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The AP-1 Sequence Is Necessary but Not Sufficient for Phorbol Induction of Collagenase in Fibroblasts†

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ABSTRACT: Collagenase, the only enzyme active at neutral pH that initiates collagen degradation, is a major gene product of fibroblasts that have been stimulated with a variety of agents, including phorbol esters. To study mechanisms controlling collagenase gene expression, we transiently transfected rabbit synovial fibroblasts with chimeric constructs containing up to 1.2 kb of the rabbit collagenase 5′-flanking DNA linked to the chloramphenicol acetyltransferase gene (CAT). Our data indicate that the magnitude of the phorbol response is directly linked to the size of the promoter fragment and that the smallest piece of promoter DNA conferring phorbol inducibility is 127 bp. Deletional and mutational analysis of this fragment revealed that the AP-1 sequence alone is insufficient for phorbol inducibility and the presence of at least two additional sequences (a PEA3-like element and a sequence that includes 5′-TTCA-3′) is required. In addition, a substantial increase in responsiveness is seen when a fragment containing 182 bp of 5′-flanking DNA is transfected, implicating a 36 bp region located between −182 and −149 as an enhancer. We conclude (1) that the AP-1 sequence is necessary but insufficient for expression of collagenase in adult fibroblasts, (2) that phorbol inducibility depends on cooperation among several sequence elements within the collagenase promoter, and (3) that regulation of this promoter is more complex than previously described.

Collagen is the body's most abundant protein and is a major component of the extracellular matrix. Collagen degradation is initiated by the enzyme collagenase (Jeffrey, 1986) and occurs in a number of normal and disease processes including wound healing, uterine resorption, tumor invasion, and arthritis. The same collagenase gene product is expressed by a variety of cell types including endothelial cells (Herron et al., 1986), keratinocytes (Lin et al., 1987), macrophages (Campbell et al., 1987), chondrocytes (Lin et al., 1988; Stephenson et al., 1987), and fibroblasts (Goldberg et al., 1986; Whitham et al., 1986; Brinckerhoff et al., 1987). Collagenase is a major gene product of fibroblasts, and, indeed, increased production of collagenase by synovial fibroblasts lining the joint is largely responsible for the extensive destruction of connective tissue seen in rheumatoid arthritis (Harris, 1985).

Previous work by a number of investigators has implicated the AP-1 sequence (also known as the TRE) in the transcriptional regulation of the human collagenase gene (Angel et al., 1987a,b; Schonthal et al., 1988; Chiu et al., 1988; Brenner et al., 1989; Conca et al., 1989; Gutman & Wasylyk, 1990). AP-1 plays an important role in the induction of collagenase gene transcription in response to agents such as phorbol esters, interleukin-1, and tumor necrosis factor. As demonstrated recently, AP-1 also has been implicated in the repression of transcription by glucocorticoids (Lucibello et al., 1990; Diamond et al., 1990; Jonat et al., 1990; Yang-Yen et al., 1990; Schule et al., 1990). Many of the previous studies on the role of the AP-1 sequence in collagenase gene expression have utilized tandem repeats of this sequence linked to reporter genes and have studied expression of these chimeric genes in immortal or tumorigenic cell lines (Angel et al., 1987a,b; Schonthall et al., 1988; Chiu et al., 1988; Brenner et al., 1989; Conca et al., 1989; Gutman & Wasylyk, 1990).

Alternatively, we have investigated collagenase gene expression with chimeric constructs containing the natural configuration of the collagenase promoter transfected into primary cultures of adult fibroblasts that express this gene

