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Novel Phorbol Ester Response Region in the Collagenase Promoter Binds Fos and Jun

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Abstract In rabbit fibroblasts the AP-1 sequence (5′-ATGAGTCAC-3′) is necessary but not sufficient for induction of collagenase transcription by phorbol esters (PMA) (Auble and Brinckerhoff: *Biochemistry* 30(18):4629–4635, 1991). In this study we identified additional sequences involved in PMA-induced transcription. Using fibroblasts transiently transfected with chimeric constructs containing fragments of the rabbit collagenase 5′-flanking DNA linked to the chloramphenicol acetyl transferase (CAT) gene, we found that deletion of nucleotides −182 to −141 in a 380 bp promoter construct resulted in about a 7-fold loss of induction by PMA. Mobility shift assays revealed that nuclear proteins from fibroblasts specifically bound to 20-bp at −182 to −161. Binding was competed completely by self and only partially by the AP-1 sequence, implying that proteins binding to the AP-1 sequence could also bind to this region. In vitro transcribed and translated c-Fos and c-Jun bound to both the AP-1 site and to the sequences from −182 to −141. DNAase I footprinting of the collagenase promoter with purified c-Jun or c-Fos/c-Jun protected the AP-1 sequence at −77 to −69 in addition to a region from −189 to −178 which overlaps a putative AP-1-like site, 5′-ATTAATCAT-3′. Finally, deletion of the −182 to −161 region in a 380-bp CAT construct resulted in a substantial reduction of PMA responsiveness. Thus, we have identified a novel phorbol-responsive region that binds c-Fos and c-Jun, and we suggest that these or similar proteins may regulate transcription of the collagenase gene by binding to sequences within and adjacent to the −182 to −161 region.

Key words: metalloproteinases, transcription, AP-1, mobility shift assay, DNAase I footprinting

The regulated degradation of interstitial collagen (types I, II, and III) is central to the maintenance and remodeling of the extracellular matrix. The metalloproteinase collagenase (MMP1) initiates collagen degradation during normal (wound healing, uterine resorption) and disease processes (tumor invasion and rheumatoid arthritis) [for reviews, see Jeffrey, 1986; Matrisian, 1990]. A variety of cell types express interstitial collagenase, including endothelial cells [Herron et al., 1986], keratinocytes [Lin et al., 1987], macrophages [Campbell et al., 1987], chondrocytes [Lin et al., 1987; Stephenson et al., 1987], vascular smooth muscle cells (T.W. James and C.E. Brinckerhoff, unpublished results), and fibroblasts [Goldberg et al., 1986; Whitham et al., 1986; Brinckerhoff et al., 1987]. The synthesis of collagenase is increased by cytokines and growth factors such as interleukin-1 [Stephenson et al., 1987; Conca et al., 1989; McCachren et al., 1989; Lafyatis et al., 1990], tumor necrosis factor-α [Dayer et al., 1985; Brenner et al., 1989], and epidermal growth factor [Partridge et al., 1987]. Protein kinase C agonists such as phorbol myristate acetate (PMA) [Stephenson et al., 1987; Brinckerhoff and Auble, 1990; Lafyatis et al., 1990] mimic the effects of these cytokines, and a number of studies have shown that induction of collagenase synthesis occurs, at least in part, at the level of transcription [Brinckerhoff et al., 1986; McCachren et al., 1989; Brinckerhoff and Auble, 1990; Lafyatis et al., 1990].

The Fos and Jun families of transcription factors interact with several DNA recognition sequences known as AP-1, TRE, or CRE sites [Bohmann et al., 1987; Franza et al., 1988; Rauscher et al., 1988; Risse et al., 1989; Cohen and Curran, 1990; Hai and Curran, 1991; Ryseck and Bravo, 1991]. Previous work has shown that PMA induction of collagenase is cooperatively mediated through the AP-1 [Angel et al., 1987b; Schonthal et al., 1988; Lafyatis et al.,

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1990] and PEA3 [Gutman and Wasylyk, 1990] binding sites which, together define a "tumor promoter oncogene-responsive unit" [Gutman and Wasylyk, 1990]. Others have shown that the AP-1 and PEA3 elements present in the collagenase gene are sufficient to confer phorbol inducibility to a minimal promoter when the chimeric constructs were transfected into tumorigenic cell lines or when the elements were used as multimeric repeats [Angel et al., 1987a,b; Gutman and Wasylyk, 1990; Jonat et al., 1990].

However, studies of the regulation of collagenase gene transcription using chimeric constructs containing the natural configuration of the collagenase promoter transfected into normal fibroblasts, a cell type that expresses the gene endogenously [Brinckerhoff et al., 1979, 1982, 1986; Brinckerhoff and Auble, 1990], reached a different conclusion [Auble and Brinckerhoff, 1991; Pan et al., 1992]. We found that transcriptional induction by phorbol esters requires 127 base-pairs of collagenase promoter DNA. We also demonstrated that the AP-1 site alone is insufficient to drive transcription in PMA-stimulated fibroblasts. At least three sequences, the AP-1 site, a PEA3-like element and a 5′-TTCA-3′ motif, contained within the first 127 base-pairs of promoter, are required to confer phorbol ester responsiveness. Thus, in normal fibroblasts, phorbol responsiveness requires cooperation among several DNA elements, and additional upstream sequences contribute further [Angel et al., 1987b; Auble and Brinckerhoff, 1991].

Previously, we identified a region of the rabbit collagenase promoter from −182 to −141 which substantially increased the level of collagenase transcription in response to phorbol esters [Auble and Brinckerhoff, 1991]. In addition, both in vitro [Angel et al., 1987b] and in vivo [Konig et al., 1992] footprinting of the human collagenase promoter with phorbol-treated cells revealed protection of several upstream regions. However, to date, no increase in transcription has been directly associated with any of these regions.

In this study, we focus on the region between −182 to −141. We correlate the DNA-protein interactions of this region with PMA-responsive sequences, and we partially characterize the nuclear factors which interact with them.

MATERIALS AND METHODS

Tissue Culture

Rabbit synovial fibroblasts were obtained from the knees of 4-week New Zealand white rabbits and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin as described [Dayer et al., 1976; Brinckerhoff et al., 1979, 1986]. Confluent cells were passaged 1:3 with 0.25% trypsin. Cells were used between passages 5 and 8 to minimize effects of passage variation in transfection experiments and nuclear extract preparations.

Site-Directed Mutagenesis/Reporter Plasmids

A previously described reporter construct containing a 380-bp HindIII fragment of rabbit collagenase 5′ flanking DNA [Auble and Brinckerhoff, 1991] fused to the promoterless chloramphenicol acetyl transferase (CAT) gene (pCCAT 380) in the vector pSV0CAT [Gorman et al., 1982] was used as a template source for site-directed deletion and substitution mutagenesis. Oligonucleotides used for site-directed mutagenesis and mobility shift assays were synthesized on a Biosearch Cyclone DNA Synthesizer and were purified by Nensorb (Dupont, Boston, MA) chromatography prior to use. Following subcloning of the 380-bp HindIII fragment into pSelect (Promega) mutagenesis was carried out using instructions provided by the manufacturer for Altered Sites (Promega, Madison, WI). Each mutation was confirmed by DNA sequencing [Sanger et al., 1977] of the mutated plasmid prior to subcloning into pSV0CAT. The orientation and verification of single-copy status of each construct was confirmed by restriction enzyme digestion.

