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Regulation of collagenase gene expression by okadaic acid, an inhibitor of protein phosphatases

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Human collagenase gene expression is regulated transcriptionally and is inducible by various mitogens in many cell types. To investigate the molecular mechanisms of this response, we examined the effects on collagenase gene expression of okadaic acid, a non-12-O-tetradecanoyl-phorbol-13-acetate (TPA)-type tumor promoter, which induces apparent "activation" of protein kinases by inhibition of protein phosphatases. Steady state levels of collagenase mRNA were markedly increased by okadaic acid treatment. We show that the AP-1 consensus sequence in the collagenase promoter is required for the induction of collagenase gene expression by okadaic acid, even though sequences upstream of the AP-1 consensus site have an additive effect. We also examined the regulation by okadaic acid of expression of the components of the AP-1 complex, c-*fos* and c-*jun*. c-*fos* expression is dramatically stimulated by okadaic acid, whereas c-*jun* expression is stimulated to a lesser extent. Induction of c-*fos* gene mRNA occurs through a region known to contain multiple regulatory elements. These results suggest that phosphorylation regulates collagenase gene expression mediated by an AP-1 binding site.

Phosphorylation has recently been suggested to be an important regulatory modification for transcription factors such as the cyclic-AMP-like responsive element binding factor (Gonzalez *et al.,* 1989), NF-kB (Sen and Baltimore, 1986), the heat shock–inducible factor (Sorger *et al.,* 1987), and AP-1 (Angel *et al.,* 1988b). The 12-O-Tetradecanoyl-phorbol-13-acetate (TPA)-inducible factor, AP-1, is a complex composed of the cJun and cFos proteins (Chiu *et al.,* 1988; Rauscher *et al.,* 1988; Angel *et al.,* 1988a), both of which are phosphoproteins containing multiple phosphorylation sites (Barber and Verma 1987; Boyle *et al.,* in preparation). The role of phosphorylation or dephosphorylation in controlling Jun and Fos activity is still not clear. However, because transcription of serum-inducible genes such as c-*fos* or c-*jun* is induced rapidly within minutes of stimulation of cell surface receptors for growth factors, even in the absence of new protein synthesis (Greenberg and Ziff, 1984; Muller *et al.,* 1984; Angel *et al.,* 1988b), it is likely that growth factor–dependent, intracellular signal transduction pathways lead to the phosphorylation and dephosphorylation of these and other transcription factors by various kinases or phosphatases. To determine whether phosphatase inhibition can, to some extent, mimic the effect of growth factors on gene expression, we examined the effect of an inhibitor of phosphatases, okadaic acid, on transcription of the human collagenase gene, the expression of which is known to be regulated by AP-1 (Angel *et al.,* 1987a,b).

Okadaic acid is a polyether derivative of a 38-carbon fatty acid that was first isolated from a marine sponge of the species *Halichondria okadai* (Tachibana *et al.,* 1981). Okadaic acid is a potent tumor promoter on mouse skin; it neither binds to the phorbol ester receptor nor activates protein kinase C (Suganuma *et al.,* 1988). It is a potent inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A) (Erdodi *et al.,* 1988; Hescheler *et al.,* 1988; Haystead *et al.,* 1989) and produces a variety of effects in physiological systems that are modulated by phosphorylation. The mechanism of action of okadaic acid is now understood as follows: okadaic acid binds to and inhibits its own receptors, protein phosphatases (Suganuma *et al.,* 1989), resulting in the apparent "activation" of protein kinases (Issinger *et al.,* 1988; Sassa *et al.,* 1989). The targets of

Abbreviations: CAT, chloramphenicol acetyltransferase; SRE, serum response element; TGF-β1, transforming growth factor-β1; TPA, 12-O-Tetradecanoyl-phorbol-13-acetate.

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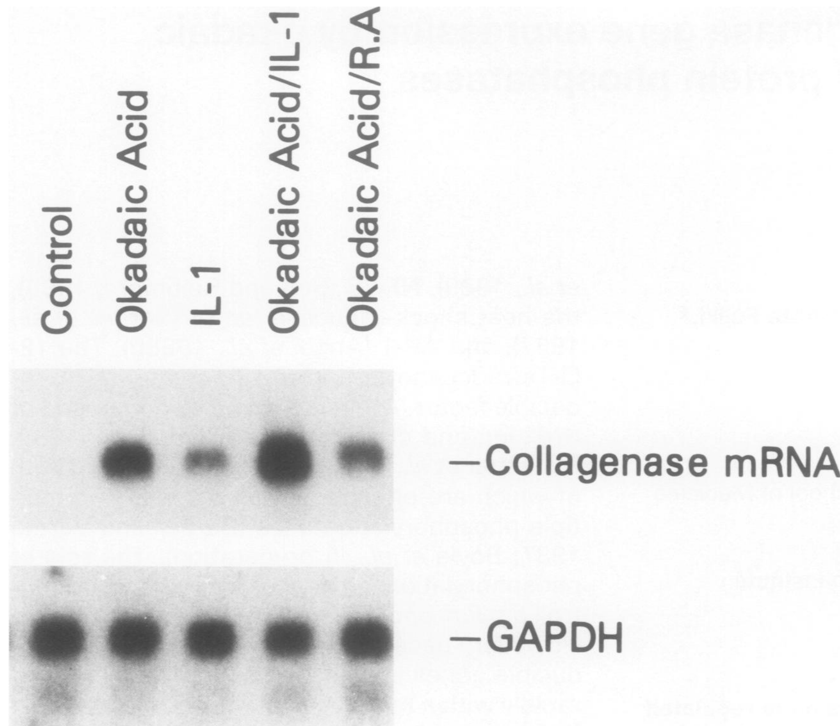

