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ARTICLES

Suppression of Collagenase Gene Expression by all-trans and 9-cis Retinoic Acid Is Ligand Dependent and Requires Both RARs and RXRs

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Abstract Retinoic acids (RA) are active metabolites of vitamin A which affect the expression of many genes involved in embryonic development, cell differentiation, and homeostasis. One important target gene for RA is matrix metalloproteinase (MMP-1, collagenase), the only enzyme active at neutral pH that can degrade interstitial collagen, a major component of extracellular matrix. Using a cell line of normal rabbit synovial fibroblasts, HIG82 cells, as a model, we report that both all-trans- and 9-cis-RA inhibit collagenase synthesis. This inhition occurs at a transcriptional level and is ligand-dependent. Constitutive levels of retinoic acid receptor (RAR) mRNA levels are low, but are increased by all-trans and by 9-cis RA. In contrast, constitutive levels of retinoid X receptor (RXR) mRNA are higher and are not affected by RA. To measure DNA/protein interactions, we used a gel mobility shift assay with oligonucleotides containing either an AP-1 site or a 40 bp region between −182/−141, nuclear extracts from RT-treated cells, and antibodies to RARs and RXRs. We found that both RARs and RXRs interact with these regions of the collagenase promoter, perhaps as part of a complex with other proteins. Our results suggest that heterodimers between RARs and RXRs mediate suppression of the collagenase gene by RA, and that RAR is a limiting factor in this negative regulation. © 1995 Wiley-Liss, Inc.

Key words: matrix metalloprotein-1, gel mobility shift assay, fibroblasts, transfection, heterodimer

Retinoids, vitamin A derivatives, are essential for growth, limb morphogenesis, development of nervous system, cell differentiation, and homeostasis [1–6]. The functions of retinoic acid are mediated by their nuclear receptors, of which two classes have been identified: retinoic acid receptors (RAR) [7,8] and retinoid X receptors (RXR) [9–11]. Each class has α, β, and γ subtypes. These receptors belong to the superfamily of thyroid hormone, steroid hormone, and vitamin D3 receptors, and their structure can be divided into several domains based on function and amino acid sequence similarities among the superfamily [12,13, and references therein]. Within a given species, the DNA binding domain and the ligand binding domain share high homology, whereas the transactivation domains show less. High conservation of a given RAR and RXR subtype is also observed across species boundaries [5–13].

These nuclear receptors are thought to function as ligand-dependent transcriptional factors. Both all-trans- and 9-cis-RA are ligands for RAR, while only 9-cis-RA activates RXR [12–17]. Several groups have reported that heterodimer formation between RXR and RAR, thyroid hormone receptor, or vitamin D_3 receptor enhances receptor binding to specific elements in the promoters of responsive genes, and mediates the hormone response [18–23]. However, homodimers of RXR or RAR have also been reported to mediate gene expression [24,25].

One important target for retinoids is the collagenase gene. Collagenase (matrix metalloproteinase-1; MMP-1) is a member of the family of matrix metalloproteinases, enzymes which play a prominant role in the degradation of the extracellular matrix [26,27]. Collagenase is a major

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gene product of fibroblasts, and it is the only family member that initiates degradation of the interstitial collagens type I, II, and III, the body's most abundant proteins [28]. Thus, this enzyme has a significant role in modeling the extracellular matrix; and understanding the mechanisms by which retinoids affect the collagenase gene is crucial to our understanding of connective tissue metabolism in both normal and disease states.

Indeed, previous studies from several laboratories, including ours, have shown that matrix metalloproteinases have an important role in embryonic development [27,29], uterine resorption [30], wound repair [31], rheumatoid arthritis [32–34], and tumor invasion [27,35]. Production of MMPs by fibroblasts is inhibited by RA, and this inhibition occurs, at least in part, at the level of transcription [36–40]. Studies on collagenase and a related MMP, stromelysin, demonstrated that transcriptional inhibition of these two enzymes is mediated by RARs and that the activator protein-1 (AP-1) site, which binds fos/jun, in the promoter of these two genes and is involved in this negative regulation [38,40–43]. However, no direct binding of RAR to the AP-1 site has been found. In vitro studies have shown that either RAR or RXR alone could bind to AP-1 protein, such as c-jun, thus sequestering it and preventing its from binding to the AP-1 site and transactivating target genes [41–43].

In a previous report [40], we examined effects of RA on collagenase gene expression by transfecting a chimeric construct containing 182 bp of the rabbit collagenase promoter linked to the chloramphenical acetyl transferase (CAT) reporter gene into HIG82 cells, a normal rabbit synovial fibroblast cell line [44]. We demonstrated that sequences in addition to the AP-1 site participated in RA-mediated repression of the collagenase gene. We also used cotransfection of an RAR expression vector with our chimeric construct to show that repression was RAR type-specific. Although RAR-α, β, and γ could each antagonize phorbol myristate acetate (PMA)-induced transcription, RAR-γ was the most potent repressor, and only it could suppress basal levels of transcription. In this study, we continue to explore the molecular mechanisms by which retinoids suppress the collagenase gene. Specifically, we report that 1) all-trans-RA and 9-cis-RA suppress collagenase synthesis and that 2) this inhibition is probably mediated by RAR and RXR, since both are present in a complex of proteins that bind to sequences within the collagenase promoter.

MATERIALS AND METHODS

Cell Culture and Treatment

HIG82 cells were originally established by spontaneous transformation of a primary culture of rabbit synovial fibroblasts [44], and were obtained from the American Tissue Culture Collection (Rockville, MD). Cells were maintained in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Sigma; St. Louis, MO) (DMEM/FCS) as described previously [40]. For experiments, cells were washed with Hanks' balanced saline three times, then switched to DMEM medium supplemented with 0.2% lactalbumin hydrolysate (DMEM/LH). Cells were treated with all-trans-RA (Roche), 9-cis-RA (Roche), or PMA (Sigma) as described in the text. The purities of stock RA solution were $>99\%$ for all-trans-RA, and 97% for 9-cis-RA.

Western Analysis

Cells were cultured in 6-well plates until confluent. After treatment with all-trans- and 9-cis-RA for 24 h, 1 ml medium was precipitated with 3% trichloroacetic acid, and the precipitates were suspended in sample buffer with mercaptoethanol, and electrophoresed on a 7.5% SDS polyacrylamide gel. The proteins were transferred to a PVDF membrane which was hybridized with sheep antiserum specific to rabbit collagenase, and stained with biotinylated goat antisheep antibody and horseradish peroxidase H complex (Vectastain ABC kit, Vector Lab.) [45].

