All-*trans*-Retinoic Acid Inhibition of Proα1(I) Collagen Gene Expression in Fetal Rat Skin Fibroblasts: Identification of a Retinoic Acid Response Element in the Proα1(I) Collagen Gene

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The current study was undertaken to determine the mechanism by which the retinoid all-*trans*-retinoic acid regulates proα1(I) collagen gene expression in fetal rat skin fibroblasts. FRS fibroblasts were stably transfected with the ColCat3.6 plasmid, which contains a portion of the 5′ flanking region of the rat proα1(I) collagen gene linked to a reporter gene, chloramphenicol acetyltransferase. The effect of t-RA on CAT activity was determined as a function of concentration and incubation time. Maximal inhibition of CAT activity by t-RA occurred at 10^{-8} M after 48 h of treatment. Transforming growth factor-β1 did not block the inhibitory effect of t-RA on CAT activity. Computer sequence analysis of the 3.6-kb DNA fragment that contains the promoter for the rat proα1(I) collagen gene identified a direct repeat RARE sequence composed of one diverse (5′-AGTAGA-3′) and one idealized (5′-GGGTCA-3′) half site located at positions −1345 and −1335, respectively.

Two nuclear retinoid receptors that were expressed in bacteria, retinoic acid receptor-γ and retinoid X receptor-α, were found to bind specifically to a double-stranded oligonucleotide containing the RARE in gel mobility shift assays. Mutation of the idealized half-site eliminated the binding of receptor proteins to the oligonucleotide. Gel mobility shift assays using nuclear protein extracts prepared from t-RA–treated FRS fibroblasts showed that binding to the oligonucleotide containing the RARE was decreased from control values. The same assays performed with the mutated oligonucleotide resulted in only slight binding. These studies indicate that t-RA downregulates the promoter activity of the rat proα1(I) collagen gene by decreasing the binding of nuclear protein to the RARE sequence in the 5′ flanking region of the gene. *Key words: keloids/procollagen/transforming growth factor-β1. J Invest Dermatol 108:476–481, 1997*

R etinoic acid, a vitamin A derivative, is an important modulator of cellular growth, differentiation, and development in vertebrate organisms. Previous studies have shown that all-*trans*-retinoic acid (t-RA) decreases procollagen production in human skin fibroblasts and in fibroblast cell lines derived from keloids (Oikarinen *et al*, 1985). Keloid-derived fibroblast cultures have been shown to have an enhanced rate of collagen synthesis (Diegelmann *et al*, 1979; Ala-Kokko *et al*, 1987; Abergel and Uitto, 1989). t-RA selectively decreases procollagen synthesis and the steady-state levels of proα2(1) collagen mRNA in keloid cell lines (Oikarinen *et al*, 1985). The steady-state levels of proα1(I) collagen mRNA in human lung fibroblast cultures are also decreased by t-RA, with a concomitant decrease of proα1(I) gene transcription as determined by nuclear run-off assays (Krupsky *et al*, 1994).

The effects of t-RA are believed to be mediated by nuclear retinoid receptors, which function as ligand-activated transcription factors and are members of the steroid/thyroid hormone receptor superfamily (Evans, 1988; Wahli and Martinez, 1991). Two families of nuclear retinoid receptors have been identified: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). The RARs and the RXRs consist of three homologous subtypes, α, β, and γ (Petkovich *et al*, 1987; Brand *et al*, 1988; Krust *et al*, 1989; Mangelsdorf *et al*, 1990; Leid *et al*, 1992a). RARs bind t-RA and 9-*cis*-retinoic acid, whereas RXRs bind only to 9-*cis*-retinoic acid (Levin *et al*, 1992; Allenby *et al*, 1993). RARs and RXRs form heterodimers that, when ligand is present, regulate the expression of specific genes (Leid *et al*, 1992; Allenby *et al*, 1993). The ligand-activated nuclear retinoid receptors modulate gene expression by binding to specific DNA sequences called retinoic acid response elements (RAREs) (De Luca, 1991; Glass *et al*, 1991). The RAREs consist of repetitive half-sites that are organized into direct repeats, palindromes, or inverted palindromes with varying spacing between the half- sites. The consensus half-site sequence is 5′-(A/

G)G(G/T)TCA-3'; however, variations in the ideal sequences have been observed (Glass et al, 1991; Gudas, 1992; Leid et al, 1992b).

In the current study, a RARE sequence was identified in the proα1(I) collagen promoter by computer sequence analysis. An oligonucleotide containing the identified sequence was found to bind bacterially expressed RARγ and RXRα in gel mobility shift assays, confirming the existence of a RARE in the proα1(I) collagen promoter. Nuclear protein extracts isolated from t-RA–treated fetal rat skin (FRS) fibroblasts showed decreased binding to the RARE as compared to binding of extracts prepared from untreated cells. The decreased binding to the RARE may in part be responsible for the t-RA–mediated downregulation of procollagen gene expression.

MATERIALS AND METHODS

Retinoids and Transforming Growth Factor-β (TGF-β) t-RA was purchased from Sigma (St. Louis, MO). TGF-β1 was purchased from R & D Systems (Minneapolis, MN). Lyophilized samples of TGF-β1 were reconstituted in 4 mM HCl containing 1 mg bovine serum albumin per ml. t-RA was dissolved in dimethyl sulfoxide and stored in liquid nitrogen.

Cell Culture Methods FRS fibroblasts were purchased from the American Type Culture Collection (CRL 1213, batch F-9707), Rockville, MD. FRS cells were grown in 90% (vol/vol) Dulbecco's modified Eagle's medium (Whittaker M.A. Bioproducts, Walkersville, MD), 10% (vol/vol) heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 units penicillin per ml, and 100 μg streptomycin per ml. Prior to treatment, the cells were washed twice with AIM V (GIBCO BRL, Grand Island, NY), a synthetic serum-free medium. The cells were treated with t-RA or TGF-β1 in AIM V medium. Control cultures were treated with the vehicle used to dissolve the respective compounds. The concentration of dimethyl sulfoxide in the medium was 0.1%. Experiments in which cells were treated with retinoids were performed under yellow lights.