***Figure 1.* Stimulation of steady state collagenase gene expression by okadaic acid and IL-1.** Human synoviocytes were left untreated (control) or treated with okadaic acid (30 ng/ml) for 17 h, IL-1 50 U/ml for 17 h, or okadaic acid and IL-1 together for 17 h or retinoic acid (10^{-6} *M*) 6 h before the okadaic acid. The Northern blot (10 μg of RNA/lane) was hybridized using human collagenase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes.

these kinases might act as regulatory proteins for tumor promotion (Fujiki *et al.*, 1989).

We report here that okadaic acid stimulates expression of the human collagenase gene, in part, through the AP-1 binding site. In addition, our results indicate that okadaic acid induces transcription of the c-*fos* gene dramatically but stimulates expression of c-*jun* to a lesser extent.

Results

Stimulation of collagenase gene expression by okadaic acid in human synoviocytes

We examined the effect of okadaic acid on expression of human collagenase mRNA in human synoviocytes in culture. As shown by Northern blot analysis in Figure 1 (*upper*), transcription of the 2.1-kb collagenase transcript (Brinckerhoff *et al.*, 1986) is markedly stimulated by okadaic acid. Because it has been reported that IL-1 also stimulates collagenase gene expression in human synoviocytes (Postlethwaite *et al.*, 1988), we examined the effect of okadaic acid on IL-1–stimulated expression. As expected, IL-1 stimulated the steady-state level of collagenase transcripts; when the cells were stimulated with both IL-1 and okadaic acid, the effects on collagenase mRNA expression in synoviocytes were additive, suggesting that these two factors act through different mechanisms.

Recently, we confirmed that pretreatment of cells with retinoic acid completely inhibited steady-state collagenase mRNA expression induced by IL-1 or TPA (Brinckerhoff *et al.*, 1986; Lafyatis *et al.*, manuscript submitted). To examine whether retinoic acid also inhibits the induction of the collagenase mRNA expression stimulated by okadaic acid, retinoic acid was added to the cells 6 h before treatment with okadaic acid. As shown in Figure 1, pretreatment of cells with retinoic acid led to partial inhibition of collagenase mRNA expression induced by okadaic acid.

Dose response of the okadaic acid effect on collagenase promoter-CAT expression

Because A-549 cells are more easily transfected than primary synoviocytes, we examined the effects of okadaic acid on collagenase promoter–chloramphenicol acetyltransferase (CAT) expression in these cells. The collagenase 5′ flanking region/CAT construct (−73/+63-CAT) was transfected into A-549 cells to study the dose-response of the effect of okadaic acid on AP-1 activity. This region of the promoter contains the TPA-responsive element of the collagenase gene, consisting of eight base pairs (−73 to −65) that constitute a binding site for the AP-1 complex (Angel *et al.*, 1987b; Lee *et al.*, 1987; Chiu *et al.*, 1988). The degree of stimulation of