Northern Blot Analysis

Total RNA from HIG82 cells was prepared by the guanidinium isothiocyanate-CsCl method [40]. Twenty micrograms RNA per sample was electrophoresed on 1% formaldehyde agarose gel and transferred to a GeneScreen Plus membrane. The membrane was hybridized with denatured [α-^{32}P]dCTP-labeled probe (2×10^6 cpm/ml) for 20 h at 56°C, then washed twice with 0.2× SSC, 0.1% SDS at room temperature for 10 min, and twice at 56°C for 30 min. Hybridization with glyceraldehyde phosphate dehydrogenase (GAPDH) or ethidium bromide staining of gels was used as loading control.

cDNA Probes

Full-length cDNA inserts of mouse RAR-γ [8], 900 bp cDNA fragment of RAR-β (EcoRI) [8],

745 bp cDNA fragment of RAR-α (EcoRI-Sac I) [8], 500 bp cDNA fragment of human RXR-α [16], 550 bp cDNA fragment of GAPDH (HindIII-XbaI) (ATCC), 630 bp fragment of collagenase (EcoRI-HaeIII) [46] were used as probes in Northern analysis. Probes were labeled with [α-^{32}P]dCTP by random priming (Pharmacia, Piscataway, NJ).

Preparation of Nuclear Extracts and Mobility Shift Assay

Nuclear extracts were prepared as described [40]. Five microgram nuclear extracts were incubated with 30,000 cpm [γ^{32}P]ATP-labeled double-stranded oligonucleotides 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) for 15 min on ice, then incubated with diluted antibody ascites (final concentration 1:15) specific for RAR-α, β, γ, and RXRs for additional 30 min on ice. The reaction products were resolved by electrophoresis on 5% nondenaturing polyacrylamide gels. Antibodies to RARs and RXRs are generous gifts from Dr. Pierre Chambon. The antibodies to RARs were raised against the F region of mouse RAR-α, β, γ, and recognize RAR-α, β, γ, respectively [47]. The antibody to RXRs was raised against the E/D region of RXRs, and recognizes all three subtypes of RXRs [10]. Antibody to a transmembrane receptor FcγRI was a gift of Dr. Paul Guyre [48].

Detection of RA Isomer

HIG82 cells were treated with 10^{-6} M all-trans- or 9-cis-RA for 6 h or 24 h. Cells were washed with Hanks' balanced saline three times, pelleted in a preweighted 15 ml tube, and stored at −70°C in dark. Retinoids within cells were extracted by the addition of 3 volume parts of plain isopropanol or isopropanol containing Ro 10-1670 (acitretin) as internal standard. Supernatant fluids were diluted with 2% acetic acid and solid-phase extracted on Bond-Elut C8 cartidges (100 mg, 1 cc). Ethanol (0.5 ml) was used for elution into an injector vial and evaporated to dryness under a stream of nitrogen at 37°C. Residues were redissolved in 0.1 ml 95% methanol, and injected on a 2 × 20 mm Spherisorb ODS/NH2 column. Acidic retinoids were transferred in backflush mode with ethanol-water-acetic acid (90:9.75:0.25) and on line dilution with water in an alternating fashion to one of two 2 × 20 mm Bondesil ODS columns. Then the sample was transferred with a gradient formed with 0.1% acetic acid in water and acetonitrile to a Spherisorb ODS columns (3 × 300 mm) for separation, and absorbance was monitored at 345 nm. When plain isopropanol was used for initial extraction, retinoid peaks were normalized to total peak area (100%). When an internal standard was added, concentration in unknown samples was calculated in ng/g of cells after calibration of the instrument using standard samples of known concentrations.

Stable Transfection

HIG82 cells were cultured in 150 mm plates until 80% confluent. RAR-γ expression vector (20 μg) (gift of Dr. Pierre Chambon) was cotransfected into HIG82 cells with 2 μg pRC/CMV selection vector (Invitrogen) using the calcium phosphate coprecipitation method. Stable transfectants were selected by culturing cells in the DMEM/FCS containing 400 μg/ml G418 for 2 months. The medium and G418 were changed twice a week, and the frequency of stable transfectant was ~10^{-6}. Three to four colonies arising from a single 150 mm plate were pooled together, passaged, and maintained in DMEM/FCS and 400 μg/ml G418.

Southern Analysis

Genomic DNAs were prepared from parent HIG82 cells and from RAR-γ stable transfectants as described [49]. Briefly, cells were digested with proteinase K overnight. After extraction with an equal volume of phenol/chloroform/isoamyl alcohol twice, DNA was precipitated by $\frac{1}{2}$ volume of 7.5M ammonium acetate and two volumes of 100% ethanol. Digested genomic DNA was electrophoresed on 0.8% agarose gel [50]. The gel was incubated with 0.25M HCl for 20 min, denaturation buffer (1.5M NaCl/0.5M NaOH) twice for 20 min, and neutralization buffer (1.5M NaCl/0.5M Tris-Cl, pH 7.0) twice for 20 min. DNA was transferred to GeneScreen plus membrane with 20 × SSC overnight, and was UV crosslinked using an UV Stratalinker 1800.

Transient Transfection and CAT Assay

Fusion of 1,176 bp of rabbit collagenase promoter to the reporter chloramphenicol acetyltransferase gene (CAT) has been described previously [51]. The chimeric CAT construct pCCAT1176 was transfected into HIG82 cells (2.5 μg DNA/60 mm plate) with RAR-γ expres-

sion vector or pSG5 (expression vector without RAR insert) by the calcium phosphate coprecipitation method. After overnight recovery, cells were cultured in DMEM/LH alone, or treated with 10^{-6}M all-trans-RA or 9-cis-RA for 26 h, with 10^{-8}M PMA for 24 h, or with 10^{-6}M RA plus 10^{-8}M PMA 24 h, after a 2 h pretreatment with RA. Cell lysates were prepared by the freeze-thaw method, and protein concentrations in the cell lysates were determined by Bradford assay (Bio-Rad). Five micrograms protein per sample was used for CAT assay [40]. The reaction products were resolved by thin-layer chromatography (TLC) and autoradiography, and were quantitated by scintillation counting of the TLC. Relative CAT activities were normalized to the CAT activity in the absence of treatment within the same transfection group.

RESULTS

Suppression of Collagenase Gene Expression by all-trans- and 9-cis-RA

Previous studies have shown that inhibition of collagenase and stromelysin gene expression by all-trans-RA is mediated by RARs [38,40–42]. We used Western analysis to examine whether RXR also plays a role in suppressing collagenase synthesis. HIG82 cells were treated for 24 h with all-trans-, 9-cis-, or all-trans-plus 9-cis-RA with the total concentration equal to that of the single agent treatment. One milliliter of culture medium was then subjected to Western analysis with anti-serum specific for rabbit collagenase. As shown in Figure 1A, both all-trans- and 9-cis-RA markedly decreased collagenase synthesis at high concentrations (10^{-6} to 10^{-7}M). Neither of these concentrations is toxic to the cells [33]. At a concentration of 10^{-8}M, all-trans- or 9-cis-RA alone did not reduce collagenase synthesis, whereas treatment with a combination of both agents did. Two other experiments gave similar results.

