The AP-1 site is required for basal expression but is not necessary for TPA-response of the human stromelysin gene

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ABSTRACT

We have studied the activity of the AP-1 site, a target for the Fos and Jun family of transcription factors, in the context of the human stromelysin promoter (–1303 to +4). In transiently transfected human HepG2, HeLa and fibroblast cell cultures, point-mutations in any position of the stromelysin AP-1 sequence TGAGTCA (–70 to –64) reduced both the basal level and TPA-induced expression from the stromelysin promoter. TPA-induction fold of the mutant promoters, however, was comparable to that of the wild-type promoter. Similarly, antisense c-Fos mRNA expression reduced basal activity but had no significant effect on the relative TPA-response of the stromelysin promoter. Further, in mouse F9 cells cotransfected with c-Fos and c-Jun expression plasmids, the transfected wild-type stromelysin promoter activity was increased 57-fold whereas no transactivation was detected for an AP-1 mutant stromelysin promoter. In gelshift assays, stromelysin promoter fragments (–101 to –11), containing the mutated AP-1 site, all failed to bind or compete for the *in vitro* synthesized Fos and Jun proteins. We interpret these data to suggest that the Fos and Jun proteins, or similar activity, and the AP-1 site are required for the basal level expression of the human stromelysin gene. Strikingly, these data also suggest that the stromelysin AP-1 site is not necessary for the TPA-response.

INTRODUCTION

Phorbol esters such as 12-O-Tetradecanoyl-Phorbol-13-Acetate (TPA) have pleiotropic effects on cells and gene expression (1, for review). These effects are initiated and sustained by the transient stimulation of protein kinase C enzymes (2). Studies on the regulation of gene expression by TPA have recently identified a number of transcription factors and nucleotide sequences that are involved in TPA responses, including factors and DNA-binding sites for AP-1 and NF- κ B. The AP-1 (Activator Protein-1) binding site is a highly conserved enhancer-like element present in many viral and cellular genes that is recognized by the Fos and Jun family of oncoproteins (3–7).

Of interest is that the effects on gene regulation of many growth factors and cytokines including interleukin-1 (8) and tumor necrosis factor (9), are thought to be mediated through the AP-1 site by the Fos and Jun proteins.

The sequence-specific DNA binding activity of the Fos and Jun proteins requires dimerization. Unlike the Fos proteins, Jun proteins (c-Jun, JunB and JunD) can form homo- and heterodimers with an equal affinity to the AP-1 site. Each member of the Jun family also forms heterodimers with the Fos protein leading to enhanced binding to the AP-1 site in DNA (10–14). Of interest is that JunB does not activate target genes regulated through the AP-1 site but rather is involved in the repression of c-Jun mediated gene activation (15, 16).

Several studies have examined the AP-1 sequence requirements for the binding of the Fos-Jun heterodimer (10–14, 17). In summary, mutations that can prevent Fos-Jun binding to the AP-1 site can also inactivate or reduce its effect on the basal and TPA-induced promoter activity. Recently, Risse *et al.* (18) made all possible point mutations in the AP-1 consensus TGAGTCA sequence and demonstrated that, in contrast to the TCA half sequence, many mutations in the TGAG portion of the sequence showed normal or significant binding of the Fos and Jun proteins. These results suggest that the palindromic AP-1 site is asymmetrical for Fos and Jun binding (18).

Collagenase and stromelysin are two major proteolytic enzymes that degrade components of the connective tissue matrix (19). Their controlled expression represents an important cellular response of the immune and inflammatory systems and is vital for tissue remodeling during development, growth, wound repair and inflammatory reactions. Of importance is that many cytokines including interleukin-1 and tumor necrosis factor can stimulate collagenase and stromelysin transcription and, conversely, retinoid and glucocorticoid hormones can repress their transcription (20–22, unpublished). Recently, several papers reported that the AP-1 site has a key role in the induction and repression of transcription from the collagenase promoter (23–25).

We are studying the role and interactions of the AP-1 site in an authentic promoter context, the human stromelysin promoter (–1303 to +4). By mutational analysis and transient transfection of cultured cells we demonstrate here that the stromelysin AP-1 site and the c-Fos and c-Jun proteins, or similar activity, are

necessary for the basal level gene expression. In contrast to these results, the AP-1 site mutants or expression of antisense c-Fos mRNA had no significant effect on the relative TPA-induced stromelysin promoter activity. Interestingly, many promoter mutants showed increased relative TPA-response as compared to that of the wild-type stromelysin promoter.

MATERIALS AND METHODS

Cell culture

Hela (ATCC CCL2), HepG2 (ATCC HB8065) and primary foreskin fibroblasts (21) were maintained and subcultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS), streptomycin (100 ug/ml) and penicillin (100 units/ml). F9 (ATCC CRL1720) cells were grown on gelatin-treated dishes in DMEM containing 10% FCS and antibiotics.