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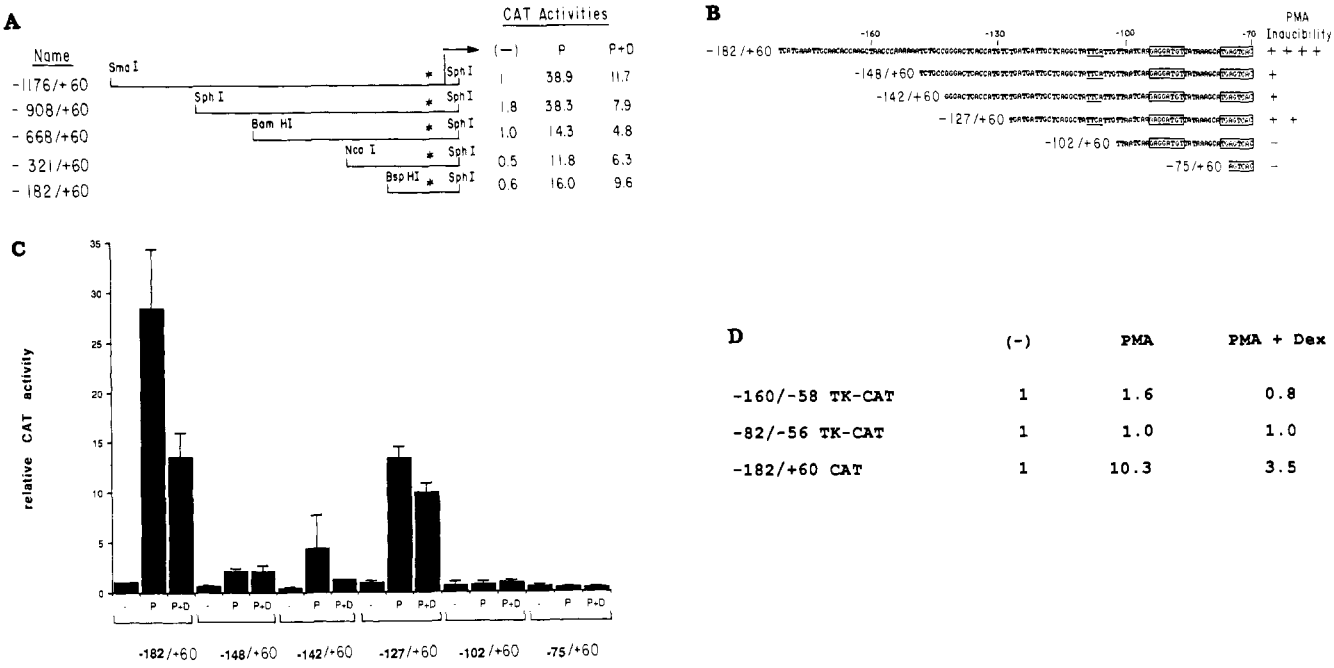

D

	(-)	PMA	PMA + Dex
-160/-58 TK-CAT	1	1.6	0.8
-82/-56 TK-CAT	1	1.0	1.0
-182/+60 CAT	1	10.3	3.5

FIGURE 1: Rabbit collagenase–CAT constructs and 5′-deletion analysis of rabbit collagenase promoter. (A) Map of the initial set of 5′-deletion constructs and summary of CAT activities. The sizes of the fragment of rabbit collagenase 5′-flanking DNA (Fini et al., 1987) which were inserted into the *Hin*dIII site of the transient expression vector pSVOCAT are indicated. The constructs are named by referring to the 5′ and 3′ ends, respectively, of the promoter fragments which were tested. Each DNA fragment terminates at the same 3′ position (+60 with respect to the start site of transcription which is marked by the arrow). The asterisk indicates the position of the AP-1 sequence. The CAT activities are expressed relative to the activity obtained in untreated (−) cells transfected with the −1176/+60 construct. "P" refers to treatment with PMA (10^{-8} M); "P+D", PMA plus dexamethasone (10^{-7} M) for 24 h prior to harvest. The reported CAT activities represent the means of at least three experiments. (B) Maps of 5′ deletions within the −182/+60 promoter fragment. These deletions were subcloned into pSVOCAT. The boxed sequence TGAGTCAC is the AP-1 sequence (Angel et al., 1987a,b), and the boxed sequence GAGGATGT is the PEA3-like sequence (Martin et al., 1988; Gutman & Wasylyk, 1990). The underlined sequence TTCA was mutated in construct 2, creating a promoter which is neither phorbol-inducible nor dexamethasone-repressible (see Figure 3). Under the column "PMA inducibility", "++++" refers to the activity of −182/+60, "++" indicates that the construct has about half the activity of −182/+60, and "+" refers to constructs with 25% or less of the activity of the −182/+60 construct. "−" indicates that the construct has no detectable PMA inducibility. (C) Relative CAT activities obtained for the constructs shown in (B). The activities are normalized to the CAT activity of the −182/+60 construct in the absence of any treatment (−). "P" and "P+D" refer to cells treated with PMA or PMA plus dexamethasone, respectively, as described above. The values represent the mean ± the standard error of at least three separate transfection experiments. (D) Rabbit collagenase–TK promoter chimeric constructs. Cells were transfected with each of the indicated plasmids and were treated as described above. The data represent the means of at least four experiments. As a positive control for the effects of PMA and dexamethasone, the −182/+60 CAT construct (see panel A) was also included. An activity of 1.0 indicates that the CAT activity is unchanged relative to the activity present in unstimulated transfectants, and the activities presented have been normalized for the effects of PMA or dexamethasone treatment on pBLCAT2 alone. In this experiment, the level of CAT activity in cells transfected with the −182/+60 construct was substantially less than that seen in (C). We attribute this variability to inherent differences in fibroblasts derived from outbred populations of rabbits (Brinckerhoff et al., 1979, 1982, 1986). However, other factors may also be involved since variations in the CAT activity have been observed in F9 teratocarcinoma cells transfected with the proenkephalin promoter (Sonnenberg et al., 1989).