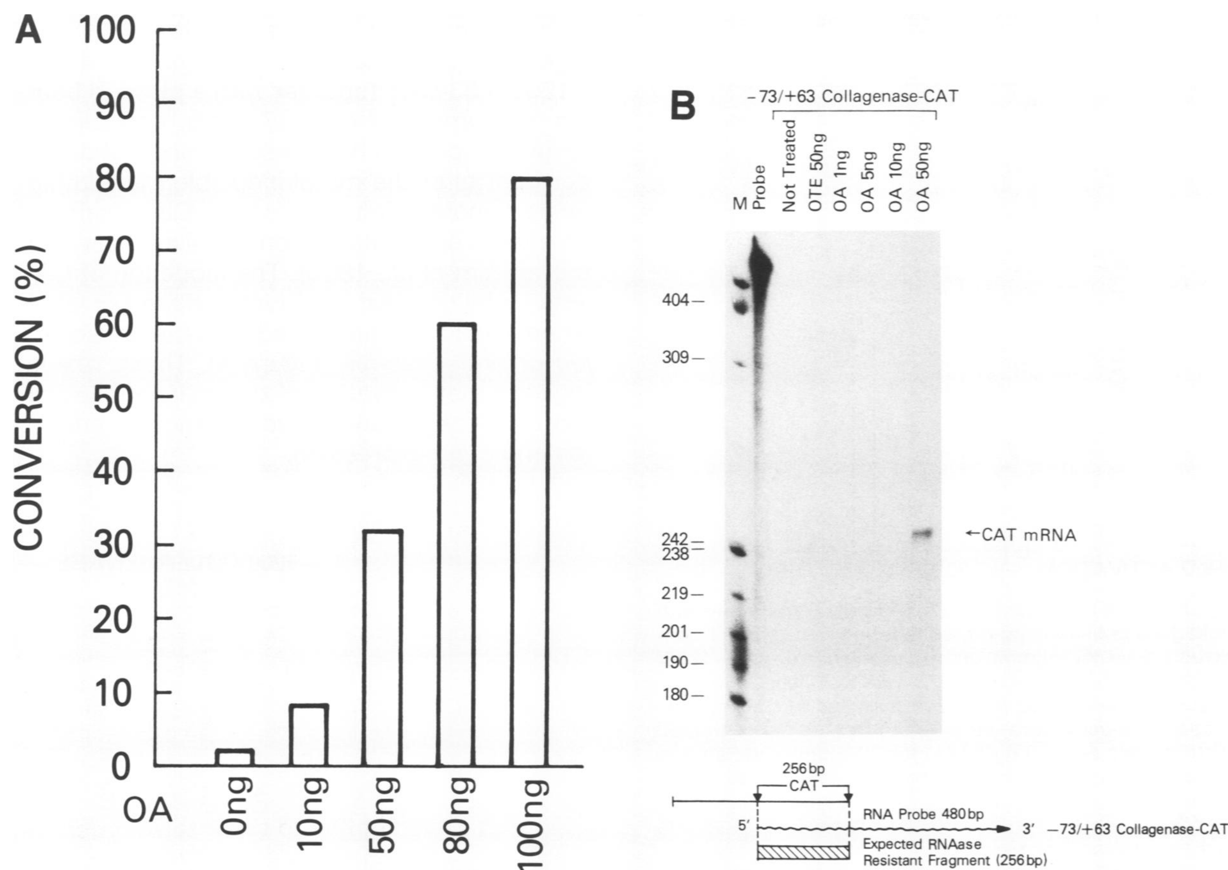

Figure 2. **Dose-dependent induction of expression of collagenase promoter-CAT construct (−73/+63-CAT) by okadaic acid.** A, dose-dependent induction of the activity of the collagenase-promoter-CAT plasmid. A-549 cells were transfected with 10 μg of plasmid, −73/+63-CAT. Okadaic acid (ng/ml) was added 24 h after the transfection; 15 h later the cells were harvested and the CAT activity was assayed. B, RNase protection analysis of CAT mRNA synthesized in A-549 cells in the absence (not treated) or presence of increasing concentrations of okadaic acid after transfection with −73/+63-CAT. *Lower panel* shows a schematic diagram of both the plasmid and RNA probes. Okadaic acid tetramethyl ether was used as a negative control. The size of the CAT-specific fragment protected from RNase digestion is 256 nucleotides.

expression of this construct by okadaic acid was dosage dependent (Figure 2A). At concentrations up to 10 ng/ml, okadaic acid showed very little effect on −73/+63-CAT expression; however, at a concentration of 100 ng/ml, it stimulated −73/+63-CAT expression dramatically. At higher concentrations, it had cytotoxic effects on the cells and caused detachment of cells from the bottom of the dishes. Sassa *et al.* (1989) showed that at this concentration the activation of protein kinases by okadaic acid reaches the maximum level. Therefore, in the rest of the experiments, the cells were treated with 50 ng/ml okadaic acid to induce CAT expression. Even this concentration of okadaic acid is cytotoxic in some cells such as human synoviocytes; thus, the concentration of okadaic acid for maximum effect varies depending on the cell type.

To establish that the increase in CAT activity of −73/+63-CAT was due to elevated levels of CAT mRNA, the steady-state levels of CAT mRNA were examined by quantitative RNase protection analysis (Figure 2B). The size of the undigested probe was 480 nucleotides, whereas the size of the CAT-specific fragment protected from RNAase digestion would be 256 nucleotides. Consistent with the results of the collagenase promoter/CAT activities presented above, the CAT mRNA level was higher in −73/+63 transfected cells treated with 50 ng/ml of okadaic acid. No CAT mRNA was detected in cells treated with okadaic acid tetramethyl ether as a control.

