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Differential regulation of collagenase gene expression by retinoic acid receptors—α, β and γ

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ABSTRACT

The mechanisms involved in retinoic acid (RA)-mediated regulation of the collagenase gene in a rabbit synovial fibroblast cell line (HIG82) were investigated. When HIG82 cells are cotransfected with expression vectors containing cDNAs for retinoic acid receptor (RAR) α1, β2, or γ1 and collagenase promoter-driven CAT reporter constructs, only RAR-γ1 represses basal CAT expression upon RA treatment, while RAR-α1, β2, and γ1 all suppress phorbol-induced CAT expression. Thus, transcriptional regulation of collagenase by RA is mediated by RARs in an RAR-type specific manner. Using mutational and deletional analysis, we find that interaction between elements within 182 bp collagenase promoter plays an important role in this process. In addition, cotreatment with RA results in a decrease of phorbol-induced mRNA levels of fos and jun, and binding of nuclear proteins to an AP-1 oligonucleotide. Furthermore, RA-induced nuclear protein(s) specifically bind to a 22 bp sequence (−182 to −161) of the collagenase promoter. We propose that RA-mediated regulation of the collagenase gene depends on the availability and interaction of specific RARs with multiple DNA elements within the promoter and with transcription factors, including AP-1 related proteins.

INTRODUCTION

The ability of retinoic acid (RA) and retinoic acid receptors (RARs) to modulate gene expression and to influence morphogenesis and deposition of the extracellular matrix has been an important topic of recent research (1–3). Hence an understanding of how RA modulates expression of the gene for collagenase, the only enzyme able to degrade interstitial collagens (type I,II and III) at neutral pH (4), is central to our understanding of connective tissue metabolism in normal development (5,6) and in disease processes (7–9). Synthesis of collagenase is increased by cytokines and growth factors, such as interleukin-1 (10–13), tumor necrosis factor-α (14,15), and epidermal growth factor (16), and these effects are mimicked by phorbol myristate acetate (PMA) (11,13,17). Previous studies by a number of investigators have shown that the regulation of collagenase synthesis by these compounds occurs at the level of transcription (11,12,17), and is mediated through the AP-1 binding site (11,18,19). More recently, Auble and Brinckerhoff (20) demonstrated that the AP-1 site, alone, could not drive transcription of the collagenase gene in PMA-stimulated fibroblasts, and that phorbol responsiveness required cooperation among DNA sequences located within 127 bp of the collagenase promoter.

All-trans retinoic acid (RA) represses PMA-induced collagenase synthesis by decreasing steady state levels of collagenase mRNA (21,22) without affecting mRNA half-life (21), indicating inhibition at the level of transcription. Recently, three groups reported that RA inhibited expression of the genes for collagenase (11,23) and a closely related enzyme, stromelysin (24), through the AP-1 binding site, although there is no evidence to suggest that RARs bind to the AP-1 site directly.

In this study, we demonstrate that suppression of basal (constitutive) and induced collagenase gene expression by RA is mediated in an RAR-type specific manner. Moreover, regulation of collagenase gene expression by RA is more complex than previously believed, and depends on the availability of specific RARs, and their requirement for DNA elements within the collagenase promoter.

MATERIALS AND METHODS

Cell culture

HIG82 cells were established by spontaneous immortalization of a primary culture of rabbit synovial fibroblasts (25), and were maintained in DMEM media supplemented with 10% fetal calf serum. HIG82 cells remain contact-inhibited, do not grow in soft agar, or form tumors in nude mice by seven months after subcutaneous injection of 5×10^7 cells (C.E.Brinckerhoff, unpublished observation).

Plasmids

The chimeric DNA constructs used in this study are shown in Fig.1A. The fusion of 182 bp or 102 bp of the rabbit collagenase promoter into the unique HindIII site of pSVOCAT (26), and the ability of these inserted fragments to drive transcription of the promoterless bacterial gene chloramphenicol acetyltransferase

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(CAT) has been described (20). Two mutations were generated in the 182 bp wild type sequence of collagenase 5′ flanking DNA by substitution mutagenesis (20). The cDNAs encoding mouse RAR-α0 (2.1 kb), β (1.95 kb) and γ (2.0 kb) have been inserted in the sense orientation into the EcoRI site of pSG5 expression vector driven by SV40 early promoter (27), and mouse RAR-γ was also inserted in the reversed orientation (rRAR-γ) as a control. Multiple isoforms of RAR-α, β and γ have been cloned, and isoforms 1 and 2 are the predominant forms of these receptors (28−30). The RAR expression vectors used in this study were subsequently named as RAR-α1, β2 and γ1 (28−30).