endogenously. We have utilized a model of cultured synovial cells derived from the knees of healthy rabbits (Brinckerhoff et al., 1979, 1982, 1986; Brinckerhoff & Auble, 1990). These fibroblasts normally synthesize very little collagenase, but expression can be greatly induced by a variety of stimuli, including phorbol myristate acetate (PMA) (Brinckerhoff et al., 1979, 1982), interleukin-1 (Dayer et al., 1986; Conca et al., 1989), tumor necrosis factor (Dayer et al., 1985; Brenner et al., 1989), epidermal growth factor (Edwards et al., 1987), and urate crystals (Brinckerhoff et al., 1982). This induction can be antagonized by cotreatment with the glucocorticoid dexamethasone (Brinckerhoff et al., 1986; Brinckerhoff & Auble, 1990; Frisch & Ruley, 1987). Sequences within the first 1 kb of 5′-flanking DNA are highly conserved between the human and rabbit collagenase genes (Fini et al., 1987; Angel et al., 1987a,b). Thus, expression of the collagenase gene in rabbit synovial fibroblasts is a suitable model for studying mechanisms controlling the expression of this gene in normal fibroblasts.

In this study, we have identified regions within 1.2 kb of 5′-flanking DNA which contribute to the transcriptional regulation of the collagenase gene. Similar to results reported for the human gene, we have shown that the AP-1 sequence is required for PMA induction of collagenase transcription. However, contrary to previously reported results, we demonstrate that the AP-1 sequence, alone, is insufficient for regulation by PMA. Rather, we find that a minimal fragment of 127 bp of promoter DNA, which contains several response elements, is needed to confer regulation by PMA in normal adult fibroblasts. Our findings indicate that mechanisms controlling collagenase gene expression are more complicated than those suggested previously.

Materials and Methods

Constructs Containing Collagenase 5′-Flanking DNA. DNA fragments containing up to 1.2 kb of 5′-flanking DNA, derived from a 2.4-kb *Eco*RI fragment of the cloned rabbit collagenase gene (Fini et al., 1987), were obtained by restriction enzyme digestion, and the purified fragments were then subcloned into pUC 19 (Yanisch-Perron et al., 1985). Each fragment contains approximately 60 bp of untranslated leader sequence and terminates at its 3′ end at an *Sph*I site which brackets the translation initiation codon. DNA fragments of 1.2, 0.9, 0.7, 0.38, and 0.24 kb (see Figure 1) were

obtained by digestion with *Sma*I and *Sph*I, *Sph*I alone, *Bam*HI and *Sph*I, *Nco*I and *Sph*I, or *Bsp*HI and *Sph*I, respectively. To prevent out-of-frame translation from the collagenase start site, the 3'-terminal *Sph*I site, which overlaps the initiation codon, was subsequently destroyed by T4 DNA polymerase treatment, and the now blunt-ended DNA was religated (Sambrook et al., 1989). Following the addition of *Hin*dIII linkers, each fragment was subcloned into the unique *Hin*dIII site of pSVOCAT (Gorman et al., 1982) in which the inserted fragments drive transcription of the promoterless bacterial gene chloramphenicol acetyltransferase (CAT). The resulting plasmids are named by referring to the 5' and 3' ends, respectively, of the promoter fragments which were tested (Figure 1). Subsequent deletions of the −182/+60 fragment were made by using appropriate restriction enzymes and subcloning into the *Hin*dIII site of pSVOCAT. The identity and orientation of each construct were confirmed by restriction enzyme digestion.

Rabbit Collagenase–Thymidine Kinase Promoter Chimeric Constructs. An oligonucleotide duplex which contains *Hin*dIII and *Bam*HI ends, a unique *Xba*I site, and sequences from −82 to −56 of the collagenase promoter was obtained by chemical synthesis of DNA on a Biosearch Cyclone DNA synthesizer:

AGCTTCTAGAAAGCATGAGTCACACAGCCCTCAGCTG
AGATCTTTCGTACTCAGTGTGTCGGGAGTCGACCTAG

The 8 bp underlined sequence indicates the AP-1 sequence, located at −70 to −78 in the rabbit gene. It is identical in seven out of eight positions with the AP-1 sequence present in the promoter of the human collagenase gene, located in approximately the same position (Fini et al., 1987; Angel et al., 1987a,b). A construct containing sequences from −82 to −56 was generated by inserting the oligonucleotide into the unique *Hin*dIII and *Bam*HI sites of pBLCAT 2 (Luckow & Schutz, 1987) just upstream of the thymidine kinase (TK) promoter–CAT gene fusion. A slightly larger construct was made from a piece of DNA spanning the region from −160 to −58 by restriction with Alu to give a 102 bp piece of DNA spanning positions −160 to −58. This was ligated to the *Hin*dIII and *Bam*HI sites just upstream of the TK promoter in pBLCAT2. Plasmids containing the insert were identified by restriction enzyme digestion, and the sequence was verified by dideoxy sequencing of the insert following subcloning into M13mp19 (Sanger et al., 1977).