We also measured the steady-state levels of collagenase mRNA after the same treatments (shown in Fig. 1B). As with the Western analysis, both all-trans- and 9-cis-RA decreased collagenase mRNA when used at concentrations of 10^{-6}M to 10^{-7}M. Here, too, the combination of all-trans- and 9-cis-RA at lower concentrations (10^{-8} to 10^{-9}M) reduced the levels of collagenase mRNA, while a single agent had no effect. The slight differences between the level of suppression seen with mRNA vs. protein reflect the fact that mRNA represents the amount of steady

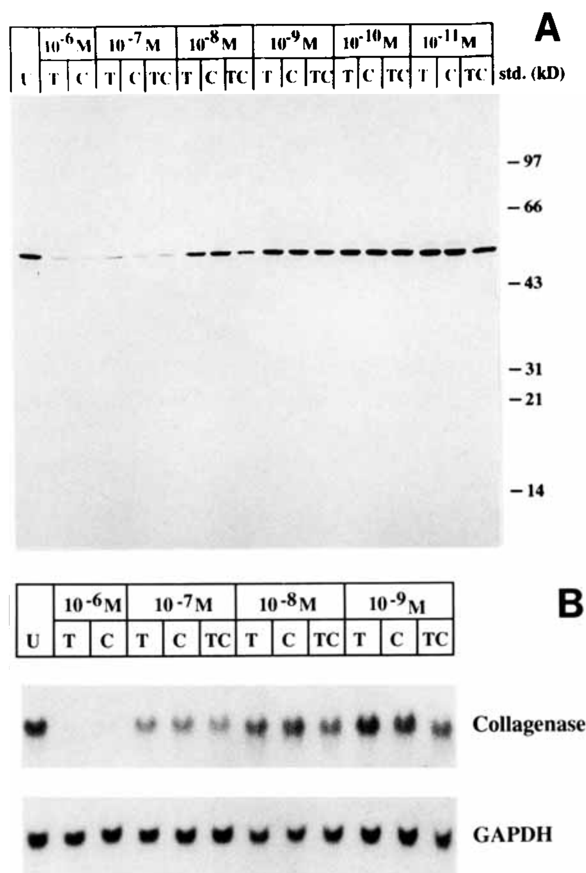


Fig. 1. Suppression of collagenase by RA. HIG82 cells were treated with all-trans-RA (T), 9-cis-RA (C), or all-trans-RA plus 9-cis-RA (TC) at the concentrations indicated for 24 h. **A:** One milliliter culture medium was subjected to TCA precipitation and Western analysis with sheep antiserum specific to rabbit collagenase. **B:** Twenty micrograms total RNA was subjected to Northern analysis. The membrane was hybridized with probes specific for rabbit collagenase, and for GAPDH as a loading control.

state mRNA at the time of harvest (24 h), while the protein levels are cumulative over the 24 h incubation period.

These data confirm that repression of collagenase mRNA by retinoids is paralleled by a decrease in collagenase protein [28,40]. Further, since only 9-cis-RA can activate RXR [12–17], it may participate in repression.

Induction of RAR mRNAs by all-trans- and 9-cis-RA

To study the possible role of RAR and RXR in mediating the effects of RA, we examined the regulation of RAR and RXR mRNAs by all-trans- and 9-cis-RA (Fig. 2). We found that mRNA for RAR-α and γ were constitutively

expressed in untreated HIG82 cells, while RAR-β mRNA was hardly detectable (Fig. 2A,B). All-trans-RA increased the steady-state levels of mRNAs for all three RARs in a dose-dependent manner, with the effective dose ranging from 10^{-6}M to 10^{-9}M (Fig. 2A). Treatment of cells with 9-cis-RA also increased mRNAs for RAR-α, β, and γ; and this increase was comparable to that seen with all-trans-RA (Fig. 2B). Induction of RAR mRNAs was observed within 3 h of treatment and peaked at 6–12 h of treatment (data not shown).

We also measured the expression of RXR-α mRNA and found that mRNA for RXR-α was constitutively expressed at a high level. Furthermore, neither 9-cis- nor all-trans-RA changed this expression (Fig. 2A,B).

The effects of retinoids on collagenase production by synovial cells are readily reversible upon removal of the drug [33,34], and we wanted to exclude the possibility that low concentrations of retinol or retinoic acid in serum-containing medium could account for constitutive expression of RAR-α, RAR-γ, and RXR-α. Therefore, we cultured HIG82 cells in serum-free medium for up to 5 days (Fig. 3). These culture conditions did not alter the steady-state levels of RAR-α, RAR-γ, and RXR-α mRNAs, although collagenase mRNA increased considerably. This finding indicates that the constitutive expression of RAR-α, RAR-γ, and RXR-α by themselves, cannot repress collagenase synthesis, and implies that suppression is ligand dependent.

Finally, we investigated the mechanisms involved in the "autoactivation" of the collagenase gene under serum-free culture condition. Since the transcription factors c-fos and c-jun are involved in activation of the collagenase gene in response to phorbol [39–42], we measured these mRNAs. Figure 3 illustrates that serum starvation increased the mRNA levels of both c-fos and c-jun in HIG82 cells, suggesting

