) and 8-Br-cAMP (500 μM) treatments were for 30 min A. Effect of cycloheximide and actinomycin D on dmPGE2-induced c-fos mRNA levels. Cycloheximide (10 μg/ml) and actinomycin D (1 μg/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 μM 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 μg/ml) and forskolin (20 μM) were added for 30 min.](image1)

![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 μg/ml) and 8-Br-cAMP (500 μM) treatments were for 30 min. H-7 (50 μM) was added for 30 min, and H89 (30 μM) for 60 min before dmPGE2 or 8-Br-cAMP treatment.](image2)
icient in cAMP-mediated intracellular signaling because they lack the Gsα subunit of the αβγ 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 × 10^5 ± 0.16 × 10^5 new cells) (P < 0.005, Student’s t test) in cell number compared with untreated control cells (2.67 × 10^5 ± 0.12 × 10^5 new cells). In S49 cyc− cells, no significant change in growth was detected (dmPGE2-treated, 1.66 × 10^5 ± 0.03 × 10^5 new cells; control, 1.94 × 10^5 ± 0.25 × 10^5 new cells). Because a significant increase in cell number was detected in wild-type cells, the data suggest that dmPGE2-caused in cAMP accumulation in wild-type but not cyc− cells, and a lack of a component 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 Gsα 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-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 Gsα 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 containing 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 CAT 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-fos225. Vero cells were transiently transfected with pGLc-fos225, treated with 4 μg/ml dmPGE2 and examined under a florescent microscope for GFP florescence (data not shown). In transfected cells that were not treated with dmPGE2, no GFP-florescence was detected; however, following the addition of dmPGE2, a fluorescent signal was present in many cells. Thus in cells that express the pGL225c-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.

**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 μg/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 lymphoma cells.
phoma cells. However, it is possible that a TPA-insensitive PKC isoform may contribute to up-regulation of c-fos by PGE2.

The finding that PGE2 signals via a cAMP pathways in MC3T3-E1 osteoblasts is in agreement with Kozawa et al. (43) who directly assayed cAMP levels following stimulation with up to 10 μm PGE2. Several studies have examined c-fos induction by PGE2 in other cell types. Increased cellular cAMP fails to up-regulate c-fos mRNA in Swiss 3T3 fibroblasts, (44, 45) although cAMP can act in synergy with other signal transduction pathways to stimulate c-fos expression and mitogenesis (46, 47, 48). Danesch et al. (29) report that the increase in c-fos mRNA is not dependent on cAMP but on a PKC-dependent mechanism. Similarly in glomerular mesangial cells, addition of 8-Br-cAMP (100 μm) and forskolin (10 μm) failed to activate c-fos and depletion of PKC blocked c-fos induction by PGE2 (29, 30). However, with the latter experiment, the concentrations of 8-Br-cAMP and forskolin may have been too low to activate c-fos. In MC3T3-E1 cells, we found that 100 μm 8-Br-cAMP was too low to activate c-fos and a higher concentration was required (500 μm) for full induction. Our studies demonstrate that PGE2 can up-regulate c-fos mRNA via a PKA-dependent, PKC-independent mechanism. Other researchers using Swiss 3T3 fibroblasts have also shown that a PKA dependent, PKC-independent mechanism is responsible for c-fos induction (47). It has been suggested (29) that, in these experiments, the induction of c-fos by PGE2 in PKC-depleted cells was not directly demonstrated leaving the possibility that c-fos induction could be mediated by PKC. Indeed taken together, our studies would support that as much as 15–20% of the c-fos induction could be through a PKC pathway.

Different second messenger systems are stimulated following binding of PGE2 to different receptor subtypes. EP1 activates phospholipase C and raises intracellular calcium levels, EP2 stimulates adenylate cyclase to elevate intracellular cAMP levels, and EP3 can either stimulate or inhibit adenylate cyclase depending on which of three splicing variants are expressed (49, 50). Our results indicate that although c-fos can be induced by activating both Ca2+-dependent and

**Fig. 6.** Prostaglandin E2 up-regulates c-fos/CAT constructs in vitro. A, Diagram of c-fos promoter constructs. B, CAT activity in Vero cells transfected with different lengths of the c-fos promoter region. The conversion of [14-C] chloramphenicol (CM) to the monoacetate forms (A and B) is shown under the conditions of indomethacin alone, indomethacin plus PGE2 and indomethacin plus octanoic acid. Transcription rates are expressed as a fold increase over the lowest basal level noted in the **Relative Transcription Rates** line and are corrected for transfection efficiency based on β-galactosidase expression.
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, 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 MTFL/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 (I). 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.

Acknowledgment

The authors appreciate the graphics produced by Chris Barnstead.

References
53. Shaw PE, Frash S, Nordheim A. 1989 Repression of c-fos transcription is mediated through p65/SRF bound to the SRE. EMBO J 8:2559–2566