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# Okadaic Acid Stimulates Osteopontin Expression Through De Novo Induction of AP-1

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**Abstract** Osteopontin, a major non-collagenous bone matrix protein, is strikingly upregulated in various tissues under certain pathologic conditions, including cancer. However, the mechanism of upregulation of the osteopontin gene in tumor cells remains unclear. Okadaic acid, a strong non-phorbol ester tumor promoter, is known to stimulate the expression of osteopontin. The aim of the present study was to understand the mechanism by which okadaic acid regulates osteopontin gene expression. Okadaic acid stimulated osteopontin mRNA expression in several cell lines within 3 h, and the increase in osteopontin mRNA was sustained for 24 h. New protein synthesis was required for the okadaic acid-elicited increase in osteopontin mRNA expression. A serial promoter deletion study showed that the okadaic acid-response element is located between positions −265 and −73, a sequence that includes the Runx2, Ets-1, and AP-1 binding sequences. Okadaic acid increased the mRNA expression of AP-1 components but not of Runx2 or Ets-1. Site-directed mutagenesis and electrophoretic mobility shift assays confirmed that protein binding of the AP-1 consensus sequence is necessary for the okadaic acid-mediated osteopontin gene upregulation. These results indicate that de novo induction of the oncoprotein AP-1 is required for okadaic acid-stimulated osteopontin gene upregulation. J. Cell. Biochem. 87: 93–102, 2002. 

**Key words:** osteopontin; okadaic acid; AP-1; promoter; tumor

Osteopontin (OP) is a multifunctional protein that is highly expressed in the extracellular matrix of bone and in various cell types such as macrophages, endothelial cells, smooth muscle cells, and epithelial cells. A secreted, usually phosphorylated glycoprotein that is involved in cell adhesion and signaling [Denhardt et al., 2001], OP can engage a number of cell surface receptors, including several α, β combinations of integrin and a splice variant of CD44 [Weber, 2001]. Among normal tissues, bone and kidney show the greatest expression of the gene, however, disease and injury lead to the striking upregulation of OP in a wide range of tissues. Pathologies associated with de novo OP expression include cancer [Tuck et al., 1997], inflammation [Ashkar et al., 2000], fibrosis [Giachelli et al., 1993], altered wound healing [Liaw et al., 1998], and granulomatous diseases [Carlson et al., 1997], whereas removal of the OP gene is associated with impaired mineralization [Yoshitake et al., 1999]. Among these reports of pathologic conditions, cancer-associated OP expression is predominant [Weber, 2001].

Okadaic acid (OKA) is a polyether compound of a 38-carbon fatty acid [Tachibana et al., 1981] and was first isolated from the marine sponges *Halichondria okadai* and *Halichondria malanocia* and is also a potent tumor promoter. Unlike the phorbol ester tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which activates protein kinase C (PKC), OKA binds to the catalytic subunits of serine/threonine

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phosphatase 1 (PP1) and 2A (PP2A) [Bralojan and Takai, 1988; Hescheler et al., 1988], and inhibits dephosphorylation of the intermediary components in signal transduction pathways, resulting in apparent increases in the phosphorylation of cellular proteins [Mumby and Walter, 1993]. The biological activities of cellular proteins are regulated by reversible phosphorylation and dephosphorylation, and many of these proteins may be components of signal transduction pathways [Karin, 1994]. Thus, perturbation of the phosphorylation state of these components by OKA greatly influences proliferation and differentiation. Regardless of the difference of their mechanism of action, both OKA and TPA are known to stimulate OP expression in normal as well as tumor cells [Su et al., 1995; Barletta et al., 2002]. Therefore, we thought that the stimulation of OP by OKA could represent a mechanism for OP upregulation in tumor tissue.

Analysis of the OP promoter has uncovered many potential sites for transcription factor interactions. Several steroid receptors, basic helix-loop-helix proteins, AP-1, Ets-1, and Runx2 are among the elements that have been implicated in OP transcription [Patarca et al., 1993; Broess et al., 1995; Bonnelye et al., 1997; Liu et al., 1997; Sato et al., 1998]. As one of the major bone marker genes, the induction of OP expression by osteotrophic agents, such as TGF-β, dexamethasone, vitamin D3, parathyroid hormone, calcitonin and retinoic acid, has been extensively investigated [Denhardt and Noda, 1998]. In contrast to the abundance of knowledge of gene regulation in osteogenesis, little is known about the mechanisms that regulate OP gene expression in tumor cells. The specific aim of the present study was to elucidate how a strong tumor promoter, OKA, regulates the transcription of OP.

