Transcriptional Induction of Collagenase-1 in Differentiated Monocyte-like (U937) Cells is Regulated by AP-1 and an Upstream C/EBP-β Site*

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Glenn A. R. Doyle‡, Richard A. Pierce, and William C. Parks¶

From the Dermatology Division, Department of Medicine, and §Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

In this report, we demonstrate that the AP-1 site and a distal promoter element regulate transcriptional induction of collagenase-1 during monocyctic differentiation. Chloramphenicol acetyltransferase expression constructs containing regions of the human collagenase-1 promoter were stably or transiently transfected into U937 cells, and reporter activity was assessed at various times after the onset of phorbol 12-myristate 13-acetate (PMA)-mediated differentiation. Rapid and strong induction of promoter activity was lost in cells subjected to a mutant AP-1 element; however, at 16–48 h post-PMA, the mutant collagenase-1 promoter displayed AP-1 independent PMA-mediated transactivation. The AP-1 mutant constructs also showed delayed transcriptional activation in PMA-treated fibroblasts. Western and supershift analyses indicated that functional Jun and Fos proteins were present in nuclear extracts of PMA-differentiated U937 cells. Promoter deletion constructs demonstrated the potential role of distal promoter sequences in regulating collagenase-1 transcription. In particular, Western, supershift, and promoter deletion analyses suggested a role for CCAAT/enhancer-binding protein-β (C/EBP-β) binding site between −2010 and −1954 in regulating transcription of collagenase-1 in monocyctic cells. Our findings suggest that distinct regulatory elements, acting somewhat independently of each other, control expression of collagenase-1. In addition, our data suggests that the rapid PMA-mediated induction of collagenase-1 transcription is controlled by a mechanism distinct from that regulating the sustained expression of this proteinase in activated macrophages.

Remodeling of the extracellular matrix during normal development and in response to tissue injury and inflammation is thought to be accomplished, in part, by the properly regulated production of matrix metalloproteinases (MMPs). As a group, these enzymes can degrade essentially all extracellular matrix components, and hence, they have been implicated in normal remodeling processes, such as uterine involution, blastocyst implantation, angiogenesis, and wound healing (for review, see Refs. 1–3). However, inappropriate expression of these proteinases is thought to contribute to the pathogenesis of various conditions, such as arthritis (4, 5), vascular disease (6–8), metastasis (3), and destructive skin diseases (9). Notably, collagenase-1 has been localized to resident and infiltrating inflammatory cells in many of these conditions (5, 7, 10–12).

Although extracellular matrix proteins can be degraded by various proteinases, fibrillar type I collagen, the most abundant protein in the body, is resistant to degradation by most enzymes. Collagen degradation is initiated by the catalytic activity of collagenases, a subgroup of the MMP gene family with the unique ability to cleave fibrillar collagens type I, II, and III within their triple helical domain (13). At physiological temperature, cleaved collagen molecules denature and become susceptible to complete digestion by other proteinases. Of the three known human metallo-collagenases, collagenase-1 (MMP-1) seems to be the enzyme that is principally responsible for collagen turnover in most human tissues. In a variety of normal and disease-associated tissue remodeling events, collagenase-1 is expressed by macrophages as well as by epithelial cells, fibroblasts, endothelial cells, and chondrocytes (14–18).

Collagenase-2 (MMP-8) is found only in neutrophils and chondrocytes (19, 20), and collagenase-3 (MMP-13), originally cloned from a breast carcinoma line (21), is also expressed in articular cartilage (22, 23) and developing bone (24).

Many agents, such as PMA, bacterial endotoxin (lipopolysaccharide), and proinflammatory cytokines, and events, such as contact with type I collagen and activated T-cells, induce or markedly stimulate collagenase-1 transcription in macrophages (25–28). Much of what is known about the transcriptional regulation of collagenase-1 points to a critical role for the AP-1 site at −72 to −66 in the human promoter. AP-1 elements bind dimers of the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) families of transcription factors (29). Angel et al. (30) first demonstrated that the AP-1 site is necessary and sufficient to confer PMA-mediated induction of the native collagenase-1 promoter or of a heterologous promoter containing this element. However, because the level of PMA-mediated induction was greater with larger collagenase-1 promoter constructs, they concluded that elements upstream of the AP-1 site might also be important in regulating collagenase-1. Indeed, the AP-1 site, the polypoma enhancer A-binding protein-3 site (−91 to −83) and the “TTCA” element (−105 to −102) are also required for full PMA-mediated induction in fibroblasts (31, 32).

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‡ Current address: Dept. of Oncology, McArdle Laboratories for Cancer Research, University of Wisconsin, Madison, WI 53706-1599. Tel.: 608-263-4767; Fax: 608-262-2824; E-mail: doyle@oncology.wisc.edu.

¶ To whom correspondence should be addressed: Div. of Dermatology, Jewish Hospital, 216 S. Kingshighway, St. Louis, MO 63110. Tel.: 314-454-7543; Fax: 314-454-5372; E-mail: bparks@imgate.wustl.edu.

