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Bone morphogenetic proteins (BMP-2 and BMP-3) induce the late phase expression of the proto-oncogene c-*fos* in murine osteoblastic MC3T3-E1 cells

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Here we report that bone morphogenetic proteins 2 and 3 (BMP-2 and BMP-3) induced marked expression of c-*fos* mRNA in a biphasic manner, i.e. the late phase (48 to 60 h) as well as the immediate-early phase (0.5 h), in murine osteoblastic MC3T3-E1 cells in vitro. The BMP-induced late phase c-*fos* gene expression was temporally associated with the onset of marked expression of the genes for osteocalcin and alkaline phosphatase, differentiation markers of mature osteoblasts. In contrast, none of TGF-β1, 10% FBS, IGF-I and IGF-II, which induced only the immediate-early c-*fos* mRNA expression, stimulated the expression of osteocalcin and alkaline phosphatase genes. These data suggest that in osteoblasts BMP-2 and BMP-3 induce the late phase expression of c-*fos*, which may play a role in transcriptional activation of the genes involved in differentiation of osteoblasts.

Bone morphogenetic protein; c-*fos*; Osteoblastic differentiation; MC3T3-E1; Alkaline phosphatase; Osteocalcin

1. INTRODUCTION

Expression of the proto-oncogene c-*fos* is known to be associated with such diverse biological processes as proliferation [1–5], cellular transformation [6], and differentiation [7–9]. Previous studies have shown that the expression of c-*fos* is differentiation stage-, tissue- and cell type-specific and occurs in a sustained manner in cells constituting the amnion and placenta [10], bone marrow [11], and differentiated macrophages [11,12], suggesting a close association of c-*fos* and cellular differentiation [11–14]. Moreover, the expression of exogenously introduced c-*fos* gene triggers cellular differentiation in F9 teratocarcinoma stem cells [7]. These lines of evidence suggest that c-*fos* is involved in the induction of differentiation at least in some cell types.

We have previously shown that c-*fos* is expressed in a sustained manner in vivo in osteoblasts in the ossifying fracture calluses of adult rat long bones. The sustained expression of c-*fos* is associated with the sequential expression of the genes for alkaline phosphatase, osteopontin, and osteocalcin, that are differentiation markers of osteoblasts. These observations have led us to hypothesize that the sustained expression of c-*fos* may have a role in the induction of osteoblastic differentiation [15]. Since the expression of c-*fos* is caused mostly by external signals [16–18], it is conceivable that the sustained c-*fos* expression in osteoblasts in the fracture calluses is elicited by some external signals transferred via fracture-triggered liberation of cytokine(s) such as bone morphogenetic proteins (BMPs) [19,20], transforming growth factor βs (TGF-βs) [21], and insulin-like growth factors (IGFs) [22–25].

BMPs refer to an activity derived from bone that induces formation of cartilage and bone in vivo [20]. BMPs are also likely to play an important role in fracture healing processes [26,27]. We and others recently showed that BMPs are involved not only in the differentiation of uncommitted mesenchymal cells to cells of chondrocytic and osteoblastic lineage but also in the differentiation of immature osteoblasts into mature osteoblasts [28–32].

In the present study we examined the effects of purified BMPs and other cytokines on the expression of c-*fos* and osteoblastic marker genes in the murine osteoblastic MC3T3-E1 cells in vitro. We show here that: (1) BMPs induce persistent c-*fos* mRNA expression in the late phase as well as the immediate-early phase; and (2) the late phase c-*fos* expression is persistent and par-

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alleled by marked expression of osteoblastic differentiation-related genes.