Transfections and CAT Assay

Plasmid DNA used for transfections was prepared by the alkaline lysis method [Sambrook et al., 1989] and was banded twice by CsCl gradient centrifugation. Transient transfection of rabbit synovial fibroblasts by the calcium phosphate coprecipitation method [Davis et al., 1986] was carried out as described [Auble and Brinckerhoff, 1991]. On the day prior to transfection, cells were seeded at 3×10^5 cells per 60-mm dish in DMEM supplemented with 10% fetal calf serum. They were transfected the following morning with 5 μg CAT reporter plasmids. Following a 6 hr incubation, the cells were shocked with 15% glycerol for 1 min. Sixteen hours later, cells were washed three times with Hank's balanced salt solution (Gibco) and 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, St. Louis, MO). Cellular lysates were prepared by the freeze-thaw method, and protein concentration was determined by Bradford assay (Bio-Rad, Richmond, CA). CAT activity was assayed as described [Gorman et al., 1982]. Three micrograms of lysate proteins were incubated with 0.20 μCi ^{14}C-chloramphenicol (Amersham, Chicago, IL) in reaction buffer (1.4 mM acetyl CoA, 14 mM Tris-HCl, pH 7.8, 3.5% glycerol) for 1 h. The reaction products were resolved by thin-layer chromatography [Sambrook et al., 1989] and autoradiography, and the percentage conversion of [^{14}C]-chloramphenicol substrate to acetylated product was determined following excision of the substrate and product spots. Acetylation of ^{14}C-chloramphenicol in assays from untreated (basal) and PMA-treated (induced) cell lysates fell within the linear range of the assay (0.5% to 30% conversion). CAT activity was normalized to the percentage acetylation of ^{14}C-chloramphenicol in extracts from untreated cells within the same transfection group. All constructs were transfected at least four times, each time in triplicate. Transfection efficiencies were equivalent for the various plasmids transfected as determined by transfected DNA content using the method of Hirt (data not shown) as previously described [Hirt, 1967; Pan et al., 1992].

Preparation of ^{32}P-Labeled DNA Fragments

One hundred picomoles of 5′-ends of single-stranded oligomers were labeled in a 20 μl reaction containing 2.5 μl [γ-^{32}P]-ATP (ICN #35020, 7,000 Ci/mmol), 2 μl of 10× reaction buffer (700 mM Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT), and 1 μl T4 polynucleotide kinase (BRL, Gaithersburg, MD). Labeled oligomers were then annealed to unlabeled complementary oligomers and the double-stranded oligoprobes were purified on a 15% polyacrylamide gel in 1× TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) for 3 h at 130 V. The gel was exposed briefly to X-ray film and the gel slice containing the labeled DNA fragments was incubated overnight on a rotary shaker at 37°C in elution buffer (0.5 M ammonium acetate, pH 7.0, 1 mM EDTA). The sample was then centrifuged and the supernatant was extracted with phenol/chloroform, ethanol precipitated, and resuspended in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) at 300,000 cpm/μl.

Preparation of Nuclear Extracts and Mobility Shift Assay

Nuclear extracts were prepared from rabbit fibroblasts as described [Pan et al., 1992], with the following modifications: Cells which were swollen in hypotonic solution at 4°C were passed through a 25 gauge needle 10 times to disrupt cell membranes. Nuclei were extracted for 30 min in 10 mM Hepes (pH 7.9), 12% glycerol, 8 mM $MgCl_2$, 500 mM KCl, 0.1 mM EDTA, 0.1 mM PMSF, and 0.25 mM DTT. Nuclear factors were then dialyzed for 1 h against dialysis buffer (10 mM Hepes [pH 7.9], 12% glycerol, 100 mM KCl, 0.1 mM EDTA, 0.1 mM PMSF, 0.25 mM DTT). Nuclear proteins (3–10 μg protein per assay) were incubated with 40,000 cpm [γ-^{32}P] ATP-labeled double-stranded oligomers (specific activity ≥2,000 cpm/fmol of 5′ ends) in binding buffer (12 mM Hepes [pH 7.9], 4 mM Tris-HCl [pH 7.9], 12% glycerol, 60 mM KCl, 1 mM EDTA, 1 μg poly dI:dC (Pharmacia, Piscataway, NJ) for 15 min at 30°C. Reaction products were resolved by electrophoresis on 5% non-denaturing polyacrylamide gels in 1XTBE. Gels were dried on Whatman 3MM paper and exposed to X-ray film overnight.

In Vitro Transcription/Translation

In vitro transcripts of full-length cDNAs encoding murine c-Fos in pGEMfos3 and human c-Jun in pGEMjun1 were generated as described in Halazonetis et al. [1988]. Briefly, the appropriately linearized plasmids were transcribed in vitro using SP6 RNA polymerase (Promega) in the presence of 5 mM m^7 GpppG (Boehringer Mannheim, Indianapolis, IN), so that 5′ capped transcripts were generated. In vitro translation was performed according to the manufacturer using 0.5 μg each of c-Fos and c-Jun transcribed mRNAs in a micrococcal nuclease-treated rabbit reticulocyte lysate (Promega) with 800 μCi/ml ^{35}S-methionine (Amersham) in a total volume of 50 μl. The in vitro translated proteins were resolved on 15% SDS-polyacrylamide gels [Laemmli, 1970] and were visualized by autofluorography of the fixed and dried gels (data not shown). One microliter of unprogrammed or programmed lysates were used in mobility shift experiments as described above. Dried gels were placed against two pieces of film separated by a piece of paper. An enhancing screen was placed against the second film. This allowed for visualization of the combined ^{32}P and ^{35}S signals and the ^{32}P signal alone from a single exposure. Films were exposed at −70°C for 16 h.

DNAase I Footprint Analyses

Pure recombinant human c-Fos was the generous gift of Dr. Tom Curran, Roche Institute of Molecular Biology (Nutley, NJ) and recombinant purified human c-Jun was obtained from Promega Corporation. DNAase I footprinting assays [Galas, 1978] were performed using the 380 bp HindIII fragment of the rabbit collagenase 5′ flanking DNA as a probe. This fragment was subcloned into pBluescript SK⁻ (Stratagene, La Jolla, CA) and the probe was prepared by end labeling with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase, either at the *Cla*1 or *Eco*R1 site in the pSK⁻ polycloning site, and recutting at either *Eco*R1 or *Cla*1, respectively. The singly end-labeled probes were purified and eluted from 12% native polyacrylamide gels. Specific activities of the probes were approximately 2,000 cpm/fmol of 5′-ends. Binding reactions contained 3 μM BSA, 10 μg poly dI:dC (Pharmacia), 0.5× binding buffer (20 mM Hepes [pH 7.9], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT), 10,000 cpm of purified end-labeled probe, and various amounts of purified c-Fos and c-Jun proteins, in a total volume of 50 μl. The binding reaction was allowed to incubate for 1 h on ice. Twenty-three milliunits of DNAase I (Worthington, DPFF grade) was added to digest the DNA in the presence of 5 mM $CaCl_2$ for 1 min. Reactions were immediately terminated by the addition of 90 μl of stop buffer (20 mM EDTA [pH 8.0], 1.0% SDS, 0.2 M NaCl, 250 μg tRNA/ml). Samples were extracted with phenol/chloroform, and the DNA was ethanol precipitated. Reaction products were electrophoresed on 8% polyacrylamide-7M urea sequencing gels along with chemically sequenced probe [Maxam and Gilbert, 1980].