Transfection and CAT assay

Transient transfection and CAT assay were performed as previously described (20). Briefly, equal amounts of DNA (2.5μg DNA per 60 mm plate, unless otherwise indicated) containing the chimeric CAT construct and RAR expression vector were cotransfected into HIG82 cells by the calcium phosphate coprecipitation method, followed by 10% glycerol shock for 3 minutes. After overnight recovery, cells were cultured in DMEM plus 0.2% lactalbumin hydrolysate (LH) alone, or treated with 10^{-6}M RA for 26h, 10^{-8}M PMA for 24h, or 10^{-6}M RA plus 10^{-8}M PMA 24h after a 2h pretreatment with RA. Cell extracts were prepared by the freeze-thaw method, and protein concentrations in the cell extracts were determined by Bradford assay (Bio-Rad). Five μg protein per sample was incubated with 0.25 μCi ^{14}C-chloramphenicol in reaction buffer (1.4mM Acetyl CoA, 14mM Tris-Cl, pH 7.8, 3.5% glycerol) for 2h, the reaction products were resolved by thin-layer chromatography (TLC) and autoradiography, and were quantitated by scintillation counting of the TLC plate. Acetylation of ^{14}C-chloramphenicol in extracts from untreated and treated cells was in the linear range of the assay (0.5% to 30% conversion). Results of CAT assays were normalized to the percentage acetylation of ^{14}C-chloramphenicol in extracts from untreated cells within the same transfection group. Student's paired t-test was used for statistical analysis.

Hirt's assay

Plasmid DNA from transfected cells was prepared as described (31). DNA extracted from 5×10^5 cells per transfection was denatured and slot-blotted onto GeneScreen Plus membrane (NEN). The membrane was hybridized with denatured [α-^{32}P]dCTP-labeled probes specifically for CAT, RAR-α, β or γ for 20h at 42°C, then washed with 0.2×SSC, 0.25% SDS twice at room temperature for 10 minutes, and twice at 56°C for 30 minutes. The membranes were exposed to a X-ray film (Kodak) overnight. Using this assay, we found that RAR-α1, β2, γ1 or rRAR-γ1 were cotransfected into HIG82 cells without altering the transfection efficiency of pCCAT182, although a low level of cross-hybridization between RARs and their parent vector pSG5 was noticed (Fig.1B).

RNAse protection analysis

RNAse protection analysis was performed as described (20). Briefly, total RNA from transfected cells was isolated by phenol extraction and isopropanol precipitation. Forty μg RNA was hybridized to a riboprobe (2×10^6cpm probe/sample) obtained by T7 RNA polymerase (Promega) transcription of an NcoI-linearized, pGEM3-derived plasmid (Promega) containing 380 bp collagenase 5′ flanking DNA fused to 251 bp of the CAT gene. The specific activity of the riboprobe was ≥ 10^8DPM/μg DNA. The reaction products were resolved on a 5% denaturing polyacrylamide gel. The gel was dried on a piece of filter paper, and then exposed to X-ray film overnight.

Northern analysis

Total RNA from HIG82 cells was prepared by the guanidinium thiocyanate-CsCl method (32). Twenty μg RNA per sample was electrophoresed on a 1% formaldehyde agarose gel. The gel was stained with ethidium bromide (1μg/ml) for loading quantitation, and then transferred to GeneScreen Plus membrane. The membrane was hybridized with denatured [α-^{32}P]dCTP-labeled probe for 20h at 56°C, then washed with 0.2×SSC, 0.25% SDS twice at room temperature for 10 minutes, and twice at 56°C for 30 minutes. Hybridization with glyceraldehyde phosphate dehydrogenase (GAPDH) was also used as a control.

cDNA probes

Full length cDNA inserts of RAR-α1, γ1, a 900 bp cDNA fragment of RAR-β2 (EcoRI) (27), a 414 bp fragment of CAT (EcoRI-ScaI) (26), a 630 bp fragment for collagenase (EcoRI-HaeIII) (33), full length cDNA inserts of c-jun (ATCC), c-fos (ATCC), and a 550 bp cDNA fragment of GAPDH (Hind III-Xba I, ATCC) were used as probes in Hirt's assay and Northern analysis. Probes were labeled with [α-^{32}P]dCTP (3000 Ci/mM, NEN) by random priming (Pharmacia). The specific activity of the probes was ≥ 1×10^6 DPM/ng DNA, and the probes were used at a final concentration of 10^6cpm probe per ml hybridization buffer in Hirt's assay and Northern analysis.