2. MATERIALS AND METHODS

2.1. *Materials*

Bone morphogenetic proteins were prepared from an osteoinductive fraction of 4 M guanidine-HCl extracts of demineralized bovine bone matrix and finally separated from contaminating osteoglycin by reversed-phase high-performance liquid chromatography as described in detail elsewhere [33,34]. Our preparation of bone morphogenetic proteins (BMPs) is a mixture of BMP-2 and BMP-3 at their relative amounts of 1:2 without other contaminants as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in both non-reducing and reducing conditions and amino acid sequence analysis of the peptides prepared by endopeptidase digestion, as described previously [32]. Osteoglycin did not have any effect on the expression of the genes in MC3T3-E1 cells analyzed in the present study (data not shown). The lyophilized BMPs were dissolved in phosphate-buffered saline containing 0.1% BSA (Calbiochem, CA). Bovine bone-derived transforming growth factor-b1 (TGF-β1) was purified as described [35]. IGF-I and IGF-II were purchased from KabiGen AB (Stockholm, Sweden). Alpha modification of minimum essential medium (αMEM) was purchased from Irvine Scientific (Santa Ana, CA, USA). Fetal bovine serum (FBS) was obtained from Bioproducts (Walkersville, MD). Other materials used were commercial products of the highest grade available.

2.2. *Cell culture*

A clonal cell line, MC3T3-E1, derived from newborn mouse calvaria was a generous gift from Dr. H. Kodama (Ohu Dental College, Kohriyama, Japan). These cells display several phenotypes characteristic of immature osteoblasts and can differentiate into mature osteoblasts in prolonged culture [36–38]. Cells were maintained in αMEM containing 3% FBS at 37°C in a humidified atmosphere of 5% CO_2 in air, with regular subculture every 3 days. Confluent cells were rinsed once with αMEM containing 0.3% FBS and incubated for 24 h in the same medium before the test substances were added. Cells were further incubated for the varying periods of time as indicated.