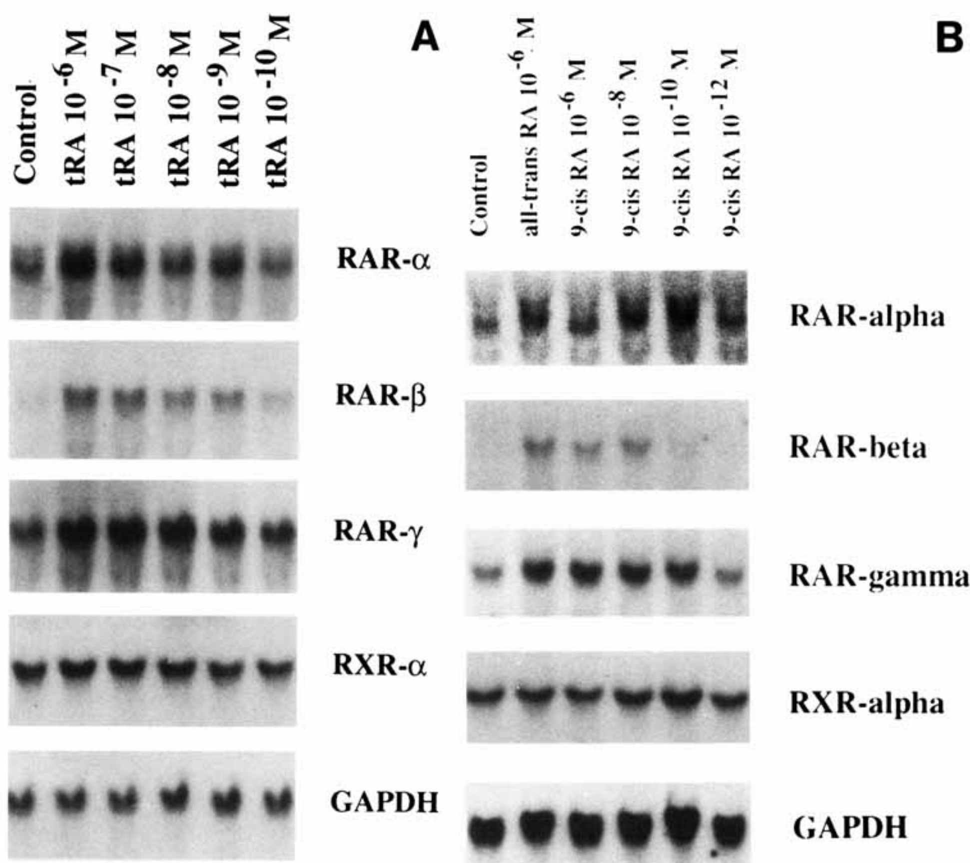


Fig. 2. Induction of RAR mRNAs by RA. HIG82 cells were treated with all-trans-RA (T) or 9-cis-RA (C) at the concentration indicated for 6 h. Total RNA was prepared using guanidium isothiocynate-CsCl method, and 20 μg RNA/sample was subjected to Northern analysis. The membrane was hybridized with probe specific for RAR-α, β, γ, RXR-α, and GAPDH for loading control. The differences in signal intensity seen between **A** and **B** are due to the differences in specific activities of the probes. Autoradiographs were exposed for 5 days.

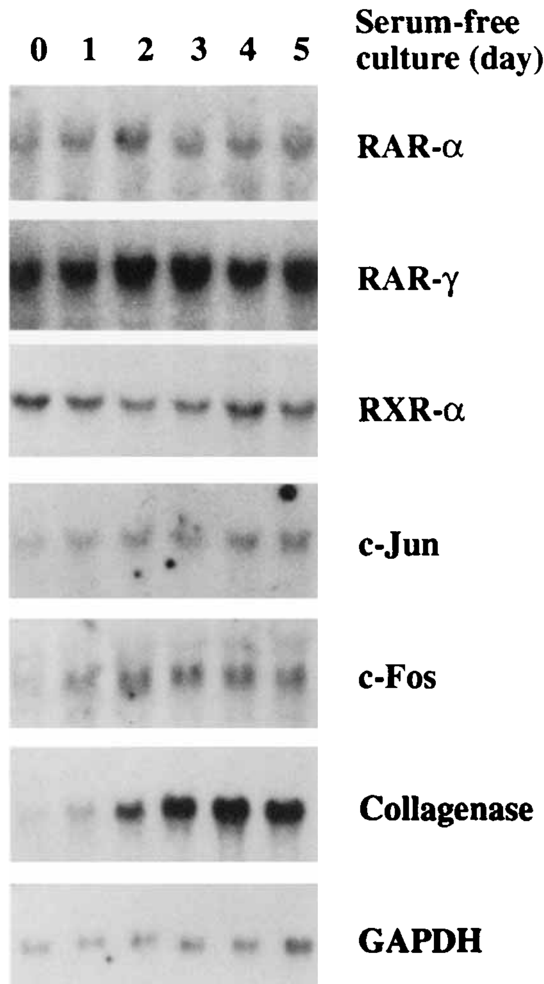


Fig. 3. Messenger RNA expression under serum starvation. Cells were cultured in serum free medium (DMEM/LH) for up to 5 days. Total RNA was prepared using the guanidium isothiocynate-CsCl method, and 20 μg RNA/sample was subjected to Northern analysis. The membrane was hybridized with probes specific for RAR-α, γ, RXR-α, c-jun, c-fos, and for GAPDH as a loading control. Autoradiographs for RARα, RARγ, and RXRα were exposed for 5 days; those for c-jun and c-fos for 3 days; those for collagenase, 1 day.

that, similar to phorbol induction [39–42], these transcription factors participate in "autoactivation" of the collagenase gene under these conditions.

Repression of Collagenase Promoter-Driven CAT Activity in Cells Stably Transfected With RAR-γ

Results from our Northern analysis illustrate that RA increases levels of RAR-α, β, and γ mRNAs without altering RXR mRNA expression, and data from our previous transient transfection assays indicate that RAR-γ is the most potent repressor [40]. However, the levels of endogenous RARs, including RAR-γ, are quite low, as evidenced by the fact that a 5 day exposure of Northern blots was required to detect RAR mRNA when 20 μg total RNA was used, and that cotransfection of an RAR expression vector along with our collagenase promoter driven-CAT reporter was necessary to obtain RA-mediated repression. These findings suggest that the availability of RAR may be a major limiting factor in RA-mediated collagenase repression. To better understand the role of RAR, we established stable transfectants expressing RAR-γ in HIG82 cells. Here, we report the characterization of these transfectants.

Stable transfectants of RAR-γ have a morphology indistinguishable from that of parent HIG82 cells, and exhibit a similar growth rate (data not shown). Figure 4A shows the Southern blot analysis of genomic DNA from these cells. EcoRI digestion, which released the RAR-γ cDNA insert (2kb) from the pSG5 expression vector, gave a 2 kb fragment as expected [8], whereas a ~8 kb fragment was observed in DNA prepared from parent HIG82 cells. Xba I digestion, which linearizes the pSG5 plasmid 3′ to the RAR-γ cDNA insert [8], resulted in a fragment of 5.5 kb. The pattern of restriction enzyme digestion indicates that the RAR-γ expression vector has successfully integrated into host genomic DNA, and is consistent with the insertion of only a single copy of the gene.

We used Northern blot analysis to measure the mRNA levels of the transfected RAR-γ gene. Constant amounts of total RNA (20 μg/sample) harvested from parent HIG82 cells that had been treated with all-trans-RA were compared to 20 μg or 5 μg of total RNA from stable transfectants (Fig. 4B). We found a constitutively expressed transcript of 2.4 kb from the exogenous RAR-γ cDNA. As expected, this is smaller in size than the full length endogenous gene transcript (3.5 kb). The expression of this transcript in control cells is much higher than that of the endogenous transcript treated with all-trans-RA for 6 h (Fig. 4B). Furthermore, the level of this exogenous transcript was not regulated by all-trans-RA, also as expected (Fig. 4C).