Preparation of nuclear extracts and mobility shift assay

Nuclear extracts were prepared according to Lee et al. (34) with the following modifications: Cells swollen in hypotonic solution at 4°C were passed through a 25 gauge needle 10 times to disrupt cell membranes. Isolated nuclei were extracted for 30 minutes at 4°C in extraction buffer (10mM Hepes pH 7.9, 12% glycerol, 8mM $MgCl_2$, 500mM KCl, 0.1mM EDTA, 0.1mM PMSF, 0.25mM DTT), then dialyzed for 1h against dialysis buffer (10mM Hepes pH 7.9, 12% glycerol, 100mM KCl, 0.1mM EDTA, 0.1mM PMSF, 0.25mM DTT). Nuclear extracts (3−10 μg protein/sample) were incubated with 40,000 cpm [γ-^{32}P]ATP-labeled double-stranded oligonucleotides (specific activity ≥2×10^6DPM/pm 5′-end oligo) in binding buffer (12mM Hepes pH 7.9, 4mM Tris-HCl pH7.9, 12% glycerol, 60mM KCl, 1mM EDTA, 1μg poly dI-dC) for 15 minutes at 30°C. Products were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel. The gel was dried on a piece of filter paper, and then exposed to a X-ray film overnight.

RESULTS

To understand the mechanisms involved in transcriptional regulation of the collagenase gene, we used the rabbit synovial fibroblast cell line, HIG82, to examine pSVOCAT expression driven by the rabbit collagenase promoter. In keeping with our previous studies in primary cultures of synovial fibroblasts (17,20), we find that the expression of promoter-less pSVOCAT in HIG82 cells is not affected by either RA or PMA treatment (data not shown). The levels of acetylation of ^{14}C-chloramphenicol in extracts from HIG82 cells transfected with the collagenase promoter driven CAT constructs range from 1−5%, and increase to as much as 20−30% upon PMA treatment.

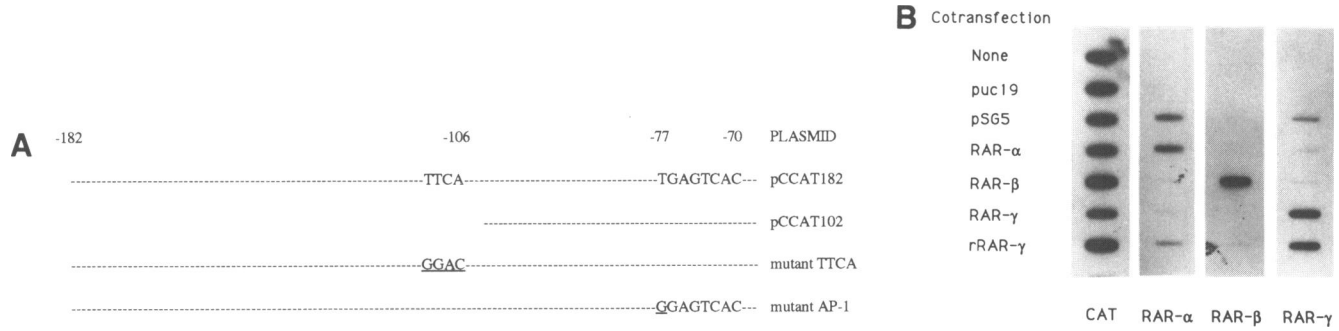

Fig. 1. A. Chimeric DNA constructs containing up to 182 bp of the rabbit collagenase promoter linked to pSVOCAT. The plasmids are designated 'pCCAT' followed by a number which indicates the size of the rabbit collagenase 5' flanking DNA (in bp) inserted. TTCA is a PMA inducible element, TGAGTCAC is the AP-1 consensus sequence. Two mutations were generated from the 182 bp wild type sequence of collagenase 5' flanking DNA. Mutated bases are underlined. B. Assessment of transfection efficiency by Hirt's assay. The pCCAT182 construct was transiently transfected into HIG82 cells. Plasmid DNAs cotransfected with pCCAT182 in equal amounts are indicated. Plasmid pUC19 is a nonrelated plasmid used as a control. After overnight recovery, cells were cultured in DMEM medium supplemented with 2% LH for 24h. Hirt's assay was performed as described in *Materials and Methods*.

Suppression of constitutive collagenase gene expression by RA is mediated through RAR-γ

We noticed that RA only slightly suppressed collagenase promoter-driven CAT expression in primary rabbit fibroblasts and HIG82 cells, although in the same cells the endogenous collagenase mRNA was markedly decreased (17). A similar finding has been reported for induction of laminin B1 gene expression by RA in F9 mouse cells, where cotransfection of exogenous human RAR-α, β, or γ was required to elucidate the RA response (35). To test whether the failure of RA to suppress collagenase promoter-driven CAT expression in HIG82 cells is due to the limited availability of RARs, we transiently cotransfected pCCAT182 or pCCAT102 into HIG82 cells with expression vectors containing cDNAs for mouse RAR-α1, β2 or γ1 at a 1:1 molar ratio (Table 1). For both constructs, only cotransfection of RAR-γ1, in the presence of RA, inhibits basal CAT expression. Neither RAR-γ1 in the reversed orientation (non-sense), nor RAR-α1 and β2 changes basal CAT expression significantly ($p > 0.05$). These data demonstrate that RA inhibits constitutive collagenase gene expression in an RAR-type specific manner.