Transient Transfection of Rabbit Synovial Fibroblasts. Rabbit synovial fibroblasts, obtained from the knees of 4-week-old New Zealand white rabbits, were isolated and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal calf serum (Gibco) as described (Dayer et al., 1976; Brinckerhoff et al., 1979, 1986). For transfections, cells were used between passages 5 and 8 and were seeded at 5×10^5 cells per 60-mm dish. The following day, they were transfected with 10 μg of CAT reporter plasmid by the calcium phosphate coprecipitation method (Davis et al., 1986). Six hours following the addition of DNA, the cells were shocked with 15% glycerol for 1 min. Sixteen hours posttransfection, the cells were transferred to serum-free medium (DMEM containing 0.2% lactalbumin hydrolysate) and incubated for an additional 24 h in serum-free medium alone or in medium containing 10^{-8} M PMA (Sigma) or PMA plus 10^{-7} M dexamethasone (Sigma). Cells were harvested, and CAT activity was assayed as described (Gorman et al., 1982). Equivalent amounts of protein (Bradford, 1976) were used in each CAT assay, and the percent conversion of [^{14}C]chloramphenicol substrate to acetylated product was determined by excision and scintillation counting of the substrate and product spots as resolved by thin-layer chromatography.

RNase Protection Analysis. Isolation of whole cell RNA from transiently transfected rabbit synovial fibroblasts was performed as described (White et al., 1982). The yield of RNA was determined spectrophotometrically, and the use of equal amounts of intact RNA for each sample was confirmed by ethidium bromide staining of agarose gels (Sambrook et al., 1989). RNase protection was performed as described by Melton et al. (1984) with modifications (Winslow et al., 1989). RNA was hybridized to a riboprobe obtained by T7 RNA polymerase (Promega) transcription of an *Nco*I-linearized, pGEM 3-derived plasmid (Promega) containing a *Bam*HI–*Eco*RI fragment of approximately 1 kb in length. This insert was obtained from pCCAT 1.2 (see Figure 1A) and contains approximately 700 bp of collagenase 5'-flanking DNA fused to 251 bp of the CAT gene.

Preparation of Nuclear Extracts. Small-scale nuclear extracts were prepared from rabbit synovial fibroblasts essentially as described (Lee et al., 1988), with the exception that 20 additional passes through a 25-gauge syringe needle were required to lyse the cells. Because a 24-h treatment with PMA has been associated with maximal response of the endogenous gene, cells were treated for this length of time prior to being harvested.

Mutation Substitutions. Mutagenesis was done with synthetic oligonucleotides 32–38 bases in length, and was carried out by using instructions provided by the manufacturer for Muta-Gene M13 (Bio-Rad). Each mutation was confirmed by DNA sequencing of the M13 template (Sanger et al., 1977) followed by subcloning into pSVOCAT.

Analysis of DNA Binding Proteins. Five micrograms of nuclear extract from PMA-treated cells was incubated with radiolabeled DNA fragments in binding buffer (12 mM HEPES, pH 7.9, 12% glycerol, 60 mM KCl, 5 mM $MgCl_2$, 0.6 mM EDTA, and 0.6 mM DTT) for 30 min at 30 °C. The reactions were analyzed by electrophoresis under nondenaturing, low ionic strength conditions, with 5% polyacrylamide gels and autoradiography (Fried & Crothers, 1981; Garner & Revzin, 1981; Carthew et al., 1985). The AP-1 oligonucleotides was radiolabeled by filling in the 5'-overhanging *Bam*HI and *Hin*dIII ends with the Klenow fragment of DNA polymerase in the presence of [α-^{32}P]dCTP (Sambrook et al., 1989).

Results

Promoter Activity of Collagenase 5'-Flanking DNA. Constructs containing fragments of rabbit collagenase 5'-flanking DNA ligated into pSVOCAT were transfected into rabbit synovial fibroblasts and were tested for their ability to drive CAT gene expression under conditions known to induce or repress the expression of the endogenous collagenase gene. Figure 1A summarizes the data obtained with plasmid constructs containing between 1200 bp (−1176/+60) and 240 bp (−182/+60) of rabbit collagenase 5'-flanking DNA. Each promoter fragment drives very little basal CAT activity, but activity is increased dramatically (nearly 40-fold for the construct −1176/+60) following incubation with PMA. The ability of PMA to induce CAT activity is substantially blocked by the simultaneous incubation with the synthetic glucocorticoid dexamethasone (Figures 1A and 2). The shorter promoter fragments are slightly less responsive to PMA and dexamethasone (compare −182/+60 to −1176/+60). This suggests that upstream regions of DNA contribute to the response mediated by these agents, as suggested by others (Angel et al., 1987a).