We measured expression of the endogenous collagenase gene in the stably transfected cells. Although similar, the level of endogenous collagenase mRNA in the transfected cells was slightly lower than that of the parent cells. This finding resembles that seen when we measured CAT expression in HIG82 cells transiently co-

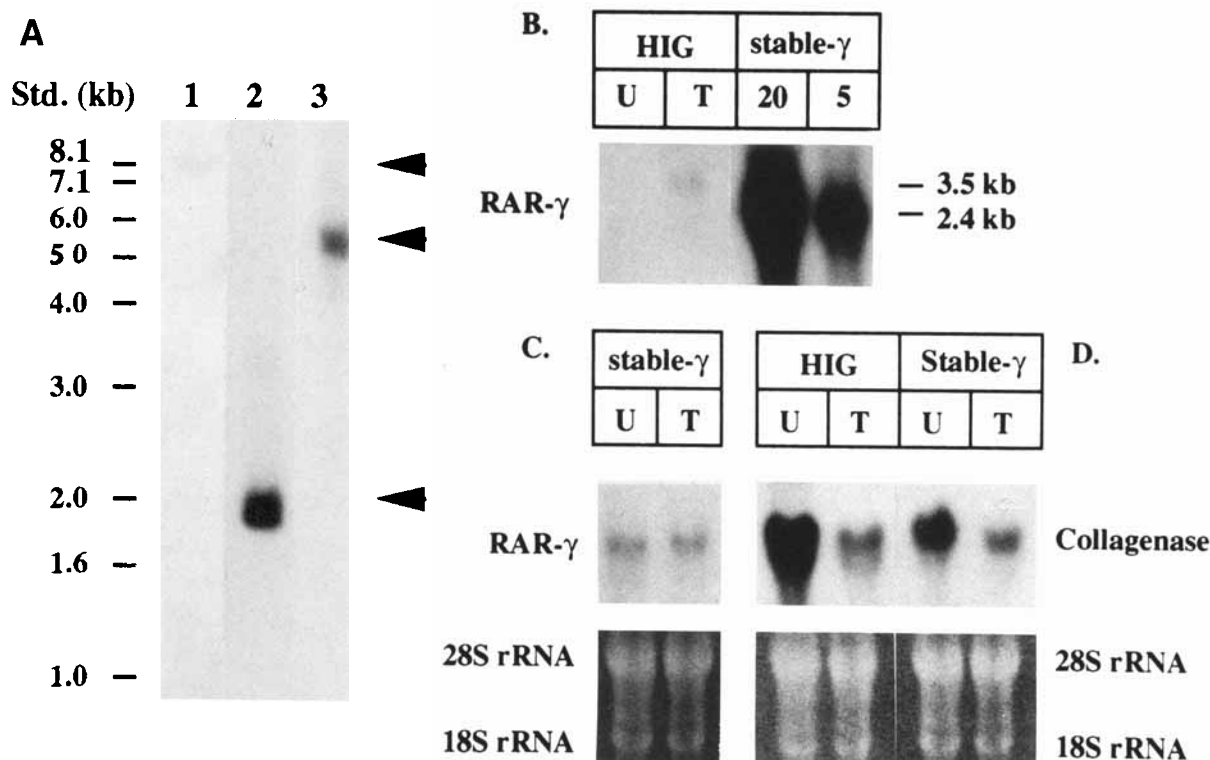


Fig. 4. Expression of exogenous RAR-γ in stable transfectants. **A:** Southern analysis. Twenty micrograms genomic DNA from parent HIG82 cells was digested with EcoRI (lane 1), 10 μg DNA from stable transfectants was digested with EcoRI (lane 2) or XbaI (lane 3). The membrane was hybridized with a probe specific for RAR-γ. **B:** Expression of transfected RAR-γ mRNA. RNA from parent HIG82 cells or RAR-γ stable transfectants was used for Northern analysis, and hybridized with a cDNA probe for RAR-γ. Twenty micrograms RNA from HIG82 cells untreated (U) or treated with 10^{-6}M all-trans-RA for 6 h (T) was used. Twenty micrograms (20) or 5 μg (5) RNA from untreated stable transfectants was used. **C,D:** Regulation of mRNA levels for RAR-γ and collagenase. Cells were cultured in DMEM/LH media (U) or treated with 10^{-6}M all-trans-RA (T) for 6 h. Twenty micrograms total RNA from RAR-γ stable transfectants (C,D) or parent HIG82 cells (D) was used for Northern analysis. Membranes were hybridized with probes specific to RAR-γ (C) or collagenase (D). Ethidium bromide staining of gels were used as a loading control.

transfected with RAR-γ and our collagenase promoter constructs [40]. Of importance, however, all-trans-RA decreased collagenase mRNA comparably in both stable transfectants and parent HIG82 cells (Fig. 4D).

Finally, we examined the function of RAR-γ using a chimeric collagenase promoter-CAT reporter containing approximately 1,200 bp of collagenase promoter DNA (pCCAT1176) [51] in these stable transfectants (Fig. 5). We measured basal and phorbol-induced transcription and assayed the ability of RAR γ to suppress transcription in the presence of 9-cis and all-trans RA. Similar to our previous findings [40], basal levels of transcription were low and were stimulated with PMA. In the presence of either 9-cis or all-trans-RA, RAR γ antagonized both basal and phorbol-induced transcription. In the parent HIG82 cells, cotransfection of an RAR expression vector was required to observe RA-mediated suppression of collagenase promoter-driven CAT activity. In RAR-γ stable transfectants, CAT activity was suppressed to levels equal to those seen when the parent cells were co-transfected with the RAR-γ expression vector. Therefore, RAR-γ is functional in these stable transfectants.

Binding of Collagenase Promoter Sequences by RAR and RXR

It is possible that homodimer formation of either RARs or RXRs, or heterodimerization between RAR and RXR, may participate in RA-mediated suppression of the collagenase gene. To determine which receptors are involved in this process, we prepared nuclear extracts from either untreated, PMA-treated, or retinoic-acid treated wild-type HIG 82 cells. We used these extracts in mobility shift assays with antibodies specific to RARs and RXRs to examine binding