Transient transfection and reporter gene assay

We use the human growth hormone as a reporter gene to study the control of expression from the linked human stromelysin promoter. Because the reporter gene product, growth hormone, is secreted into the cell culture medium, its level of expression can be measured by a sensitive radioimmunoassay. Accordingly, we routinely use a transfection protocol for cells grown on 96-well microtiter plates (26). For transient transfection experiments, human skin fibroblasts and mouse F9 embryonal carcinoma stem cells were plated on 24 or 48-well plates and human HeLa and HepG2 cells were plated on 96-well plates. When indicated, before transfection, F9 cells were treated with retinoic and dibutyryl cyclic AMP (36) for three days to induce cellular differentiation. For the skin fibroblasts, transfections were carried out overnight using calcium phosphate as described (21); all other cells were transfected overnight with the cell transfect kit (Pharmacia). Cultures were then rinsed twice and further incubated in DMEM containing 10% FCS plus antibiotics for two days after which the culture medium was removed and assayed for secreted growth hormone using a solid phase radioimmunoassay kit (Nichols Institute). Experiments were carried out in triplicate or quadruplicate wells for given samples using two different plasmid preparations and repeated at least three times for each cell line. The amount of transfected DNA was adjusted according to the well-surface so that 50 ng per well was routinely used on 96-well plates. No carrier DNA was used except for the skin fibroblasts where the transfected DNA was composed of 50% reporter and 50% calf thymus DNA. All DNA preparations were purified by centrifugation twice through a CsCl gradient.

Oligonucleotide site-directed mutagenesis

Point mutations M1-M7 of the AP-1 site (Table I), the AP-1 deletion mutant, and the insertion of unique SmaI site (+4) for the stromelysin promoter were made by site-directed mutagenesis of single stranded template essentially as described in the MutaGene kit (Bio Rad) using T4 or T7 DNA polymerase (27, 28). All mutants were isolated and characterized by oligonucleotide screening and DNA sequencing. Oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer and used without further purification. In all mutant oligonucleotides the target site was in the middle of the sequence. To construct a unique Sma I site (+4) in the human stromelysin promoter,

a plasmid derived from a SstI/partial XbaI digest of a human stromelysin gene clone (21) was cut with SstI and Hind III (which cuts in the vector polylinker) to isolate a 2.1 kb fragment containing promoter region, the first exon and first intron of the gene. This was cloned into M13mp18 vector and mutated with a 34-mer oligonucleotide that inserted four nucleotides (CCCG) after the +4 base of the human stromelysin gene (21). The mutated DNA was cut with SstI (−1303) and Sma I (+4) and, using Hind III (5') and Bam HI (3') octamer linkers, was inserted into the human growth hormone reporter gene pφGH (29) and into M13mp19 vector to construct the stromelysin promoter mutants. In mutants M1-M7, each nucleotide in the AP-1 sequence TGAGTCA was changed (see Table I) using 17-mer oligonucleotides. To construct the mutant in which the AP-1 site is absent, the AP-1 sequence was removed using a 44-mer oligonucleotide.

Plasmid constructs

The stromelysin promoter linked to chloramphenicol acetyltransferase (CAT)-gene was created by inserting the wild-type or M1 mutant fragment Sst I (−1303) to Sma I (+4) into pGCAT-C vector (34). Col-TRE1X/TK-CAT (30) is derived from pBLCAT2 (35) and contains the AP-1 site from human collagenase gene (−73 to −65) inserted upstream of the thymidine kinase (TK) gene (−105 to +51). p1XBLGH and pBLGH were derived from Col-TRE1X/TK-CAT and pBLCAT2, respectively, by EcoRI digestion and replacement of the CAT gene with human growth hormone (GH) gene. The plasmids pSVsof, expressing antisense mouse c-Fos RNA (30), pSV-c-Jun, expressing mouse c-Jun (33), pBK28, expressing human c-Fos (32) and rat cDNA clones for c-Fos and c-Jun (11) have been described.

Chloramphenicol acetyl transferase (CAT) assay

For CAT assay, 7×10^5 cells were transiently transfected in 100 mm plates with 20 ug reporter and 20 ug carrier DNA and treated as above. Aliquots of cell extract containing equal amounts of total protein (32 ug) were assayed in a cocktail containing 400 nCi ^{14}C -chloramphenicol, 5.3 mM acetyl CoA and 250 mM Tris-Cl, pH 7.5. After an incubation of 45 minutes at 37°C, the entire reaction mixture was extracted with ethyl acetate and analyzed by thin layer chromatography (33).

RNase protection assay

The fragment of the stromelysin promoter-growth hormone reporter gene construct extending from −70 to the end of the first exon of growth hormone gene (see Fig. 2) was amplified by polymerase chain reaction and cloned into sp73 vector (Promega). The construct was characterized by nucleotide

WT	TGAGTCA
M1	@GAGTCA
M2	T ⁺ TAGTCA
M3	TG@GTCA
M4	TGAT ⁺ TCA
M5	TGAG@CA
M6	TGAGT ⁺ AA
M7	TGAGTCC

Table I. AP-1 site mutations. M1-M7 refer to the point mutations (highlighted) in the wild-type (WT) stromelysin AP-1 site (−70 to −64).

sequencing. Labeled antisense RNA was generated using T7 RNA polymerase and ^{32}P -UTP (800 Ci/mmol, NEN) according to the protocol provided by Pharmacia. The labeled RNA (approx. 2×10^5 counts) was then hybridized with 20 ug of total RNA from transiently transfected HepG2 cells and treated with RNase A and T1 as described (33). The reaction products were analyzed on 8% acrylamide-7M urea sequencing gel.