Localization of regions for okadaic acid regulation in the 5′ flanking region of the human collagenase gene

To determine whether the AP-1 site is the only element of the collagenase promoter responsive to okadaic acid, chimeric plasmids containing

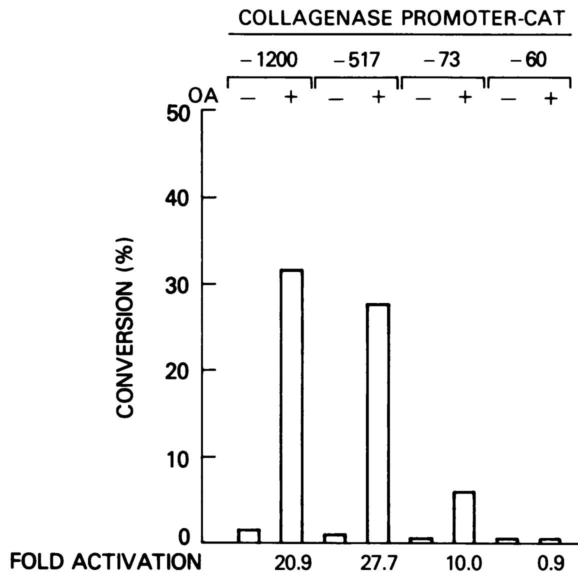

Figure 3. **CAT expression by A-549 cells transfected with collagenase 5'-flanking region deletion mutant/CAT constructs.** Five micrograms of the plasmid constructs −1200/+63-CAT, −517/+63-CAT, −73/+63-CAT, and −60/+63-CAT were transfected into A-549 cells, and 24 h after transfection, the cells were either left untreated (−) or treated with okadaic acid 50 ng/ml (+) for 20 h under serum-free conditions. Values expressed are the mean of three experiments.

various segments of the collagenase 5' flanking region ligated to the CAT gene were used. The recombinant plasmids were transfected into A-549 cells, and the cells were then incubated in the absence or presence of okadaic acid (50 ng/ml). All transfections were transient expression experiments in which okadaic acid was added 24 h after transfection. After a predetermined interval, the cells were harvested and CAT activity measured. In the presence of okadaic acid, the sequences −1200 to +63, −517 to +63, and −73 to +63 stimulated expression 20.9-, 27.7-, and 10-fold, respectively (Figure 3). The response was lost when the deletion continued beyond position −73; mutant −60/+63-CAT was not inducible, indicating that sequences responsive to okadaic acid induction are located in that region. This region contains the TPA-responsive element, which is the binding site for the AP-1 complex, suggesting that this site is one of the targets for okadaic acid stimulation.

Because okadaic acid stimulation decreased about five- to sevenfold after deletion of sequences between −517 and −73, other okadaic acid–responsive elements may be present in this upstream region of the promoter. To establish whether the TPA-responsive element motif might also be involved in the okadaic acid stimulation of this upstream region of the collagenase promoter, we cotransfected the −1200/+63-CAT together with a plasmid bearing a fragment of c-*jun* (R. Chiu, unpublished results) or c-*fos* in the antisense orientation under control of the metal-inducible metallothionein promoter (Holt *et al.,* 1986). Cd^{2+} itself had no effects on the activation of collagenase promoter (Kim *et al.,* 1990). The induction of CAT activity by okadaic acid was reduced or abolished in cells cotransfected with the antisense-*jun* or antisense-*fos* (Figure 4). These results indicate that activation of the collagenase promoter-CAT by okadaic acid depends on prior c-*fos* and c-*jun* expression.

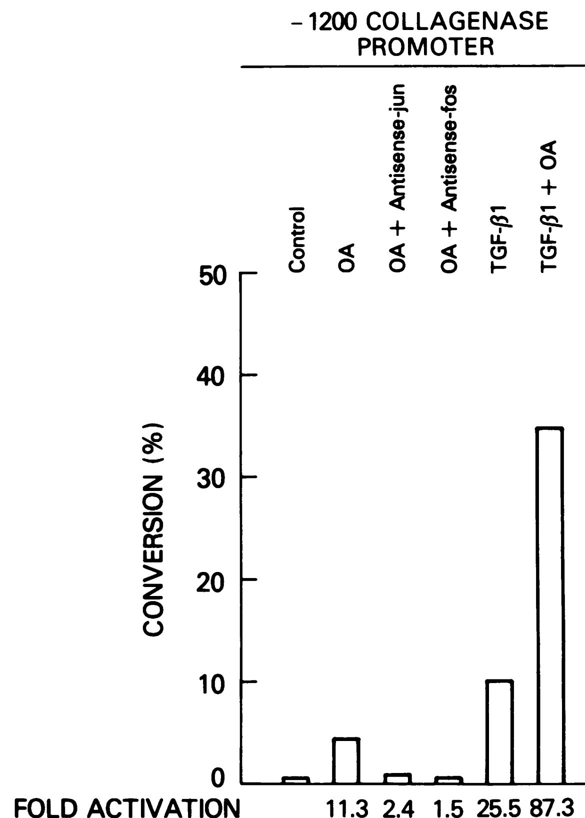

Figure 4. **Inhibition of okadaic acid induction by antisense-jun or antisense-fos.** One microgram of the plasmid −1200/+63-CAT was transfected into A-549 cells with 2 μg of either RSV-antisense-jun or antisense-fos under control of the metal-inducible mouse MT-IIA promoter. Twenty-four hours after the transfection, the cells were either treated with okadaic acid (50 ng/ml) or left untreated. The cells cotransfected with the fos construct were treated with 5 μM Cd^{2+} for 24 h to induce expression of antisense-fos. Twenty-four hours after the okadaic acid treatment, the cells were harvested, and CAT enzyme activity was determined. For the TGF-β1 treatment, cells were treated with TGF-β1 (5 ng/ml) alone or in combination with okadaic acid 24 h after transfection.