Sequences within 182 bp of the collagenase promoter are required for RA-mediated suppression

We also examined the regulation of PMA-induced collagenase transcription by RA using the pCCAT182 construct (Fig.2A). Treatment with PMA increases CAT activity by four fold, and cotreatment with RA does not antagonize this induction. When the RAR-γ1 expression vector is cotransfected with pCCAT182, the induction of CAT activity by PMA is similar to that seen in the cells without cotransfection. However, cotreatment with RA decreases the PMA-induced CAT activity by 60%. This antagonism is not observed when RAR-γ1 in the reversed orientation (non-sense) is cotransfected with pCCAT182. Cotransfection of RAR-α1 or β2 with pCCAT182 also results in a decrease of PMA-induced CAT activity upon cotreatment with RA, which is not seen with cotransfection of parent vector pSG5 without the RAR insert (Table 2). These data suggest that repression of PMA-induced collagenase gene expression can be mediated by all three types of RAR (α, β and γ), and only RAR-γ inhibits constitutive collagenase gene expression upon RA treatment.

Table 1. Repression of basal CAT activity by RAR-γ upon RA treatment

Cotransfection	pSG5	RAR-α	RAR-β	RAR-γ	rRAR-γ
pCCAT182	0.89±0.11	0.98±0.10	0.94±0.11	0.33±0.06	0.87±0.09
pCCAT102	1.10±0.07	1.00±0.07	0.95±0.03	0.37±0.05	1.21±0.12

The pCCAT182 or pCCAT102 constructs were transiently cotransfected into HIG82 cells with RAR expression vectors or the parent vector without RAR inserts (pSG5) at 1:1 molar ratio. Cells were then treated with 10^{-6}M RA for 26h. Five μg protein from each sample was used for CAT assay. The values of each construct represent percentage acetylation of ^{14}C-choloramphenicol in extracts from RA-treated cells divided by percentage acetylation of ^{14}C-choloramphenicol in extracts from untreated cells. Results shown are the mean of four independent transfections ±S.E.

Table 2. Regulation of CAT activity by RARs upon RA plus PMA treatment

Cotransfection	pSG5	RAR-α	RAR-β	RAR-γ	rRAR-γ
pCCAT182	1.00±0.14	0.68±0.06	0.47±0.04	0.42±0.05	1.13±0.09
pCCAT102	3.15±0.27	1.01±0.10	1.04±0.12	0.93±0.05	3.28±0.29

The pCCAT182 or pCCAT102 constructs were transiently cotransfected into HIG82 cells with RAR expression vectors or the parent vector without RAR inserts (pSG5) at 1:1 molar ratio. Cells were then treated with PMA or RA plus PMA. Five μg protein from each sample was used for CAT assay. The values of each construct represent percentage acetylation of ^{14}C-choloramphenicol in extracts from cells cotreated with RA plus PMA divided by percentage acetylation of ^{14}C-choloramphenicol in extracts from cells treated with PMA only. Results shown are the mean of four independent transfections ±S.E.

Previous studies by other investigators on the regulation of metalloproteinase genes (collagenase and stromelysin) by RA have focused mainly on the AP-1 site (11,23,24). However, transfection of pCCAT102, which contains an intact AP-1 sequence in its native promoter configuration, results in a loss of CAT induction upon PMA treatment (Fig.2B), a finding in agreement with our earlier studies in primary rabbit fibroblasts (20). Interestingly, the combination of RA and PMA treatment results in a synergistic enhancement of CAT expression. Cotransfection of RAR-γ1 abolishes this enhancement, while cotransfection of RAR-γ in the reversed orientation does not (Fig.2B). Cotransfection of RAR-α1 or β2 with pCCAT102 also diminishes this synergism (Table 2). These findings suggest that sequences between −182 to −102 bp of collagenase promoter are able to repress this enhanced CAT activity.