In vitro synthesis of Fos and Jun

Rat cDNAs for c-Fos and c-Jun were linearized with Bam HI in the polylinker site and transcribed *in vitro* using SP6 polymerase and m7 'GpppG' as recommended by the supplier

(Promega). Synthesis of the mRNA was monitored by agarose gel. *In vitro* translation of the Fos and Jun mRNAs (3 μg) was carried out in nuclease treated rabbit reticulocyte lysate (Promega) using ^{35}S -methionine. The translation products were characterized and assayed by SDS-gel electrophoresis and by TCA precipitation.

Gelshift

The AvaII-AvaI (−101 to −11) fragment of stromelysin promoter was isolated by agarose gel electrophoresis and end-labeled using Klenow DNA polymerase and ^{32}P dCTP. The binding reaction mixture (20 μl) contained 0.4 ng of probe (8000 c.p.m.), 1–3 μl of the *in vitro* synthesized Fos and Jun without preincubation and 17 μl of binding reaction buffer (Stratagene). In competing reactions double-stranded 22-mer oligonucleotides containing the SP-1 and AP-1 sites (Stratagene) were used. For greater resolution, protein-DNA complexes were resolved on 6% polyacrylamide gel in 1 $\times$ gelshift buffer (Stratagene) at 4°C and constant current of 25 mA. Autoradiography was performed on dried gels using an extra film to quench radioactivity arising from the ^{35}S -labelled Fos and Jun proteins.

Fig. 1. Basal level and TPA-induced stromelysin promoter activity. Human stromelysin promoter (−1303 to +4), the AP-1 site mutants M1-M7 and the promoter lacking the AP-1 site (Δ) were inserted into the human growth hormone (hGH) reporter gene and transiently transfected into HepG2, fibroblast and HeLa cells. One set of cultures was treated with TPA (100 ng/ml) for two days. The superimposed bars show the activity of the wild-type (wt) and mutant (M1-M7 and Δ) stromelysin promoters for the basal level (filled bars) or TPA-induced expression (open bars). Numbers refer to the fold-increase in the promoter activity induced by TPA. The promoter activity is expressed as c.p.m. of secreted growth hormone as determined by radioimmunoassay. 10,000 c.p.m. corresponds to 5 ng/ml of hGH in the medium. In HeLa transfections we noted large variation in the TPA-response (up to 77-fold, see Table II). However, these circumstances do not affect the suggestions and conclusions presented in this paper. The level of expression of the promoterless growth hormone gene (pGH), indicated by the left hand bar, has not been subtracted from the experimental values. Note that in many cases this activity exceeds the basal level expression from the mutant promoters. Further, this activity is increased in TPA treated cells, presumably due to increased random transcription of the promoterless growth hormone gene (see text). Due to different transfection efficiencies of the cells, promoter activities are not comparable between the different cell types. Data are from one representative set of experiments.

Fig. 2. RNase protection assay. Twenty micrograms of cytoplasmic RNA isolated from transfected HepG2 cells, was hybridized with antisense RNA probe (2×10^5 cpm) and digested as described in Materials and Methods. Cells on 15 cm plates were transiently transfected with 20 ug of promoterless growth hormone gene pGH (lane 3) or with stromelysin M1 mutant promoter linked to the reporter gene of human growth hormone (lanes 4 and 5). The size of the protected fragment is illustrated on top of the figure and shown by the arrow. The 216 nt RNA probe from T7 transcription covering the first exon of growth hormone gene is also illustrated and shown in lane 2. The dark spot halfway on the lane is an artifact of the autoradiogram. The size of the RNA markers is shown in lane 1. '−' and '+' refer to TPA treatment (100 ng/ml) of the transfected cells for 18 hrs.

RESULTS

The AP-1 site is required for basal level promoter activity but it is not necessary for TPA-induction

To study the transcriptional role of the AP-1 site in an authentic promoter context, the TGAGTCA sequence (−70 to −64) of the human stromelysin promoter (21) was mutated in all seven positions to generate the point mutants M1-M7 (Table 1). These mutant promoters (−1303 to +4) were then inserted into the reporter gene of human growth hormone, p ϕ GH (29). Using transiently transfected human HepG2, HeLa and fibroblast cell cultures, we then assayed their basal level and TPA-induced activity. Fig. 1 represents results from a typical set of experiments and shows that in all cell lines tested the basal level expression of the mutant promoters was reduced in comparison with the wild-type activity. This result was most pronounced in HepG2 cells where the basal level activity of all the mutant promoters was only 5%-10% of the wild-type activity. In fibroblasts and HeLa cells, depending on the AP-1 site mutation, the mutant promoters showed about 10–50% of the wild-type expression.