1 The abbreviations used are: MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; C/EBP-β, CCAAT/enhancer-binding protein-β; kb, kilobase(s); TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; CAPS, 3-cyclohexylamino)propanesulfonic acid.
We assessed the requirement of the AP-1 site and more distal promoter sequences to collagenase-1 gene activation during subsequent to monocytic differentiation. We used PMA-promoter sequences to collagenase-1 gene activation during transcription, other upstream elements, including a though the AP-1 site is required to mediate strong collagenase-1 transcription, a 2.5-kb pair human Alu repeat fragment derived from the absence of a functional AP-1 site. We conclude that all collagenase-1 promoter activity is induced and maintained in the absence of a functional AP-1 site. We report that differentiation of monocytes into macrophages (33) and because the 94

e-globin gene (42) was blotted as well. Human collagenase-1 promoter linked to the thymidine kinase (TK) promoter of pBLCAT2. pAPCAT2a contains 3 collagenase-1 AP-1 sites in tandem upstream of the thymidine kinase promoter of pBLCAT2.

EXPERIMENTAL PROCEDURES

Cell Culture—U937 cells (35) were obtained from the American Type Culture Collection (CRL 1593) and maintained in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% low endotoxin fetal calf serum (Life Technologies, Inc.), non-essential amino acids, L-glutamine, sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. For induction of cell differentiation, U937 cells were plated at 5 × 10^5 cells/ml and exposed to 8 × 10^{-8} M PMA (Sigma). Human skin fibroblasts were grown in Dulbecco's modified Eagle's medium, 10% fetal calf serum (Life Technologies, Inc.) containing the same supplements listed above.

RNA and Transcription Analyses—Total RNA was isolated by the guanidinium phenol extraction method (36). Conditions for Northern hybridization and washes were as described (27). Blots were hybridized with the wild-type AP-1 element.

Plasmids and Constructs—Fig. 1 shows maps of all collagenase-1 promoter constructs used in this study. pBLCAT2 contains the −105/+51 region of the herpes simplex virus thymidine kinase (TK) promoter fused to a CAT reporter gene (43). pAPCAT2a is derived from pBLCAT2, and contains a tandem triplet of the collagenase-1 AP-1 site subcloned 5' of the TK promoter. A plasmid containing the −2278/+36 region of the human collagenase-1 promoter (44) was generously provided by Dr. Steven Frisch (La Jolla Cancer Research Foundation, La Jolla, CA). To eliminate the possibility of transcriptional differences due to the vector backbone, all collagenase-1 promoter sequences were subcloned into pBLCAT2. The TK promoter of pBLCAT2 was removed during the synthesis of the collagenase-1 promoter deletion constructs (Fig. 1A). The p-2278CAT, p-2278MCAT, p-511CAT, p-179CAT, p-59CAT, and p-72CAT vectors were generated by PCR as described (26). Constructs p-2010CAT, p-1954CAT, p-1689CAT, p-1552CAT, p-1197CAT, and p-997CAT were made by digestion of p-2278CAT with BsaHI, HpaI, XmnI, EcoRV, BglII, or BamHI, respectively. The digestion products were blunted (when necessary) with the Klenow fragment of DNA polymerase, further digested with XhoI, and the appropriate blunt end XhoI fragment was subcloned into blunt HindIII/XhoI digested pBLCAT2.

The internal deletion construct, p-1197A-997CAT, was created by cutting p-2278CAT with BglII and BamHI followed by ligation of the vector. Internal deletion construct p-2010A-1954CAT was created by recombinant, whole plasmid PCR (45) using the 5' (ATAGcactgACCCCT-GGAAGAGTCTCAT) and the 3' (CGGcagctgCAATTAACCTCCT- GTG) primers (deleted/mutated sequences in lowercase). The PCR product was digested with SphI and ligated. The resultant construct was digested with HindIII and BamHI, and the HindIII/BamHI fragment was subcloned into HindIII/BamHI cut p-2278CAT. All PCR was performed with either Vent™ or Deep Vent™ DNA polymerase (New England Biolabs, Beverly, MA) to minimize unwanted mutations. All newly created plasmids were sequenced to verify that only the desired alterations were introduced during PCR steps. Sequencing reactions were done using a Sequenase™ Kit (U. S. Biochemical Corp., Cleveland, OH).

Heterologous promoter constructs contain collagenase-1 promoter sequences upstream of −997 linked to the TK promoter. These were constructed by subcloning the BamHI/XhoI TK promoter fragment of pBLCAT2 into deletion constructs that had been digested with BamHI and XhoI. For example, p-2278CAT gives rise to p-2278TKCAT, in which −2278 is the most 5' and −997 (at the BamHI site) is the most 3' nucleotide of the collagenase-1 promoter fragment (Fig. 1B).