RESULTS

DNA Sequences From −182 to −141 of the Collagenase Promoter Induce Transcription by Phorbol Esters

Figure 1A compares the sequences in the 5′-flanking regions of the rabbit [Fini et al., 1987] and human [Angel et al., 1987a] collagenase genes. The sequences are 79% identical from position −182 to −1 of each promoter relative to the transcription start site of the rabbit gene and imply a conserved role in transcriptional regulation. This region contains several well-conserved sequences that are required for phorbol responsiveness of the rabbit collagenase promoter, including an AP-1 site, a PEA-3-like element, and a 5′-TTCA-3′ sequence [Auble and Brinckerhoff, 1991]. The presence of an additional PMA-responsive element within the region −182 to −141 of the rabbit collagenase promoter is suggested, since a 5′ deletion of these sequences reduces phorbol inducibility tenfold [Auble and Brinckerhoff, 1991].

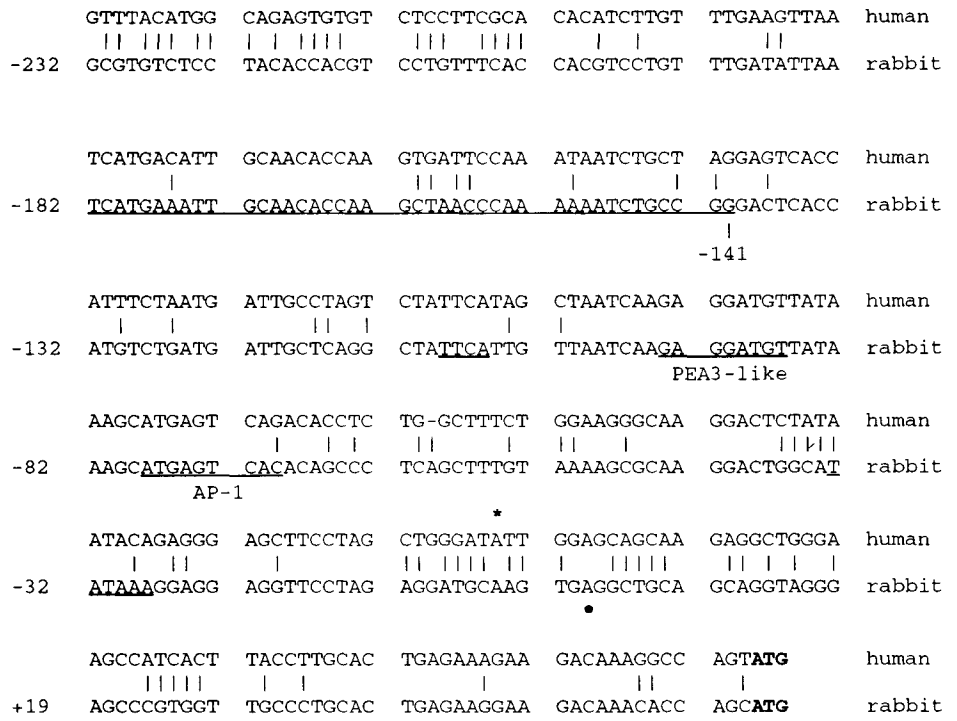
To confirm the significance of these sequences in mediating the phorbol ester response, the 42-bp region between −182 to −141 of the wild-type promoter (pCCAT380) was deleted internally (pCCATΔ−182/−141, Fig. 1B). We found that basal (untreated) CAT activity in fibroblasts transfected with pCCATΔ−182/−141 was reduced to 30% of the level from untreated cells transfected with pCCAT380 (Fig. 2). Upon addition of PMA to fibroblasts transfected with pCCAT380, CAT activity was induced nearly 30-fold (Fig. 2), but CAT activity in cells transfected with pCCATΔ−182/−141 was induced only 4-fold above the untreated control. This 7-fold loss in phorbol responsiveness is similar to that seen previously with a 5′ deletion mutation of this region [Auble and Brinckerhoff, 1991]. Thus, our results confirm the functional importance of sequences located between −182 to −141 of the collagenase promoter.

Nuclear Proteins Specifically Bind to Sequences Within −182 to −141 of the Collagenase Promoter

To localize the sequences within this 42 bp region which interact with nuclear proteins, we constructed three double-stranded oligomers

Fig. 1. DNA sequence of the 5′ flanking regions of the human and rabbit collagenase genes. **A:** Vertical lines indicate non-identical bases between the sequences. Asterisks (*) mark the single start-site of transcription of the rabbit gene [Fini et al., 1987], and the major start-site in the human gene [Angel et al., 1987a]. 62 bases 3′ of the start-site is the initiation codon (ATG) of the rabbit gene in bold. The TATA, AP-1, PEA-3-like, and TTCA sequences and the region from −182 to −141 of the rabbit gene are shown underlined. **B:** Sequences of synthesized deoxynucleotides used for electrophoretic mobility gel shifts and site-directed mutagenesis. Numbers indicate 5′ and 3′ ends of the rabbit collagenase promoter fragments relative to the start site of transcription. The AP-1 and AP-2 sequences are shown in bold. Δ marks the position of the deleted sequences as indicated by the number designations, in the mutagenic oligonucleotides. The mutagenic oligonucleotides used for substitution mutation are shown as sequences flanked by dashes. Above each dash is the corresponding wild-type sequence.

A



B

Deoxynucleotides used for gel shift experiments

Fragment	Nucleotide sequence
-182/-141 region	5'TCATGAAATTGCAACACCAAGCTAACCCAAAAAATCTGCCGG3' 3'AGTACTTTAACGTTGTGGTTCGATTGGGTTTTTTAGACGGCC5'
-182/-161 region	5'TCATGAAATTGCAACACCAAGC3' 3'AGTACTTTAACGTTGTGGTTCG5'
-172/-153 region	5'GCAACACCAAGCTAACCCAA3' 3'CGTTGTGGTTCGATTGGGTT5'
-160/-140 region	5'TAACCCAAAAAATCTGCCGGG3' 3'ATTGGGTTTTTTAGACGGCCC5'
Consensus AP-1	5'AGCTTCTTAGAAAGC**ATGAGTCA**CACAGCCCTCAGCTG3' 3'TCGAAGAATCTTTCG**TACTCAGT**GTGTCGGGAGTCGAC5'
Consensus AP-2	5'GATCGAACTGA**CCGCCCGCGG**CCCGT3' 3'CTAGCTTGACT**GGCGGGCGCC**GGGCA5'

Deoxyoligonucleotides used for mutagenesis

Mutagenic oligo/ **mutant CAT construct**	Nucleotide sequence
del. -182/-141 region/ **pCCATΔ-182/-141**	ACGTCCTGTTTGATATTAAΔGGACTCACCATGTCTGATG
del. -161/-141 region/ **pCCATΔ-161/-141**	CATGAAATTGCAACACCAAGΔACTCACCATGTCTGATGATT
del. -182/-162 region/ **pCCATΔ-182/-162**	CACGTCCTGTTTGATATTAAΔCTAACCCAAAAAATCTGCCG
-180/-177 region/ **pCCATmATGA**	GATATTAATCATGAAATTGCAACA ----------**GACG**----------
-188/-183 region/ **pCCATmTATTAA**	CCACGTCCTGTTTGATATTAATCATGAAATTGCAAC ----------**GCGGCC**----------

Figure 1.