Transfection of FRS Fibroblasts and Stable Selection Cells were transfected by using the calcium phosphate co-precipitation method (Chen and Okayma, 1987). Plasmids were purified by double CsCl banding according to standard methods. ColCat3.6 (supplied by D. Rowe and A. Lichtler, University of Connecticut Health Science Center, Farmington, CT) containing the rat proα1(I) collagen promoter was transfected into FRS cells. The pSV2neo plasmid was co-transfected for stable cell selection (Gorman et al, 1982; Southern and Berg, 1982; Lichtler et al, 1989; Ritzenthaler et al, 1991). Stable transfections were used since reproducible results were not obtained by using transient transfections for glucocorticoid-treated cells. Cells were selected with G418 (200 μg per ml).

Preparation of Cell Lysates and Assay of Chloramphenicol Acetyltransferase (CAT) Activity At the appropriate time after treatment, cells were placed on ice and washed three times with ice-cold phosphate-buffered saline (GIBCO BRL, Grand Island, NY). Cells were harvested in 1 ml of 40 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, 150 mM NaCl, and 1 mM ethylenediamine tetraacetic acid at 4°C as described (Gorman et al, 1982). CAT activity was determined by modification of an established method (Neumann et al, 1987). The same amount of protein was added to each reaction mixture contained in 50 μl of 100 mM Tris-HCl, pH 7.8. Two hundred microliters of 1.25 mM chloramphenicol in 100 mM Tris-HCl, pH 7.8, were added along with 0.5 μCi of [³H]acetyl-CoA (200 mCi/mmol, DuPont NEN, Boston, MA) and 22.5 μl of 1 mM unlabeled acetyl-CoA (Sigma, St. Louis, MO). The entire reaction mixture was overlaid with liquid scintillation fluid (Econofluor-2, DuPont NEN). This assay allows multiple time point determinations by liquid scintillation counting. The acetylated chloramphenicol product is miscible in the aqueous-immiscible scintillation counting mixture. Time course activity of each sample was determined and only activity in the linear range of the time course was used. Within each assay, data from the same time point were reported for all samples. Protein concentrations of cell lysates were determined. (Lowry et al, 1951).

DNA Mobility Shift Assays Confluent FRS fibroblasts were washed twice with AIM V medium. The cells were treated with t-RA (final concentration, 10⁻⁶ M) in AIM V medium. Control cells received the vehicle dimethyl sulfoxide (0.1%) used to dissolve t-RA. Nuclear extracts were prepared according to the method of Andrews and Faller (1991) and the protein concentration was determined (Lowry et al, 1951).

Single-stranded oligonucleotides containing the consensus RARE half-site sequence (5'-GGGTCA-3') or a sequence in which the six bases were changed (5'-TATGAC-3') were synthesized by Integrated DNA Technologies (Coralville, IA). The sequence containing the altered bases is referred
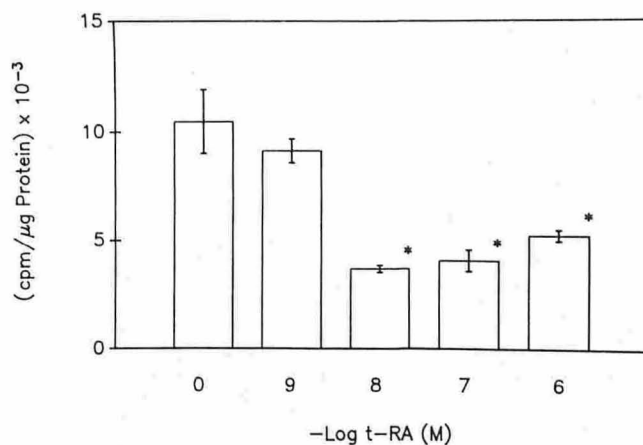

Figure 1. Maximal inhibition of proα1(I) collagen promoter activity occurs at 10⁻⁸ M t-RA. Stable FRS fibroblasts transfected with ColCat3.6 were examined for dose dependence to t-RA. Confluent cells were exposed to 10⁻⁶, 10⁻⁷, 10⁻⁸, or 10⁻⁹ M t-RA for 48 h. Cell lysates were assayed for CAT activity, which is expressed as counts per min per μg of protein. *Significantly different from control values at $\rho < 0.05$. *Error bars*, SEM (n = 3).

to as the mutated retinoic acid response element (mRARE). The entire sequences of the oligonucleotides are listed in the legends to **Figs 4–7**. Twenty micrograms of the single-stranded oligonucleotides were annealed with 20 μg of their complementary strands in 200 mM NaCl by heating to 95°C for 7 min and then slowly cooled to 4°C. The double-stranded oligonucleotides were stored at −20°C. Double-stranded oligonucleotides, RAR consensus oligonucleotide, and RXR consensus oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA) were also used as DNA probes. The sequences of these probes are listed in the legends to **Figs 5** and **7**.