## MATERIALS AND METHODS

### Cell Culture

Osteoblastic MC3T3-E1 cells were maintained in alpha-minimum essential medium (α-MEM; GibcoBRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GibcoBRL) and 100 U/ml penicillin/streptomycin (USB, Cleveland, OH) at 37°C in a humidified atmosphere containing 5% $CO_2$. Murine bone marrow-derived stromal cells were cultured in RPMI-1640 (GibcoBRL) containing 10% FBS; NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL) containing 10% calf serum; C3H10T1/2 cells and ROS17/2.8 cells were cultured in DMEM containing 10% FBS. The cells were grown until they were 75–80% confluent. OKA (Roche Molecular Biochemicals, Manheim, Germany) dissolved in dimethylformamide (DMF; Sigma Chemical Co., St. Louis, MO) was added to the growth medium to a final concentration of 50 ng/ml for the final 24 h. Controls were treated with equivalent amounts of DMF alone (less than 0.1% of the final concentration). Cycloheximide (CHX; Sigma Chemical Co.) was pretreated for 30 min and then OKA or DMF were added to the medium.

### RNA Extraction and Northern Blot Analysis

Total RNA was isolated as previously described [Lee et al., 1999] and quantified by spectrophotometry. For Northern blot analysis, 10 μg of total RNA was separated by agarose gel electrophoresis and blotted onto a Hybond-N + membrane (Amersham, Arlington, IL) in 20 × SSPE. The RNA was cross-linked by exposure to ultraviolet light and then air-dried. The probes were labeled with [α-$^{32}$P]-dCTP (Amersham) using the Megaprime DNA labeling system kit (Amersham). Prehybridization and hybridization were performed using Express Hyb solution (Clontech, Palo Alto, CA). After hybridization, the membrane was washed in 2 × SSC, 0.1% SDS at room temperature and then in 0.1 × SSC, 0.1% SDS at 55°C and exposed to Agfa X-ray film at −70°C with an intensifying screen.

### Construction of Plasmids

Chromosomal DNA was prepared from MC3T3-E1 cells using the DNAzol$^{TM}$ reagent (GibcoBRL). Serial deletion mutants of the mouse OP promoter were PCR amplified from mouse chromosomal DNA using the synthetic primers listed in Table I. Each PCR product was digested with *XhoI* and *HindIII* and cloned upstream of the firefly luciferase gene of the pGL2-basic vector (Promega, Madison, WI). The constructs were named OP-1983, OP-955, OP-521, OP-265, and OP-73, and contained the sequence of the mouse OP promoter from −1983, −955, −521, −265, and −73 to +75, respectively. The PCR products were confirmed by their size as determined by electrophoresis and by sequencing.

**TABLE I. Primer Sequences for Construction of OP Deletion Mutants and Site Directed Mutagenesis**

| | Oligonucleotide sequences |
|---|---|
| OP-1983 | 5′-AGATctcgagTCCTGGAAGGGTCATA-3′ (forward primer) |
| OP-955 | 5′-ATTActcgagCACTGCTTACCGAAGG-3′ (forward primer) |
| OP-521 | 5′-TGCActcgagTACAAAACAGAGCCAC-3′ (forward primer) |
| OP-265 | 5′-TTActcgagTGGATCCCTGATGCTCT-3′ (forward primer) |
| OP-73 | 5′-GActcgagACACATCACTCCACCTCCTG-3′ (forward primer) |
| Reverse primer | 5′-CTGCaagcttGTTTCCTCCGAGAATG-3′ |
| OP-TREm | 5′-GAGTGAT***a***T***a***TCATGAGG-3′ (reverse primer) |
| OP-OSE2m | 5′-CTCTGGTTTTGT***tc***TTTAA-3′ (reverse primer) |
| GL1 Primer | 5′-TGTATCTTATGGTACTGTAACTG-3′ |
| GL2 Primer | 5′-TGGAAGACGCCAAAAACATAAAG-3′ |

The lower case letters correspond to restriction enzyme sites for *XhoI* (forward primers) or *HindIII* (reverse primers). The lower case italic bold letters designate the substitution of nucleotide.

### Transfection and Luciferase Assays

The reporter vectors were transfected using the LipofectAMINE PLUS™ Reagent (GibcoBRL) according to the manufacturer's instructions. MC3T3-E1 cells were plated into six-well dishes at a density of $1 \times 10^5$ cells/well. Twenty-four hours later, the cells were transfected with reporter plasmids (1 μg/well) and expression vectors (1 μg/well). OKA (50 ng/ml) was added to the cells 24 h after the transfection, and the cells were cultured for an additional 24 h; controls were treated with media containing an equivalent amount of DMF. Cell lysates were prepared with the Luciferase Assay System kit (Promega). The luciferase activity was determined with a luminometer (EG&G Berthold, Walloc, Finland).

### Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear extracts and labeled probes were prepared as described previously [Ryoo et al., 1997], and protein content was determined using a Bradford Protein Assay Kit (Bio-Rad, CA). The probes used for electrophoretic mobility shift assays (EMSA) are listed in Table II. The probes were labeled with [α-$^{32}$P] dCTP. Approximately 1 ng of labeled oligonucleotide probe was incubated with 10 μg of nuclear extract for 30 min at room temperature in the presence of 1 × gel shift binding buffer containing 15 mM Tris pH 7.5, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 50 mM NaCl, 10% glycerol, and 2 μg of poly (dI-dC). Competition was performed with either 50, 100, or 1000-fold molar excess of unlabeled AP-1 binding site (TRE). For the supershift assay, FosB (H-75X), JunD (SC-74X), and c-Jun (SC-45X) antibodies (Santa Cruz Biotech. Inc., CA) were incubated with the protein-DNA complex. Reactions were separated in a 5% non-denaturing polyacrylamide gel. After electrophoresis, the gel was dried on Whatman 3MM paper and subjected to autoradiography.

### Site-Directed Mutagenesis of AP-1 and the Runx2 Binding Site

The OP-TRE and OP-OSE2 mutant constructs were generated by a two-step PCR strategy. For the first set of PCR reactions, the GL1 primer (Promega) was used as the forward primer and the TRE mutant primer (Table I; bases −80 to −63 of the OP promoter) was used as the reverse primer to create OP-TREm (272 bp), and the GL1 primer was used as the forward primer and the OSE2mut primer (bases −139 to −121 of the OP promoter) was used as the reverse primer to create the OP-OSEm construct (214 bp). The OP-265 construct was used as the template. Next, each product was used as the forward primer and the GL2 primer (Promega) was used as the reverse primer for the second PCR reaction, generating products of 458 bp. These products were digested with *XhoI* and *HindIII* and the resulting 338 bp fragment was ligated to the pGL2-Basic vector. Using the OP-OSE2mut construct as a template, we generated an OP-OSE2 and TRE double mutant construct. The GL1 primer and TREmut primer were used for the first PCR reaction, and the PCR product (272 bp) and GL2 primer were used for the second reaction. The accuracy of each construct was confirmed by sequencing.

## RESULTS

### Treatment With OKA Upregulates OP mRNA Levels

The concentration of OKA that we used in this experiment was determined to be optimal in our

**TABLE II. Probes for Electrophoretic Mobility Shift Assays**

| | Probe sequences |
|---|---|
| OP-TRE wild | Upper: 5′-GGGACCTCATGACACATCACTC-3′<br>Lower: 5′-GGGGAGTGATGTGTCATGAGGT-3′ |
| OP-TRE mut | Upper: 5′-GGGACCTCATGA***t A t***ATCACTC-3′<br>Lower: 5′-GGGGAGTGAT***a***T***a***TCATGAGGT-3′ |
| OP-OSE2 wild | Upper: 5′-GGTTTTAAACCACAAAACCAGAGGAGGAAGTGTA-3′<br>Lower: 5′-GGTACACTTCCTCCTCTGGTTTTGTGGTTTAAAA-3′ |
| OP-OSE2 mut | Upper: 5′-GGTTTTAAA***ga***ACAAAACCAGAGGAGGAAGTGTA-3′<br>Lower: 5′-GGTACACTTCCTCCTCTGGTTTTGT***tc***TTTAAAA-3′ |
| AP-1-consensus | Upper: 5′-GGGGCTTGATGACTCAGCCGGA-3′<br>Lower: 5′-GGGAGGCCGACTCAGTAGTTCG-3′ |

The lower case italic bold characters indicate the substitution of nucleotide.

previous studies [Choi et al., 1995, 1996]. Northern blot analysis of mRNAs from several normal and tumor cells indicated that OP expression was consistently stimulated by OKA treatment regardless of cellular origin. OP was upregulated by OKA treatment in normal cells such as murine bone marrow-derived stromal cell lines (ST2), fibroblastic NIH3T3, mesenchymal progenitor cells (C3H10T1/2), and osteoblastic MC3T3-E1 cells, as well as in tumor cell such as rat osteosarcoma (ROS 17/2.8) (Fig. 1A). In order to determine exactly when OP mRNA expression was induced, the cells were treated with OKA for different time periods. Though the initial stimulatory effect occurred as early as 3 h after OKA treatment was initiated, the most prominent increase began at 12 h and was sustained until 24 h (Fig. 1B). To determine whether the OP up-regulation by OKA is differentiation-stage specific, cells were treated with OKA at different stages of osteoblast differentiation. OKA (50 ng/ml) consistently upregulated OP mRNA expression at all stages of osteoblast differentiation (Fig. 1C). As the upregulation of OP gene expression by OKA was an early event, we examined whether OKA-mediated upregulation of OP mRNA requires the synthesis of new protein. MC3T3-E1 cells were treated with OKA (50 ng/ml) alone or in combination with cycloheximide (10 μg/ml), a protein synthesis inhibitor. Although MC3T3-E1 cells continued to express OP mRNA slightly in the presence of cycloheximide, treatment with cycloheximide effectively diminished the increase in OP mRNA expression that resulted from stimulation by OKA (Fig. 1D). This implies that increases in phosphorylation of some proteins by OKA stimulates the synthesis of new protein(s), probably transcription factors, that in turn stimulate OP mRNA expression.