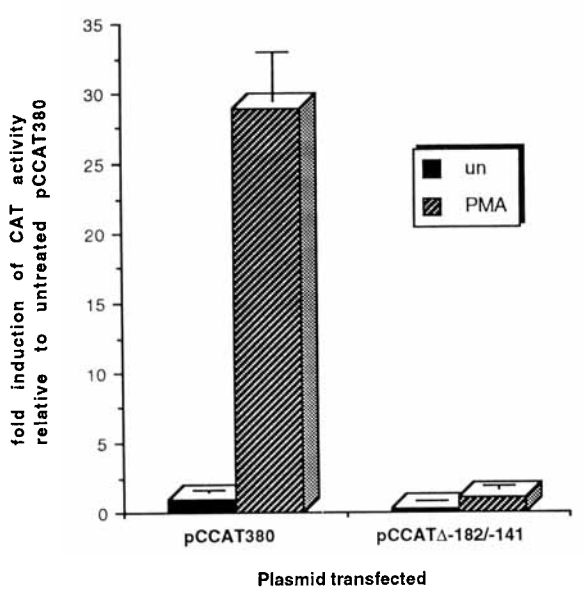


Fig. 2. PMA inducation is markedly reduced with a 42-bp deletion of the collagenase promoter. The wild-type pCCAT 380 and the mutant pCCAT Δ−182/−141 were transfected into rabbit fibroblasts. Cells remained either untreated (un) or were treated with 10^{-8} M phorbol myristate acetate (PMA). CAT assays were carried out as described in Materials and Methods. Acetylation of ^{14}C-chloramphenicol by extracts from untreated cells was 2% ± 0.2% (pCCAT 380), and 0.6% ± 0.05% (pCCAT Δ−182/−141). Results shown are the means of four independent transfections, each in triplicate, ± 1 standard deviation. The value of the untreated (control) CAT activity of pCCAT 380 is 1.0.

representing the 5′ half (−182/−161), 3′ half (−160/−140), and middle (−172/−153) of the −182 to −141 region and complexed them with nuclear extracts from both untreated and PMA-treated fibroblasts (Fig. 3). Two of these probes, −160/−140 and −172/−153, bound little or no protein in both extracts. However, the sequences from −182 to −161 bound proteins in extracts from untreated cells and this binding was increased upon treatment of cells with PMA.

Using nuclear extracts from PMA-treated cells, we examined the specificity of binding (Fig. 3B). Similar to Figure 3A, there was little to no complex formation with the −160/−140 or −172/−153 probes. However, the −182/−141 probe bound substantial amounts of protein and this binding was specific since competition was seen only with self sequences; neither the region from −160 to −140 nor an oligo containing an unrelated transcription factor binding site, the AP-2 sequence [Jones et al., 1988], competed the shifted complexes. The subtle variations in shift patterns between Figure 3A and 3B may represent slight variations in nuclear extracts prepared from outbred populations of fibroblasts [Dayer et al., 1976, 1985; Brinckerhoff et al., 1979, 1986; Jeffrey, 1986]. Taken together these findings implicate involvement of sequences within −182 to −161 in the transcriptional response to PMA.

Nuclear Proteins Which Specifically Bind to the Region From −182 to −161 Can Also Bind to the AP-1 Sequence

Using mobility shift assays, we confirmed the ability of nuclear proteins from fibroblasts to bind specifically to the AP-1 sequence, and we examined the ability of sequences within the −182 to −161 region to compete for these proteins (Fig. 4). As expected, PMA treatment increased the amount of protein bound to the AP-1 site. This binding was competed by an excess of self-oligo (compare lanes 1 and 2), but was not competed by 100-fold molar excess of −182/−141 or −182/−162 oligos (Fig. 4, lanes 3, 4, 7, and 8) or by a mutant AP-1 oligo which abolishes Fos/Jun binding (data not shown, [Risse et al., 1989]). Proteins from these extracts also bound to the region from −182 to −161 (lanes 9 and 13), and this binding was competed completely by an excess of self competitor (lanes 10 and 14), but not by the −160/−140 oligo (lanes 12 and 16). However, in contrast to shifts with the AP-1 oligo which were not competed by the −182/−161 oligo (lanes 4 and 8), proteins bound to the −182/−161 probe were slightly competed by a 100-fold molar excess of unlabeled AP-1 oligonucleotide (lanes 11 and 15). PMA treatment of cells resulted in enhanced binding to the −182/−161 region and this was accompanied by a modest change in complex mobility (compare lanes 9 and 13). The fact that the AP-1 oligo could compete for specific complex formation on the −182/−161 oligoprobe suggests that proteins which bind specifically to the AP-1 site, such as those related to Fos and Jun [Rauscher et al., 1988], might also interact with sequences between −182 to −161.

In Vitro Translated c-Fos and c-Jun Proteins Specifically Bind to the AP-1 Sequence and to the Region From −182 to −141 of the Collagenase Promoter

We examined the possibility that Fos and Jun could bind to sequences within the region from −182 to −141 of the collagenase promoter. For these studies we performed gel shift experiments with murine c-Fos and human c-Jun pro-

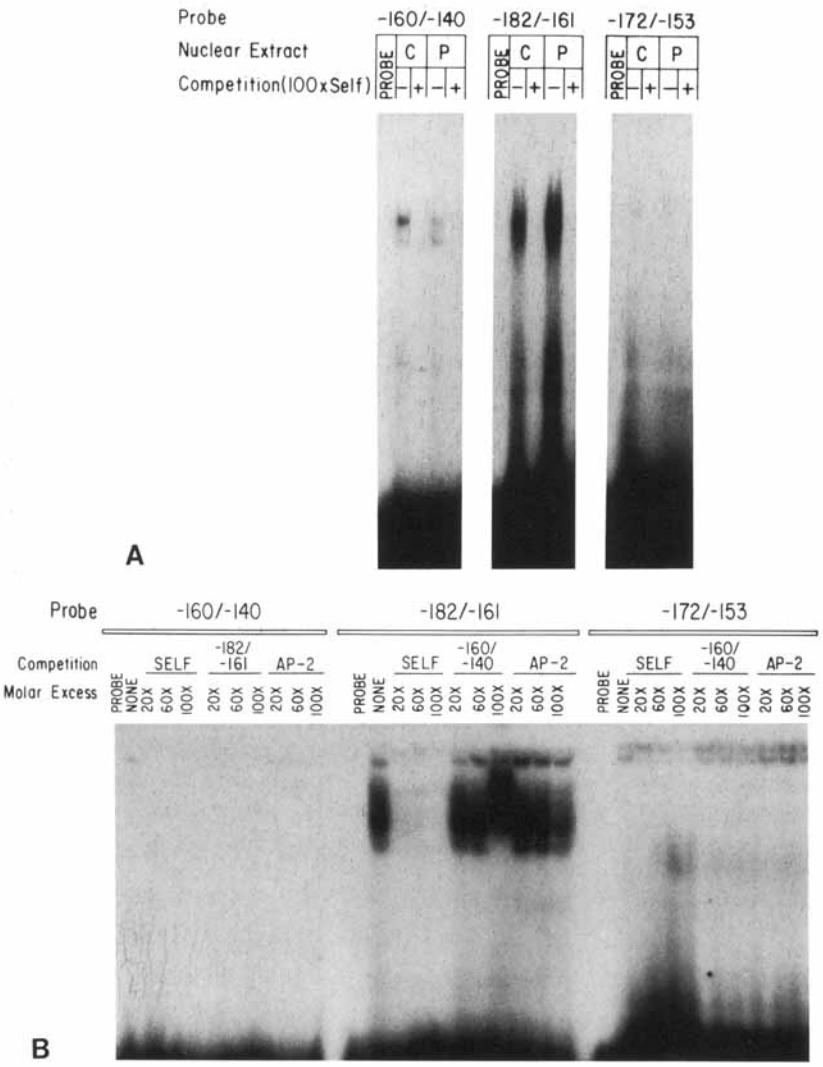


Fig. 3. Gel shift analysis with nuclear proteins and radiolabeled fragments of the collagenase promoter Complexes were formed by binding of 10 μg nuclear extract proteins with three collagenase 5 -flanking sequence oligoprobes −160/−140, −182/−161, and −172/−153 (see Fig 1B) **A:** Rabbit fibroblasts were cultured in serum-free medium alone (C) or treated with 10^{-8} M PMA for 2 h (P) 100-fold molar excess of unlabeled self oligonucleotides were omitted (−) or added (+) to binding reactions Shown are data from a single representative gel **B:** Competition analysis of specific complex formation with 2 h PMA-treated nuclear extracts Competitor sequences as depicted in Figure 1B were titrated at 20- (20×), 60- (60×), or 100-fold (100×) molar excess in binding reactions with the probes Complexes formed in the lane marked 100× with the −160/−140 competitor probed with −182/−161 migrated aberrantly The lane does not impact the results and is included to show contiguity of the gel