Stable U937 Transfectants—U937 cells (10^5 in 0.5 ml) were transfected with 5 μg of linearized pRSV-Neo and 50 μg of linearized pBLCAT2, pAPCAT2a, p-72CAT, p-511CAT, p-2278CAT, or p-2278MCAT. Cells were electroporated at 250 V and 600 microfarads in a 0.4-cm gap cuvette using a BTX 3000 electroporator (Biotechnologies and Experimental Research, Inc., San Diego, CA). Place on ice for 10 min, added to 9 ml of culture medium, centrifuged to pellet the cells, and plated in 10 ml of fresh medium. After 24 h, cells were shifted to medium supplemented with 400 μg/ml Geneticin® (Life Technologies, Inc.). After 2 weeks, G418-resistant cells were subcloned by limiting dilution or maintained as a pooled population of clones in medium containing 200 μg/ml Geneticin®. To minimize insertion effects, two groups of stable clones, consisting of 6 clones and the other of 12
clones, were pooled. Southern hybridization with $^{32}$P-labeled CAT cDNA was done on individual clones and demonstrated that incorporated DNA was roughly equivalent among clones (data not shown).

**Transient Transfections**—U937 cells were transfected by a modification of the DEAE-dextran method essentially as described (26, 46). Human skin fibroblasts were transiently transfected by calcium phosphate precipitation. After transfection, cells were allowed to recover for 24 h prior to treatment with PMA. After recovery, cultures were divided equally and cells were plated in medium with or without PMA. After transfection, cells were given fresh medium and allowed to recover for 24 h prior to treatment with PMA. Hirt extraction (47) and Southern hybridization with $^{32}$P-labeled CAT cDNA was done to determine transfection efficiency.

**CAT Assays**—At the indicated times, cells were harvested, washed, and lysed in 200 µl of 250 mM Tris, pH 7.8, by freeze-thawing. Lysates were incubated at 65 °C for 5 min and then cleared of debris by centrifugation. Equivalent amounts (25–100 µg) of cleared lysate, normalized to total protein (Bradford protein assay; Bio-Rad), were assayed for CAT activity using acetyl-CoA (Sigma) and [14C]chloramphenicol essentially as described (48). Reaction products were separated by thin layer chromatography and visualized by autoradiography. Results were quantified by cutting out the appropriate spots from the chromatography plate. Relative induction was obtained by dividing percent acetylation of treated versus untreated samples.

**Electrophoretic Mobility Shift and Supershift Assays**—Nuclear extracts were prepared by the method of Dignam et al. (49). The integrity of all nuclear extract preparations was assessed by determining the ability of proteins to bind a radiolabeled Oct-1 double-stranded oligomer (data not shown). Oct-1 protein binding was constitutive and, thus, served as an internal control. Only extracts without apparent protein degradation were used. For AP-1 studies, double-stranded oligomers containing either wild-type (GATCAAGACCAGGAGACACCT) or mutant (GATCAAGACCAGGAGACACCT) human collagenase-1 promoter sequence were used as probes and competitors. For the binding mutant (GATCAAAGCAcccgggAGACACCT) human collagenase-1 promoter sequence was demonstrated (26). Equivalent amounts of nuclear protein (5 µg) and probe counts were used in all reactions.

**Western Analysis of Nuclear Proteins**—Nuclear lysates. The results shown are representative of four separate experiments. A similar time course for post-PMA nuclear run-off assays demonstrated that collagenase-1 transcription was detectable at 12 h of PMA differentiation and remained at a constant level thereafter (Fig. 2B). The mRNA levels were increased, and maximal and sustained levels of CAT activity were achieved by 8 h post-PMA. Consistent results were obtained in four separate experiments. A similar time course for induction of collagenase-1 promoter activity was observed in stable transformants (Figs. 3, −2278). Full induction of the wild-type collagenase-1 promoter was detected at 6 h after PMA treatment, and the levels were maintained for up to 96 h (Figs. 2C and D). These data indicate that events necessary for maximal induction of collagenase-1 are activated within 4–6 h post-PMA. Nuclear run-off assays demonstrated that collagenase-1 transcription was detectable at 12 h of PMA differentiation and remained at a constant level thereafter (Fig. 2B). These observations demonstrate that the onset of collagenase-1 induction occurs earlier than reported previously (34). CAT activity conferred by the full-length collagenase-1 promoter construct (p-2278CAT) in transiently transfected, PMA-treated U937 cells paralleled the pattern of induction of the endogenous gene (Fig. 2C). Only slight background CAT activity was seen in untreated cells. By 4 h post-PMA, promoter activity was increased, and maximal and sustained levels of CAT activity were achieved by 8 h post-PMA. Consistent results were obtained in four separate experiments. A similar time course for induction of collagenase-1 promoter activity was observed in stable transformants (Fig. 3, −2278). Full induction of the wild-type collagenase-1 promoter was detected at 6 h after PMA treatment, and the levels were maintained for up to 96 h (Figs. 2C and D). These data indicate that events necessary for maximal induction of collagenase-1 are activated within 4–6 h post-PMA.
not bind nuclear proteins (data not shown). In two groups of pooled clones, mutation of the AP-1 site (p-2278MCAT) eliminated the rapid (i.e. by 4–6 h) and strong transactivation observed with the wild-type construct (p-2278CAT) (Fig. 3). Between 0 and 8 h post-PMA, no CAT activity was detected in U937 cells stably transfected with the mutant AP-1 construct (data not shown). However, transcriptional induction of the mutant AP-1 collagenase-1 promoter was consistently detected at 16 h post-PMA (Fig. 3, −2278M). Although CAT activity expressed by −2278MCAT was much lower than that conferred by the wild-type promoter, the level of CAT activity was maintained for up to 96 h after PMA differentiation, similar to the sustained activity from the wild-type promoter (Fig. 3). Experiments with individual stable clones showed the same patterns of induction with both the wild-type and mutant promoters (data not shown). Southern hybridization demonstrated that incorporated DNA was roughly equivalent among stable lines (data not shown).