The oligonucleotides were labeled with ³²P by using the 5' DNA Terminus Labeling System (GIBCO BRL, Gaithersburg, MD). Gel shift binding reactions (20 μl) contained ³²P-end-labeled double-stranded oligonucleotide with the RARE or mRARE sequence (2–3 × 10⁶ cpm per pmol), 10 μg of nuclear protein extract, 1.5 μg of poly[d(I-C)] (Pharmacia, Piscataway, NJ), 90 mM KCl, 1 mM ethylenediamine tetraacetic acid, 1 mM dithiothreitol, and 5% glycerol. In some assays, 50 ng of the bacterial-expressed receptor proteins RARγ or RXRα (obtained from Dr. Jen-Fu Chiu, University of Vermont, Burlington, VT) were used instead of nuclear protein extract. In other experiments, RARγ protein purified from bacterial lysates was obtained from Santa Cruz Biotechnology. Reaction mixtures were incubated for 30 min at room temperature and separated on 6% polyacrylamide gels (19:1 acrylamide to bisacrylamide) in 0.25× TBE buffer [89 mM Tris borate, 89 mM boric acid, 2 mM ethylenediamine tetraacetic acid (pH 8.0)] for 4 h at 100 V. The gels were air-dried and autoradiographed. The band intensities of the bound protein–DNA complexes were measured by densitometric analysis using a Shimadzu dual-wavelength thin-layer chromatography scanner, model CS-930 (Shimadzu, Kyoto, Japan). Competition experiments were performed by the addition of unlabeled RARE or RAR consensus oligonucleotide to reaction mixtures. In gel supershift assays, RARγ antibody (Santa Cruz Biotechnology) was added to reaction mixtures subsequent to a 20-min incubation of the oligonucleotide probe RARE with RARγ protein. Incubation was continued for an additional 45 min at room temperature.

Statistical Analysis Significance of differences in means was determined by Student's t test.

RESULTS

t-RA Decreases Rat Proα1(I) Collagen Promoter Activity in Stable FRS Fibroblast Cell Lines FRS fibroblasts stably transfected with ColCat3.6 showed a decrease in CAT activity when treated with t-RA for 48 h **(Fig 1)**. At 10⁻⁸ M t-RA, there was a 65% decrease in CAT activity. Increasing the concentration of retinoid to 10⁻⁷ M and 10⁻⁶ M did not increase the inhibitory effect of t-RA. At 10⁻⁹ M t-RA, the cell line did not demonstrate a statistically significant decrease in proα1(I) collagen promoter
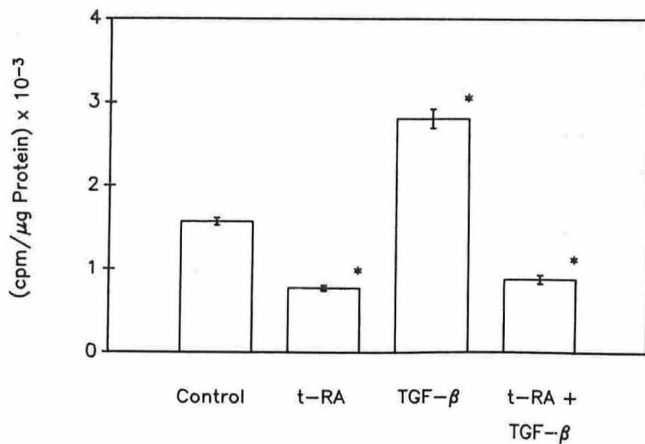
Figure 2. t-RA decreases and TGF-β1 increases proα1(I) collagen promoter activity in stable FRS fibroblasts. Stable FRS fibroblasts transfected with ColCat3.6 were examined for responsiveness to t-RA, TGF-β1, or t-RA plus TGF-β1. Confluent cells were treated with 10^{-6} M t-RA, 5.0 ng TGF-β1 per ml, or 10^{-6} M t-RA plus 5.0 ng TGF-β1 per ml for 48 h. Cell lysates were assayed for CAT activity, which is expressed as counts per min per μg of protein. *Significantly different from control values at $\rho < 0.01$. *Error bars,* SEM (n = 3 or 4).
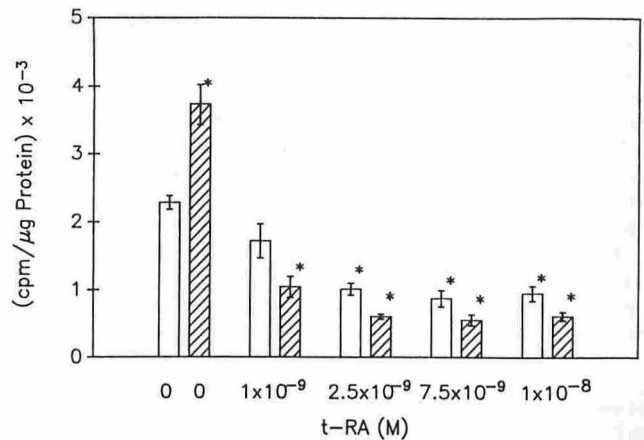

Figure 3. TGF-β1 does not block the t-RA-mediated decrease in proα1(I) collagen promoter activity in stable FRS fibroblasts at various concentrations of t-RA. Stable FRS fibroblasts transfected with ColCat3.6 were examined for responsiveness to t-RA, TGF-β1, or t-RA plus TGF-β1. Confluent cells were treated with 10^{-9} M, 2.5×10^{-9} M, 7.5×10^{-9} M, or 10^{-8} M t-RA plus or minus 5.0 ng TGF-β1 per ml for 48 h. Cell lysates were assayed for CAT activity, which is expressed as counts per min per μg of protein. □, t-RA-treated cell cultures; ▨, t-RA + TGF-β1 (5.0 ng per ml)-treated cell cultures. *Significantly different from control values (no addition of t-RA or TGF-β1) at $\rho < 0.01$. *Error bars,* SEM (n = 3 or 4).

activity. Similar results were obtained with a second stable cell line transfected with ColCat3.6 (data not shown). When a stable cell line transfected with ColCat3.6 was treated with 10^{-6} M t-RA for 24 h, there was no significant decrease in promoter activity. The significant decrease in promoter activity observed after 48 h of incubation with 10^{-6} M t-RA was maintained for an additional 48 h (data not shown).