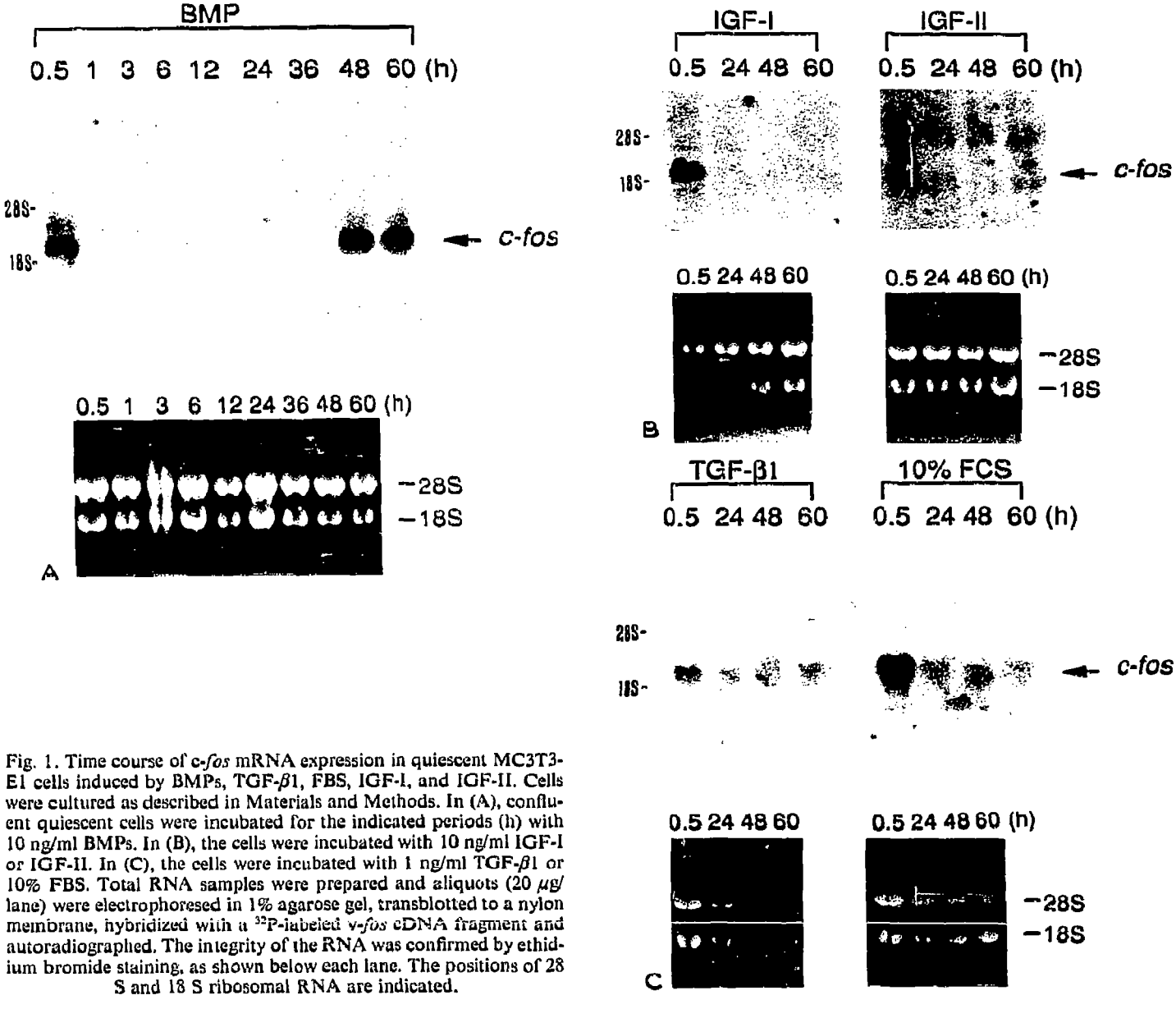

Fig. 1. Time course of c-*fos* mRNA expression in quiescent MC3T3-E1 cells induced by BMPs, TGF-β1, FBS, IGF-I, and IGF-II. Cells were cultured as described in Materials and Methods. In (A), confluent quiescent cells were incubated for the indicated periods (h) with 10 ng/ml BMPs. In (B), the cells were incubated with 10 ng/ml IGF-I or IGF-II. In (C), the cells were incubated with 1 ng/ml TGF-β1 or 10% FBS. Total RNA samples were prepared and aliquots (20 μg/lane) were electrophoresed in 1% agarose gel, transblotted to a nylon membrane, hybridized with a ^{32}P-labeled v-*fos* cDNA fragment and autoradiographed. The integrity of the RNA was confirmed by ethidium bromide staining, as shown below each lane. The positions of 28 S and 18 S ribosomal RNA are indicated.

2.3. *Total RNA extraction and Northern blot analysis*

Total RNA was extracted by the acid guanidinium–phenol–chloroform method [39]. Denatured total RNA (20 mg) was separated by 1% agarose gel electrophoresis and transferred to a nylon membrane (BioTrace, Ann Arbor, MI). Hybridization was performed at high stringency [15]. The membrane was exposed to Kodak X-Omat film at −80°C for 24 h with a Cronex Lightning Plus intensifying screen (DuPont, Wilmington, DE). The following hybridization probes were used: v-*fos*, a 1 kb *Pst*I fragment of pv-fos-1 [40]; purified synthetic oligonucleotides of 50-mer corresponding to Ile^{146}–Ala^{162} (human/rat conserved region) of rat liver/bone/kidney alkaline phosphatase cDNA [41] and of 51-mer corresponding to the Cys^{19}–Lys^{35} of mouse osteocalcin cDNA [42]. The v-*fos* cDNA was labeled with (α-^{32}P)-dCTP (New England Nuclear, Boston, MA) using a commercially available random primed labeling kit (Amersham, Tokyo, Japan) and the oligonucleotides were end-labeled with [γ-^{32}P]ATP (New England Nuclear, Boston, MA).

3. RESULTS AND DISCUSSION

The quiescent MC3T3-E1 cells were stimulated by BMPs, TGF-β1, IGF-I, IGF-II or FBS, and the time-course of the c-*fos* mRNA level was monitored by Northern blot hybridization. Intriguingly, BMPs (10 ng/ml) induced expression of the c-*fos* gene in a biphasic fashion (Fig. 1A): BMPs stimulated the immediate-early expression of c-*fos* mRNA which was characterized by a rapid and transient increase within half an hour in the mRNA level. Expression of the c-*fos* mRNA became undetectable by one hour after stimulation. After a long cessation, abundant expression of c-*fos* mRNA became once again detectable by 48 h after stimulation and persisted at least for an additional 12 h. Fig. 1B and C show that, IGF-I (10 ng/ml), IGF-II (10 ng/ml), TGF-b_1 (1 ng/ml) and 10% FBS all induced the immediate-early expression of c-*fos* mRNA. Merriman et al. recently reported that IGF-I and IGF-II stimulated the immediate-early c-*fos* mRNA expression in osteoblasts which preceded cellular proliferation [43], corroborating the generally accepted concept that the immediate early expression of the c-*fos* gene is related to growth–response of the cells. Unlike this early phase *c-fos* expression, however, the late phase expression of c-*fos* in osteoblasts in our study was BMPs-specific.