Treatment of the transiently transfected cells with the phorbol ester TPA increased the wild-type stromelysin promoter activity about 2 to 5 fold depending on the cell line used. Interestingly, the TPA-response of the mutant promoters appeared to be complex and cell type-specific. In comparison with the wild-type promoter, all the mutant promoters showed an absolute reduction in their response to TPA treatment. However, taking into account the lower basal level expression of the mutant promoters, the fold of TPA-induction (relative TPA-response) was generally not reduced (Fig. 1). In fact, the relative TPA-response for many of the mutant promoters exceeded that of the wild-type promoter.

Fig. 3. Gelshift with Fos and Jun. The 90-bp stromelysin promoter fragment was incubated with *in vitro* synthesized Fos and Jun proteins and the binding was assayed by gelshift. Left side of figure: Reactions with Jun, Fos, Fos + Jun or reticulocyte lysate alone (RL) demonstrate that both Fos and Jun are needed for the complex formation (denoted by FJ). Right side of figure: Reaction with or without competing 22-mer oligonucleotides (250 $\times$ molar excess) containing the AP-1 or the SP-1 site show that Fos and Jun complex recognized the AP-1 site in the promoter fragment (sequence is shown on the bottom of Fig. 3).

For example, in HepG2 cells and fibroblasts, the promoter activity of the mutants M6 and M7 was highly inducible by TPA compared to that of the wild-type promoter. In contrast, the stromelysin promoter mutant M5 showed little or no TPA-response in any of the cell lines tested. These results were consistently seen in three separate transfection experiments done in triplicate or quadruplicate samples and with two different preparations of DNA. Finally, the data shown in Fig. 1 demonstrate that only in HeLa cells, all the AP-1 site mutations reduced both the basal expression and relative TPA-response of the stromelysin promoter.

We have shown elsewhere (26) that when measured from the same transiently transfected HepG2 cell culture, the growth hormone gene expression as measured by radioimmunoassay and by RNase protection assay (using total cellular RNA) give compatible data for the stromelysin promoter activity and reflects correctly initiated transcripts from the transfected promoter. As shown in Fig. 2, using HepG2 cells transfected with the M1 stromelysin promoter mutant, RNase protection assay of the untreated cells produced a distinct band of 79 nucleotides in length. The intensity of this protected band was significantly increased in cells treated with TPA. These data clearly demonstrate that the growth hormone gene expression from the linked stromelysin mutant promoter, albeit low, represents real and correctly initiated transcripts in the transfected cells. In similar experiments using HepG2 cells transfected with the promoterless growth hormone reporter gene we consistently saw a very faint RNase protected signal corresponding to the size of the first exon of the growth hormone gene (not visible in Fig. 2). This result, presumably due to a random initiation of transcription, could explain the low level of growth hormone expression of the cells transfected with the promoterless reporter gene (Fig. 1).

Fig. 4. Stromelysin promoter mutants do not bind Fos and Jun. The mutated 90-bp promoter fragments are tested here for their binding activity to Fos and Jun. Panel A shows that none of the 32 P labeled fragments (WT to M7) at the same specific activity (20,000 cpm/ng) bind to Fos and Jun complex except for the wild-type (WT) indicated by the arrow. The faint gelshift band that comigrates with the Fos-Jun complex, and the low molecular weight bands seen with some of the mutants, represent non-specific endogenous binding proteins from reticulocyte lysate. These complexes could not be competed with the AP-1 oligonucleotide nor were they consistently seen with different lots of reticulocyte lysate. Panel B shows that none of the AP-1 mutant fragments can compete for Fos and Jun complex when they are used as competitors (50 $\times$ molar excess) with 32 P WT fragment. In contrast, efficient competition is seen with the wild-type (WT) fragment.

We interpret the above results to suggest that, in the human stromelysin promoter context (−1303 to +4), the AP-1 site is required for basal level expression but, apparently, is not essential in the mechanism of TPA-induction. In order to study this notion further, we removed the AP-1 site (TGAGTCA) from the stromelysin promoter by oligonucleotide site-directed mutagenesis and studied the expression and TPA-response of such an AP-1-less promoter in transiently transfected cells in culture. As shown in Fig. 1, the activity of this mutant promoter was similar to that of the M1-M7 mutants in all cell lines tested. Although its basal level expression was reduced, its relative TPA-response in HepG2 cells and fibroblasts was about the same or higher, respectively, than that of the wild-type stromelysin promoter. Again, only in HeLa cells was the TPA-inducibility of this promoter mutant reduced. These results clearly indicate that TPA can stimulate human stromelysin gene expression by a mechanism independent of the AP-1 site.