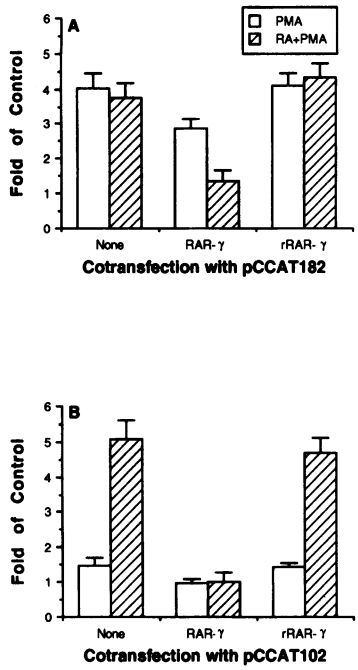

Fig. 2. RAR-γ inhibits collagenase promoter-driven CAT expression. The pCCAT182 (A) or pCCAT102 (B) were cotransfected with RAR-γ1 or rRAR-γ1 into HIG82 cells. Cells were then treated with PMA or RA plus PMA. CAT assays were carried out as described in *Materials and Methods*. Acetylation of ^{14}C-choloramphenicol in extracts from untreated cells was in the range of 2–5% (pCCAT182), or 1–2% (pCCAT102). Results shown are the mean of four independent transfections ± S.E. A value of 1 represents the levels of CAT activity in extracts from untreated cells.

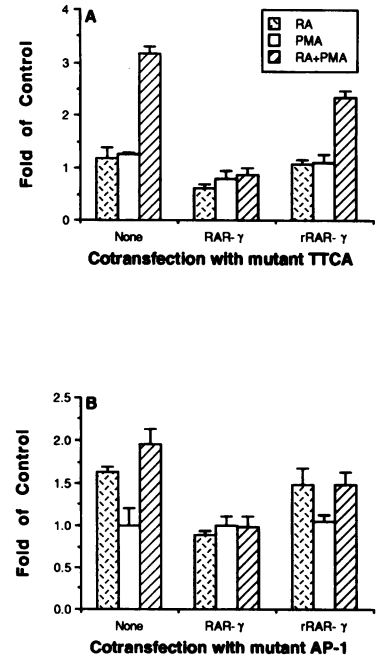

Fig. 3. Mutational analysis of pCCAT182 construct. The mutant TTCA (A) or mutant AP-1 (B) was cotransfected with RAR-γ1 or rRAR-γ1 into HIG82 cells. Treatments and assays were carried out as described in *Materials and Methods*. Acetylation of ^{14}C-choloramphenicol in extracts from untreated cells was in the range of 1–2%. Results shown are the mean of four independent transfections ± S.E. A value of 1 represents the levels of CAT activity in extracts from untreated cells.

To further localize the promoter region for this response, we examined the expression of mutant TTCA construct (four base pair mutation at −109 to −106 bp in wild type pCCAT182, see Fig.1A). Previously, we have shown that this TTCA element was necessary for induction of collagenase gene expression by PMA in primary rabbit fibroblasts (20). Now, we find that this mutation also results in a loss of PMA-inducibility in HIG82 cells (Fig.3A). As with the pCCAT102 construct, in the presence of both RA and PMA, a synergistic enhancement of CAT activity is observed (Fig.3A), although either RA or PMA alone does not increase CAT activity significantly ($P > 0.05$). Cotransfection of RAR-γ1 abolishes this synergism completely (Fig.3A), whereas cotransfection of RAR-γ1 in the reversed orientation does not greatly change this enhancement (Fig.3A). These results imply that the TTCA element is required for suppression of this enhancement by RA and PMA.

We also examined the role of AP-1 site using a construct containing a single base pair mutation in wild type pCCAT182, which is known to prevent binding of the Fos/Jun heterodimer (18, see Fig.1A). As expected, this construct results in a loss of PMA inducibility in HIG82 cells (Fig.3B). Surprisingly, CAT activity of this construct increases upon RA treatment ($p < 0.05$), and cotransfection with RAR-α1 or β2 fails to abrogate this increase (data not shown). Only cotransfection with RAR-γ1 in the sense orientation brings the CAT activity back down to the control level (Fig.3B), thus indicating that RAR-γ1 can exert its effects in the absence of an intact AP-1 site.

To ensure that the CAT activity measured in the above experiments (Fig.2) reflected transcriptional regulation, we examined the steady state level of CAT mRNA in transfected HIG82 cells by RNAse protection analysis (Fig.4A). Induction of CAT mRNA by PMA is greater in the pCCAT182 construct than that in pCCAT102. RA, in combination with PMA, further increases the CAT mRNA in cells transfected with pCCAT102, but reduces PMA-induced CAT mRNA in cells transfected with pCCAT182. Thus, levels of CAT mRNA parallel the levels of CAT protein activity presented in Fig.2. In addition, the modulation of endogenous collagenase mRNA by RA and PMA is similar in both transfection groups (pCCAT182 vs pCCAT102) as determined by Northern analysis (Fig.4B). Furthermore, in agreement with our previous findings (17), collagenase protein in culture media of these cells is decreased by RA as determined by Western analysis and by a ^{14}C-collagen fibril assay (data not shown). Thus, the disparate regulation of endogenous gene/pCCAT182 vs pCCAT102 by RA suggests the presence of RA-response elements within 182 bp of collagenase promoter.