AP-1 Independent Induction of the Collagenase-1 Promoter Is Not Restricted to Monocytic Cells—PMA treatment stimulated activation of the wild-type collagenase-1 promoter in human skin fibroblasts (Fig. 4, −2278). Basal activity of the wild-type collagenase-1 promoter was high in these cells, likely due to constitutive c-Jun expression (data not shown), but at 8 and 24 h post-PMA, CAT activity increased. Mutation of the AP-1 site eliminated the high basal activity seen with the wild-type collagenase-1 promoter (Fig. 4, −2278M). However, similar to that observed in differentiated U937 cells, p-2278MCAT conferred transcriptional induction in transiently transfected fibroblasts at 8 and 24 h post-PMA (Fig. 4, −2278M). Thus, the collagenase-1 promoter can be transcriptionally induced by PMA in the absence of a functional proximal AP-1 element. Time matched controls incubated without PMA had the same level of CAT activity for p-2278CAT or p-2278MCAT as did the 0 h cells (data not shown).

c-Jun and c-Fos Are Present in Early and Late U937 Nuclear Extracts—The kinetics of c-fos and c-jun expression were assessed by Northern analysis (data not shown). In contrast to the delayed kinetics of collagenase-1 induction (Fig. 2) and in full agreement with data from others (50–52), c-fos and c-jun transcripts were detected as early as 15 min post-PMA, peaked between 1 and 2 h after PMA addition, and were sustained at low levels over the next 48 h (data not shown). Because c-Fos protein expression may not correlate with expression of its mRNA in U937 cells (50) and because its subcellular localization is regulated (53), we used an immunoblotting assay to detect Fos family proteins in nuclear extracts from untreated and PMA-differentiated U937 cells. c-Fos protein was detected in both 1- and 24-h post-PMA nuclear extracts using pan-Fos or c-Fos-specific antibodies (Fig. 5). The upward shift in the c-Fos band seen in the 24-h extract may be due to increased protein phosphorylation (54). While the presence of c-Fos in nuclear extracts at 1 h post-PMA was anticipated, the clear abundance of c-Fos protein in the 24-h extract was not. A previous report indicated that c-Fos protein could not be detected in PMA-differentiated U937 cells after 2 h of treatment (50). Because these authors immunoprecipitated metabolically-labeled protein from whole cell extracts, they may have underestimated c-Fos protein levels during periods of low c-Fos protein synthesis. We detected no FosB protein by immunoblotting nuclear extracts from untreated or PMA-treated U937 cells with a FosB-specific antibody (data not shown). Proteins distinct from c-Fos were detected by the pan-Fos antibody in the nuclear extract from untreated cells, but not in those from PMA-treated cells. The identity of these proteins is uncertain, but their sizes are consistent with the Fos-related proteins Fra-1 (29.4 kDa) and Fra-2 (35.2 kDa) which are expressed in U937 cells (55).

Regardless of the identity of the bands in basal cell extracts, they may have underestimated c-Fos protein levels during periods of low c-Fos protein synthesis. We detected no FosB protein by immunoblotting nuclear extracts from untreated or PMA-treated U937 cells with a FosB-specific antibody (data not shown). Proteins distinct from c-Fos were detected by the pan-Fos antibody in the nuclear extract from untreated cells, but not in those from PMA-treated cells. The identity of these proteins is uncertain, but their sizes are consistent with the Fos-related proteins Fra-1 (29.4 kDa) and Fra-2 (35.2 kDa) which are expressed in U937 cells (55).

We also used the immunoblotting assay to detect Jun family proteins in nuclear extracts from untreated and PMA-differentiated U937 cells. Using a c-Jun-specific antibody, c-Jun pro-
Because the mutant AP-1 collagenase-1 promoter was induced by PMA, we assessed the influence of regions upstream of the proximal AP-1 site during collagenase-1 induction in U937 cells. Cells were transfected with the various promoter constructs, and CAT activity was assessed 24 h after addition of PMA. Southern hybridization of Hirt extracted DNA indicated equivalent transfection efficiency among constructs and that the level of plasmid DNA remained constant after addition of PMA (Fig. 5). The high molecular weight bands detected in all extracts may be due to ubiquitination (56) or altered phosphorylation of c-Jun (57). Because c-Jun is not expressed in basal U937 cells, the low molecular mass bands between 30 and 20 kDa seen in all samples are likely nonspecific products. No additional bands were detected with the pan-Jun or JunB antibodies (data not shown).