teins cotranslated in vitro using reticulocyte lysate and ^{35}S-methionine, thus producing ^{35}S-labeled Fos/Jun heterodimers [Halazonetis et al., 1988]. Both c-Fos and c-Jun migrated on an SDS polyacrylamide gel in accordance with their molecular weights (data not shown) [Halazonetis et al., 1988]. Figure 5A shows a representative gel shift experiment in which c-Fos and c-Jun were incubated with ^{32}P-labeled AP-1 oligoprobe. Mobility shift gels were autoradiographed so as to visualize both the combined [^{35}S + ^{32}P] signals or just the [^{32}P] signal alone (Fig. 5) [Halazonetis et al., 1988]. Lysates from unprogrammed reticulocytes did not bind either the AP-1 sequence (Fig. 5A, lanes 1–3 and 7–9) or the 42 bp oligomer from −182 to −141 (data not shown). However, lysates programmed with mRNAs for c-Fos and c-Jun formed specific complexes with the labeled probes (Fig. 5A, lanes 4–6 and 10–12). This binding was efficiently competed by self (lane 11) and slightly competed by the −182/−161 oligomer (Fig. 5, lane 12).

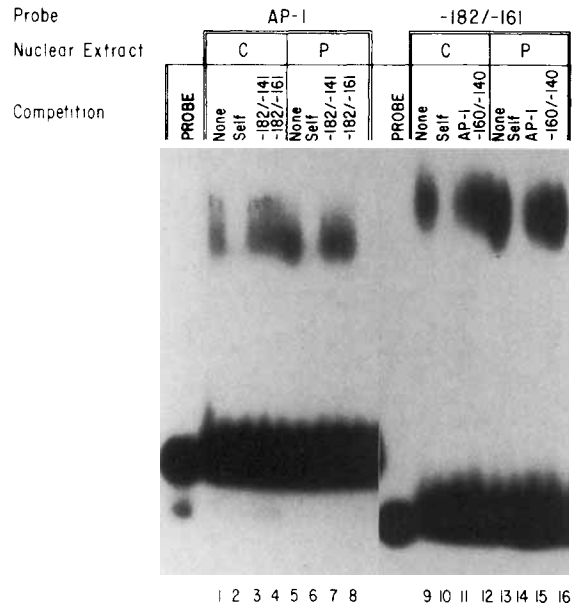


Fig. 4. Specificity of DNA-protein complexes formed with either the AP-1 sequence or with the region from −182 to −161 of the collagenase promoter. Specific complex formation with 10 μg nuclear extract proteins from rabbit fibroblasts cultured in serum-free medium (C) or treated for 2 h with 10^{-8} M PMA (P), and labeled oligonucleotide probes representing the AP-1 sequence or the −182/−161 region. Unlabeled competitor oligonucleotides (see Fig. 1B) were used at a 100-fold molar excess.

The in vitro translated c-Fos/c-Jun heterodimer was then bound to the −182/−141 oligoprobe (Fig. 5B) and its binding ability was compared to that of the AP-1 probe. Both probes bound c-Fos/c-Jun and these DNA-protein complexes migrated at different mobilities (Fig. 5B, compare lanes 7 and 10, and see Discussion). In addition, the c-Fos/c-Jun heterodimer shifted more of the AP-1 probe compared to the −182/−141 probe (compare intensity of lanes 7 and 10). In contrast to shifts obtained with extracts from fibroblasts, which showed limited cross-competition (Fig. 4), cross-competition with c-Fos/c-Jun heterodimer showed that each oligomer could compete as effectively for proteins bound to the other sequence (Fig. 5B, lanes 9 and 12). These data support our earlier hypothesis that AP-1-related proteins can bind to both the AP-1 sequence and to sequences within the region from −182 to −141 (see Discussion).

Purified c-Jun Homodimers and c-Fos/c-Jun Heterodimers Footprint a Region in the Vicinity of −182 of the Collagenase Promoter

To examine the interaction of Fos and Jun proteins with the collagenase promoter at the level of nucleotide sequence, DNAase I footprinting experiments were performed using purified human c-Fos and c-Jun. As expected, c-Jun homodimer protected the AP-1 site on either the coding or non-coding strands (Fig. 6). In addition, a protected region was seen from −189 to −178 on the coding strand (Fig. 6A,B), and a DNAase I hypersensitive site located at A −177 (Fig. 6A,B) also provided evidence of a DNA-protein interaction [Goodisman and Dabrowiak, 1992]. The sequence 5′-ATGA-3′, an apparent AP-1 half-site located at −180 to −177, is a portion of the sequence protected by c-Jun homodimer. This sequence is also found downstream between −125 to −122 (Fig. 6A,B), but there is no evidence of protection at this region. Figure 6B shows a detail insert of the region that is footprinted by purified c-Jun homodimer on both the coding and non-coding strands of the collagenase promoter. On the non-coding strand c-Jun protects the AP-1 sequence (Fig. 6A) and evidence for upstream interactions was seen at sites which become increasingly DNAase I hypersensitive upon titration of c-Jun protein: G-195, A-158, G-145, and G-142 (Fig. 6B) in addition to a protected A at −184. Together, these data suggest that sequences flanking the ATGA at −180 to −177 contribute to binding of c-Jun.

We then performed DNAase I footprinting using equimolar amounts of purified c-Fos and c-Jun heterodimer (Fig. 6C). Titration of increasing amounts of the heterodimer protected the same sequences as described above as well as additional nucleotides. The G at −178 was protected at 0.5 μM c-Fos/c-Jun heterodimer where only partial protection was observed at the same concentration of c-Jun homodimer. The A at −177 was hypersensitive to DNAase I cleavage when bound by c-Jun homodimer but was protected from DNAase I cleavage by the c-Fos/c-Jun heterodimer, suggesting protection of a larger region upon binding of the heterodimer. This may also represent differences in the conformation of the DNA template resulting from the binding of c-Jun/c-Jun or c-Fos/c-Jun binding to this region. There are several DNAase I hypersensitive sites flanking this region on the non-coding strand: G-195, A-158, G-145, and G-142. Thus, the non-coding strand showed a similar pattern of DNA-protein interaction with the c-Fos/c-Jun heterodimer and with the c-Jun homodimer. These results confirm sequence-specific binding by c-Jun and c-Fos/c-Jun within and just upstream of the phorbol ester respon-