Roles of Fos/Jun and the AP-1 binding site in RA-mediated collagenase gene regulation

Since previous studies showed that negative regulation of collagenase gene expression by RA was mediated through the AP-1 site which binds Fos/Jun (11, 23), we also examined the roles of AP-1 site and fos/jun in our system. Using Northern analysis, we find that RA decreases the PMA-induced mRNA levels of c-fos and c-jun. This inhibition of fos/jun mRNAs by RA continues for at least 24h (Fig.5).

The decrease of AP-1 related proteins by RA is supported by mobility shift experiments. A 33mer, representing the sequence from −88 to −56 of the collagenase promoter containing the

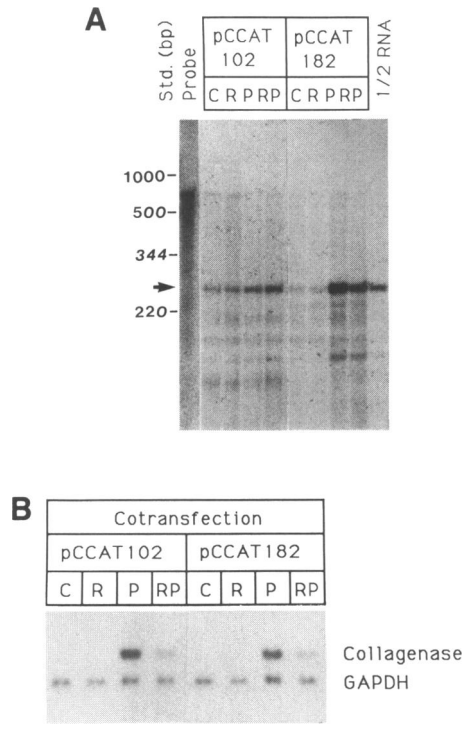

Fig. 4. Regulation of CAT mRNA (A) and endogenous collagenase mRNA (B) by RA and PMA. HIG82 cells were transfected with pCCAT102 or pCCAT182 (10 μg/100 mm plate). Cells were cultured in DMEM plus 2% LH (C), or treated for 24h with 10^{-6}M RA (R), 10^{-8}M PMA (P), or 10^{-6}M RA plus 10^{-8}M PMA (RP). (A) RNAse protection analysis. Forty μg total RNA per sample was used. The protected band is indicated by the arrow. A reaction containing 20μg RNA from PMA-treated cells transfected with pCCAT182 is indicated as 1/2 RNA. (B) Northern analysis. Four μg total RNA per sample (same RNA as used in Fig.4A) was used. Specific mRNA hybridization to ^{32}P-labeled cDNAs for collagenase or GAPDH is indicated.

AP-1 site, was used as the probe in the experiment shown in Fig.6A. The nuclear extracts derived from PMA-treated cells bind specifically to this AP-1 oligonucleotide (33mer) as expected, and this binding is competed by a 100 fold molar excess of self, but not by the 42mer oligonucleotide (−182 to −141 of the collagenase promoter). However, when extracts from cells treated with PMA plus RA are used, the amount of complex formed decreases markedly. Further, a 10 fold molar excess of self and a 100 fold excess of the 42mer are able to compete this complex formation. These results suggest that RA decreases the amount and/or affinity of protein binding to an AP-1 oligonucleotide, and that RA-induced nuclear proteins may bind to sequences within the 42mer and to PMA-induced proteins which bind to an AP-1 oligonucleotide.

The possibility that RA-induced proteins bind to both collagenase promoter sequences and AP-1 related proteins is further illustrated in the mobility shift experiment shown in Fig.6B. The 22mer (−182 to −161 of the collagenase promoter) is used as a probe in this representative experiment. Three complexes are formed with nuclear extracts from cells treated with RA, while little complex formation is observed with extracts from PMA-treated cells. All three complexes are competed by 100 fold excess of self, while two complexes are competed partially by 100 fold excess of the AP-1 oligonucleotide, suggesting that these two complexes may contain AP-1 related proteins.

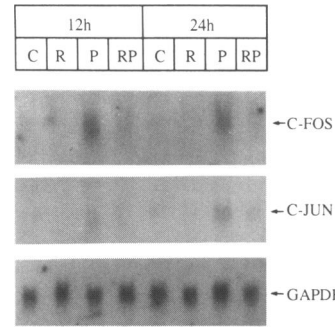

Fig. 5. Inhibition of c-fos, c-jun mRNAs by RA. HIG82 cells were treated with 10^{-6}M RA (R), 10^{-8}M PMA (P), or 10^{-6}M RA plus 10^{-8}M PMA (RP) for 12h or 24h. Total RNA was prepared by the guanidinium thiocyanate-CsCl method. Twenty μg RNA per sample was used. Specific mRNA hybridization to ^{32}P-labeled cDNAs for c-fos, c-jun or GAPDH is indicated.