Nuclear Proteins c-Fos and c-Jun Bind the Collagenase-1 AP-1 Site—Electrophoretic mobility shift and supershift assays were done to confirm the presence of active AP-1 binding Jun/Fos dimers. Nuclear extracts from untreated cells did not support binding to a double-stranded oligomer containing the native collagenase-1 AP-1 site, where as extracts from 4, 8, and 24 h PMA-treated cells exhibited strong binding activity (Fig. 6). The binding activity was competed by excess unlabeled wild-type AP-1 oligomer (Fig. 6, 24C) but not by excess oligomer containing the mutated AP-1 site or by an unrelated sequence (data not shown). In addition, radiolabeled, double-stranded mutant AP-1 oligomers showed no binding to nuclear proteins (data not shown).

Supershift analysis demonstrated that JunD and c-Fos were present in the shifted complexes (Fig. 6). We consistently detected a weak supershifted complex with the c-Jun antibody and a very weak, if any, complex for JunB. Neither the relative amounts of shifted complexes nor their composition changed at any time after the onset of PMA differentiation. A FosB-specific antibody did not supershift complexes formed in extracts from PMA-treated cells (data not shown). The lack of other Fos family proteins agrees with our immunoblotting data (Fig. 5). Although we cannot definitively determine the identity of the Jun component, these results suggest that heterodimers of c-Fos and Jun family proteins contribute to maximal collagenase-1 transcriptional induction at early and late times of U937 differentiation.

Transient Transfection of Collagenase-1 Promoter Constructs in U937 Cells—Because the mutant AP-1 collagenase-1 promoter was induced by PMA, we assessed the influence of regions upstream of the proximal AP-1 site during collagenase-1 induction in U937 cells. Cells were transfected with the various promoter constructs, and CAT activity was assessed 24 h after addition of PMA. Southern hybridization of Hirt extracted DNA indicated equivalent transfection efficiency among constructs and that the level of plasmid DNA remained constant up to 72 h after transfection (48 h post-PMA, data not shown). For presentation, we have divided the collagenase-1 promoter into upstream (−2278 to −511) and downstream (−511 to +36) regions.

Relative to p-2278CAT (number 1), induction of construct p-511CAT (number 11) was reduced by 50% in response to PMA differentiation (Fig. 7), and p-511CAT had similar activity in PMA-treated stable transformants (data not shown). Deletion of sequences between −511 and −179 (p-179CAT, number 12) resulted in no further decrease in PMA responsiveness relative to p-511CAT (number 11). However, PMA responsiveness was further reduced when sequences between −179 and −95 were deleted (−95CAT, number 13). Weak, yet reproducible transcriptional induction was observed with the smallest AP-1 containing collagenase-1 promoter construct, p-72CAT (number 14). CAT activity from this construct was also stimulated to a similar degree in PMA-treated stably transfected U937 cells (data not shown).

The AP-1 site, in the absence of upstream sequences, was sufficient for minimal PMA responsiveness. However, because the level of induction observed with p-72CAT (number 14) is extremely low compared with most other constructs, other upstream elements are needed for full activation of collagenase-1 transcription by PMA differentiation. Although mutation of the AP-1 site, in the context of the full-length promoter (p-2278MCAT, number 2) resulted in a loss of detectable PMA responsiveness in transiently transfected U937 cells, this construct was induced in stably transfected cells (−2278M; Fig. 3). Hirt extraction (47) verified that p-2278MCAT entered transiently transfected cells with the same efficiency as other constructs (data not shown). The seemingly contradictory transfection data with construct p-2278MCAT (Figs. 3 and 7) can be reconciled by the greater sensitivity inherent in the use of stable lines versus transient transfections.

In constructs containing the wild-type AP-1 element, deletion of sequences −2278 to −997 (p-997CAT, number 9) diminished both baseline and PMA-induced transcription (Fig. 7), and the fold induction was decreased about 2-fold relative to the wild type (p-2278CAT, number 1) construct (Fig. 7B). Deletion of sequences between −2278 and −2010 (p-2010CAT, number 3) and between −1197 and −997 (p-1197Δ-997CAT, number 10) had only a minor effect on promoter activity in basal U937 cells or induction in PMA-differentiated cells (Fig. 7). In contrast, promoter activity was markedly reduced and fold induction decreased relative to p-2278CAT (number 1) upon deletion of sequences between −2010 and −1954 from the collagenase-1 promoter (p-1954CAT to p-997CAT, and p-2010Δ-1954CAT, numbers 4–9).