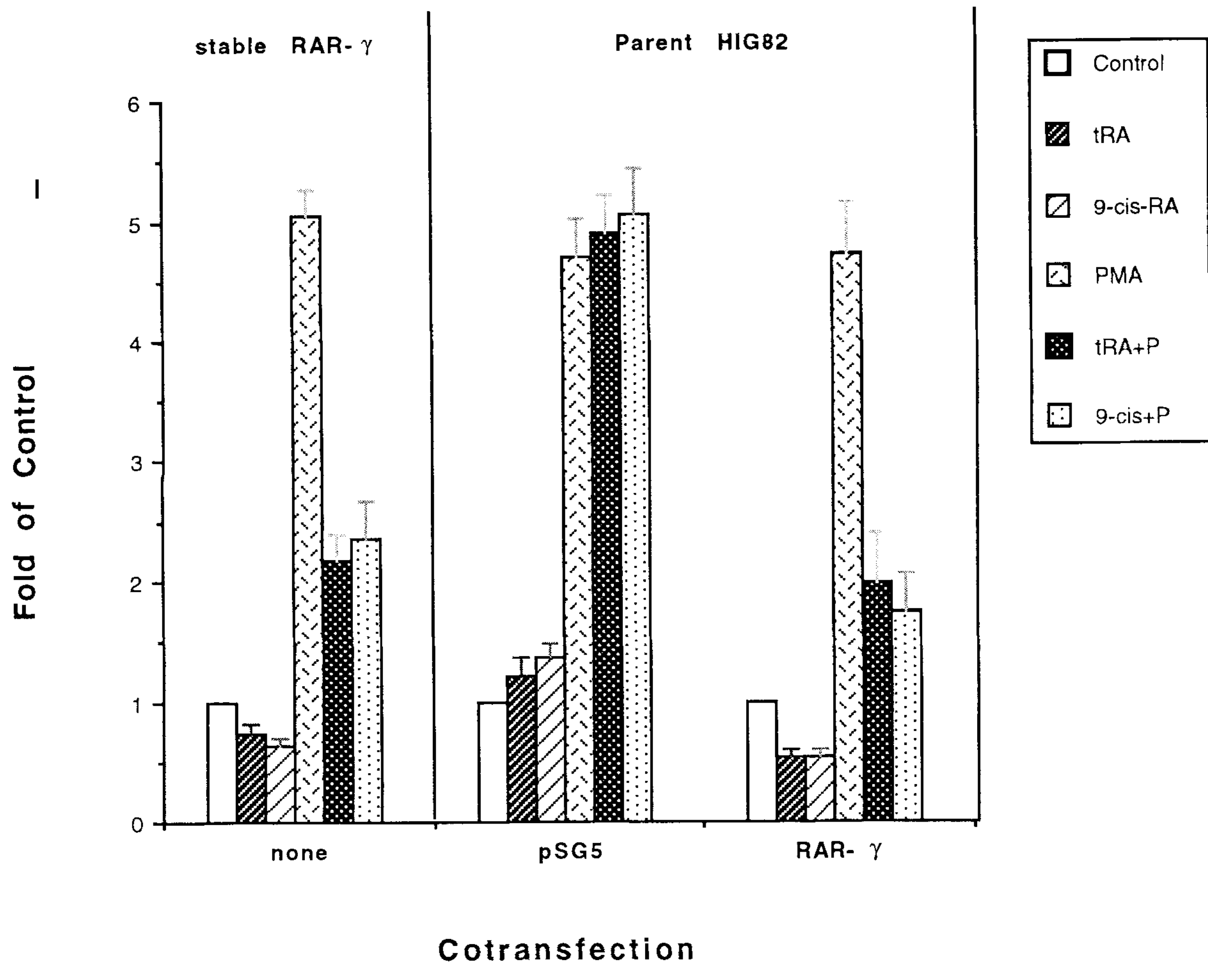


Fig. 5. Suppression of CAT activity driven by 1,176 bp of the rabbit collagenase promoter. A chimeric CAT construct was transfected into parent HIG82 cells, along with either pSG5, the parent vector alone, or RAR-γ expression vector, or into RAR-γ stable transfectants. Cells were left untreated, or were treated with PMA (10^{-8}M) and/or 9-cis or all-trans RA (10^{-6}M). CAT activities from cells in treatment groups are presented as fold of CAT activity from untreated cells.

of RAR and RXR to collagenase promoter sequences.

In our previous studies, we showed that a 182 bp fragment of the collagenase promoter mediated suppression of collagenase transcription by RA [40]. We also defined at least two regions within this 182 bp which participated in this RA and PMA response, a 40 bp region between −182 bp and −141 bp, and the region between −83 bp and −57 bp, which contains an AP-1 site at −77 [40,51,52]. In this study we measured binding of nuclear proteins to each of these regions. We treated cells with all-trans- or 9-cis-RA, with PMA, or with each retinoid plus PMA for 24 h to ensure enough time for new synthesis of RAR proteins, since induction of RAR mRNAs by RA requires at least 3–6 h of treatment. When the −182/−141 oligo was used as a probe, nuclear proteins from untreated cells or from cells treated with either all-trans- or 9-cis-RA have similar binding patterns (bands 1 and 2; Fig. 6A), whereas nuclear proteins from cells treated with PMA show less binding (Fig. 6A). However, nuclear extracts from cells treated with either retinoid plus PMA show substantially enhanced binding, and we observed complexes with distinctive mobilities (bands 3 and 4) (Fig. 6A). This binding is specific, since it is competed by a 100-fold molar excess of self (S) but not of nonself (N). Of importance, addition of antibodies specific to each RAR and to RXRs to the binding reactions at the recommended concentrations [10,47] gave rise to a supershifted band (band 5), as shown in Figure 6A. Like others [53], we found that these supershifts did not cause a diminution of the shifted

protein/DNA complex (bands 3 and 4), perhaps because the amount of shifted material is small relative to the total amount (see Discussion).

Controls in which antibodies to RAR or RXR were added without nuclear extracts, or addition of either of two antibodies of the same isotype as that of the RAR/RXR antibodies but which are directed against a transmembrane receptor (FcγRI), did not give supershifts (Fig. 6A), even when higher concentrations were used (data not shown). Also, no supershifts were seen using nuclear extracts from untreated cells, or from cells treated with RA for only 1–3 h (data not shown). These data demonstrate specific binding of antibodies to RARs and RXRs and suggest that both RAR and RXR are present in the nuclear proteins binding to −182/−141 sequences.

We also measured binding of nuclear extracts to the −83/−57 oligo which contains an AP-1 binding site at −77. In contrast to the data presented in Figure 6A where, compared to extracts from untreated cells, extracts from RT-treated cells showed only a minimal increase in binding to the −182/1–41 oligo, nuclear proteins derived from all-trans- and 9-cis-RA-treated cells bound to the oligo with an AP-1 site, and binding was competed by a 100-fold molar excess of self (Fig. 6B, bands 1, 2). Two other bands are also present, but they do not appear to be regulated by retinoids. Addition of antibodies to RARs and RXRs in the binding reaction gave rise to a supershifted band (Fig. 6B, band 3), again suggesting that the complex contains both RARs and RXRs. A nonspecific oligonucleotide failed to compete for binding, and control antibodies did not supershift the complexes formed between the AP-1 oligo and nuclear extracts from RA-treated cells (data not shown).

Similar to other investigators, who found that this mutation abolished binding of Fos and Jun [54], we found that a single T to G basepair mutation at position −77 of the AP-1 site in this oligo prevented binding of all proteins (data not shown). This indicates that even though RARs and RXRs may not bind directly to the AP-1 site [37,38,41,42] an intact AP-1 sequence is necessary (see Discussion).