### OP Promoter-Luc Reporters are Activated by OKA

The largest deletion mutant of mouse OP promoter contained the sequence from the −1983 position to the +75 position, and was named OP-1983. Because OKA stimulates OP-1983 promoter activity (Fig. 2B), OKA stimulation of OP expression may occur primarily through transcriptional regulation, with newly synthesized proteins as the central regulators. To determine the *cis*-acting elements that are responsible for the binding of this OKA-stimulated transcription factor, we used a series of 5′ deletion mutants of the OP promoter (Table I). Significant basal promoter activity was seen in OP-265 and in the longer constructs. Of these, OP-955 demonstrated the strongest basal promoter activity. To find the OKA-response element in the promoter, cells were transfected with each of the reporter plasmids. After overnight culture, cells were treated with OKA (50 ng/ml) for 24 h. OKA stimulated the expression of each reporter gene about three to four-fold, except for the OP-73 construct (Fig. 2B). This serial promoter deletion study indicates that the OKA-responding *cis*-acting element is located between the −73 and −265 positions of the OP proximal promoter. A computerized search of this sequence showed that it contains binding consensus sequences for Runx2, Ets-1, and AP-1 (Fig. 2A).

### OKA Stimulates AP-1 Expression but has no Effect on Runx2 or Ets-1 Expression

Our next experiment addressed whether the expression of Runx2, Ets-1, or AP-1 is regulated by OKA treatment. A Northern blot analysis showed that Ets-1 expression was not significantly changed by OKA treatment until OP

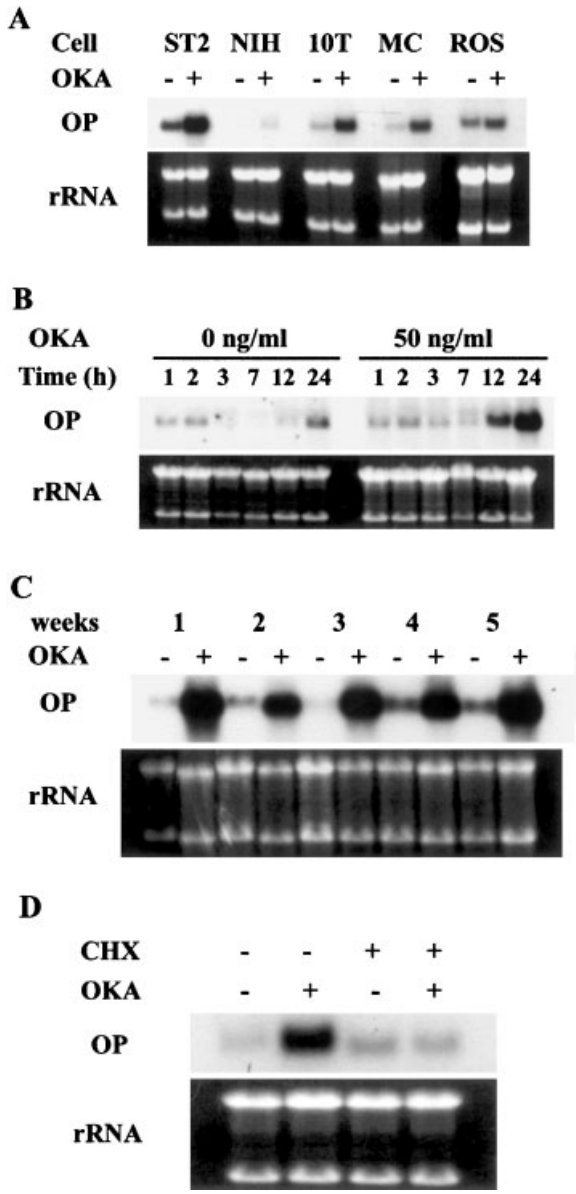


**Fig. 1.** Okadaic acid increases osteopontin (OP) gene expression, which requires new protein. **A**: Mouse bone marrow stromal cells (ST2), NIH3T3 cells (NIH), C3H10T1/2 cells (10T), MC3T3-E1 cells (MC), and ROS 17/2.8 cells (ROS) were cultured for 3 days. Confluent cells were treated for the final 24 h with 50 ng/ml OKA or vehicle, dimethylformamide (DMF). **B**: Confluent MC3T3-E1 cells were treated with 50 ng/ml OKA for the indicated period. **C**: MC3T3-E1 cells were cultured for 1–5 weeks and treated with 50 ng/ml OKA for the final 24 h. **D**: MC3T3-E1 cells were cultured for 3 days and treated for the final 24 h with OKA alone or in combination with cycloheximide (CHX) (10 μg/ml). Ten micrograms of total RNA was analyzed by Northern blot hybridization with a cDNA probe for OP. Equivalent amounts of intact RNA were loaded in each lane as indicated by ethidium bromide staining of ribosomal RNA.