In a previous study, we showed that dexamethasone down-regulates procollagen gene expression by measuring the decrease in CAT activity in cell lysates prepared from dexamethasone-treated FRS fibroblasts stably transfected with ColCat3.6. We proposed that glucocorticoids coordinately regulate procollagen gene expression through both the glucocorticoid response element and the TGF-β element (Meisler *et al,* 1995). A glucocorticoid response element sequence is located at positions −655 to −650 and a TGF-β element is located at position −1628 upstream from the start site of transcription in the rat proα1(I) collagen promoter (Lichtler *et al,* 1989; Ritzenthaler *et al,* 1991, 1993). Computer sequence analysis of the portion of the 5′ flanking region of the rat proα1(I) collagen gene in the ColCat3.6 plasmid revealed a sequence matched to the consensus RARE half-site, 5′-(A/G)G(G/T)TCA-3′ (Leid *et al,* 1992b). The 6-bp sequence is 5′-GGGTCA-3′ and is located at positions −1335 to −1330 from the start site of transcription in the rat proα1(I) collagen promoter. There is a closely related 6-bp sequence to the consensus RARE half-site, 5′-AGTAGA-3′, located at positions −1345 to −1340. These two sequences are separated by 4 bp. The sequence from positions −1345 to −1330 is referred to as the RARE.

TGF-β Does Not Block the Decrease in Proα1(I) Collagen Promoter Activity Caused by t-RA In a previous study, concurrent TGF-β1 treatment was able to bring the observed dexamethasone-mediated decrease in CAT activity back to the control value (Meisler *et al,* 1995). In this study, addition of TGF-β1 to the culture medium did not block the inhibitory effect of various concentrations of t-RA on CAT activity. TGF-β1 (5.0 ng per ml) treatment alone caused a 1.8-fold increase in CAT activity. In a previous studying using NIH 3T3 cells, TGF-β1 treatment alone resulted in an approximately 2-fold increase in proα1(I) collagen promoter activity (Jimenez *et al,* 1994). t-RA at a concentration of 10^{-6} M caused a decrease in CAT activity of 51%, whereas the combination of 10^{-6} M t-RA plus 5.0 ng TGF-β1 per ml elicited a 44% decrease in CAT activity (**Fig 2**). Furthermore, addition of 5.0 ng TGF-β1 per ml to cells treated simultaneously

with t-RA at concentrations between 10^{-8} M and 10^{-9} M did not block the t-RA–mediated decrease in CAT activity (**Fig 3**). At 10^{-8} M t-RA, there was a 59% decrease in proα1(I) collagen promoter activity, and in the presence of TGF-β1, the decrease in CAT activity was 73%. Similar decreases in promoter activity were found, respectively, when the concentration of t-RA was 7.5×10^{-9} M or 2.5×10^{-9} M. At 10^{-9} M t-RA, there was no significant decrease in CAT activity, which agrees with the data shown in **Fig 1**, but the combination of 5.0 ng TGF-β1 per ml and 10^{-9} M t-RA caused a 54% decrease in proα1(I) collagen promoter activity. Thus even at concentrations of t-RA below the physiologic serum concentrations of 10^{-7} M to 10^{-8} M (Adamson *et al,* 1992), addition of TGF-β1 did not abrogate the t-RA–mediated decrease in proα1(I) collagen promoter activity. In a previous study using human lung fibroblasts, t-RA treatment inhibited basal and TGF-β1–stimulated collagen synthesis. When fibroblasts were incubated with TGF-β1 and t-RA, there was a decrease in α1(I) collagen mRNA accumulation and gene transcription (Redlich *et al,* 1995).

RARγ and RXRα bind to a *cis*-Element in the 5′ Flanking region of the Rat Proα1(I) Collagen Gene Bacterially expressed receptor proteins RARγ and RXRα were used in gel mobility shift assays to identify a RARE in the rat α1(I) collagen promoter. When the ^{32}P-labeled oligonucleotide 5′-A<u>AGTA</u>-<u>GA</u>GTGG<u>GGGT</u><u>CA</u>GGCAGAGCACC-3′ (located in the promoter from positions −1346 to −1319) was used as a probe, binding was observed with both RARγ and RXRα protein (**Fig 4**). There was an absence of binding with unprogrammed bacterial lysate. When the 6-bp sequence 5′-GGGTCA-3′ (consensus RARE half-site sequence) was replaced with the sequence 5′-TATGAC-3′ (mutated RARE half-site sequence), the RARγ and RXRα proteins no longer bound to the oligonucleotide. These results indicate that the sequence from positions −1335 to −1330 (5′-GGGTCA-3′) in the proα1(I) collagen promoter is a RARE half-site. Bacterially expressed RARγ and RXRα bound in a similar pattern to a RARE identified in the 5′ flanking region of the rat α-fetoprotein gene (Liu *et al,* 1994).

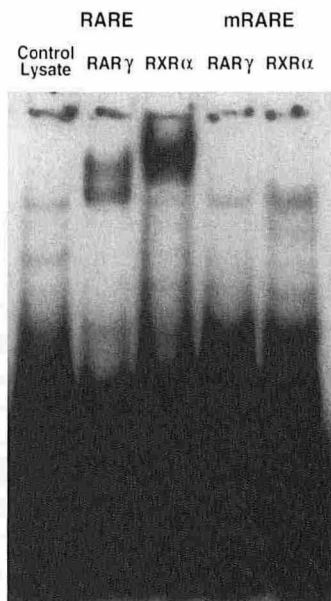**Addition of RARγ Antibody Causes a Supershift of the RARγ-RARE Complex** RARγ-purified protein produced in

Figure 4. Bacterially synthesized RARγ and RXRα binds to a RARE in the proα1(I) collagen promoter. Gel mobility shift assays were performed with ^{32}P-end-labeled double-stranded oligonucleotide containing the RARE half-site sequence (5′-GGGTCA-3′) (2.3 × 10^6 cpm per pmol) or the mRARE half-site sequence (5′-TATGAC-3′) (2.8 × 10^6 cpm per pmol) and 50 ng of bacterially expressed RARγ or RXRα. The ^{32}P-labeled probe containing the RARE sequence was also incubated with unprogrammed bacterial lysate as control. In addition, each assay mixture contained 0.2 μg of poly[d(I-C)] and 6 μg of bovine serum albumin in a total volume of 20 μl. Complete sequence of the oligonucleotide containing the RARE in the rat proα1(I) collagen promoter from positions −1346 to −1319 (5′-AAGTAGAGTGGGGGTCAGGCAGAGCACC-3′); complete sequence of the oligonucleotide containing the mRARE (5′-AAGTAGAGTGGTATGACGGCAGAGCACC-3′).