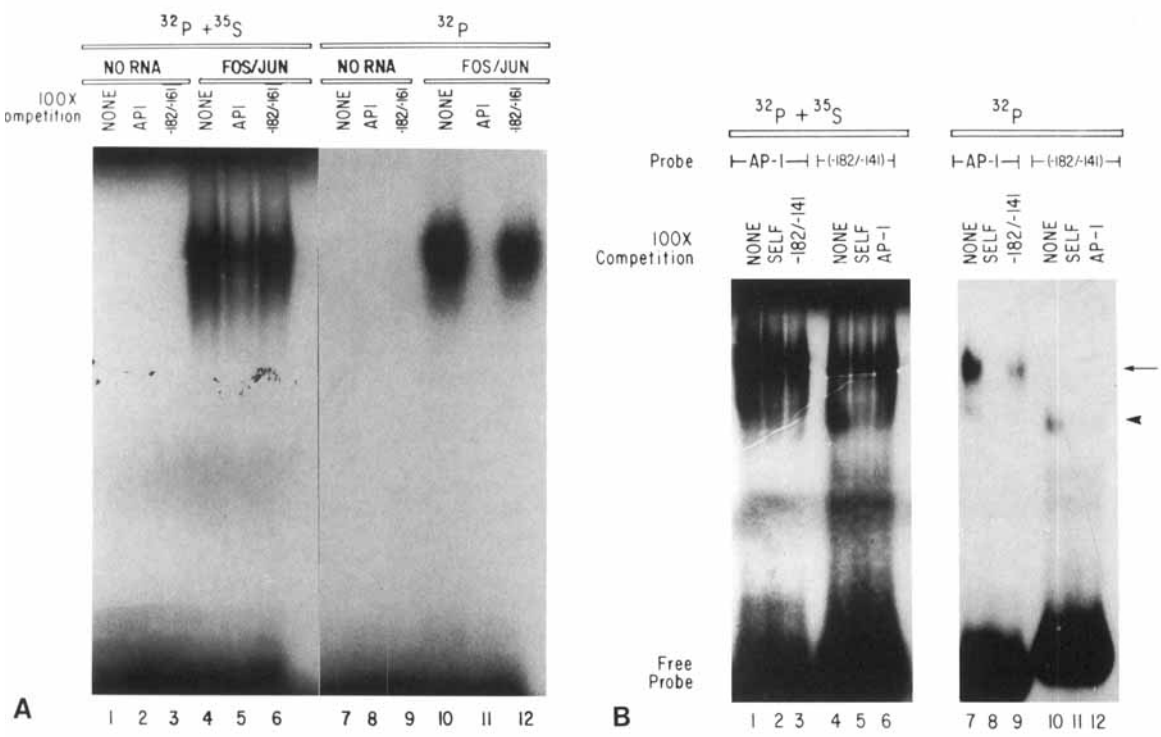


Fig. 5. Gel shift analysis with in vitro translated c-Fos and c-Jun proteins and the AP-1 and −182/−141 sequences of the collagenase promoter **A:** In vitro translated c-Fos/c-Jun heterodimer binds to the rabbit collagenase AP-1 sequence Unprogrammed (NO RNA) or programmed (FOS/JUN) rabbit reticulocyte lysates were incubated with ^{32}P-labeled oligonucleotide probe AP-1 Unlabeled competitor oligonucleotide sequences were used at 100-fold molar excess **B:** In vitro translated c-Fos/c-Jun heterodimer binds to the AP-1 sequence and to the rabbit collagenase region from −182 to −141 The positions of the c-Fos/c-Jun AP-1 probe complex (arrow) and the c-Fos/c-Jun −182/−141 probe complex (arrowhead) are shown Competition analysis used 100-fold molar excess of either unlabeled self- or cross-competitors

sive region of the collagenase gene from −182 to −141.

Deletional Mutation of the Collagenase Promoter From −182 to −161 Results in a Substantial Loss of Phorbol Ester Responsiveness

To examine the function of the nucleotide sequences implicated by mobility shift and footprinting analyses, several substitution and deletion mutations were generated in the 380 bp fragment of the collagenase 5′ flanking sequence (see Fig. 1B). The basal levels of CAT activity in cells transfected with substitution mutations altering the apparent AP-1 half-site (pCCATmATGA), or an upstream site protected by c-Jun (pCCATmTATTAA), and with the deletion mutant PCCATΔ−182/−162 were equivalent to the wild type pCCAT380 (Fig. 7A). However, the deletion mutant pCCATΔ−161/−141 elevated basal levels of CAT activity 6-fold over the wild-type, and this finding suggests the presence of an inhibitory element in this region of the wild-type promoter.

Figure 7B demonstrates the ability of these mutant constructs to increase expression of CAT activity upon treatment with phorbol esters. Relative to wild-type activity (100%, Fig. 2), neither the deletion mutant pCCATΔ−161/−141 nor the two substitution mutants, pCCATmATGA or pCCATmTATTAA, showed any change in PMA inducibility. However, the 21-bp internal deletion of the region from −182 to −162 (PCCATΔ−182/−162) showed an 80% loss of phorbol responsiveness, thus confirming the presence of sequences within this region that enhance transcription by PMA.

DISCUSSION

Our earlier studies suggested the presence of phorbol-responsive sequences between −182 to −141 of the rabbit collagenase promoter [Auble and Brinckerhoff, 1991], and our work reported here demonstrates that this region contains a novel element, to which members of the Fos and Jun families of protooncogenes bind. An internal deletion from −182 to −141 in a 380-bp fragment of the collagenase promoter confirmed