DNA binding of the Fos and Jun proteins

In order to examine the interaction of Fos and Jun proteins with the wild-type and mutant stromelysin promoters, we assayed by gelshift the binding of *in vitro* synthesized Fos and Jun¹ proteins to a 90-bp *Ava*II-*Ava*I restriction fragment (−101 to −11) containing the AP-1 site. Results of these experiments are shown in Figs. 3 and 4. In line with several earlier reports (10–14), both the Fos and Jun proteins (Fos-Jun heterodimer) were required for efficient binding to the AP-1 site (Fig. 3). This binding apparently is specific because it could be competed with a 22-bp double stranded oligonucleotide containing the AP-1 site but not with a similar oligonucleotide containing the SP-1 site (Fig. 3). In order to test whether or not the mutant promoter fragments can bind the Fos-Jun complex, we used two different approaches. First, in the binding assay (Fig. 4A) we used the wild type and mutant fragments, labeled to the same specific activity, to assay for their binding to the Fos and Jun protein complex. As shown, none of the AP-1 mutant fragments could bind to the *in vitro* synthesized Fos-Jun heterodimer whereas effective binding was observed with the wild-type promoter fragment. Second, in the competing binding assay (Fig. 4B) we used the mutant promoter fragments (50× molar excess) to measure their capacity to inhibit the Fos and Jun complex formation with the wild-type fragment. This assay indicated that, in comparison with the wild-type fragment, all seven fragments from M1-M7 mutant promoters showed little or no competition for the Fos-Jun heterodimer complex. These data, taken together with the results of the above transient gene expression studies, suggest that the low basal level activity of the stromelysin promoter mutants is due to the lack of Fos-Jun binding to the mutated AP-1 site.

Antisense Fos RNA expression

To further study the involvement of the Fos and Jun proteins in the basal level or TPA-induced expression of the stromelysin promoter, the wild-type promoter was cotransfected into HepG2, HeLa and fibroblast cells with control plasmid or a plasmid expressing antisense c-Fos mRNA. As shown in Table II, in the presence of antisense Fos mRNA, basal stromelysin promoter expression was reduced to 67% (HepG2), 57% (HeLa) and 56% (fibroblasts) of the control activity. Strikingly, however, in

HepG2 cells and fibroblasts the relative TPA-response of the stromelysin promoter was not affected by antisense Fos expression. Of interest and consistent with the other data above is that only in HeLa cells was the relative TPA-response reduced. These results, therefore, provide independent support for the view that c-Fos protein and, by implication the AP-1 site, is involved in the basal level expression but is not essential for the mechanism of TPA-induced promoter activity.

Transactivation by the Fos and Jun proteins

If the AP-1 site and the Fos and Jun proteins are required for the basal level activity of the stromelysin promoter, then overexpression of the Fos and Jun proteins should transactivate the wild-type promoter but not the AP-1 site mutant promoters.

Table II. Antisense Fos mRNA inhibits the basal level but not TPA-induced stromelysin promoter activity. Cells were transiently transfected with the growth hormone reporter gene containing human stromelysin promoter (−1303 to +4) together with the plasmid KSV10 (control) or a plasmid expressing antisense mouse c-Fos mRNA (sof). One set of cultures was treated with TPA (100 ng/ml) for two days before assaying promoter activity. Numbers on the left hand column indicate the decrease of promoter activity in comparison with the control basal level (normalized to value 1) upon transfection of antisense Fos (sof). Numbers on the right hand columns refer to the promoter activity and fold of induction after TPA treatment. Data are from one representative experiment and an average of four separate transfections.

	Plasmid	-	TPA +	Induction
HepG2	Control	1	14	14
	Sof	0.67	10	15
HeLa	Control	1	77	77
	Sof	0.57	18	32
Fibroblast	Control	1	3.4	3.4
	Sof	0.56	2.0	3.6

Fig. 5. Fos and Jun transactivate the stromelysin promoter in F9 cells. Wild-type or the M1 mutant stromelysin promoters in the growth hormone reporter gene were cotransfected into F9 embryonal carcinoma stem cells with equal amounts (0.5 µg/well) of KSV10 control plasmid (−) or with plasmids pSV-C-Jun and pBK-28 expressing mouse c-Jun and human c-Fos proteins, (+FJ), respectively. The activity of promoterless growth hormone gene (pOGH) has not been subtracted from experimental values. Numbers refer to the fold of transactivation by Fos and Jun.

¹ In all of the experiments reported in this paper we used c-Fos and c-Jun.

Fig. 6. Transcriptional activation of stromelysin gene in F9 cells. Undifferentiated and differentiated F9 cells were transfected with WT stromelysin promoter (filled bars) and cotransfected with c-Jun and c-Fos expression vectors (open bars). One set of each culture was treated with TPA for two days after transfection. The induction-fold following TPA treatment is indicated in the text. The transactivation fold in different experiments was between 20 and 100-fold. A table showing TPA-response and differential mRNA expression for Fos and Jun proteins in differentiated and undifferentiated F9 cells is based on the Northern hybridization data of Yang-Yen *et al.* (36). The three levels of expression are low (+), medium (++) and high (+++).

Accordingly, the wild-type stromelysin promoter was transfected into mouse F9 embryonal carcinoma stem cells together with a control plasmid or plasmids expressing human Fos and mouse Jun proteins. As demonstrated previously, undifferentiated F9 cells have no endogenous c-Fos or c-Jun proteins (15,16,36). As shown in Fig. 5, cotransfection of the Fos and Jun expression plasmids resulted in a dramatic increase (57-fold) in the basal level stromelysin promoter activity. In contrast, and consistent with our other data reported here, Fos and Jun could not transactivate the stromelysin promoter mutant M1.