Escherichia coli for use as a positive control for gel shift studies formed a complex with the RARE in the rat proα1(I) collagen promoter. Addition of an antibody to RARγ gave rise to a supershifted band **(Fig 5A)**. In the presence of the antibody, an antibody–protein–DNA complex was formed that resulted in a further reduction in the mobility of the RARγ–RARE complex. In a competition experiment, RARγ was incubated with a 100-fold molar excess of unlabeled oligonucleotide before the addition of ^{32}P-labeled RARE. The unlabeled oligonucleotide contains the consensus binding site for the RAR protein. As shown in **Fig 5B**, the unlabeled RAR consensus oligonucleotide competed effectively with the ^{32}P-labeled RARE from the rat proα1(I) collagen promoter for binding to the RARγ protein.

t-RA Treatment of FRS Fibroblasts Decreases Nuclear Binding to the RARE We used the gel mobility shift assay to study the binding of nuclear proteins isolated from control and t-RA–treated fibroblasts to the RARE sequence **(Fig 6)**. Fig 6A and **B** represent two different experiments. t-RA treatment of non-transfected FRS fibroblasts decreased the binding of isolated nuclear proteins to the ^{32}P-labeled oligonucleotide (5′-AAGTAGAGTGGGGGTCAGGCAGAGCACC-3′) containing the RARE half-site (5′-GGGTCA-3′) to 30% of the binding observed when cells were untreated **(Fig 6A)** and in a second experiment to 33% of control values **(Fig 6B)**. When the 6-bp sequence 5′-GGGTCA-3′ in the oligonucleotide was replaced with the sequence 5′-TATGAC-3′ (mRARE), the binding of the nuclear proteins isolated from t-RA–treated fibroblasts decreased to 5% of control value and to 9% of the value found with the nonmutated probe. There was a 45% decrease in binding of nuclear protein from untreated cells to the probe containing the mutated RARE whereas
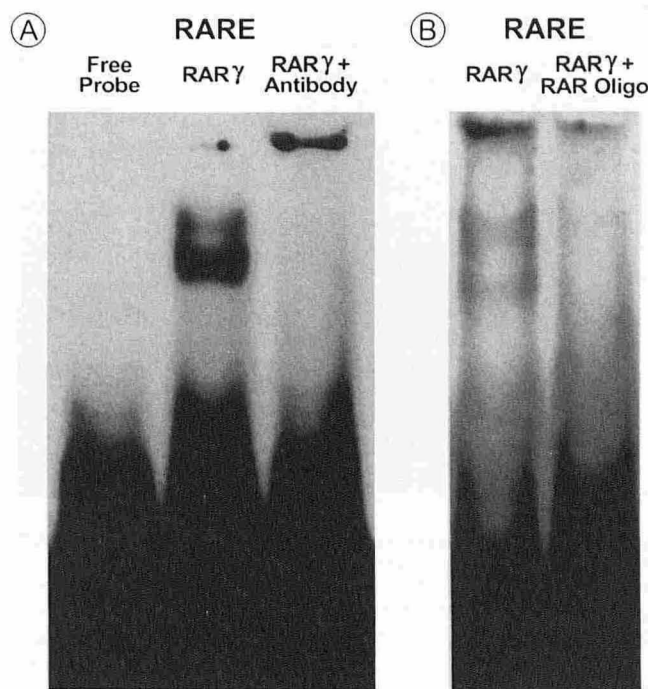

Figure 5. Purified RARγ binds to a RARE in the proα1(I) collagen promoter. *(A)* ^{32}P-end-labeled double-stranded oligonucleotide containing the RARE half-site sequence (5′-GGGTCA-3′) (1.2 × 10^{6}M cpm per pmol) was incubated with 200 ng of purified RARγ. In addition, each assay mixture contained 0.2 μg of poly[d(I-C)] and 6 μg of bovine serum albumin in a total volume of 20 μl. After a 20-min incubation at room temperature, 2 μl of RARγ antibody (1 mg per ml) were added to the reaction mixture and incubation was continued for an additional 45 min at room temperature. In a competition experiment *(B)*, 100 ng of purified RARγ were incubated with 100-fold molar excess of unlabeled RAR consensus oligonucleotide before the addition of labeled RARE from the proα1(I) collagen promoter. Complete sequence of the oligonucleotide containing the RARE in the rat proα1(I) collagen promoter from positions −1346 to −1319 (5′-AAGTAGAGTGGGGGTCAGGCAGAGCACC-3′); complete sequence of the RAR oligonucleotide that contains the binding site for the RAR (5′-AGGGTAGGGTTCACCGAAAGTTCACTC-3′).

(Fig 4) the binding of the nuclear receptors RARγ and RXRα was eliminated with the mutated RARE. Because nuclear extracts from untreated cells are composed of many proteins, some may react with the mutated RARE sequence. RARγ and RXRα are pure protein preparations and thus would not react with a oligonucleotide containing a mutated RARE.