To further characterize the upstream regions of the collagenase-1 promoter (−2278 to −997), we constructed a series of heterologous promoter constructs containing various fragments of the distal collagenase-1 promoter linked to the TK promoter of pBLCAT2 (Fig. 1B). CAT activity from the TK promoter of pBLCAT2 was not changed by PMA treatment of U937 cells (Figs. 7 and 8). Based on the fold induction observed with p-2278MCAT (number 2), p-1954MCAT (number 4), and p-2010Δ-1954CAT (numbers 4–9) on the basis of fold induction, we have focused on the promoter construct p-2278MCAT, which confers the greatest PMA responsiveness. This construct was induced to a similar degree in PMA-treated stable transformants (data not shown).

C/EBP-β Is Present in Nuclear Extracts of U937 Cells and Interacts with Collagenase-1 Promoter Sequences between −2010 and −1954—The data with the mutant AP-1 construct (Fig. 3, −2278M) and the drop in PMA-mediated transactivation between p-2010TKCAT and p-1954TKCAT (Fig. 8) suggest the existence of functional AP-1-independent element between −2010 and −1954 of the collagenase-1 promoter. We inspected this region of the promoter for known transcription factor
DNA-binding elements. A putative C/EBP-binding site (TTAG-GCAATT) and NF-κB-like site (GGCAATTCC) were identified between 22013 and 21990. Because the C/EBP family of transcription factors can regulate cellular differentiation (58), we looked for the presence of C/EBP proteins in U937 nuclear extracts. Immunoblotting of nuclear extracts with a pan-C/EBP antibody detected only one band of about 42 kDa (Fig. 9). Detection with a specific antibody verified that this band was C/EBP-β (Fig. 9). Furthermore, these analyses demonstrated that the relative abundance of C/EBP-β in U937 nuclear extracts increased with time of PMA treatment. The C/EBP-β-specific antibody detected a doublet in which the upper band may be the phosphorylated form of the lower band (59).

To determine if C/EBP-β could bind the sequences between 22013 and 21990 of the collagenase-1 promoter, we performed gel shift and supershift analyses with a double-stranded oligomer encompassing this region (Fig. 10). Gel shift analysis demonstrated that a nuclear factor in untreated and PMA-

![Image](image-url)
C/EBP-β is in nuclear extracts of U937 cells. U937 cells were treated with 8 × 10⁻⁶ M PMA, and nuclear extracts were prepared from control cells (0) and from cells at 4, 8, and 24 h post-PMA. Proteins were separated by SDS-polyacrylamide gel electrophoresis, and the gels were processed for immunoblotting with C/EBP-β-specific or pan-C/EBP antibodies. Both antibodies detected a band at 42 kDa (arrows), consistent with the size of C/EBP-β. The numbers between the gels indicate the migration of molecular weight standards. Identical results were obtained with extracts from cells in three separate experiments.

FIG. 8. Upstream collagenase-1 promoter regions respond to PMA in the absence of the AP-1 element. Heterologous constructs containing upstream (−2278 to −997) regions of the human collagenase-1 promoter linked to the thymidine kinase promoter of pBLCAT2 (see Fig. 1). pAPCAT2a contains 3 tandem repeats of the human collagenase-1 AP-1 site upstream of the thymidine kinase promoter of the parental plasmid pBLCAT2. A, shown are representative CAT data for each heterologous construct. U937 cells were transiently transfected, and half of the cells were treated with PMA for 24 h. CAT activity in cell lysates (50 μg) was assessed for untreated (−) and PMA-treated (+) cells. All procedures and manipulations were identical for each construct series. B, the percent acetylation was determined by scintillation counting and these data are shown in the histogram. The results shown are the mean ± S.E. of at least four to six determinations for each construct.

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FIG. 9. C/EBP-β is in nuclear extracts of U937 cells. U937 cells were treated with 8 × 10⁻⁶ M PMA, and nuclear extracts were prepared from control cells (0) and from cells at 4, 8, and 24 h post-PMA. Proteins were separated by SDS-polyacrylamide gel electrophoresis, and the gels were processed for immunoblotting with C/EBP-β-specific or pan-C/EBP antibodies. Both antibodies detected a band at 42 kDa (arrows), consistent with the size of C/EBP-β. The numbers between the gels indicate the migration of molecular weight standards. Identical results were obtained with extracts from cells in three separate experiments.