Recent data indicate that BMPs induce differentiation of osteoblast precursor cells into mature osteoblasts [28–32]. Indeed, BMPs were able to induce marked expression of osteocalcin mRNA 48–60 h after exposure in MC3T3-E1 cells (Fig. 2). Moreover, the expression of osteocalcin mRNA closely paralleled the late phase c-*fos* expression (Fig. 1A). None of TGF-β1, IGF-I, IGF-II and 10% FBS were able to stimulate expression of osteocalcin mRNA (Fig. 2). BMPs are the most potent stimulator of alkaline phosphatase activity in MC3T3-E1 cells among the cytokines so far tested [32]. Fig. 3 shows that BMPs enhanced expression of alkaline phosphatase mRNA which again paralleled the late phase c-*fos* expression. In contrast, TGF-β1 suppressed the expression of alkaline phosphatase mRNA, confirming our previous observations that TGF-β1, as

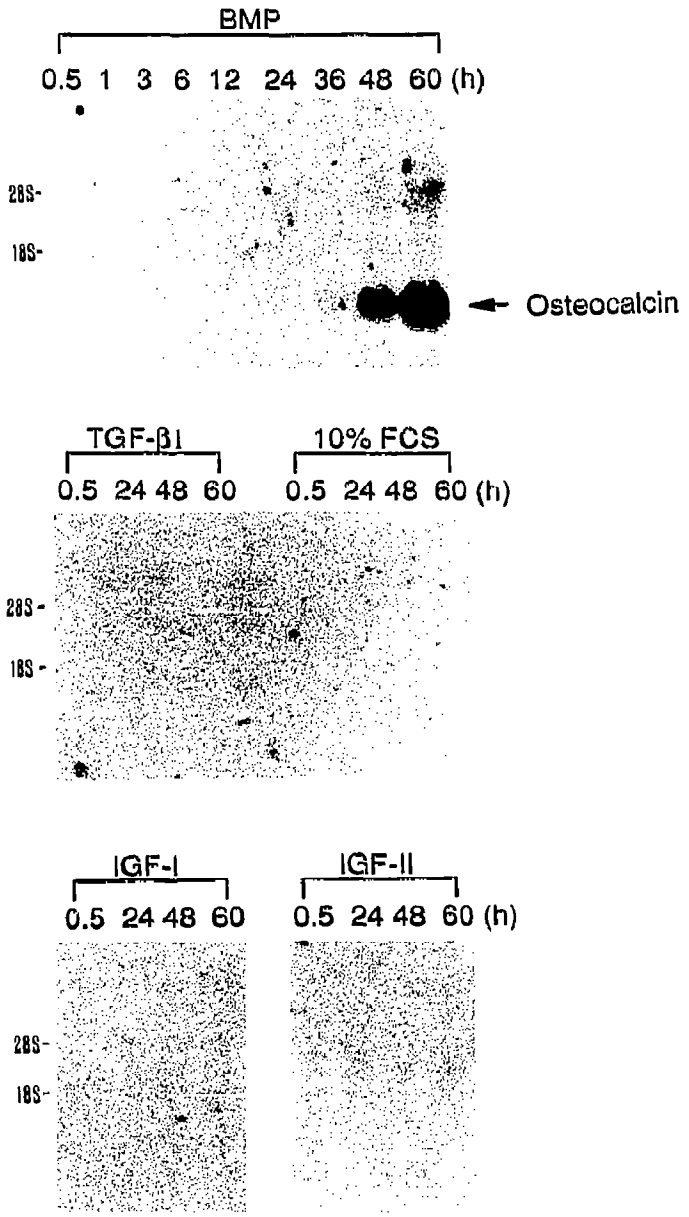

Fig. 2. Time course of osteocalcin mRNA expression in quiescent MC3T3-E1 cells induced by BMPs, TGF-β1, FBS, IGF-I, and IGF-II. The membrane shown in Fig. 1 was dehybridized, and then rehybridized with the radiolabeled probe for osteocalcin mRNA. The positions of 28 S and 18 S ribosomal RNA are indicated.

well as FBS, suppressed the alkaline phosphatase activity while it stimulated the cellular proliferation of MC3T3- E1 cells [32,44]. Thus, BMPs induced the late phase c-*fos* expression which was temporally associated with the marked expression of osteoblastic marker genes in MC3T3-E1 cells. Our results provide in vitro evidence

for the hypothesis that the sustained late phase expression of the c-*fos* gene plays a role in osteoblastic differentiation.