DISCUSSION

Differential regulation of the collagenase gene by RA

Our study demonstrates that RA-mediated suppression of CAT expression driven by the collagenase promoter is dependent on the availability of functional RARs, and that RAR-α, β and γ differentially regulate collagenase transcription. Upon RA treatment, only RAR-γ1 represses the basal CAT expression driven by the collagenase promoter (Table 1), whereas all three RARs (α1, β2 and γ1) suppress PMA-induced CAT expression driven by 182 bp collagenase 5′-flanking DNA (Fig.2A, Table 2). Thus, suppression of basal and induced collagenase gene expression by RA is mediated in trans by RAR-type specific mechanisms. These results differ with those reported by Schüle et al. (23) who found that all three RARs (α, β and γ) repressed basal/constitutive collagenase promoter expression. However, their study was carried out in HeLa cells with a 1200 bp promoter construct, while we used normal fibroblasts and a 182 bp promoter construct. The difference between these two studies may be due to cell-type specific gene regulation, although the size of promoter constructs cannot be ruled out.

It has been reported that RAR-γ is predominantly expressed in embryonic tissue and in adult tissues such as skin and lung, whereas RAR-α and β are expressed in a variety of tissues (27). We find that only RAR-γ mRNA is detectable by Northern analysis in untreated HIG82 cells. However, the mRNAs for all three RARs are increased markedly within 3h of RA treatment (unpublished observation). Since RARs play distinct roles during cell differentiation (3,36,37) and morphogenesis (3,28,38−40), it is conceivable that RAR-specific modulation of collagenase transcription may be important during normal development and in disease processes where the modeling and remodeling of connective tissue is involved.

Interaction of AP-1 related proteins and RA-induced proteins with 182 bp of collagenase promoter is involved in RA-mediated collagenase gene regulation

The importance of the AP-1 binding site in RA-mediated negative regulation of metalloproteinase genes (collagenase and stromelysin) has been demonstrated (11,23,24), and our data support a role for AP-1 in RA-mediated repression of collagenase.

We also find that RA decreases the steady-state levels of both c-fos and c-jun mRNAs (Fig.5), and this decrease in AP-1 related