Isomerization of RA Within HIG82 Cells

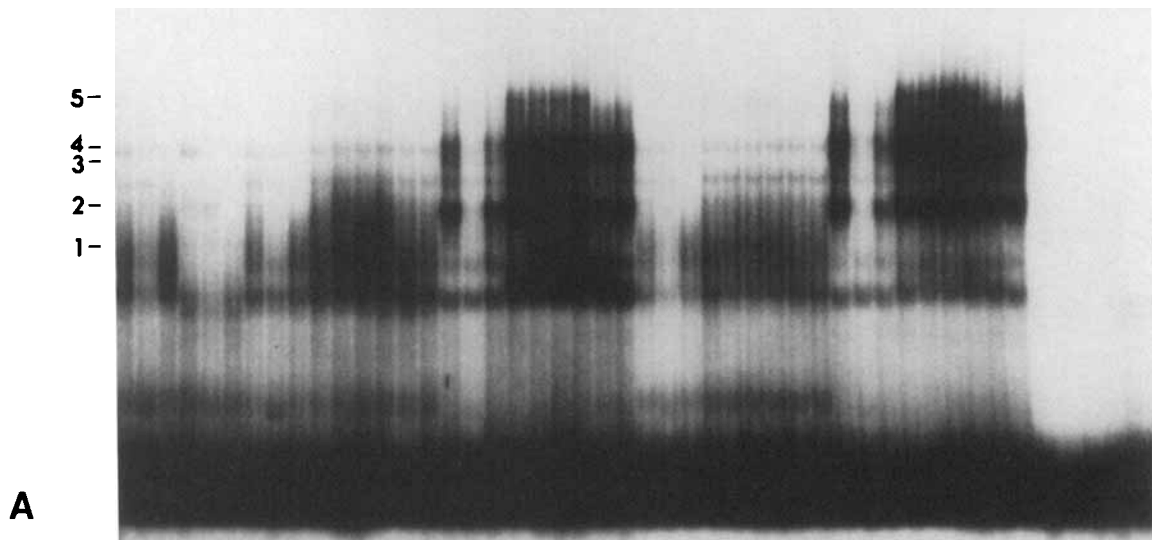
Our data indicate that high concentrations of all-trans-RA can mediate suppression of the collagenase gene (Figs. 1 and 2) and that RXR is involved in this suppression (Figs. 5 and 6). Since all-trans-RA cannot activate RXR, it is possible that isomerization of all-trans-RA to 9-cis-RA within HIG82 cells may be an important mechanism by which all-trans-RA-mediates repression. To test this hypothesis, we examined the isomers of RA within HIG82 cells after treatment of cells with all-trans-RA or 9-cis-RA for 6 h or 24 h. As shown in Table I, after treatment with all-trans-RA for 6 h, the predominant isomer within HIG82 cells was all-trans-RA (90%). However, after 24 h treatment, some accumulation of 9-cis-RA (20%), and of 13-cis-RA, was found. Thus, the conversion from all-trans-RA to 9-cis-RA may explain how high concentrations of all-trans-RA could achieve maximum suppression. In contrast, a combination of both all-trans-RA and 9-cis-RA was required for inhibition of collagenase synthesis at lower concentrations (details in Discussion). The conversion of RA in cells treated with 9-cis-RA was more rapid. After 6 h, 50% of total RA within cells was in the all-trans-RA isoform, and this reached 70% after 24 h.

We also measured the concentration of total RA within HIG82 cells after treatment for 6 h and 24 h. The initial concentration of either all-trans- or 9-cis-RA in culture medium was 10^{-6}M. After 6 h, the total concentration of RA in cells treated with all-trans-RA was ~5 μg/g cells, and this decreased to ~260 ng/g cells after 24 h treatment. The total concentration of RA in cells treated with 9-cis-RA was ~2.15 μg/g cells after 6 h, and decreased to 55 ng/g cells after 24 h. This result indicates that 9-cis-RA decayed at a faster rate than all-trans-RA. Since the percentage of all-trans-RA increased over time in cells treated with 9-cis-RA, conversion of 9-cis-RA to all-trans-RA may be a mechanism by which cells slow the decay of total RA (see Discussion).

DISCUSSION

This paper demonstrates that both all-trans and 9-cis retinoic acid suppress collagenase gene expression in normal fibroblasts. Induction of endogenous RARs by RA treatment of cells, or the introduction of exogenous RARs in the presence of RA, is required for this suppression. In addition, both RAR and RXR bind to collagenase promoter sequences, as shown by mobility shift assay with specific antibodies. The fact that all three RARs are present in the shifted complexes, although RAR γ is the most potent repres-

Treatment	none	PMA	all-trans RA	all-trans RA+PMA	9-cis RA	9-cis RA+PMA	no extracts
Competitor	- S N	- S N	- S N - - - - - -	- S N - - - - - -	- S N - - - - - -	- S N - - - - - -	- - - - - -
Ab to RAR	- - -	- - -	- - - α β γ - - -	- - - α β γ - - -	- - - α β γ - - -	- - - α β γ - - -	α β γ - - -
Ab to RXR	- - -	- - -	- - - - - - + - -	- - - - - - + - -	- - - - - - + - -	- - - - - - + - -	- - - + - -
Ab to FcγRI	- - -	- - -	- - - - - - - 1 2	- - - - - - - 1 2	- - - - - - - 1 2	- - - - - - - 1 2	- - - - 1 2



Treatment	none	all-trans RA	9-cis RA
Competitor	- +	- + - - - -	- + - - - -
Ab to RAR	- -	- - α β γ -	- - α β γ -
Ab to RXR	- -	- - - - - +	- - - - - +

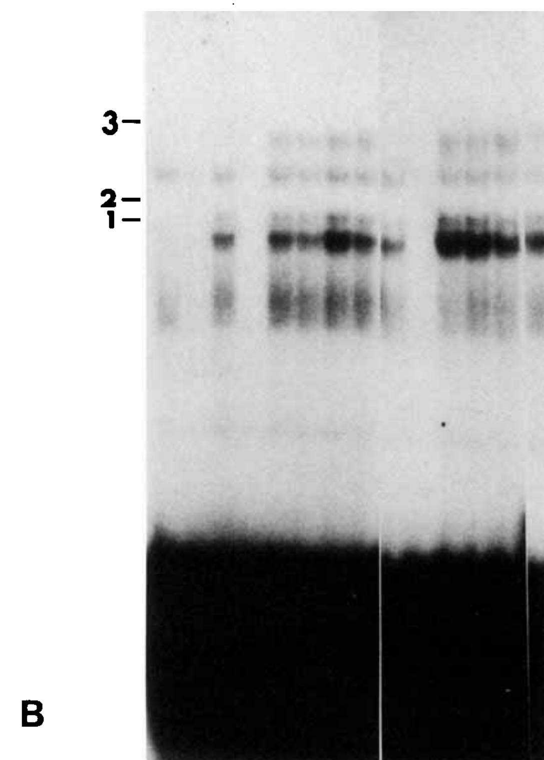


Fig. 6. Specific complex formation between nuclear proteins and radiolabeled fragments of the rabbit collagenase promoter. HIG82 cells were cultured in DMEM/LH media (none) or treated with 10^{-6}M all-trans-RA, 10^{-6}M 9-cis-RA, 10^{-8}M PMA, PMA plus all-trans-RA, or PMA plus 9-cis-RA for 24 h. Five micrograms nuclear extract proteins were incubated with oligoprobe either alone (−) or in the presence of 100× molar excess of unlabeled competitors of self (S) or a non-specific oligo (N) (5′-AGCTTGCTCAGGCTAT-3′) for 15 min on ice, then antibodies to RARs and RXRs were added to the binding reaction for another 30 min incubation. The reaction products were resolved by electrophoresis on 5% nondenaturing polyacrylamide gels. **A:** Specific complex formation with the −182/−141 oligoprobe. The oligo represents rabbit collagenase promoter sequence from −182 to −141 (5′-TCATGAAATTGCAACACCAAGCTAACCCAAAAAATCTGCCG-3′). **B:** Specific complexes formation with the −83/−57 oligoprobe. The oligo represents rabbit collagenase promoter sequence from −83 to −56 (5′-GAAAGCATGAGTCACACAGCCCTCAGCT-3′). AP-1 consensus was underlined.