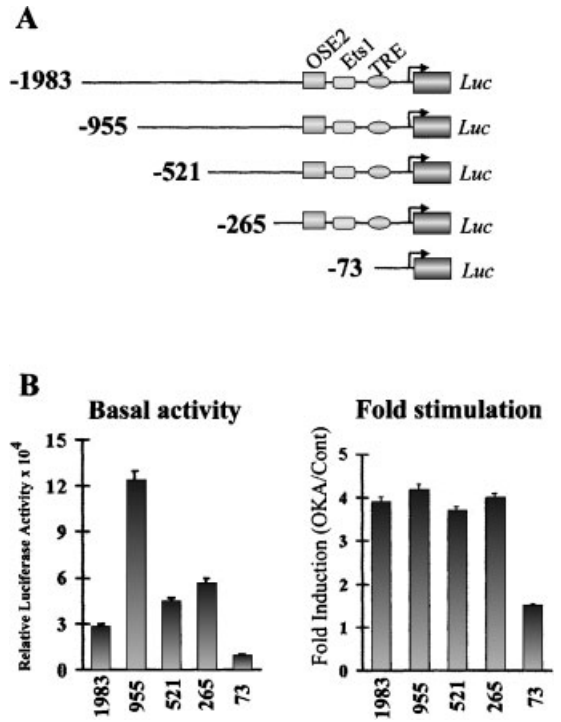


**Fig. 2.** Osteopontin promoter-luciferase chimeric genes are activated by okadaic acid. The serial deletion constructs of the OP promoter shown in (**A**) were transfected into MC3T3-E1 cells (**B**). The cells were treated with OKA for the final 16–24 h. Cells were harvested and assayed for luciferase activity. Luciferase activity was normalized by the total protein content in the lysates.

expression increased, and that OKA treatment actually decreased the Runx2 mRNA level. In contrast, the expression of AP-1 components, such as FosB, c-Jun, and JunD, was strongly increased 1 h after treatment with OKA (Fig. 3). Because transcriptional activity depends mainly on the interaction between transcription factors and their cognate *cis*-acting elements, we next examined the specific binding activities of Runx2 and AP-1. OKA treatment did not greatly influence the binding of Runx2 to Runx2 binding site (OSE2) (Fig. 4A). Consistent with the Northern analysis, AP-1 binding to AP-1 binding site (TRE) greatly increased from 3–6 h after the OKA treatment (Fig. 4B). The specificity of the AP-1 binding was confirmed with a competition assay. A molar excess of cold TRE effectively competed with the TRE site for the binding of the AP-1 complex (Fig. 4C, lanes 4, 5, and 6), but a molar excess of cold TREm did not successfully compete (Fig. 4C, lanes 7, 8, and 9). AP-1 binding did not occur on the labeled

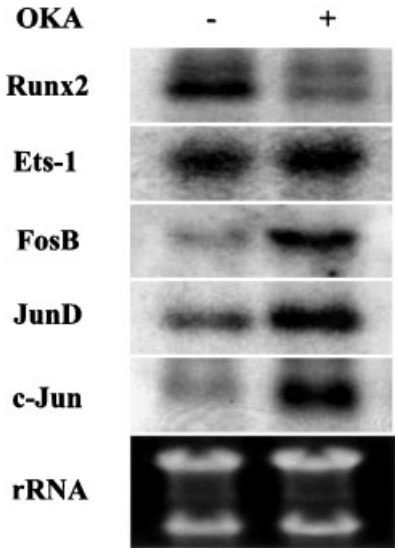


**Fig. 3.** Okadaic acid induces the components of activator protein-1 (AP-1). MC3T3-E1 cells were cultured for 3 days and treated with OKA for the final 1 h. Ten micrograms of total RNA was analyzed by Northern blot hybridization with cDNA probes for Runx2, Ets-1, FosB, JunD, and c-Jun. Equivalent amounts of intact RNA were loaded in each lane as indicated by ethidium bromide staining of ribosomal RNA.

TREm (Fig. 4C, lanes 10 and 11). The size of the binding complex was supershifted by antibodies against FosB, c-Jun or JunD (Fig. 4C, lanes 12, 13, and 14).