Specific Complex Formation Between Nuclear Proteins from FRS Fibroblasts and RAR and RXR Consensus Oligonucleotides Gel mobility shift assays were used to show that nuclear extracts contain RAR and RXR proteins. We found that nuclear proteins derived from untreated cells bound to the ^{32}P-labeled oligonucleotide that contains the consensus binding site for the RAR (5′-AGGGTAGGGTTCACCGAAAGTTCACTC-3′) **(Fig 7A)**. Two bands were found and were similar to those found when recombinant and purified recombinant RARγ were reacted with the RARE from the proα1(I) collagen promoter **(Figs 4 and 5**, respectively). The binding of nuclear extract to the ^{32}P-labeled RAR consensus oligonucleotide in the presence of 100-fold molar excess of the unlabeled RARE from the rat proα1(I) collagen promoter changed the binding pattern. A single band was found. The nuclear extract derived from untreated cells also bound to a ^{32}P-labeled oligonucleotide that contains the consensus binding site for the RXR (5′-AGCTTCAGGTCAGAGGTCAGAGAGCT-3′) **(Fig 7B)**. A 100-fold molar excess of the unlabeled RARE from the rat proα1(I) collagen promoter reduced the binding of nuclear
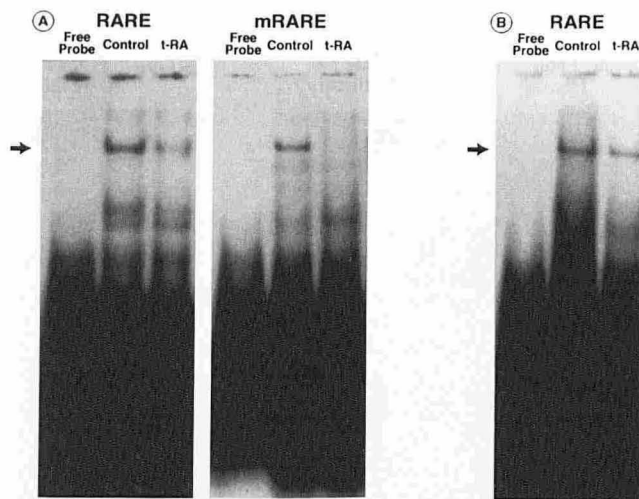
Figure 6. t-RA decreases nuclear protein binding to the RARE.
Confluent FRS fibroblasts were treated with 10^{-6} M t-RA for 48 h. *(A)*
^{32}P-end-labeled double-stranded oligonucleotide containing the RARE
half-site sequence (5'-GGGTCA-3') (2.3×10^6 cpm per pmol) or the
mRARE half-site sequence (5'-TATGAC-3') (2.8×10^6 cpm per pmol)
was incubated with 10 μg of nuclear protein extract and 1.5 μg of
poly[d(I-C)] in buffer described in *Materials and Methods*. In a second
experiment *(B)*, 10 μg of nuclear protein extract were incubated with
^{32}P-end-labeled double-stranded oligonucleotide (2.6×10^6 cpm per pmol)
containing the RARE half-site sequence (5'-GGGTCA-3'). The nuclear
protein–oligonucleotide complex is indicated by the →. Complete se-
quence of the oligonucleotide containing the RARE in the rat proα1(I)
collagen promoter from positions −1346 to −1319 is 5'-AAGTAGAGT-
GGGGGGTCAGGCAGAGCACC-3'; complete sequence of the oligonucle-
otide containing the mRARE is 5'-AAGTAGAGTGGTATGACGGCA-
GAGCACC-3'.

**Figure 7. Nuclear proteins bind to RAR and RXR consensus
oligonucleotides.** *(A)* ^{32}P-end-labeled double-stranded oligonucleotide
containing the consensus binding site for the RAR (3.5×10^5 cpm per
pmol) was incubated with 10 μg of nuclear protein extract and 1.5 μg of
poly[d(I-C)] in buffer described in *Materials and Methods*. In a competition
experiment, 10 μg of nuclear extract were incubated with 100-fold molar
excess of unlabeled RARE from the proα1(I) collagen promoter before the
addition of labeled consensus RAR oligonucleotide. The → indicates the
band corresponding to the nuclear protein–oligonucleotide complex that is
eliminated in the competition experiment. *(B)* ^{32}P-end-labeled double-
stranded oligonucleotide containing the consensus binding site for the RXR
(4.9×10^5 cpm per pmol) was incubated with 10 μg of nuclear protein
extract and 1.5 μg of poly[d(I-C)] in a total volume of 20 μl. In a
competition experiment, 10 μg of nuclear extract were incubated with
100-fold molar excess of unlabeled RARE from the proα1(I) collagen
promoter before the addition of labeled consensus RXR oligonucleotide.
Complete sequence of the RAR oligonucleotide that contains the binding
site for the RAR is 5'-AGGGTAGGGTTCACCGAAAGTTCACTC-3';
complete sequence of the RXR oligonucleotide that contains the binding
site for RXR is 5'-AGCTTCAGGTCAGAGGTCAGAGAGCT-3'; com-
plete sequence of the oligonucleotide containing the RARE in the rat
proα1(I) collagen promoter from positions -1346 to -1319 is 5'-AAGTA-
GAGTGGGGGTCAGGCAGAGCACC-3'.

extract to the ^{32}P-labeled RXR consensus oligonucleotide. Incom-
plete competition of the unlabeled RARE from the proα1(I)
collagen promoter with a labeled oligonucleotide that contains the
RAR consensus sequence could be due to the high affinity of RAR
proteins with RAREs that are direct repeats and are separated by
five nucleotides (Mangelsdorf *et al*, 1994). Likewise, incomplete
competition of the unlabeled RARE from the proα1(I) collagen
promoter with a labeled oligonucleotide that contains the RXR
consensus sequence could be due to the high affinity of RXR
proteins with retinoid X response elements that are direct repeats
and are separated by one nucleotide (Mangelsdorf *et al*, 1994).

DISCUSSION

In the current study, we explored the molecular mechanism by
which t-RA regulates proα1(I) collagen gene expression. Previous
studies have shown that t-RA decreases collagen synthesis in
normal and disease states. The mechanism by which retinoids
decrease proα1(I) collagen gene expression may also apply to
hypertrophic scars and keloids, two cutaneous disorders character-
ized by excessive collagen deposition (Janssen, 1980). Retinoids
have been found to stimulate collagen synthesis when skin is
recovering from injuries such as those induced by wounding,
ultraviolet radiation, or glucocorticoid-induced skin atrophy (as
described in a review by Gendimenico and Mezick, 1993).