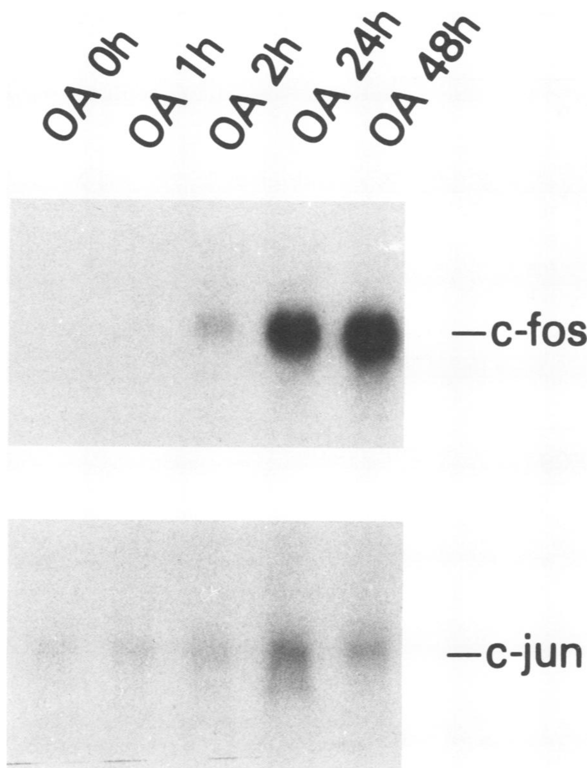

Figure 5. **Kinetics of c-fos and c-jun expression in A-549 cells after treatment with okadaic acid.** Total RNA was isolated from cells following 1, 2, 24, or 48 h treatment in serum-free medium containing 50 ng/ml okadaic acid. RNA was hybridized to radiolabeled c-*jun* or c-*fos* probes.

Because transforming growth factor-β1 (TGF-β1) also has been shown to act through the AP-1 site in the collagenase promoter (Kim *et al.*, 1990), we investigated whether okadaic acid might synergize with TGF-β1 to induce collagenase promoter-CAT expression. A-549 cells transfected with the plasmid −1200/+63-CAT were treated with TGF-β1. As shown in Figure 4, TGF-β1 stimulated CAT gene expression significantly and synergized with okadaic acid; this suggests that even though both of these factors are acting through AP-1 sites, they may use different activating mechanisms.

Regulation of c-fos and c-jun expression by okadaic acid in A-549 cells and human synoviocytes

To explore how okadaic acid might regulate TRE-mediated gene expression, we examined its effects on the transcriptional regulation of both c-*fos* and c-*jun* genes, the protein products of which are the components of the AP-1 complex. Incubation of A-549 cells with okadaic acid resulted in prolonged induction of c-*fos* mRNA, first detectable at 24 h and lasting at least 48 h (Figure 5). The stimulatory effect of okadaic acid on *jun* transcription was much weaker; maximum levels (two- to threefold) of induction are observed within 24 h in these cells. In contrast, okadaic acid treatment results in a more transient increase in the c-*fos* mRNA level in human synoviocytes. One hour after stimulation of synoviocytes with okadaic acid, c-*fos* mRNA levels were markedly stimulated (Figure 6). c-*fos* mRNA levels returned to baseline within 2 h. Interestingly, after 30 h, the c-*fos* mRNA level was increased again. Similar to our results in A-549 cells, okadaic acid had a minimal effect on c-*jun* gene expression (data not shown).

Parallel to their effects on stimulation of collagenase gene expression (Figure 1), IL-1 and okadaic acid also acted additively to stimulate c-*fos* mRNA expression. When the same blot was probed with the collagenase cDNA probe, we could detect a marked induction of collagenase mRNA 6 h after treatment with okadaic acid and IL-1 together. Stimulation of collagenase mRNA expression by okadaic acid alone was detectable only after longer treatment of

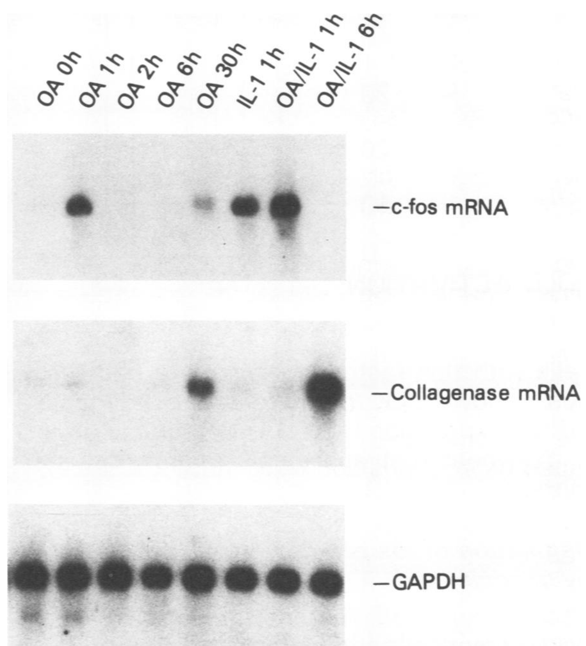

Figure 6. **Induction of fos mRNA by okadaic acid in human synoviocytes.** Confluent fourth-passage human synoviocytes were placed in serum-free media for 16 h and then treated with okadaic acid (30 ng/ml) for 1, 2, 6, or 30 h. The cells were also treated with IL-1 either alone or together with okadaic acid for the predetermined time period. After treatment, RNA was isolated, blotted onto Nytran paper, and hybridized to c-*fos*, human collagenase, or glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probes.