### Site-Directed Mutagenesis of the AP-1 Binding Site Abrogates OKA Stimulation of the OP Promoter

To investigate the effect of AP-1 binding on OP promoter activity, we performed site-directed mutagenesis of the Runx2 and AP-1 binding sites of the OP-265 construct. OKA treatment of OP-265-Luc-transfected cells increased luciferase activity by three to fourfold. Mutation of OSE2 did not influence OKA-stimulated OP transcription. In contrast, the TRE mutation and the TRE/OSE2 double mutation almost completely abolished OKA-stimulated promoter activity (Fig. 5A). As FosB expression was

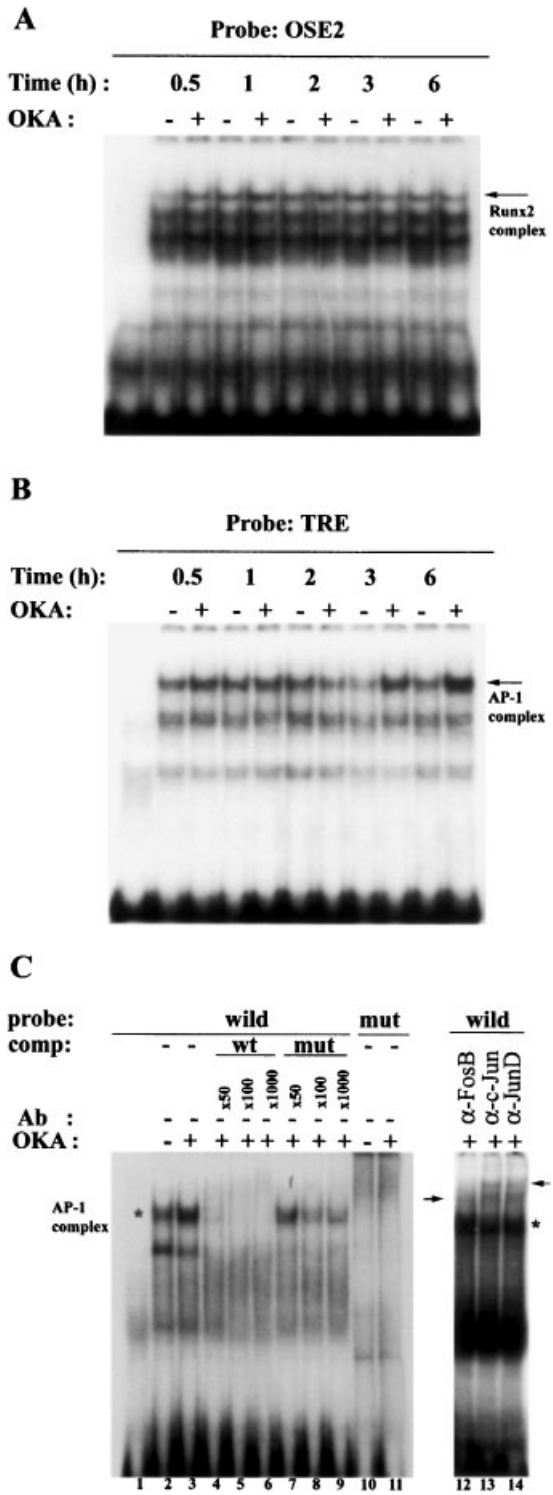


**Fig. 4.** Okadaic acid increases AP-1 binding activity on the OP promoter. ROS17/2.8 cells were treated for various times with or without OKA, and then nuclear extracts were prepared from the cells. **A** and **B**: $^{32}$P-labeled oligonucleotides corresponding to the wild-type Runx2 (A) or wild-type AP-1 (B) binding sites in the OP promoter (see Table II) were incubated with the nuclear extracts. **C**: Nuclear extracts from ROS17/2.8 cells treated for 6 h with or without OKA were incubated with $^{32}$P-labeled probes containing the TRE site or a TRE mutant of the OP promoter. Unlabeled wild-type or mutant competitors were used for competition, and FosB, c-Jun, and JunD antibodies were used for the supershift assay. Asterisk shows AP-1 complexes; arrows indicate supershifted bands of AP-1 components.