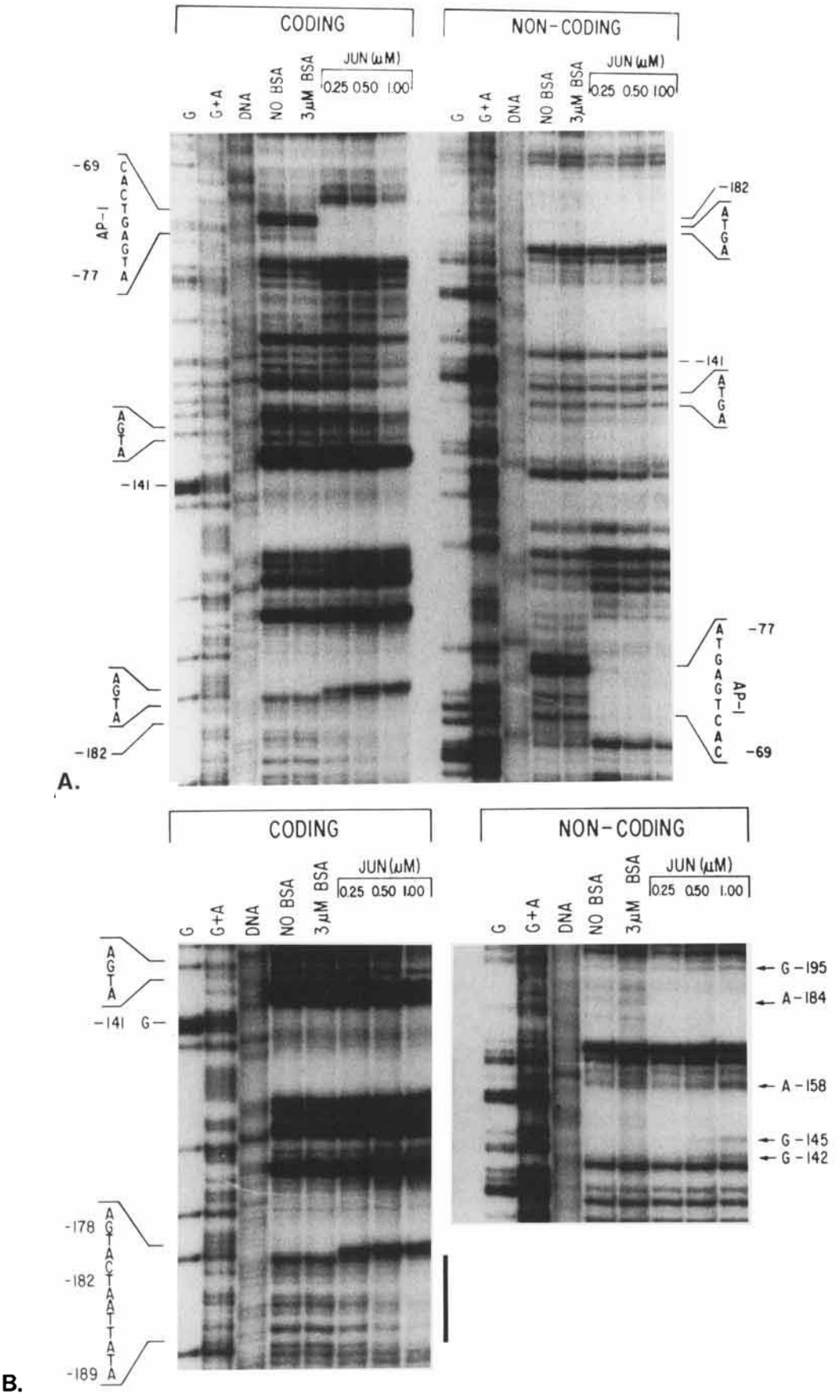

A.
CODING
NON-CODING
G
G+A
DNA
NO BSA
3μM BSA
JUN (μM)
0.25 0.50 1.00
-69
AP-1
CACTGAGTA
-77
AGTA
-141
AGTA
-182
-182
ATGA
--141
ATGA
ATGAGTCAC
-77
AP-1
-69
B.
CODING
NON-CODING
AGTA
-141 G
AGTACTAATTATA
-178
-182
-189
G -195
A -184
A -158
G -145
G -142

Figure 6

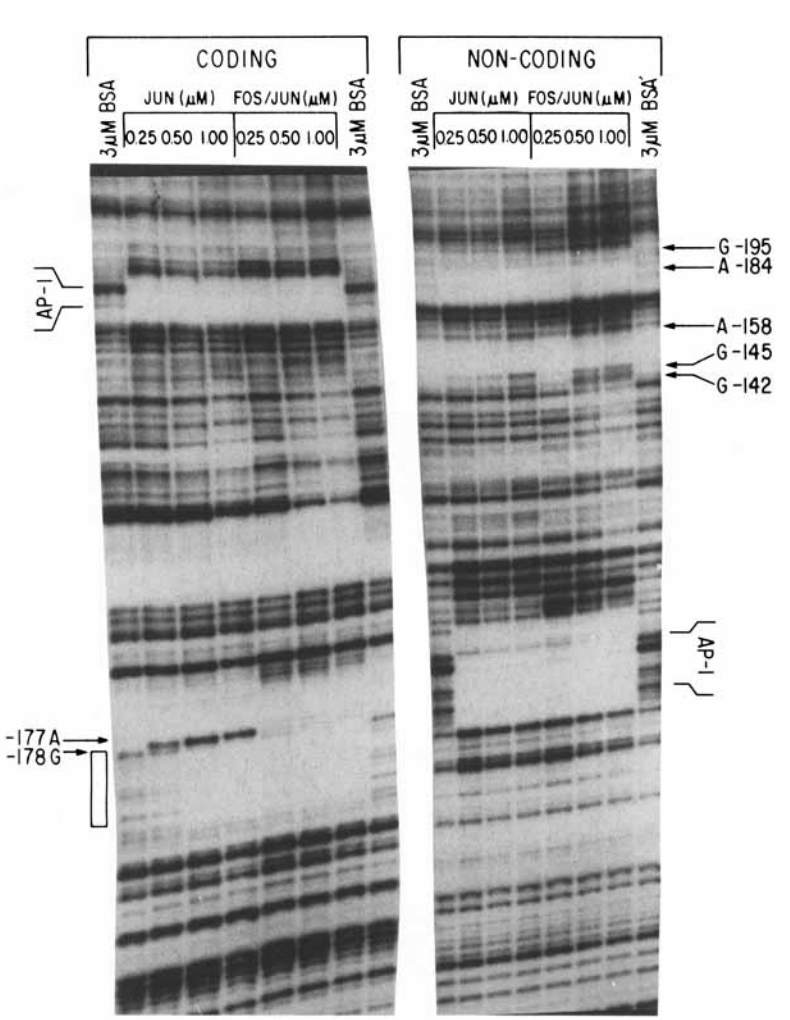


Fig. 6. Binding of purified c-Fos and c-Jun to the rabbit collagenase promoter region DNAase I footprinting assays were performed on the 380-bp HindIII fragment end-labeled on either the coding or non-coding strand with purified recombinant c-Jun alone or c-Fos and c-Jun The rabbit collagenase promoter contains an AP-1 sequence located at −69 to −77 as shown on the coding and non-coding strands Two ATGA motifs are shown located at −180 to −177 and −125 to −122 Each reaction mixture contained 10,000 cpm of ^{32}P-end-labeled probe, 3 μM BSA (except where indicated), and the indicated amounts of either c-Jun of c-Fos DNAase I was added to the mixture and the partially cleaved 380-bp collagenase fragment was electrophoresed through an 8% polyacrylamide-7 M urea sequencing gel The addition of BSA did not alter the pattern of DNAase I cleavage Numbered, arrowed bases in panels B and C indicate sites protected from or hypersensitive to DNAase I treatment upon binding by c-Fos and/or c-Jun **A:** Footprinting assays with various amounts of c-Jun G and G + A represent chemical sequencing tracks DNA, purified template not subjected to DNAase I treatment **B:** Detail inset of panel A of the region centered on −182 to −141 upon binding by c-Jun Bar indicates the −189/−178 region protected on coding strand by purified c-Jun **C:** Binding with various amounts of c-Jun homodimer or c-Fos/c-Jun heterodimer to the collagenase promoter region The rectangle indicates the region protected by c-Jun as in panel B

the importance of sequences within this region in mediating phorbol inducibility. Furthermore, gel shift analysis localized the PMA-responsive region to 20-bp extending from −182 to −161. Competition experiments with an AP-1 oligomer and nuclear extracts from PMA-treated cells suggested that Fos- and Jun-like proteins bound to this region. Gel shift and DNAase I footprinting analyses with recombinant c-Fos and c-Jun provided confirmatory data.