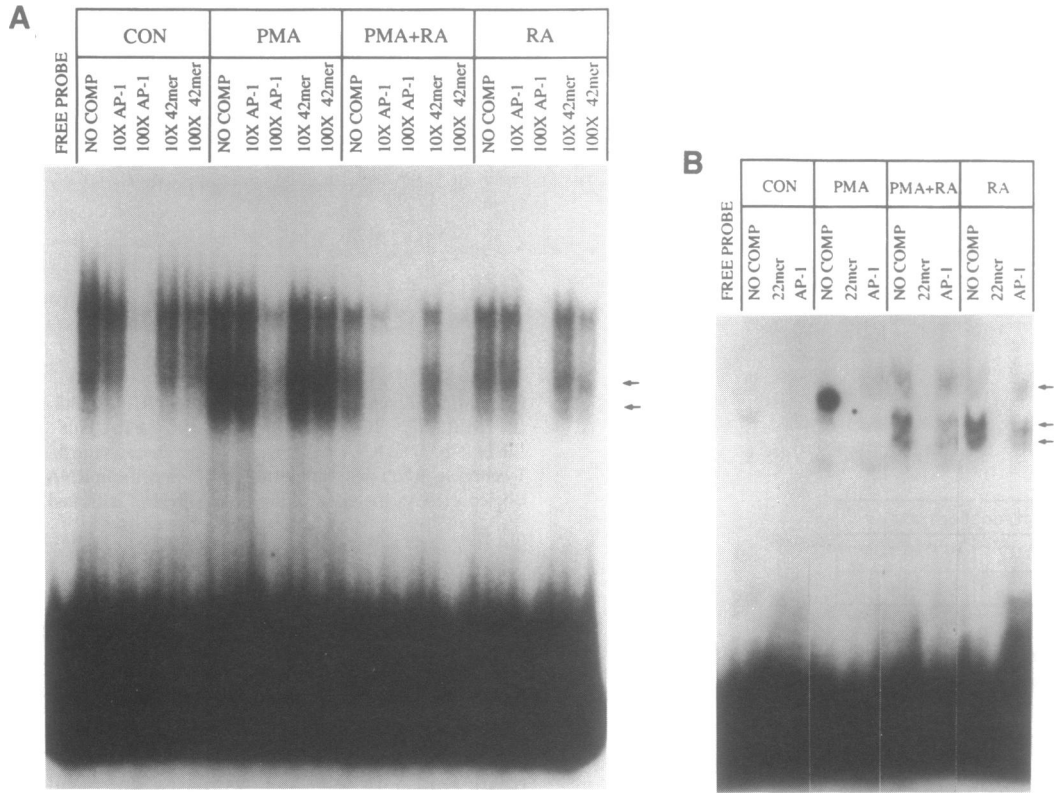

Fig. 6. Specific complex formation between nuclear proteins and radiolabeled fragments of the rabbit collagenase promoter. HIG82 cells were cultured in DMEM plus 2% LH (CON) or treated with 10^{-8}M PMA, 10^{-6}M RA, or PMA+RA for 2h (A), or 6h (B). A. Specific complexes formation with the AP-1 oligoprobe. The oligoprobe represents sequence from −88 to −56 of the collagenase promoter containing the AP-1 consensus (underlined) 5′-AGCTTCTAGAAAGCATGAGTCACACAAGCCCTCAGCTG-3′. Three μg nuclear extract proteins were reacted with double stranded oligoprobe alone (NO COMP) or in the presence of 10× or 100×molar excess of unlabeled competitors of self (AP-1) or '42mer', a 42 bp fragment from −182 to −141 of the collagenase promoter (5′-TCATGAAATTGCAACACCAAGCTAACCCAAAAAATCTGCCG-3′). B. Specific complex formation with a 22 bp double-stranded oligoprobe (22mer) from −182 to −161 of the rabbit collagenase promoter (5′-TCATGAAATTGCAACACCAAGC-3′). Ten μg nuclear extract proteins were reacted with oligoprobe either alone (NO COMP) or in the presence of 100×molar excess of unlabeled competitors of self or AP-1 oligo as described above.

proteins is supported by the mobility shift experiment shown in Fig.6A. These mobility shift experiments (Fig.6A,6B) also provide evidence that RA-induced nuclear proteins can bind to the nucleotides between −182 and −141 bp of the collagenase promoter, and compete binding of nuclear proteins to an AP-1 oligonucleotide. These results illustrate the importance of cooperation between DNA elements within the collagenase promoter. The possibility that RA-induced protein(s) could form complexes with AP-1 related protein(s) is supported by *in vitro* experiments in which the binding of RAR-α to Jun protein can reduce the binding of Jun to the AP-1 site (23). Studies with glucocorticoid receptors have also demonstrated that negative gene regulation mediated by the glucocorticoid receptor is due to the direct binding of the receptor to the c-Jun protein and to the AP-1 site (41−44). All these studies suggest a potential mechanism of repression via protein-protein interaction in addition to protein-DNA interaction.

Cooperation of DNA elements within collagenase promoter and availability of specific RARs play a central role in RA-mediated collagenase gene regulation

Several retinoic acid response elements (RARE) have been reported, and most of them involve palindomes or direct repeats (3,45−48). Although we do not find any elements within 182 bp collagenase promoter identical to the reported RAREs (3,45−48), we demonstrate that interaction between DNA elements within the promoter contributes substantially to the ability of RA and PMA to modulate collagenase gene expression.

Sequences within 182 bp of the collagenase 5′-flanking DNA are required to repress both constitutive and PMA-induced gene expression by RA in HIG82 cells. The presence of both the TTCA element and the AP-1 consensus sequence are neccessary for PMA-induced collagenase transcription (20, Fig.2B,3A,3B). The TTCA element also plays an important role in RA-mediated suppression, since mutation or deletion of this element gives a synergistic enhancement of CAT expression in the presence of RA and PMA (Fig.2B,3A). The fact that cotransfection of RAR-α1, β2, and γ1 can antagonize this enhancement (Fig.2B,3A, Table 2) suggests that it may be mediated by RA-induced factor(s) that are distinct from the transfected RARs. For example, retinoid X receptor (RXR) can form heterodimers with RARs, then enhance RAR response by increasing ligand sensitivity and DNA binding (49−52). On the other hand, RXR could also act as a competitor of RAR by binding to the same response element (53). Antagonism between RAR-β and RAR-γ1 or different isoforms of RAR-γ has also been reported (54). In combination with studies reported by others, our results suggest that availability and interaction of these receptors with DNA elements within the collagenase promoter play a central role in mediating the retinoid response.

In summary, our data suggest that sequences in addition to the AP-1 site play a role in mediating RA repression. Further, the sequences associated with RA repression are also involved in PMA induction. Mutation or deletion of these PMA-response elements abolishes RA repression, and even confers a positive regulation by RA. This positive regulation can be abrogated by cotransfection of RARs, suggesting a quantitative contribution of these receptors in mediating RA effects. Thus, transcriptional regulation of collagenase is likely to depend on the availability of specific RARs and other RA-induced proteins, as well as interactions between DNA elements and various transcriptional factors, including Fos and Jun.

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