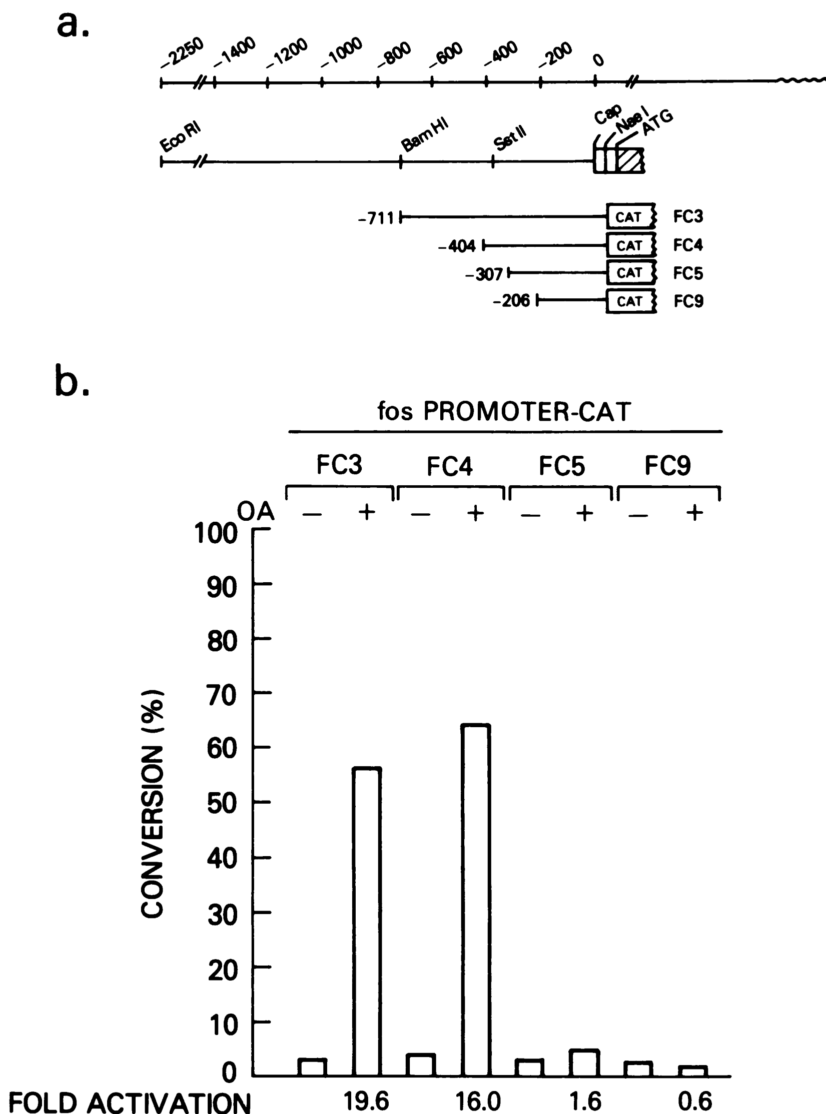

Figure 7. **Identification of the regions of the c-fos promoter region required for okadaic acid activation.** a, the schematic diagram of the human c-fos gene. FC3, FC4, FC5, and FC9 (Fujii *et al.,* 1988; Sassone-Corsi *et al.,* 1988) are fos-CAT fusions in which c-fos promoter deletions from positions −711, −404, −307, and −206 to the +42 (site Nae I) were linked to CAT sequences. b, A-549 cells were transfected with the indicated construct (10 μg of plasmid). Twenty-four hours after transfection, the cells were incubated for 20 h in the absence (−) or presence (+) of 50 ng/ml of okadaic acid.

cells with this factor; however, we had previously shown that the increase of collagenase mRNA expression by IL-1 is detectable after 3 h treatment (Lafyatis *et al.,* manuscript submitted).