To study further the role of Fos and Jun in the stromelysin gene regulation, we carried out transfection experiments in differentiated and undifferentiated F9 cells in the presence or absence of TPA. As shown in Fig. 6, very little stromelysin expression is detected in both cell types and, significantly, the promoter response to TPA-treatment appears minimal. In undifferentiated F9 cells, TPA induces the stromelysin expression 1.9 fold and in differentiated F9 cells the induction is 1.4 fold. Again, a striking transactivation of the stromelysin promoter occurs in the presence of cotransfected c-Fos and c-Jun expression plasmids. The effect of TPA in these experiments is intriguing. Taking into account the minimal TPA-response of the stromelysin promoter alone, TPA seems to inhibit the relative transactivation by Fos and Jun. In the experiment shown in Fig. 6 this inhibition is about 50% in both types of F9 cells (see Discussion).

As shown in Fig. 7, the Fos and Jun proteins can also transactivate the stromelysin promoter in HepG2 cells. In these experiments, cotransfection with Fos and Jun expression plasmids resulted in a consistent 3-fold increase in the basal level promoter

Fig. 7. Fos and Jun transactivate the stromelysin promoter in HepG2 cells. HepG2 cells were cotransfected with the wild-type stromelysin promoter and either the control plasmid or the Fos and Jun expression plasmids (+FJ) as in Fig. 5. One set of cultures was treated (+) with TPA (100 ng/ml) for two days before assaying the promoter activity. Numbers refer to the induction fold by TPA and the fold of transactivation by Fos and Jun. The activity of promoterless reporter gene (p0GH) has not been subtracted from experimental values.

Fig. 8. TPA-induced CAT expression from wild-type and mutant stromelysin promoters. Primary human skin fibroblasts were transiently transfected with pGCAT-C (promoterless CAT reporter gene), WTCAT (CAT gene with wild-type stromelysin promoter) or M1CAT (CAT gene containing promoter with mutant AP-1 site) and treated with TPA 24h post-transfection for an additional 48h. CAT activity and the percentage of acetylation was determined as in Materials and Methods. Induction fold was determined after subtracting from the experimental values the background % acetylation contributed by pGCAT-C alone. Basal wild-type CAT activity was assigned a value of 1 to facilitate comparisons. The value of 4.3 refers to the induction fold relative to basal wild-type activity. The value of 3.5 refers to the induction fold relative to basal mutant activity. Data are from one representative experiment.

activity. A similar increase in the promoter expression was obtained by TPA-treatment of control cells that were transfected with the stromelysin promoter alone. Interestingly, TPA treatment of the Fos and Jun transactivated HepG2 cells neither inhibited nor enhanced the basal level stromelysin promoter activity.

Reporter gene context

We were concerned about the possibility that the results for the stromelysin AP-1 site presented here may have reflected some 'cryptic' contribution of the hGH reporter gene used. For example, the hGH gene contains elements that can allow response to glucocorticoids in certain reporter gene constructs (29). Therefore, the wild-type and M1 mutant stromelysin promoters (-1303 to +4) were inserted into the reporter gene of chloramphenicol acetyl transferase, GCAT-C, (34). As shown

Fig. 9. The role of AP-1 in minimal TK promoter. *Panel A* shows the TPA response of the CAT reporter gene (Col-TRE1X/TK-CAT) containing minimal TK promoter with the AP-1 site from human collagenase gene (–73 to –65). pGCAT-C is promoterless CAT gene. HepG2 cells were transiently transfected with the reporter genes and cultured with or without TPA for 48 hr before CAT assay. *Panel B* shows the TPA response of the growth hormone reporter gene containing minimal TK promoter with (p1XBLGH) or without (pBLGH) the AP-1 site from human collagenase gene. p0GH is promoterless growth hormone gene. Human skin fibroblasts were transiently transfected with the reporter genes and cultured with or without TPA for 48 hr before growth hormone assay.

in Fig. 8, in transiently transfected skin fibroblasts, the relative TPA-responses of the wild-type (4.3-fold induction) and the M1 mutant (3.5-fold) promoter were comparable. We also saw these results using similarly transfected HepG2 cells (data not shown). Based on the results of these experiments we conclude that the sequence contribution of the reporter gene is insignificant. Rather, the transient gene expression in the experiments reported here is due to the transcriptional activity of the stromelysin promoter.

We have studied here the transcriptional activity of AP-1 site in the context of the human stromelysin promoter. Therefore, it was of interest to study the activity of one copy of the AP-1 site inserted into an unrelated gene promoter, the minimal promoter (–105 to +51) of the thymidine kinase (TK) gene. The AP-1 site linked to the TK promoter was used in two different reporter genes, CAT and hGH, and its role in basal expression and TPA-response was analyzed in transiently transfected skin fibroblasts and HepG2 cells in culture. As shown in Fig. 9B, the AP-1 site could only marginally stimulate basal expression from the TK promoter. Its role as a TPA response element appeared to be marginal as well. In several experiments using the reporter gene of growth hormone, one copy of the AP-1 site increased the relative TPA-response of the TK promoter about 1.4-fold. Furthermore, this result is in line with the minimal TPA-response (Fig. 9A) seen using the CAT reporter gene.