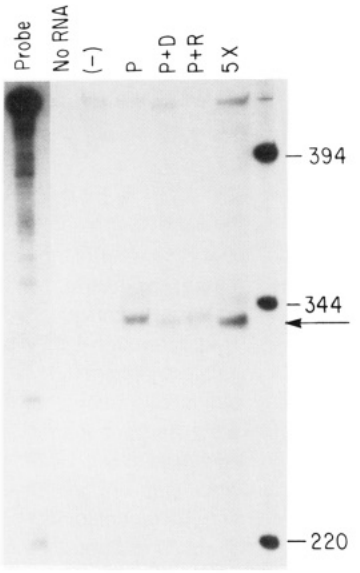

FIGURE 2: RNase protection analysis of CAT mRNA. Rabbit fibroblasts were transfected with the −668/+60 construct, treated as indicated with PMA (10^{-8} M), PMA plus dexamethasone (10^{-7} M), or PMA plus retinoic acid (10^{-6} M). At 24 h, whole cell RNA was harvested as described. CAT mRNA was detected by RNase protection analysis. The arrow indicates the position of the specifically protected band which migrates at the expected position for RNA which has been properly initiated at the collagenase start site (Fini et al., 1987). The lane marked "Probe" contains a portion of the radiolabeled antisense RNA used in the RNase protection analysis. The "No RNA" lane represents a hybridization reaction containing riboprobe but no whole cell RNA. The "5X" lane resulted from a reaction containing RNA from PMA-treated cells and 5 times the amount of riboprobe present in the "P" lane. The similar intensity of the bands present in the "P" and "5X" lanes indicates that the riboprobe was present in molar excess over unlabeled CAT mRNA.

Measurement of CAT mRNA. To ensure that the CAT activities measured in the above experiments reflected the steady-state levels of CAT mRNA, we compared the amounts of CAT mRNA present in transfected cells by RNase protection analysis. Results of one such experiment are shown in Figure 2. The riboprobe specifically protects a fragment of about 330 bases, the expected size of transcripts which have been properly initiated at the collagenase start site (Fini et al., 1987). The level of CAT mRNA in untreated transfected cells is barely detectable, but is induced by treatment with phorbol esters. In addition, the phorbol induction is blocked by dexamethasone. We note, parenthetically, that the vitamin A analogue retinoic acid (10^{-6} M) also blocked the induction of CAT mRNA as has been reported for the endogenous gene (Brinckerhoff et al., 1986; Brinckerhoff & Auble, 1990).

Activity of the AP-1 Sequence. Since the −182/+60 construct represented the smallest fragment tested so far which conferred phorbol responsiveness and dexamethasone repression, we further characterized it by 5′-deletional analysis and by measuring the ability of the deletions to drive CAT expression. The sequence of this fragment and the locations of 5′-deletion end points are shown in Figure 1B. The CAT activities obtained when rabbit fibroblasts were transfected with these constructs in the presence of phorbol or phorbol and dexamethasone are shown in Figure 1C. Compared to the −182/+60 fragment, all the deletion constructs show reduced phorbol inducibility. However, we found that the −127/+60 fragment was more phorbol-inducible than were the larger fragments, −142/+60 and −148/+60. This suggests the presence of a negative regulatory element between positions −148 and −127. We also found that the −127/+60 construct is the smallest fragment that is both phorbol-inducible and dexamethasone-repressible, indicating that the sequences responsible for this dual regulation colocalize to a relatively small piece of DNA. Finally, the increase in PMA-induced CAT activity seen with the −182/+60 construct compared to the −148/+60 construct suggests the presence of an enhancer in the 36 bp between −182 and −148. Taken together, these results indicate that a series of sequences within the −182/+60 construct contribute significantly to phorbol inducibility and dexamethasone repressibility.

We found that the −102/+60 construct has basal activity but, unexpectedly, is not induced by PMA. This result is surprising since the rabbit collagenase promoter contains an AP-1 sequence motif between −78 and −70 (Fini et al., 1987). This is analogous to the AP-1 sequence found in the highly conserved human collagenase promoter, where it functions independently as a major phorbol-inducible element in HeLa and a variety of other cells (Angel et al., 1987a,b). However, we found that in synovial fibroblasts, the AP-1 sequence alone (−82/−56 TK–CAT) failed to support phorbol-induced gene expression when ligated to the heterologous TK promoter (Figure 1D) as did a slightly larger fragment of DNA (from −160 to −58). These TK–CAT constructs containing the AP-1 sequence showed a 3–8-fold increase in basal level CAT activity, relative to the promoterless pSVOCAT. This enhancer activity suggests that the AP-1 site is the target of a transcriptional activator in rabbit fibroblasts. However, since the RSV and SV40 promoters drive higher levels of CAT transcription (200-fold and 25-fold, respectively) than the unstimulated collagenase promoter (Brinckerhoff & Auble, 1990), the collagenase promoter constructs tested here are not saturating the transcriptional machinery.