Gonda et al. showed that granulocyte-colony stimu-

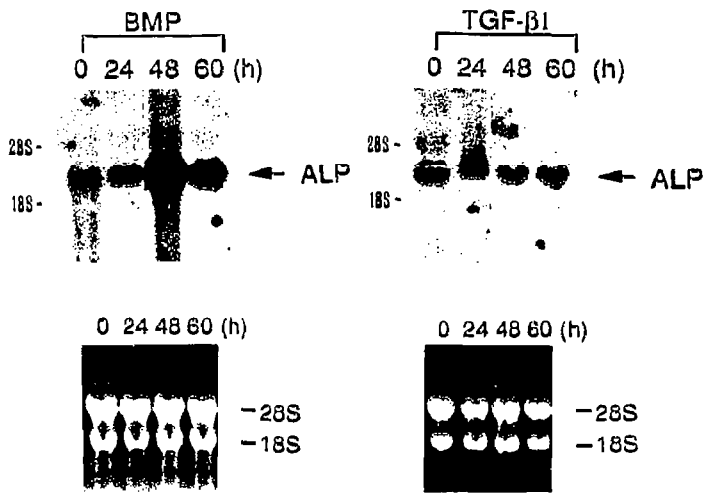

Fig. 3. Alkaline phosphatase (ALP) mRNA expression in quiescent MC3T3-E1 cells induced by BMPs and TGF-β1. Cells were cultured and incubated as described in the legend to Fig. 1. Northern blot hybridization with the radiolabeled probe for alkaline phosphatase mRNA was carried out as described in Materials and Methods. The integrity of the RNA analyzed was confirmed by ethidium bromide staining, as shown below each lane. The positions of 28 S and 18 S ribosomal RNA are indicated.

lating factor (G-CSF) induces the expression of c-*fos* at the late stages of differentiation (more than 48 h after G-CSF treatment) in murine myelomonocytic precursor cells [12]. Differentiation of cultured monomyelocytic cells is so far the only in vitro model where the c-*fos* gene is constitutively turned on [13,14]. Mitchel et al. subsequently reported that the c-*fos* expression is neither sufficient nor obligatory for differentiation of monomyelocytes to macrophages, suggesting that one possible role of the *fos* gene product during the differentiation may be to signal growth arrest of cells so that they can enter the macrophage differentiation pathway [45].

It is not known whether the BMP-induced late phase expression of the c-*fos* gene in osteoblastic MC3T3-E1 cells is a step necessary to induction of the osteoblastic marker genes. However, the following in vivo evidence suggests a specific role of the c-*fos* gene in bone development: (1) deregulated over-expression of the c-*fos* gene in transgenic mice results in multiple lesions of increased bone formation in newborns without apparent abnormalities in the other tissues [46]; (2) expression of c-*fos* mRNA is observed in the growth regions of fetal bone and mesodermal web tissue which are the sites of major changes in the orientation of fingers and toes [47]; and (3) as we previously reported [15], the ossifying soft callus and periosteal hard callus markedly express c-*fos* mRNA in a sustained manner at the fracture site of adult rat tibiae in vivo. This persistent c-*fos* expression was temporally followed by sequential expression of the genes for alkaline phosphatase, osteopontin and osteocalcin in each type of fracture calluses. Further, BMPs are thought to play an important local regulatory role in fracture healing [26,27]. Taken together, it is conceivable that BMPs induce the constitutive expression of c-*fos* gene in committed immature osteoblasts which then confers on these cells the ability to pass through a predetermined differentiation pathway, by inducing the expression of a particular set of genes.

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