DISCUSSION

Originally, AP-1 was defined as a DNA-binding activity in HeLa cell extracts that recognized sequences in the basal level enhancer of the metallothionein IIA gene and the 72-bp enhancer of SV40-virus (4). These sequences or AP-1 sites as they are now known in many cellular and viral genes play a role in the activation or repression of gene expression (3). The AP-1 site is one of the TPA response elements (TRE) and a major target for the binding of the Fos and Jun family of transcription factors (5–7).

We have studied here the transcriptional role of the AP-1 site in the context of the human stromelysin promoter (–1303 to +4).

Our results from transient transfection experiments show that the stromelysin AP-1 site (–70 to –64) is required for basal expression of the promoter. Because the promoter with mutated AP-1 sites showed reduced transcriptional activity and did not bind or compete for *in vitro* synthesized c-Fos and c-Jun proteins (Figs. 1–4) it seems likely that the basal expression is regulated by Fos and Jun. In support of this view, we have shown here that c-Fos and c-Jun expression in transiently transfected mouse F9 cells transactivates the wild-type stromelysin promoter but not the AP-1 site mutant promoter tested. Furthermore, we speculate that if the Fos and Jun proteins are needed for basal expression of the stromelysin gene then the heterodimer of the c-Fos and c-Jun proteins should play a major role in the regulation. This notion is based on the data shown here and that reported by Yang-Yen *et al.* (36) as summarized in Fig. 6. In the F9 embryonal carcinoma stem cells only Jun D is constitutively expressed and at increased levels in differentiated F9 cells. Upon cell differentiation c-Jun becomes expressed as well. It is interesting to note that, although TPA induces the expression of c-Fos and Jun B in undifferentiated F9 cells, in no circumstances are c-Fos and c-Jun coexpressed. These data are in line with the low level of stromelysin expression in transfected F9 cells as well as with the minimal increase of promoter activity in the TPA treated F9 cells (see Fig. 6).

An intriguing result of the present study was the observation that a functional AP-1 site apparently is not necessary for the relative TPA-response of the stromelysin promoter. First of all, Fig. 1 shows that the TPA-response of the AP-1 site mutant stromelysin promoters and, strikingly, the promoter mutant lacking the AP-1 site is similar to that of the wild-type promoter in transfected fibroblasts and HepG2 cells. However, it is important to point out that in HeLa cells both the basal expression and TPA-response apparently are affected by the AP-1 site mutations. It is also intriguing that one of the AP-1 site mutations (M5) almost completely abolished the TPA-response of the stromelysin promoter in all cell lines tested (Fig. 1). Understanding these differences and exceptions in results may provide insight into the mechanism of TPA-response.

Using the terminology for strong versus weak promoters, the above results can be restated as follows. If the wild-type stromelysin promoter is considered as a strong promoter then the AP-1 site mutant promoters represent weak promoters because of their reduced basal activity in the transfected cells. As we have shown here, both of these promoter types, however, can be similarly induced by TPA treatment. Importantly, since in these cases the only difference between the strong and weak promoters is the presence or absence of a functional AP-1 site, respectively, we conclude here that the AP-1 site is not necessary for TPA-response. Results from cotransfection experiments with antisense c-Fos mRNA expression plasmid are in line with this notion. As shown in Table II, antisense c-Fos transfection reduces the basal level stromelysin expression but has little or no effect on the relative TPA-response of the stromelysin promoter. Accordingly, these results provide totally independent evidence for the idea that the Fos protein and, by implication, the AP-1 site is not necessary for TPA-induction mechanism.

We have recently identified, in the human stromelysin promoter, three additional AP-1 like sites (B. G., unpublished). In gelshift experiments, in comparison with the proximal AP-1 site, these elements display weak or moderate binding for the *in vitro* synthesized Fos-Jun heterodimer. Our preliminary results from mutational analysis and transient gene expression studies

suggest that none of these three AP-1 like elements affect the level of TPA-induced stromelysin promoter activity. However, results from similar experiments indicate that each one of them can regulate the basal promoter activity. Irrespective of their putative role in the stromelysin gene regulation we conclude from the above data that these elements for the Fos-Jun binding can not explain the TPA-response of the stromelysin promoter mutants studied here.

We also tested the effect of one copy of the AP-1 site on the heterologous thymidine kinase (TK) gene promoter. In transfected fibroblasts and HepG2 cells the AP-1 site had little or no effect on basal expression or TPA-response of the TK promoter. Furthermore, we have consistently seen these results using two different reporter genes, the CAT and hGH genes. These data which have been reported by others as well (15,30,36) suggest that the AP-1 site alone is not sufficient for transcriptional control of genes. Yet significant TPA-response and stimulation of transcription is obtained with promoter constructs containing multimerized (three or five copies) AP-1 sites (15,30,36). In respect of promoter structure and mechanism of gene regulation, these observations, however, are difficult to interpret. Presumably, the true organization of regulatory elements, such as the AP-1 site, plays an important role in gene expression comparable to that of the elements themselves. It follows that the transcriptional activity of the AP-1 site may change depending on its precise DNA sequence environment (see below).