We also used rabbit fibroblasts to test constructs in which either one or five copies of the human collagenase AP-1 sequence (Angel et al., 1987a,b) were ligated to the TK promoter (constructs kindly provided by H. J. Rahmsdorf). Others have shown that constructs such as these were sufficient to confer phorbol inducibility to the TK promoter in a variety of immortalized cell lines and in human fetal fibroblasts (Angel et al., 1987a,b; Schonthal et al., 1988; Brenner et al., 1989). In contrast, we found that (1) even five copies of the human TRE sequence conferred only a 2-fold increase in CAT activity in phorbol-treated rabbit fibroblasts (data not shown), and (2) one copy of the rabbit AP-1 sequence linked to the TK promoter and assayed in HeLa cells did confer phorbol inducibility to the TK promoter (H. J. Rahmsdorf, personal communication). These results indicate that the differences we observed were not due to differences between the rabbit and human AP-1 sequences. Multiple DNA sequences are required for the phorbol response in normal fibroblasts, but they are not required in HeLa cells, suggesting that there may be differences between the quantity and, possibly, the complement of transcription factors in these two cell types (see Discussion).

Substitutional Mutagenesis of the Collagenase Promoter. We further analyzed the sequences in the region from −116 to −78 for their response to PMA and dexamethasone. A series of plasmids containing short DNA substitutions in the −182/+60 fragment was constructed by site-directed mutagenesis, and these are designated mutants 1–9 (Figure 3A). The constructs were cloned into PSVOCAT, and CAT activities driven by the mutant templates were determined (Figure 3B). We made a single base pair change at position −78 (mutant 9), analogous to a mutation in the human collagenase AP-1 sequence which prevents binding of the FOS-JUN heterodimer (Angel et al., 1987b). This mutation completely obliterates the PMA response in rabbit fibroblasts,

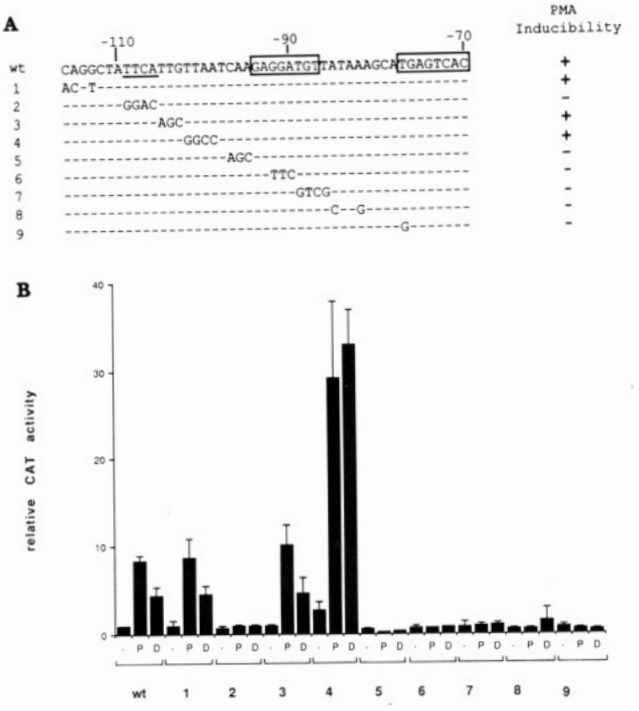

FIGURE 3: Substitution mutagenesis of the rabbit collagenase promoter. (A) Substitution mutants. A portion of the wild-type sequence is shown on the top line, and the short substituted sequences which were introduced by site-directed mutagenesis are shown on subsequent lines. The dashes indicate DNA sequences which were not changed. The data to the right summarize the results presented in (B) in which "+" indicates that CAT activity driven by the construct was induced by PMA whereas "−" indicates that it was not. (B) Summary of data obtained with the constructs containing substitutions of DNA in the collagenase promoter. The activities are expressed relative to the activity of the −182/+60 ("wt") construct present in untreated (−) transfected cells. The relative CAT activities in cells treated with PMA (P) or PMA plus dexamethasone (D) are also indicated. The data represent the mean ± the standard error of at least three independent transfection experiments.