Regulation of fos promoter-CAT expression by okadaic acid

Because we had demonstrated that okadaic acid dramatically stimulates expression of c-*fos* mRNA in both synoviocytes and A-549 cells (Figures 5 and 6), we also examined its effects on the regulatory region of the 5′ flanking region of the c-*fos* gene. To determine the regions that are responsive to okadaic acid, we tested a series of 5′ deletion mutants of the c-*fos* gene transfected into A-549 cells. The regions contained in these plasmids are shown in Figure 7a. Increased CAT activities were observed when plasmids FC3 and FC4 were transfected and treated with okadaic acid, but little or no stimulation was observed with constructs FC5 and FC9 (Figure 7b). Thus, the sequences responsive to induction by okadaic acid are located between −404 and −307 (Figure 7, a and b). This region contains multiple regulatory elements, including a dyad symmetry element, a v-*sis*-conditioned medium responsive element, and an AP-1 binding site (Sassone-Corsi *et al.,* 1988). To determine whether the AP-1 site is involved, we cotransfected the FC3 construct with antisense *fos* or antisense *jun* constructs and treated with okadaic acid. In contrast to the effects of these constructs on okadaic acid-stimulated collagenase promoter-CAT expression, neither antisense *jun* or antisense *fos*

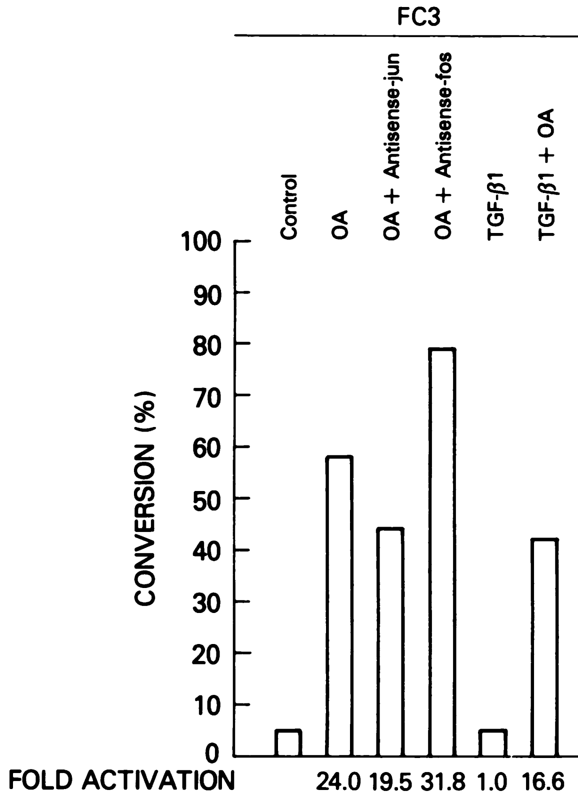

Figure 8. **Effect of antisense-jun or antisense-fos on okadaic acid–stimulated expression of the FC3 fos promoter/CAT plasmid.** The plasmid, FC3, was transfected into A-549 cells with 2 μg of either antisense-jun or antisense-fos as described in legend of Figure 4. For the TGF-β1 treatment, cells were treated with TGF-β1 (5 ng/ml) alone or in combination with okadaic acid 24 h after transfection. Twenty-four hours after the okadaic acid treatment, the cells were harvested and CAT enzyme activity was determined.

blocked the stimulation by okadaic acid of the *fos* constructs (Figure 8). On the other hand, cotransfection of antisense *fos* actually increased the CAT activity of FC3; this may be due to the prevention of the autorepression of c-*fos* expression by its own product (Sassone-Corsi *et al.*, 1988; Schönthal *et al.*, 1989). These results suggest that one of the other sequence elements flanking the gene, and not the AP-1 binding site, is involved in the okadaic acid stimulation of c-*fos* expression. The lack of effect of TGF-β1 on c-*fos* expression further supports the different mechanisms of action of TGF-β1 and okadaic acid.

Discussion

In the present studies, we have demonstrated that okadaic acid, an inhibitor of protein phosphatases, regulates expression of the collagenase gene through AP-1 complex binding sites and expression of the c-*fos* gene through distinct sites in the multiple regulatory region. Cotransfection with antisense-*jun* or antisense-*fos* abolished the okadaic acid effects on collagenase promoter-CAT expression, clearly indicating that the major components of the AP-1 complex are responsible for the induction by okadaic acid. In contrast, activation of the c-*fos* promoter by okadaic acid requires sequences located between positions −404 and −307 that are insensitive to antisense *jun* or *fos;* this suggests that other regulatory elements in this region, including the serum responsive element (SRE) (Treisman, 1986), a v-*sis*-conditioned medium responsive element (Hayes *et al.*, 1987), or a cyclic-AMP-like responsive element, might be involved.

The nuclear protooncogenes c-*fos* and c-jun encode nuclear phosphoproteins (Fos and Jun), which play a pivotal role in the transduction of extracellular and intracellular signals into changes in gene expression. Analysis of Fos and Jun proteins has showed that these proteins contain multiple phosphorylation sites (Barber and Verma, 1987; Boyle *et al.*, in preparation). It remains to be determined whether okadaic acid exerts its effects through modulation of cFos or cJun phosphorylation. However, the present results suggest that the primary target for okadaic action is another protein interacting with upstream region of the c-*fos* promoter.