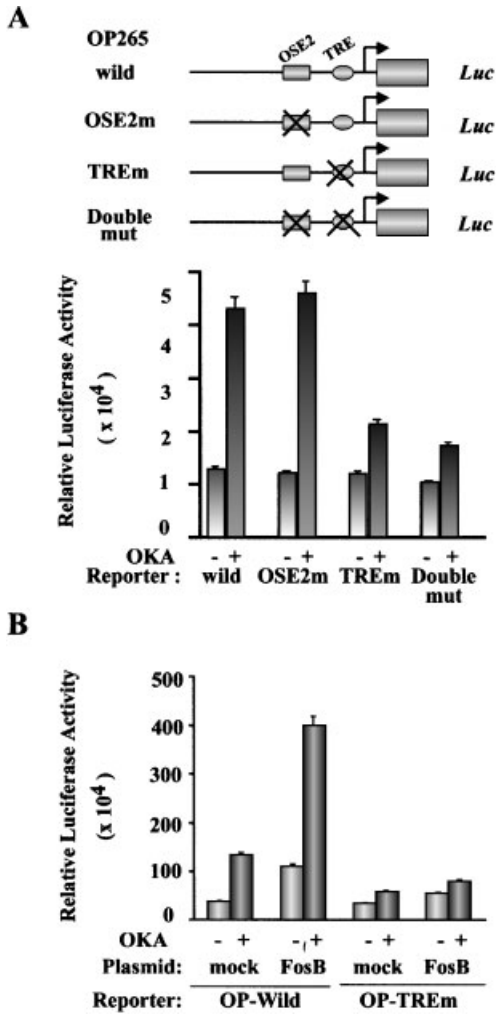


**Fig. 5.** The stimulation of OP expression by okadaic acid is mediated by a TRE site in the proximal OP promoter. **A**: Mutations in the proximal OP promoter are illustrated. The constructs were transfected into MC3T3-E1 cells, followed by treatment with OKA for 16–24 h. **B**: ROS17/2.8 cells were transiently cotransfected with 1 μg of either wild type or mutated OP promoter constructs (OP-wild or OP-TREm) and 1 μg of FosB expression vector as indicated. Reporter activities were determined 16–24 h after the treatment with OKA. Luciferase activity was normalized by the total protein concentration in the lysates.

strongly stimulated by OKA treatment, we cotransfected the FosB expression vector with the reporter vectors. This forced expression of FosB stimulated the OP-265 promoter activity even in the absence of OKA, and OKA treatment further stimulated OP promoter activity. Consistent with the results of the OKA treatment, the TRE mutant did not respond at all to the FosB transfection, either in the presence or absence of OKA treatment (Fig. 5B).

## DISCUSSION

### OKA-Stimulated OP Expression Requires De Novo Protein Synthesis

In this study, OKA strongly upregulated the steady-state expression of OP mRNA regardless of its cellular origin. OKA-induced OP gene expression is thus not restricted to tumor cells but occurs commonly in cells expressing OP. Moreover, the stimulation of OP expression by OKA was not dependent on the differentiation state of the cells. Our previous reports showed that the growth factors PDGF-BB [Kim et al., 1999] and FGF-2 [Bae et al., 2000] stimulate OP expression in the early stages of MC3T3-E1 cell differentiation but suppress it strongly in the later mineralization stage. Nuclear matrix components and their organization change dramatically with cell differentiation, and the changes would greatly influence the use of the transcriptional machinery [Lindenmuth et al., 1998]. Thus, OP regulation by growth factors relies on the cellular environment. In contrast, as a potent tumor promoter, the OKA signal might be strong enough to overcome the unfavorable nuclear matrix organization resulting from cell differentiation, which may lead to tumor progression.

OP induction occurred as early as 3 h and the induction level was sustained until 24 h after OKA treatment, which suggests the induction is not an immediate response. Pretreatment with a protein synthesis inhibitor, cycloheximide, completely blocked OKA-stimulated OP expression in the cells. Thus, the stimulation of OP expression by OKA could not be the result of direct phosphorylation of nuclear proteins (Fig. 1D). Inhibition of protein phosphatases by OKA seems to lead to new protein synthesis that regulates the transcription of the OP gene. OKA is known to be a strong inhibitor of PP1 and PP2A, whose role in the cell is recovery of the serine or threonine moieties of cellular proteins from their phosphorylated state [Bialojan and Takai, 1988]. Recent reports indicate that OKA stimulates several protein kinases such as PKC, MEK1, ERK and SAPK, through the inactivation of PP2A, which recovers these kinases from their activated state [Schonthal, 2001]. The sustained activation of these kinases has been known to stimulate several transcription factors such as AP-1, NFκB, and SP1 [Sontag, 2001]. Therefore, these transcription factors could be candidates for the OKA-induced

newly synthesized proteins that stimulate OP transcription.