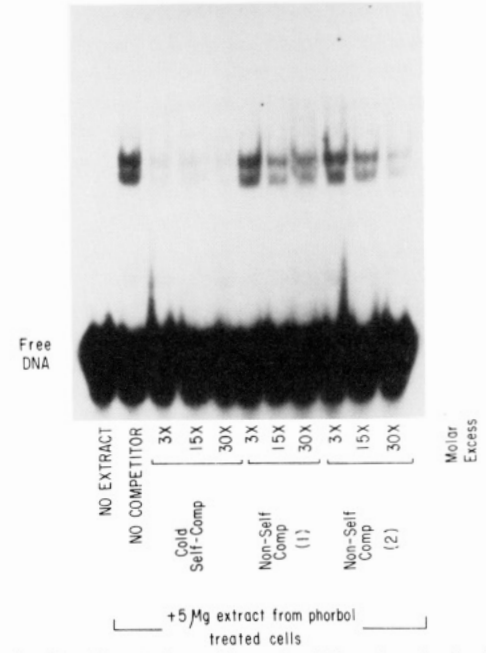

FIGURE 4: Nondenaturing polyacrylamide gel analysis of nuclear factors interacting with the rabbit AP-1 sequence. A radiolabeled 37 bp oligonucleotide containing the rabbit AP-1 sequence was incubated with 5 μg of nuclear extract from PMA-treated cells, and the reaction products were analyzed by polyacrylamide gel electrophoresis and autoradiography. The band at the bottom of the gel is the free DNA as indicated. The incubation of the DNA fragment with the nuclear extract resulted in the formation of two more slowly migrating species. The formation of these two complexes could be competed with a 3-, 15-, or 30-fold molar excess of unlabeled AP-1 37-mer (indicated as "Cold Self-Comp") but was not competed to nearly the same extent by two other unlabeled competitors of unrelated sequence [indicated as "Non-Self Comp (1)" and "Non-Self Comp (2)"]. The first non-self-competitor is an oligonucleotide duplex containing an Sp1 binding site (Stratagene), and the second non-self-competitor has the top strand sequence: 5′-GATCCACGCGTCGACTCTATCTATCTATCTATCTATCT-ACGCGTG-3′.

indicating that while the AP-1 sequence is insufficient for the response to PMA, it is nonetheless required.

To confirm that the AP-1 sequence is involved, we tested its ability to bind nuclear proteins from PMA-treated rabbit fibroblasts. Gel mobility shift analysis showed that these extracts interact specifically with the AP-1 sequence in vitro (Figure 4), findings that are consistent with the observations of others (Angel et al., 1987a,b; Schonthall et al., 1988).

We then made additional substitutions throughout the region from −97 to −83 (mutants 5–8) and found they also destroy the PMA response. The effects of these substitutions can be partially explained by the presence of the sequence 5′-GAG-GATGT-3′ from −94 to −87. This PEA3-like sequence was originally identified in the polyoma virus enhancer (Martin et al., 1988; Gutman & Wasylyk, 1990). Factors binding to the PEA3 sequence act in conjunction with factors bound to the AP-1 sequence to synergistically activate transcription (Martin et al., 1988; Gutman & Wasylyk, 1990). The fact that mutations in this region of DNA abolish phorbol responsiveness implicates the PEA3-like element in collagenase induction. We also found a third DNA element which is located from −109 to −106 and which includes the sequence 5′-TTCA-3′. A mutation in this region (mutation 2) abolishes phorbol inducibility. Of particular interest is mutation 4, which substitutes four base pairs between positions −102 and −99. This construct displays increased basal expression and enhanced phorbol inducibility, but no dexamethasone repressibility. This result suggests that phorbol inducibility and dexamethasone suppressibility can be dissociated, perhaps by preventing binding of either activator proteins mediating induction by PMA and/or glucocorticoid receptor, and supports several recently described models for glucocorticoid modulation of gene expression (see Discussion).

Discussion

Collagenase is a major gene product of fibroblasts, and expression of this gene by these cells throughout the body plays a role in the modeling and remodeling of connective tissue (Harris, 1985; Jeffrey, 1986). Because of this prominent biological role, we have chosen to study the regulation of collagenase gene expression in the fibroblasts that express this gene endogenously. Compared to the phorbol inducibility seen with a 1.2-kb fragment of promoter DNA, we found that progressively smaller fragments of DNA gave progressively smaller responses to PMA. We found that the smallest fragment capable of responding to phorbol contained 127 bp of promoter DNA (the −127/+60 construct; see Figure 1B). The AP-1 site is necessary but not sufficient to mediate phorbol-induced collagenase gene expression, since a mutation in the AP-1 sequence abolishes phorbol induction.

Mutational substitutions of sequences within 46 bp of the −182/+60 bp fragment indicate that there is functional cooperativity among the AP-1 sequence and at least two additional sequence elements. First, the rabbit collagenase gene contains a PEA3-like element, and mutations in this region abolished activity. A second element with the sequence 5′-

TTCA-3′ is also necessary since a 4 bp substitution gave a greatly diminished response to phorbol. In addition to these sequences, we note that another 36 bp region located upstream at −182 to −149 substantially increases the level of phorbol inducibility (Figure 1a,b), suggesting the presence of an enhancer sequence.