Induction of human c-*fos* transcription by serum growth factors is mediated through the SRE (Gilman *et al.*, 1986; Treisman, 1986; Gilman, 1988), which contains the dyad symmetry element, a binding site for the serum response factors (Gilman *et al.*, 1986; Prywes and Roeder, 1986; Treisman, 1986). The SRE is a multifunctional element that is the target of at least two different signal transduction pathways that activate c-*fos* transcription (Gilman, 1988). Of particular interest is the possibility that the SRE may also be the target for repression of c-*fos* transcription following induction with serum (Greenberg *et al.*, 1986; Sassone-Corsi *et al.*, 1988; Schönthal *et al.*, 1989). Microinjection of oligonucleotides representing the SRE into serum-depleted cells blocks c-*fos* induction by serum (Gilman *et al.*, 1988), showing clearly that a positively acting factor must also bind to the SRE. A detailed examination of the SRE-protein interaction has demonstrated that two distinct cellular proteins (p67 and p62) (Schroter *et al.*, 1987; Treisman, 1987; Ryan *et al.*, 1989; Shaw *et al.*, 1989a) bind to the c-*fos* SRE, suggesting that a ternary complex between $p67^{SRF}$, the SRE, and p62 is functionally involved in the induction of c-*fos* expression by serum (Shaw *et*

al., 1989a,b). Recently, it has been demonstrated that $p67^{SRF}$ is phosphorylated in vivo on serine residues, and that phosphatase treatment of this factor in vitro abolishes its DNA-binding activity (Prywes *et al.,* 1988). Moreover, it has also been demonstrated that p62 and p67 are phosphorylated in vivo (Ryan *et al.,* 1989). These results suggest that okadaic acid may activate the c-*fos* promoter by inhibiting the activities of the serine-specific protein phosphatases, and thereby stabilizing the active, phosphorylated form of the serum responsive factor.

Okadaic acid has recently been demonstrated to regulate expression of human immunodeficiency virus (HIV) LTR-CAT through the NF-kB binding site. NF-kB binding was inducible in Jurkat cells by okadaic acid treatment (Thevenin *et al.,* manuscript submitted). These results further support the suggestion that phosphorylation might be involved in the transcriptional regulation of a variety of genes.

Methods

Cell culture

Human synoviocytes were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10% human serum as described previously (Lafyatis *et al.,* 1989). Human lung adenocarcinoma (A-549) cells were maintained in Dulbecco's modified Eagle's medium with high glucose supplemented with 5% fetal bovine serum.

RNA isolation and blotting

A-549 cells or fourth-passage synoviocytes were grown to confluence in 175-cm^2 flasks; washed with phosphate-buffered saline; and placed in Dulbecco's modified Eagle's medium plus penicillin, streptomycin, glutamine, and bovine serum albumin, 1 mg/ml. After 24 h, this medium was replaced and additions of okadaic acid, TPA, or TGF-β1 made as described in the text. Following stimulation, RNA was isolated as described (Chomczynski and Sacchi, 1987). Ten micrograms of each sample of total RNA was run on a 1.0% agarose/1.1 M formaldehyde gel and blotted onto a Nytran membrane (Schleicher and Schuell). Prehybridization, hybridization, and washing of the filters was as described (Church and Gilbert, 1984). Human collagenase, c-*jun*, c-*fos*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes were prepared by random primed labeling of excised insert cDNAs (Fort *et al.,* 1985; Angel *et al.,* 1987a,b).

Cell transfection and CAT assays

Transfections were performed by the $CaPO_4$ precipitation method (Luse and Roeder, 1980). Following incubation with the $CaPO_4$-DNA coprecipitates, the cells were incubated in media containing 0.5% fetal bovine serum; okadaic acid, TPA, or TGF-β1 were added as described in the text. After 48 h, cells were harvested, and extracts were assayed for CAT activity according to the method of Gorman *et al.* (1983). The collagenase 5′ flanking region/CAT plasmids −1.2 K/+63-CAT, −0.5 K/+63-CAT, −73/+63-CAT, and −60/+63-CAT used in these experiments were previously described (Angel *et al.,* 1987a). The *fos*-CAT constructs FC3, FC4, FC5, and FC9 were kindly provided by Dr. Masaharu Fujii (Kanazawa Cancer Center, Japan). The antisense *jun* and antisense *fos* constructs were obtained from Dr. R. Chiu (University of California, San Diego) and Dr. J. T. Holt (Vanderbilt University), respectively. Transfection frequencies were monitored by cotransfection with 1 μg of pSVGH, a growth hormone expression vector.

Ribonuclease protection assays

RNA probes were synthesized according to the instructions of the manufacturer (Promega). Ten micrograms of −73/+63 collagenase-CAT construct was transfected into A-549 cells, and 24 h after transfection the cells were treated with the indicated amounts of okadaic acid or okadaic acid tetramethyl ether, used as a negative control. Twenty hours later, the cells were analyzed for the level of correctly initiated CAT transcript by RNAase protection. The size of the CAT riboprobe was 480 nucleotides, whereas the size of the fragment protected from RNAase digestion is 256 nucleotides (Angel *et al.,* 1987b).

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