DISCUSSION

Data presented here, as well as in other studies (27, 34), show that once collagenase-1 production is induced in macrophages, enzyme expression remains active for days. In this report, we characterized regions of the collagenase-1 promoter which are involved in both activation and maintenance of collagenase-1 transcription during and subsequent to U937 differentiation. Our findings, in agreement with others (30, 31), indicate that the proximal AP-1 element is necessary but not sufficient to confer maximal transcriptional activation of collagenase-1. We also report that mutation of the AP-1 element reduces and delays but does not eliminate maintained collagenase-1 promoter induction in U937 cells or fibroblasts. The sustained nature of the delayed, AP-1-independent induction of collagenase-1 suggests that the mediating factor(s) may be important in regulating maintained collagenase-1 expression by macrophages actively involved in tissue remodeling events associated with inflammation. As is discussed, C/EBP-β may mediate the AP-1-independent response and maximize AP-1-dependent responses in differentiated monocytes.
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NF-κB and c-EBPβ binding to an upstream element of the collagenase-1 promoter. Nuclear extracts were isolated from U937 cells treated with PMA for 0, 4, 8, or 24 h and incubated with a radiolabeled, double-stranded oligomer containing the collagenase-1 NF-κB and C/EBP consensus sites (region 1 in schematic). **Left panel,** free probe (FP) was shifted (S) by proteins in nuclear extracts from control (0) U937 cells, and binding activity was increased in extracts from PMA-treated cells. Binding was competed by co-incubating extracts and probe with a 50-fold molar excess of unlabeled, wild-type NF-κB consensus (W). Binding was not decreased by co-incubation with a mutant C/EBP sequence (M) or with a wild-type C/EBP consensus oligomer (not shown). Weak, nonmodulated, and no binding activity were detected with radiolabeled, double-stranded oligomers to regions 2 and 3, respectively (data not shown). **Right panel,** addition of either a pan-C/EBP or C/EBP-β-specific antibody to the binding reactions resulted in a supershift (SS) of the shifted probe (S). Identical results were obtained with a second set of extracts.

**Fig. 10.** C/EBP-β binds to an upstream element of the collagenase-1 promoter. Nuclear extracts were isolated from U937 cells treated with PMA for 0, 4, 8, or 24 h and incubated with a radiolabeled, double-stranded oligomer containing the collagenase-1 C/EBP and NF-κB-like sites (region 1 in schematic). Left panel, free probe (FP) was shifted (S) by proteins in nuclear extracts from control (0) U937 cells, and binding activity was increased in extracts from PMA-treated cells. Binding was competed by co-incubating extracts and probe with a 50-fold molar excess of unlabeled, wild-type C/EBP consensus (W). Binding was not decreased by co-incubation with a mutant C/EBP sequence (M) or with a wild-type NF-κB consensus oligomer (not shown). Weak, nonmodulated, and no binding activity were detected with radiolabeled, double-stranded oligomers to regions 2 and 3, respectively (data not shown). Right panel, addition of either a pan-C/EBP or C/EBP-β-specific antibody to the binding reactions resulted in a supershift (SS) of the shifted probe (S). Identical results were obtained with a second set of extracts.

role for JunD cannot be excluded, we suggest that c-Jun/c-Fos heterodimers are involved in induction of collagenase-1 expression in U937 cells and stimulated monocytes.

Comparable to findings in HeLa cells (30), but in contrast to those in fibroblasts (31), we found that the AP-1 element confers a minimal response to PMA in U937 cells (Fig. 7B). However, in association with the AP-1 site, the region between −179 and −95 of the collagenase-1 promoter was needed for strong PMA responsiveness in U937 cells (Fig. 7B). In fibroblasts, PMA- and oncogene-mediated transactivation of collagenase-1 is enhanced when a complete “12-O-tetradecanoylphorbol-13-acetate/oncogene responsive unit,” polyoma enhancer A-binding protein-3, and AP-1 elements in tandem, is present in promoter constructs (32). In monocytic cells, the polyoma enhancer A-binding protein-3 site (−91 to −83) may not be critical for up-regulating collagenase-1 expression since only a small difference in promoter induction is seen between constructs p-95CAT and p-72CAT, with both responses being relatively weak (1.5- to 2-fold; Fig. 7B). Indeed, other studies have shown that the 12-O-tetradecanoylphorbol-13-acetate/oncogene responsive unit is not sufficient to maximally stimulate collagenase-1 transcription in response to PMA (30, 31). In contrast to the weak induction of p-72CAT and p-95CAT, p-179CAT confers much of the PMA responsiveness (5-fold) observed in U937 cells (Fig. 7B). In the analogous region of the rabbit collagenase-1 promoter, a “TTCA” element (−105 to −100) and less characterized sequences at −182 to −161 are necessary to confer strong PMA responsiveness in fibroblasts (31, 62). There is extensive homology (95–100% identical in the areas mentioned) between the human and rabbit promoters within this downstream region. This fact, together with the decreased PMA-mediated induction observed when sequences −179 to −95 are deleted (Fig. 7B) suggests the possibility that factors binding these sequences may play some role in activating collagenase-1 expression in monocytic cells. Nonetheless, although this downstream region conferred much of the PMA responsiveness, distal upstream promoter elements were needed to achieve a maximal response (Fig. 7B).