Functional cooperativity between TREs or between TREs and another element has been described (Okuda et al., 1990; Gutman & Wasylyk, 1990). The promoter of the rat glutathione transferase P gene contains two imperfect TREs (Okuda et al., 1990). When transfected into F9 teratocarcinoma cells, each TRE sequence had no activity by itself, but the two elements acted synergistically to form a strong enhancer which was active even though F9 cells contain a very low level of AP-1 activity. Similarly, in transfected LMTK$^-$ fibroblasts, the TRE of the human collagenase gene cooperated with the PEA-like motif that is present in this promoter and that resembles the PEA3 sequence originally described in the polyoma virus (Gutman & Wasylyk, 1990). These investigators used four tandem repeats of these motifs and found that maximum levels of activity were seen only with chimeric constructs that had adjacent PEA3 and AP-1 elements. Our studies also demonstrate the importance of interactions between adjacent PEA3 and TRE motifs. In contrast, however, by utilizing only the single copy of each element in the native configuration of the collagenase promoter, we found that the presence of a third element, 5′-TTCA-3′, was required for induction. Thus, by demonstrating interaction among multiple sequence elements found in the natural configuration of the rabbit collagenase promoter, our results extend this model of cooperativity.

Our findings also support those of others who report a major role for the TRE in mediating phorbol induction of the collagenase gene. Numerous investigators have studied the responsiveness of this element under a variety of conditions and in several cell types (Angel et al., 1987a,b; Schonthal et al., 1988; Brenner et al., 1989; Gutman & Wasylyk, 1990). Multimers of the TRE have often been used, either by themselves or linked to other response elements, such as the PEA3 sequence mentioned above. These constructs are generally very responsive to induction with phorbol, particularly when transfected into immortal or tumorigenic cell lines, such as HeLa cells and HepG2 cells. Consequently, a model has developed which depicts the AP-1 site, a target for the transcription factors FOS and JUN, as a single major force in the transcriptional activation of the collagenase gene.

What is often not appreciated, however, is the fact that one copy of the TRE is often remarkably unresponsive, giving only about a 3-fold increase in CAT activity in phorbol-stimulated HepG2 cells, while the 5X TRE gave 78-fold induction in these same cells (Brenner et al., 1989). When this 5X TRE was transfected into phorbol-stimulated primary human fetal fibroblasts, it gave only a 25-fold increase in CAT (Brenner et al., 1989). Perhaps the cellular complement of transcription factors plays a key role in mediating gene expression, and this expression may be both qualitatively and quantitatively different in normal diploid fibroblasts vs immortal or tumorigenic cell lines (Curran & Franza, 1988). Our data in which the rabbit and human TRE constructs were unresponsive to PMA in normal fibroblasts but responsive in HeLa cells support this concept. Reasons for this disparate behavior are probably complex. It is known, for example, that FOS and JUN can form both homo- and heterodimers and that heterodimeric complexes have affinities for consensus sequences that differ from homodimers (Curran & Franza, 1988; Halazonetis et al., 1988; Abate et al., 1990). Moreover, JUN-related and FOS-related proteins are induced and are turned over with different kinetics (Curran & Franza, 1988). The rate of transcription of certain genes may vary according to the available concentrations and affinities of these transcription factors and according to the relative abundance of homo- and heterodimers. Furthermore, it is likely that in some cells, additional factors may enhance the DNA binding properties of FOS and JUN. Since the complement of transcription factors may vary from one cell type to another, the mechanisms regulating gene expression may also vary. Consequently, mechanisms governing expression of the collagenase TRE in HeLa cells may not be identical with those in fibroblasts.

In nearly all of our constructs, phorbol inducibility is coupled with dexamethasone repressibility. This observation is consistent with the recent findings from a number of laboratories that support a general model for negative regulation by glucocorticoids (Lucibello et al., 1990; Diamond et al., 1990; Jonat et al., 1990; Yang-Yen et al., 1990; Schule et al., 1990). In this model, the target DNA sequence, denoted as a "composite" glucocorticoid response element (Diamond et al., 1990), binds both to the glucocorticoid receptor and to the FOS and JUN proteins that interact with each other and with the DNA. However, our mutation (4) that substitutes GGCC for TTAA at positions −102 to −99 uncouples phorbol inducibility and dexamethasone repressibility. Because this site is distinct from the TRE, it suggests that an additional mechanism may contribute to glucocorticoid repression.

Since we are assaying the collagenase promoter in mature fibroblasts which express the endogenous collagenase gene during normal remodeling and disease states, our results are physiologically relevant to mechanisms controlling collagenase expression. Our data indicate that the collagenase gene is transcriptionally regulated by a more complex interaction of factors than has yet been described. Further insights into the tissue-specific expression of the collagenase gene will emerge through the continued analysis of the collagenase promoter in fibroblasts.

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