We have demonstrated that t-RA downregulates proα1(I) col-
lagen gene transcription in a time- and concentration-dependent
manner in stably transfected FRS fibroblasts. t-RA at an equivalent
molar concentration was more effective in reducing CAT activity
than dexamethasone (data not shown). A previous study from our
laboratory (Meisler *et al*, 1995) demonstrated that glucocorticoids
were able to bring the TGF-β–induced increase in CAT activity
back to control values. Unlike the glucocorticoid, dexamethasone,
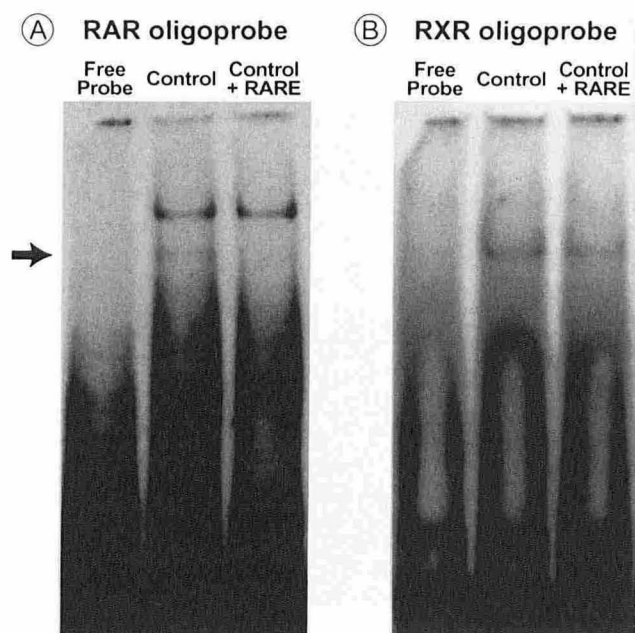t-RA used in combination with TGF-β lowered CAT activity

below the control value at all concentrations tested. This latter
finding suggests that t-RA may have some utility as an agent to
reduce fibrosis induced by TGF-β. Whether or not t-RA can
reduce excessive collagen production *in vivo* will need to be verified
with models of keloids, fibrosis, or scarring.

This study indicates by gel mobility shift assay that t-RA
decreases receptor bound to the RARE. The RARE was shown to
bind to both recombinant RARγ and RXRα in gel mobility shift
assays. Mutation of the consensus RARE half site (5'-GGGTCA-
3') eliminated receptor binding **(Fig 4)**. It is not surprising that both
RARγ and RXRα bind to the RARE, as RARs and RXRs form
heterodimeric complexes in the absence or presence of ligand (Leid
et al, 1992a; Allenby *et al*, 1993; Mangelsdorf *et al*, 1994). The fact
that nuclear proteins isolated from untreated cells bind to the
RARE **(Fig 6)** and to RAR and RXR consensus oligonucleotides
(Fig 7) implies that there is an endogenous level of retinoid
receptor present in FRS fibroblasts. Pan and Brinckerhoff (1994), in
studying t-RA inhibition of collagenase gene expression in synovial
fibroblasts, showed that RXRα mRNA is constitutively expressed
and that the mRNAs for RARα, β and, γ are inducible by the
addition of t-RA. They suggest that RARs and RXRs repress
transcription of the collagenase gene by interfering with the binding

of the transcription factor AP-1 (c-*jun*/c-*fos*) to the 5′ regulatory region of the collagenase gene. The interference by retinoid receptors with other positive-acting transcription factors is thought to be the mechanism by which genes are negatively regulated by t-RA (Gudas *et al*, 1994; Mangelsdorf *et al*, 1994). Our studies indicate, however, that the downregulation of proα1(I) gene expression is associated with a decrease in binding of nuclear proteins isolated from t-RA–treated fibroblasts to the RARE sequence in the 5′ flanking region of the gene.

We have previously demonstrated, using gel mobility shift assays, that glucocorticoid treatment of FRS fibroblasts decreased glucocorticoid receptor binding to the glucocorticoid response element and TGF-β activator protein to the TGF-β element contained in the 5′ flanking region of the proα1(I) collagen gene (Meisler *et al*, 1995). Since retinoid receptors are members of the steroid/thyroid hormone receptor superfamily, one would expect that genes that are negatively regulated by t-RA would also show decreased binding of receptors to the RARE. Additional support of our hypothesis comes from studies on the human K5 keratin gene (Ohtsuki *et al*, 1992). The promoter sequence for the human K5 keratin gene has several individual consensus half-sites recognized by RARs. HeLa cells were co-transfected with plasmid constructs containing the half-sites and vectors expressing RARs. Treatment with t-RA decreased promoter activity of the CAT constructs. Thus regulation of the K5 keratin gene by retinoids is effected by interaction of nuclear receptors for t-RA with RARE sequences within the promoter.

REFERENCES

Abergel RP, Uitto J: Dermal fibrosis in cutaneous disease: keloids as a model to study the mechanisms of collagen deposition in tissues. In: Uitto J, Perejda AG (eds.). *Diseases of Connective Tissue: The Molecular Pathology of the Extracellular Matrix.* Marcel Dekker, New York, 1989, pp 345–366

Adamson PC, Balis FM, Smith MA, Murphy RF, Godwin KA, Poplack DG: Dose-dependent pharmacokinetics of all-*trans* retinoic acid. *J Natl Cancer Inst* 84:1332–1335, 1992

Ala-Kokko L, Rintala A, Savolainen ER: Collagen gene expression in keloids: analysis of collagen metabolism and type I, II, IV, and V procollagen mRNA in keloid tissue and keloid fibroblast cultures. *J Invest Dermatol* 89:238–244, 1987

Allenby G, Bocquel M-T, Saunders M, Kazmer S, Speck J, Rosenberger M, Lovey A, Kastner P, Grippo JF, Chambon P, Levin AA: Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. *Proc Natl Acad Sci USA* 90:30–34, 1993

Andrews NC, Faller DV: A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 19:2499, 1991