Our footprinting results show differences in protection of the region around −182 depending on whether c-Jun alone or c-Fos/c-Jun were assayed. These differences in DNAase I cleavage patterns are potentially due to differences in bending conformations of the DNA template upon binding of either the Fos/Jun heterodimer or the Jun/Jun homodimer, as has been suggested [Kerppola and Curran, 1991]. However, because this region also binds Fos- and Jun-like

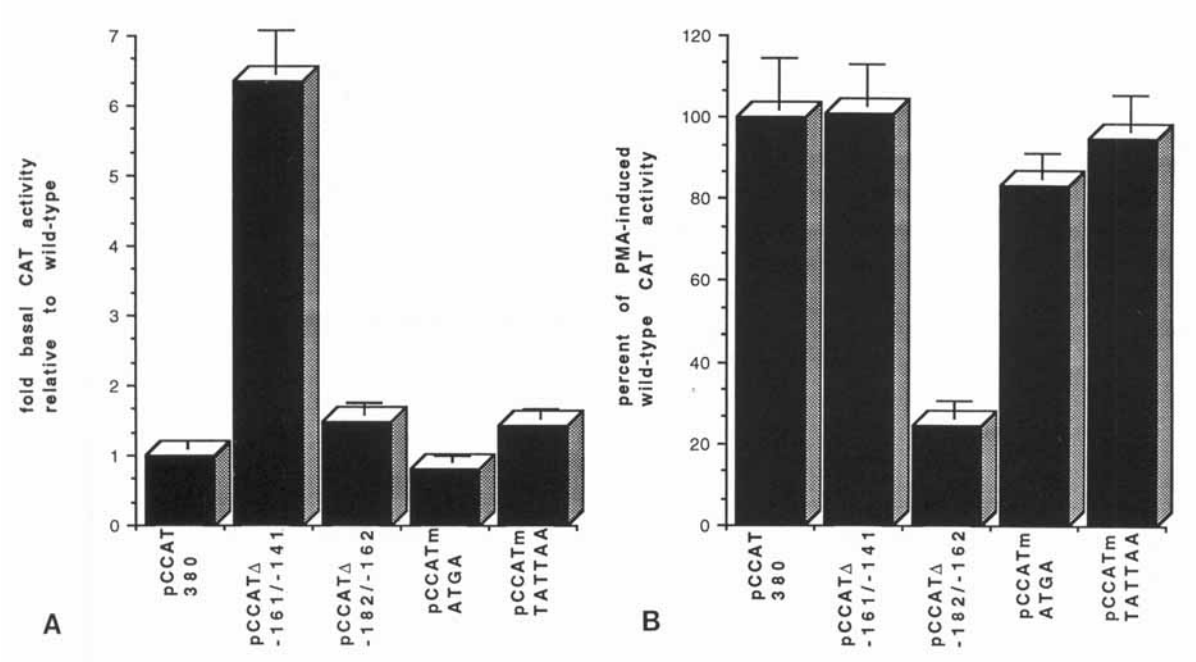


Fig. 7. PMA induction and basal CAT expression of site-directed mutations in the phorbol responsive region of the collagenase promoter Rabbit fibroblasts were transiently transfected with mutant collagenase-CAT constructs as in Figure 1B and were assayed for CAT activity after no treatment or treated with 10^{-8} M PMA for 24 h in serum-free medium Each plasmid was transfected at least four times in triplicate and CAT activity was normalized to total protein of transfected cell lysates Error bars indicate 1 standard deviation **A:** Basal CAT activity of wild-type (2% ± 0 2% ^{14}C-chloramphenicol acetylation) and mutant collagenase constructs **B:** PMA-inducible CAT activity of mutant promoter constructs relative to the PMA inducibility of the wild-type promoter (pCCAT 380) Percentage ^{14}C-chloramphenicol acetylation of PMA-treated vs untreated lysates was used to calculate fold of phorbol ester inducibility for each CAT construct Fold-induction for each mutant is expressed as a percentage of the wild-type PMA-induced activity (100%)

proteins, it is possible that it contains an AP-1-like element. Indeed, a computer search of the Transcription Factors Database [Ghosh, 1991, 1992] revealed at −186 to −180 the presence of the sequence 5′-TTAATCA-3′ which is identical in 5 of 7 positions to the traditional AP-1 consensus sequence. Upon transfection of two constructs containing mutations in this region, we found phorbol responsiveness equivalent to the wild-type. We conclude that either these particular mutations did not prevent the binding of Fos- and/or Jun-like factors [Risse et al., 1989] or that these sequences are not absolutely required for promoter activation. Indeed, little difference in phorbol inducibility was observed previously between constructs containing either 380-bp or 240-bp of collagenase 5′ flanking DNA and this latter construct did not contain sequences 5′ of −182 [Auble and Brinckerhoff, 1991].

Previous footprinting studies of the human collagenase promoter both in vitro and in vivo revealed protection of a region known as "Box 5" [Angel et al., 1987b; Konig et al., 1992] which corresponds to sequences located within the region from −182 to −161 of the rabbit gene. This region exhibits 95% nucleotide sequence identity between promoters (Fig. 1A). While these protected sequences in the human gene coincide with PMA-responsive sequences we have identified in the rabbit gene, they do not correspond completely to the area of protection we visualized with purified c-Fos and c-Jun. This difference in protection and the results of cross-competition experiments (Fig. 4) suggests that proteins in addition to those related to Fos and Jun may be interacting with the −182 to −161 sequence. The possibility that these factors share sequence similarities with the DNA binding domains of Fos and Jun cannot be ruled out. Alternatively, the affinity of Fos- and Jun-like proteins for sequences within this region may be somewhat less than for the traditional AP-1 sequence, since the cross-competitions revealed that AP-1 oligomers could compete with −182/−161 probe but that the reverse was not true.

Some of these factors binding to the −182 to −161 PMA-response region may be other members of the Fos and Jun families [Verma et al., 1990; Herdegen et al., 1991; Ryseck and Bravo,

1991]. Jun-B and Jun-D have extensive amino acid sequence similarity to the DNA binding domain of c-Jun [Ryder et al., 1988] and form homodimers and heterodimers with each other and with Fos [Nakabeppu et al., 1988]. The relative binding of various mutant AP-1 sites to c-Jun, Jun-B, or Jun-D was similar, with or without Fos, and it has been proposed that differences either in binding or biologic activity between Jun proteins are likely due to interaction with non-Jun components of the transcription machinery [Nakabeppu et al., 1988].

The classical AP-1 sequence has been well documented for the important role it plays in enhancing transcription in a wide variety of genes [Lee et al., 1987; Angel et al., 1987a,b; Curran and Franza, 1988]. Indeed, its presence in the genes for the metalloproteinases stromelysin and collagenase has allowed these promoters to be used as model systems in which to study the role of AP-1 transactivation [Angel et al., 1987a,b; Buttice et al., 1991; Conca et al., 1991; Sirum and Brinckerhoff, 1991]. However, it has become increasingly clear that the AP-1 sequence, although important and even essential, is not the only contributor. In normal fibroblasts, the AP-1 sequence in the collagenase promoter requires cooperation with two other elements all located within 127-bp of 5′ flanking DNA [Auble and Brinckerhoff, 1991]. In addition, Buttice et al. [1991], working with 1,300-bp of the human stromelysin promoter, showed that a single point mutation in the AP-1 sequence could abolish basal levels of transcription but did not reduce phorbol ester responsiveness, suggesting a role for upstream elements.

The discovery of a second putative AP-1 site in the collagenase promoter that is functionally silent during the PMA response in Hela and other tumorigenic cells [Angel et al., 1987b] but that is active in fibroblasts is significant. Our previous work clearly showed that the AP-1 site was, alone, insufficient for induction of collagenase transcription by phorbol esters [Auble and Brinckerhoff, 1991]. Yet work of other investigators concluded that the AP-1 site was sufficient to confer the phorbol ester response. The different requirements for the PMA response between cells of various origins and phenotypes underscore the value of determining the mechanisms that regulate transcription in a tissue-specific manner.

In summary, we have localized a novel region of the collagenase promoter that is transcriptionally responsive to induction by phorbol esters. We propose that an AP-1-like site located adjacent to −182 and −161 of the collagenase promoter binds Fos- and Jun-like proteins and, in conjunction with additional factors, contributes to transactivation of collagenase gene expression. Possibly, this upstream AP-1-like sequence represents one in a series of cis-elements throughout the promoter that can respond to an assortment of transcription factors and modulate transcriptional rates under a variety of physiologic and pathologic conditions.

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