Recently, three papers have reported data which agree with the results and conclusions presented here. First, Timmers *et al.* (38) identified an AP-1 like site in the rat JE gene that was essential for the basal expression but not for TPA-response. Mutations on the JE AP-1 site (TGACTCC -52 to -46) significantly reduced the basal activity of the promoter (-543 to +54) and interestingly, resulted in increased relative TPA-response as compared to that of the wild-type promoter. Their results also indicate that the JE AP-1 site is not sufficient for the basal level expression because minimal promoter constructs with the JE AP-1 site showed little or no basal level expression or TPA induced activity. It is unclear, however, whether the Fos and Jun proteins are involved in the activation of the JE AP-1 site because the sequence, which is similar to the M7 mutant described here, does not bind *in vitro* synthesized Fos and Jun proteins. Second, by studying the effects of antisense Fos mRNA expression in NIH 3T3 cells, Kerr *et al.* (39) suggested that the growth factors PDGF (platelet derived growth factor) and EGF (epidermal growth factor) induce rat stromelysin gene expression by Fos-dependent and Fos-independent pathways, respectively. Significantly, however, the stimulatory effect of both PDGF and EGF on stromelysin transcription seemed to involve factors recognizing the AP-1 site. In addition, their data indicated that in cells expressing antisense c-Fos mRNA the basal level activity of transfected stromelysin promoter (-751 to -1) was reduced to 65% of the control promoter activity. However, in these cells no significant effect was seen on the relative TPA-response of the stromelysin promoter. Third, as shown by van Dam *et al.* (40), the AP-1 site of the c-Jun gene is important for basal expression but does not affect the TPA-response of the gene. Similar to our results, mutational inactivation of the c-Jun AP-1 site abolished stimulation by TPA of the -79 to +170 gene construct, whereas this did not affect the TPA inducibility of the -1600 to +740 gene for c-Jun. Thus, the AP-1 site functions differently in the contexts of the full-length c-Jun promoter and of smaller promoter fragments (40).

Furthermore, we note here that the AP-1 site sequence, TTA-GTCA, of the stromelysin promoter mutant M2 is the same as that in the P motif of SV40 enhancer (41). While mutation of this sequence completely abolished the AP-1 site DNase footprint it, remarkably, had no effect either on the basal expression from the linked B-globin reporter gene or its induction by TPA. From the results of these experiments it seems that the P motif AP-1 binding site is not required for induction of SV40 enhancer activity by TPA (41). Interestingly, however, five copies of the P motif alone was as active and displayed similar transcriptional response to TPA as the complete SV40 enhancer. It is tempting to speculate that these results may be due to sequence context effects so that different or additional transcription factors are needed to distinguish the P motif when it is part of the SV40 enhancer or when it is alone. Alternatively, these data may reflect other interacting elements in the enhancer sequence or in the B-globin reporter gene used in these studies. These notions, furthermore, are consistent with the recent data on the differential effects of the adenovirus E1A oncoprotein on the transcriptional activity of the c-Jun and collagenase AP-1 sites (42). Indeed, Deutsch *et al.* (43) have presented direct evidence for the role of sequence context on the transcriptional activity of the AP-1 site. For example, different sequence contexts of only a few bases flanking the AP-1 site had pronounced effects on the TPA-response of the human gonadotropin gene in transiently transfected JEG-3 cells. Finally, Schule *et al.* (44) have demonstrated the importance of promoter architecture in the transcriptional control of genes. The results of their experiments suggest that the glucocorticoid receptor can interact with many different transcriptional factors and synergistically activate gene expression.

We also note here that the stromelysin promoter mutants M2 and M3 contain mutations in the AP-1 site (TTAGTCA and TGCGTCA, mutations underlined) identical to those prepared by Risse *et al.* (18). In their studies, in comparison with the wild-type sequence, these point mutations had little or no effect on the Fos-Jun heterodimer binding to a double stranded 17-mer oligonucleotide. In contrast, when we examined these sequences in the context of 90-bp fragment from the stromelysin promoter, we could not detect any binding or competition for the Fos-Jun heterodimer. Importantly, in transient gene expression studies these same mutants showed reduced basal level activity similar to that of the other promoter mutants studied here. At present we have no explanation for this striking variation in results but different sequence contexts may be important for the Fos-Jun binding.

The AP-1 site mutants studied here were constructed to be symmetrical in the palindromic sequence. As an example, the M3 mutant reads TGCGTCA on the top strand. Its complementary sequence on the bottom strand is TGACGCA which is similar to that of the M5 mutant top strand sequence TGAGGCA. Therefore, the M3 mutant is analogous to the M5 mutant when turned upside down. Note that in many genes the central nucleotide of the AP-1 site is either C or G. Because of these considerations we expected to see similar responses for the M3 and M5 stromelysin promoter mutants. Thus, it was intriguing to find out that the M5 mutant promoter consistently showed little or no TPA-response whereas the M3 mutant could be induced by TPA in relative terms similarly to that of the wild-type promoter (Fig.1). We interpret these data to suggest that the AP-1 site activity *in vivo* can be polarized to reflect the contribution of the sequence context of the gene.

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