Brand N, Petkovich M, Krust A, Chambon P, de Thé H, Marchio A, Tiollais P, Dejean: Identification of a second human retinoic acid receptor. *Nature* 332:850–853, 1988

Chen C, Okayma H: High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 7:2745–2752, 1987

De Luca LM: Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J* 5:2924–2933, 1991

Diegelmann RF, Cohen IK, McCoy BJ: Growth kinetics and collagen synthesis of normal skin, normal scar and keloid fibroblasts *in vitro*. *J Cell Physiol* 98:341–346, 1979

Evans RM: The steroid and thyroid hormone receptor superfamily. *Science* 240:889–895, 1988

Gendimenico GJ, Mezick JA: Pharmacological effects of retinoids on skin cells. *Skin Pharmacol* 6:24–34, 1993

Glass CK, DiRenzo J, Kurokawa R, Han Z: Regulation of gene expression by retinoic acid receptors. *DNA Cell Biol* 10:623–638, 1991

Gorman CM, Moffat LF, Howard BH: Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* 2:1044–1051, 1982

Gudas LJ: Retinoids, retinoid-responsive genes, cell differentiation, and cancer. *Cell Growth Differ* 3:655–662, 1992

Gudas LJ, Sporn MB, Roberts AB: Cellular biology and biochemistry of the retinoids. In: Sporn MB, Roberts AB, Goodman DS (eds.). *The Retinoids: Biology, Chemistry and Medicine.* Raven Press, New York, 1994, pp 443–520

Janssen De Limpens AMP: The local treatment of hypertrophic scars and keloids with topical retinoic acid. *Br J Dermatol* 103:319–323, 1980

Jimenez SA, Varga J, Olsen A, Li L, Diaz A, Herhal J, Koch J: Functional analysis of human α1(I) procollagen gene promoter. *J Biol Chem* 269:12684–12691, 1994

Krupsky M, Fine A, Berk JL, Goldstein RH: Retinoic acid-induced inhibition of type I collagen gene expression by human lung fibroblasts. *Biochim Biophys Acta* 1219:335–341, 1994

Krust A, Kastner P, Petkovich M, Zelent A, Chambon P: A third human retinoic acid receptor, hRAR-γ. *Proc Natl Acad Sci USA* 86:5310–5314, 1989

Leid M, Kastner P, Chambon P: Multiplicity generates diversity in the retinoic acid signalling pathways. *Trends Biochem Sci* 17:427–433, 1992b

Leid M, Kastner P, Lyons R, Nakshatri H, Saunders M, Zacharewski T, Chen J-Y, Staub A, Garnier J-M, Mader S, Chambon P: Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* 68:377–395, 1992a

Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzeisen C, Rosenberger M, Lovey A, Grippo JF: 9-*cis* retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. *Nature* 355:359–361, 1992

Lichtler A, Stover ML, Angilly J, Kream B, Rowe DW: Isolation and characterization of the rat α1(I) promoter. *J Biol Chem* 264:3072–3077, 1989

Liu Y, Chen H, Chiu J-F: Identification of a retinoic acid response element upstream of the rat α-fetoprotein gene. *Mol Cell Endocrinol* 103:149–156, 1994

Lowry OH, Rosebrough NJ, Fall AL, Randall RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275, 1951

Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM: Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* 345:224–229, 1990

Mangelsdorf DJ, Umesono K, Evans RM: The retinoid receptors. In: Sporn MB, Roberts AB, Goodman DS (eds.). *The Retinoids: Biology, Chemistry, and Medicine.* Raven Press, New York, 1994, pp 319–349

Meisler N, Shull S, Xie R, Long GL, Absher M, Connolly JP, Cutroneo KR: Glucocorticoids coordinately regulate type I collagen proα1 promoter activity through both the glucocorticoid and transforming growth factor β response elements: a novel mechanism of glucocorticoid regulation of eukaryotic genes. *J Cell Biochem* 59:376–388, 1995

Neumann JR, Morency CA, Russian KO: A novel rapid assay for chloramphenicol acetyltransferase gene expression. *BioTechniques* 5:444–447, 1987

Ohtsuki M, Tomic-Canic M, Freedberg, IM, Blumenberg, M: Nuclear proteins involved in transcription of the human K5 keratin gene. *J Invest Dermatol* 99:206–215, 1992

Oikarinen H, Oikarinen AI, Tan EML, Abergel RP, Meeker CA, Chu M-L, Prockop DJ, Uitto J: Modulation of procollagen gene expression by retinoids: inhibition of collagen production by retinoic acid accompanied by reduced type 1 procollagen messenger ribonucleic acid levels in human skin fibroblast cultures. *J Clin Invest* 75:1545–1553, 1985

Pan L, Brinckerhoff CE: Inhibition of collagenase gene expression in synovial fibroblasts by all-*trans* and 9-*cis* retinoic acid. *Ann NY Acad Sci* 732:335–347, 1994

Petkovich M, Brand NJ, Krust A, Chambon P: A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 330:444–450, 1987

Redlich CA, Delisser HM, Elias JA: Retinoic acid inhibition of transforming growth factor-β-induced collagen production by human lung fibroblasts. *Am J Respir Cell Mol Biol* 12:287–295, 1995

Ritzenthaler JD, Goldstein RH, Fine A, Lichtler A, Rowe DW, Smith BD: Transforming-growth-factor-β activation elements in the distal promoter regions of the rat α1 type I collagen gene. *Biochem J* 280:157–162, 1991

Ritzenthaler JD, Goldstein RH, Fine A, Smith BD: Regulation of the α11(I) collagen promoter via a transforming growth factor-β activation element. *J Biol Chem* 268:13625–13631, 1993

Southern PJ, Berg P: Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J Mol Appl Genet* 1:327–341, 1982

Wahli W, Martinez E: Superfamily of steroid nuclear receptors: positive and negative regulators of gene expression. *FASEB J* 5:2243–2249, 1991