Consistent with the kinetics of c-Fos and c-Jun expression, the AP-1 element is needed for rapid and strong PMA-mediated induction of collagenase-1 transcription in U937 cells. However, our data indicate that, distal upstream elements are required to maximize AP-1-dependent induction and can induce and maintain collagenase-1 expression in an AP-1-independent manner. Most compelling is that the AP-1 mutant construct, p-2278MCAT, displayed delayed, yet maintained, PMA-mediated activation in U937 cells and fibroblasts (Figs. 3 and 4). While Buttice et al. (63) showed that mutation of the analogous AP-1 site in the related stromelysin-1 promoter did not fully eliminate PMA responsiveness in fibroblasts, to our knowledge this is the first report of AP-1 independent PMA-mediated collagenase-1 promoter induction in any cell type. We detected no CAT activity in PMA-treated U937 cells transfected with either a −511 or −179 deletion construct containing the AP-1 mutation (26). Similarly, Jonat et al. (64) found no evidence of PMA-mediated induction of a −517/+6 collagenase-1 promoter fragment containing an AP-1 mutation when assayed in HeLa cells. Thus, the delayed AP-1-independent response is likely controlled by a regulatory element(s) upstream of position −517.

While AP-1 factors are needed for the rapid and strong induction of collagenase-1 in U937 cells (Fig. 3), our data suggest that cooperation between distal upstream and downstream factors maintains gene expression over extended periods. We observed that collagenase-1 promoter activity was suppressed by sequences between −1954 and −179 (Fig. 7). This suppression was not seen with p-2278CAT, p-2010CAT, and p-1197Δ-997CAT, nor was it seen with smaller constructs (p-179CAT, p-95CAT, and p-72CAT). Furthermore, the p-2010Δ-1954CAT internal deletion construct had diminished...
transcriptional activity relative to p-2278-TKCAT. Thus, we suggest that a suppressive element is located between -511 and -179 and that factors bound between -2010 and -1954 overcome this suppression to enhance AP-1 dependent responses. Imai et al. (65) have proposed a model in fibroblasts in which the collagenase-1 promoter is brought into an active conformation by the interplay of regulatory factors which bind elements between -1705 and -1595. Similarly, we speculate that factors bound between -2010 and -1954 might help maintain collagenase-1 expression in PMA-differentiated U937 cells by interacting with downstream factors to overcome the potential inhibitory effect of the intervening sequences.

Heterologous promoter constructs p-2278-TKCAT and p-2010-TKCAT were stimulated by PMA differentiation of U937 cells independent of any downstream collagenase-1 AP-1 site, with the majority of this response lost once sequences -2010 to -1954 were deleted (Fig. 8). The proserpine-binding site located at -1704 to -1689 (65) may function in monocytyic cells because weak PMA responsiveness is lost when this sequence is deleted from heterologous promoter constructs (Fig. 8). However, this effect is minimal compared with the decreased PMA responsiveness caused by deletion of sequences between -2010 and -1954 from heterologous promoter constructs. Notably, no PMA responsiveness from heterologous constructs containing upstream collagenase-1 promoter regions was observed in HeLa cells (30). In the context of the wild-type collagenase-1 promoter, sequences between -2010 to -1954 were necessary to achieve maximal collagenase-1 promoter induction in U937 cells (Fig. 7B). In addition, deletion of this region caused decreased collagenase-1 promoter activity in untreated and PMA-differentiated U937 cells (Fig. 7B). In contrast, deletion of -2010 to -1954 did not decrease basal collagenase-1 promoter activity when assayed in fibroblasts (65). We identified a putative C/EBP-binding site (-2006 to -1997) within this distal promoter region. Importantly, C/EBP-β protein capable of binding this collagenase-1 promoter site is present in untreated U937 nuclear extracts, and levels are increased in PMA-differentiated U937 cells (Figs. 9 and 10). Together, these data suggest that C/EBP-β may mediate, in part, the observed AP-1-independent activation and maintenance of collagenase-1 expression and specifically enhance AP-1 dependent responses in monocyte/macrophage cells.

The C/EBP family consists of five proteins (α, β, δ, γ, and CRP-1) which are ZIP transcription factors that form homo- and heterodimers to bind the consensus sequence (N(T/T/ GINNGAA(T/G)) (66). Although C/EBP-β is found in many tissues (67), it seems to play a prominent role in activating and regulating gene expression in monocytes and macrophages (59, 68, 69). C/EBP-β expression is induced during later stages of monocytyic, but not granulocytyic differentiation (69), is constitutively low in monocyte/macrophages (68, 70), and is strongly induced in macrophages by inflammatory mediators, such as lipopolysaccharide (71). Therefore, C/EBP-β may be a necessary factor for normal monocyte/macrophage development and function. Notably, C/EBP-δ, which forms heterodimers with C/EBP-β to synergistically activate transcription, is also strongly induced in monocytyic cells by lipopolysaccharide (72), an agent that potently stimulates collagenase-1 expression in macrophages (27). In addition, C/EBP-β and C/EBP-δ expression is stimulated or induced in various other tissues by inflammatory mediators such as interleukin-1, interleukin-6, and tumor necrosis factor-α (71, 72). Thus, while our data suggest that C/EBP-β is involved in the induction of collagenase-1 expression in monocytes, C/EBP-β may also be involved in activating collagenase-1 transcription in other cell types once C/EBP-β expression has been induced by inflammatory factors.
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Glenn A. R. Doyle, Richard A. Pierce and William C. Parks

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