TABLE I. Isomers of RA in HIG82 Cells Treated With all-trans- or 9-cis-RA*

Treatment	Time (h)	Isomer of retinoic acid (%)			
		all-trans	9-cis	13-cis	Others
all-trans	6	80.0	8.1	10.8	1.1
	24	68.7	17.3	10.6	3.4
9-cis	6	50.8	37.5	8.9	2.8
	24	64.3	24.1	6.4	5.1

*Cells were treated with 1 μM all-trans- or 9-cis-RA for the time indicated. After washing three times with Hanks' balanced saline, isomers of RA in the cell pellets were examined. Results are expressed as percentage of each RA isomer. No RA was detected in untreated cells.

sor [40], suggests that promoter sequences in addition to those described here participate in transcriptional repression [39,40].

Signals of all-trans- and 9-cis-RA are transduced through specific nuclear receptors, RARs and RXRs. Both all-trans-RA and 9-cis-RA bind RARs with similar affinity (kd ~0.2–0.7 nM), whereas only 9-cis-RA binds RXRs with a kd ranging from ~14 to 18 nM or 1.6 to 2.4 nM [55,56]. In this study, we demonstrate that conversion from all-trans-RA to 9-cis-RA occurs within HIG82 cells. This chemical modification of RA within HIG82 cells could explain how, at higher concentrations (10^{-6} to 10^{-7}M), there is sufficient conversion of all-trans-RA to 9-cis-RA to activate both RAR and RXR, thus resulting in a maximum repression of collagenase synthesis. However, at lower concentrations (10^{-8}M) both all-trans- and 9-cis-RA are required to achieve suppression.

The concentration of intracellular all-trans-RA and its metabolism are controlled by cellular RA binding proteins (CRABPI, CRABPII), which bind all-trans-RA with high affinity [57–61]. So far, no cellular binding protein for 9-cis-RA has been reported; thus the majority of intracellular 9-cis-RA may exist as a free form [57,58, references therein]. In this study, we also observe a faster conversion of 9-cis-RA to all-trans-RA compared to that seen in all-trans-RA treatment. This is probably due to the presence of intracellular 9-cis-RA in a free form. In contrast, the majority of all-trans-RA is bound to cellular RA binding proteins and this may prevent the chemical modification.

RAR and RXR mediate RA functions by binding to the retinoic acid responsive elements (RARE) within the promoter of target genes [62–66]. RARE is generally composed of a direct repeat of the half-site core motif pu(G/T)TCA which is spaced by 2(DR-2) or 5(DR-5) basepairs [13,67,68]. Recent studies from several groups showed that activation of RA-responsive elements often occurs through formation of RAR/RXR heterodimers. The asymmetric nature of RAREs, the availability of RAR and RXR, and the ligand all determine how specific target genes are regulated [69–74]. Although a computer search did not reveal any perfect sequence match with the RARE half-site core motif within 1,200 bp of the rabbit collagenase gene promoter, results of our mobility shift assay support the presence of RAR and RXR in a complex of proteins which binds specifically to two regions within 182 bp of the rabbit collagenase promoter, regions which we know participate in RA and PMA responsiveness [40,51,52]. One is the region around the AP-1 concensus sequence at −77. We found that nuclear extracts from cells treated with RA bind to an oligo containing collagenase promoter sequences from −83 to −57 bp with an intact AP-1 binding site (−77 to −70). "Supershift" experiments with antibodies to RARs and RXRs suggest that both RAR and RXR are involved in complex formation, perhaps as heterodimers (Fig. 6B). Indeed, heterodimer formation of RXR with RAR, with the vitamin D3 receptor, or with the thyroid hormone receptor mediates hormones function and may enhance the binding affinity of the receptors to their respective response elements [18–23,75–77].

However, other studies have shown that RAR or RXR alone did not bind directly to the AP-1 consensus in the collagenase and stromelysin promoters [37,38,41,42]. Instead, they appeared to blunt transactivation of target genes by binding AP-1 proteins, such as c-jun, through protein-protein interactions. Our findings suggest the existence of heterodimers between RXR and RAR, and it is conceivable that these heterodimers, probably as part of a complex of additional proteins [37,38,41,42], are binding directly to the DNA. This idea is supported by the fact that a) treatment of cells with RT, alone, increased the binding and b) a single base pair mutation abolished binding of all proteins. Thus, it is possible that heterodimer formation increases the affinity of the receptors for the AP-1 site. Alternatively, neighboring sequences near the AP-1 site may facilitate binding of RAR and RXRs. However, even if neighboring se-

quences are involved, an intact AP-1 site is necessary.

Another region of the collagenase promoter which binds RAR and RXR is located at −182 to −141 bp, and it seems clear that proteins in addition to RARs and RXRs play a role in mediating this binding. In contrast to the AP-1 oligo, treating cells with RT alone does not change the binding pattern substantially and addition of antibodies results in only a very weak supershift, at best. In addition, nuclear extracts from cells treated with PMA do not show increased binding to this oligo above that seen with extracts from untreated cells. However, nuclear proteins from cells treated with both RA and PMA bind to this region with high affinity, and these proteins contain RAR and RXR (Fig. 6A). The difference in binding patterns between this upstream region and the AP-1 site at −77 suggests that different protein components may be involved in complex formation at each site [52]. Further, the finding that the region from −182 to −142 did not show increased binding with extracts from cells treated with either PMA or RA, but did show an increase when cells were treated with both compounds indicates a possible mechanism for RA action. Repression of PMA-induced, but not basal, transcription may be mediated through these sequences, and may well involve a complex of multiple proteins, including RARs and RXRs.

Despite the appearance of a specific "supershifted" band in the presence of antibodies to the RARs and RXRs, there was no diminution in the signal given by the complex of shifted DNA/protein (Fig. 6A and B). This is similar to a report by Boylan et al. who used these same antibodies with extracts of F9 teratocarcinoma cells [53]. Our results may be attributable to the relatively low level of endogenous RAR protein present in both the F9 cells [78] and in the synovial fibroblasts [40]. Indeed, transient transfection experiments with either the laminin promoter in F9 cells [78] or the collagenase promoter in fibroblasts [40] required co-transfection of RARs in order to measure the effects of these receptors on transcription.

In examining expression of endogenous RARs and RXR α, we found that levels of mRNA for the RARs were comparatively low, but increased upon treatment with either all-trans- or 9-cis-RA. In contrast, mRNA for RXR α was constitutively high and was not changed by RA treatment (Fig. 2). These results suggest that the availability (amount) of RAR may be a limiting factor and that RXR may facilitate this negative regulation. Furthermore, suppression was ligand dependent, since expression of RARs and RXRs in the absence of retinoic acid was unable to repress the collagenase gene (Figs. 3, 5, and ref. 40). Thus, our data indicate that suppression requires both RARs and RXRs and involves, but is not limited to, the AP-1 site.

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