### Runx2 is not Involved in the OKA-Stimulated OP Expression

Because the OKA-stimulated OP gene expression seemed to be regulated by newly synthesized transcription factors, we tried to identify the responsible regulators by analyzing the OP promoter. The serial OP promoter deletion assay indicated that the binding of newly synthesized transcription factors to the promoter sequence between positions −265 and −73 is required for the induction of OP expression by OKA. A computerized search of possible transcription factor binding sites indicated that there are three consensus sequences for transcription factor binding in this portion of the OP promoter: those for Runx2, Ets-1, and AP-1. As previously demonstrated [Ducy et al., 1997], most osteoblast marker genes, including OP, are strongly stimulated by Runx2 transfection. Consistent with this, bone marker genes such as osteocalcin, bone sialoprotein, and type I collagen have a typical Runx2 binding site (AACCACA) in their proximal promoter [Banerjee et al., 1997; Ducy et al., 1997; Xiao et al., 1998]. In addition, Sato et al. [1998] reported that the Runx2 and Ets-1 binding sites in the promoter region of the OP gene, and their spatial relationships, might be important for OP gene expression. However, our data clearly demonstrated that Runx2 is not involved in OKA-stimulated OP expression. First, not only did OKA fail to stimulate Runx2 expression before the major peak of OP expression (3–24 h), it actually strongly suppressed Runx2 expression. Second, no significant change in Runx2 binding was detected with the Runx2 binding site of OP using EMSA. Finally, mutation of OSE2 site did not abrogate OKA-stimulated OP promoter activity. Thus, Runx2 is not likely to be responsible for OP induction in this experimental system. As Ets-1 seemed quite dependent on the interaction with Runx2 protein for the regulation of OP expression [Sato et al., 1998] and its expression was not changed by OKA treatment in this experiment, we did not investigate the role of Ets-1 in this study. However, because translational regulation or posttranslational modification are also known to be important steps in the regulation of Runx2 [Xiao et al., 2000; Sudhakar et al., 2001] and Ets-1 [Yang et al., 1996] activity, we cannot completely rule out the involvement of the two transcription factors in OP regulation by OKA.

### AP-1 is Involved in OKA-Stimulated OP Expression

In contrast to Runx2 and Ets-1, the expression of AP-1 components, such as FosB, JunD, and c-Jun, was stimulated 1 h after OKA treatment. As previously described [Rosenberger et al., 1999], the expression of all of the AP-1 components is stimulated by OKA treatment. Thus, there is a strong possibility that the other AP-1 components could be upregulated by OKA treatment in our system as well. Consistent with the increase in the mRNA levels of the AP-1 components, a gel mobility shift assay also indicated that the nuclear extract from OKA-treated cells had protein components that strongly bound with the TRE consensus sequence of the OP promoter. Previously, another tumor promoter, TPA, was also reported to stimulate OP expression through the activation of AP-1, but another TRE downstream of the OP transcription initiation site was thought to be responsible for the induction [Craig et al., 1989; Patarca et al., 1993]. However, in this study the OP-73 reporter vector, which included the downstream TRE, did not respond to OKA treatment (Fig. 2B), and the gel mobility shift assay did not show a significant increase in protein binding with the downstream TRE probe following OKA treatment (data not shown). This suggests that the downstream TRE may not be responsible for the regulation of OP expression by OKA. The site-directed mutagenesis of the OP promoter indicated that AP-1 binding to the TRE ($-69 \sim -75$) would be the most important step in the induction of OP expression by OKA. The binding activity of the TRE in this system was somewhat weaker than that of the conventional TRE consensus sequence, but the binding specificity was very similar. The importance of the binding between AP-1 and the TRE was also demonstrated by the transfection study with the OP-265 TREm mutant reporter vector, whose promoter activity was not stimulated by OKA treatment.

OP promoter activity was stimulated by exogenous expression of FosB and was further stimulated by OKA treatment (Fig. 5B). This may be because OKA further stimulated endogenous expression of AP-1 components, OKA stimulated the activity of the exogenously

introduced FosB protein, or a combination of both. It has been demonstrated that PD98059, an ERK-1/2-specific inhibitor, completely abrogates okadaic acid-induced AP-1 transactivation without altering AP-1 expression, DNA binding, or complex composition [Rosenberger et al., 1999]. Phosphorylation analyses have indicated that inhibition of ERK1/2 decreased okadaic acid-elevated phosphorylation of JunD and FosB while the other AP-1 components were not influenced [Rosenberger et al., 1999]. Together, these results suggest that not only the expression but also the phosphorylation of AP-1 is crucial for the regulation of OP by OKA. OP expression increases in tumor tissues, particularly at the invasive edge of the tumor. OKA and TPA, strong tumor promoters, also greatly enhance OP expression in normal and tumor cells, indicating that OP could be a marker of tumorigenesis. If so, what is the role of OP in tumorigenesis? Recent reports indicate that OP normally expressed in bone is incorporated into the extracellular matrix but tumor-driven OP is soluble and not matrix-associated [Rittling et al., 2002]. Elevated levels of OP in the plasma of patients are associated with metastatic activity of the tumors [Singhal et al., 1997]. These results strongly suggest that the OP protein synthesized in tumorigenesis has different characteristics compared to the protein found in normal tissue. As tumor cells usually have a perturbation in their phosphorylation and/or dephosphorylation machineries, the posttranslational modification of the OP protein may differ in normal and tumor tissues, which might affect the solubility and function of the protein. Therefore, further investigation of the phosphorylation state of the OP protein is needed. In summary, we have examined the mechanisms by which OP gene expression is regulated by a strong tumor promoter, OKA. Our data indicate that the increase in OP expression by OKA requires de novo synthesis of the oncoprotein AP-1.

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