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Glucocorticoid and Retinoid Regulation of Alpha-2 Type I Procollagen Promoter Activity

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Abstract Glucocorticoids decrease type I procollagen synthesis by decreasing the steady state levels of procollagen mRNAs and mRNA synthesis. The present studies were undertaken to determine the functional sequences of the proα2(I) collagen gene required for the glucocorticoid-mediated decrease of type I procollagen mRNA synthesis. Embryonic mouse fibroblasts were stably transfected with the pR40 DNA CAT construct containing the 5′ flanking region fragment from −2048 to +54 and the intronic fragment from +418 to +1524 of the mouse α2 (I) collagen gene. Dexamethasone treatment of these pR40 transfected fibroblasts resulted in a significant decrease in CAT activity which agrees with the glucocorticoid-mediated decrease of the steady state levels of type I procollagen mRNAs. To determine the possible role of the first intron fragment in the dexamethasone-mediated decrease of CAT activity, pR36, a CAT plasmid containing the first intron fragment and the SV40 early promoter, was transfected into mouse fibroblasts and treated with dexamethasone. No significant decrease in CAT activity was observed. The dexamethasone-mediated response was then localized within the 5′ flanking region by preparing a series of constructs containing internal deletions and transfecting these plasmids into mouse fibroblasts. The regions −2048 to −981 and −506 to −351 were required for the dexamethasone response of gene activity. However, the DNA stretch from −981 to −506 was not. Analysis of the DNA sequences of these regions revealed a single GRE at −1023 to −1018 and a modified doublet at −873 to −856. The doublet GRE contains an A/T strand switch of the third base pair as compared to the single GRE and is not necessary for dexamethasone regulation of gene activity. All-trans-retinoic acid increased CAT activity of the same pR40 CAT construct transfected in the mouse fibroblasts. DNA sequencing revealed a RARE and a modified RARE in the stretch of DNA from −981 to −506. Deletion of only the latter DNA region eliminated the elevation of CAT activity elicited by all-trans-retinoic acid. Our results indicate that the singlet GRE and the RARE are required for glucocorticoid and retinoic acid regulation of proα2(I) collagen gene activity, respectively.

Key words: collagen gene regulation, collagen gene expression, glucocorticoid collagen gene regulation, retinoid collagen gene regulation, dexamethasone, trans-retinoic acid

Glucocorticoids decrease collagen synthesis in connective tissues and fibroblast cell culture [Cutroneo et al., 1986]. The decrease of type I collagen synthesis has been attributed to a decrease of the steady state level of total cellular type I procollagen mRNAs [Cutroneo, in press]. In glucocorticoid-treated chick skin fibroblasts that were pulse labeled with radioactive uridine, nuclear type I procollagen mRNA synthesis was selectively decreased [Cockayne et al., 1986]. This mechanism of glucocorticoid regulation was also demonstrated in the rat small intestine [Walsh et al., 1987] and in rat hepatocytes [Weiner et al., 1987] using the nuclear run-off system. Although a number of studies have reported a glucocorticoid-mediated decrease of procollagen mRNA synthesis, other studies have attributed the decrease of total cellular procollagen mRNA to destabilization of procollagen mRNAs [Hamalainen et al., 1985; Raghow et al., 1986]. However, we have demonstrated in chick

Abbreviations used: CAT, chloramphenicol acetyl transferase; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; GRE, glucocorticoid response element; PBS, phosphate buffered saline; PCI, phenol/chloroform/isoamyl alcohol (25:24:1); TBE, 89 mM Tris/89 mM boric acid/2 mM EDTA (pH 8.0); T.E., 10 mM Tris/1 mM EDTA (pH 7.4–8.0); TNE, 10 mM Tris/100 mM NaCl/1 mM EDTA (pH 7.5); TRIS, tris(hydroxymethyl)aminomethane; RARE, retinoic acid response element.

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skin fibroblasts that the degradation of chick type I procollagen mRNAs was not affected by glucocorticoid treatment [Cockayne et al., 1986].

Retinoids such as vitamin A and all-trans-retinoic acid stimulate new connective tissue synthesis. Retinoids induce differentiation of mouse fibroblasts in culture [Demetriou et al., 1985], which is manifested as a decrease in cell growth rate, enhanced collagen deposition, and morphological differentiation. Retinoid effects on connective tissue in vivo occur in photodamaged mouse skin and during wound healing. In photodamaged mouse skin, topically applied all-trans-retinoic acid stimulates the deposition of a subepidermal band of collagen which is observed histologically and biochemically as an increase in the aminopropeptide of type III collagen [Schwartz et al., 1991]. Retinoids also enhance wound healing in animals by stimulating the deposition of collagen [Lee et al., 1973; Hunt, 1986].

Retinoids antagonize the anti-anabolic effects of glucocorticoids on connective tissue synthesis. All-trans-retinoic acid can prevent skin atrophy induced by topical glucocorticoids [Lesnick et al., 1989]. Vitamin A has been shown to have the opposite effects of glucocorticoids on collagen synthesis and collagen deposition [Ehrlich and Tarver, 1971; Ehrlich et al., 1973; Wehr et al., 1976; Salmela, 1981; Salmela and Ahonen, 1981]. The glucocorticoid-mediated inhibition of granuloma growth, collagen content, and collagen synthesis are reversed or retarded by co-administration of Vitamin A. Glucocorticoid-mediated reductions in inflammatory cell infiltration, fibroplasia, capillary budding, and deposition of collagen fibers are prevented by concurrent Vitamin A therapy [Ehrlich et al., 1973]. In addition, we have shown that Vitamin A blocks the inhibitory effects of glucocorticoids on collagen, total protein, and DNA in granulomas [Wehr et al., 1976].

Glucocorticoid and retinoid effects on cellular processes are mediated by specific receptors in target tissues and cells. Steroid hormone action is mediated through a steroid-receptor complex that binds to sensitive genes at specific GREs. The glucocorticoid-induced decrease of collagen synthesis is mediated by a receptor mechanism [Cockayne et al., 1986]. The dexamethasone-mediated decrease of collagen synthesis in chick skin fibroblasts is blocked by progesterone and RU486, glucocorticoid antagonists, and potentiated by 5β-dihydrocortisol, a glucocorticoid agonist. Oikarinen et al. [1987] also demonstrated a down regulation of [H^3] dexamethasone binding to glucocorticoid receptors in dexamethasone treated human skin fibroblasts. Nuclear receptors for all-trans-retinoic acid have been identified that act to mediate the effects of retinoids on gene transcription by the same fundamental mechanism as steroid hormones [Lotan and Clifford, 1991]. A number of retinoic acid response elements (RAREs) on various genes have been identified [Lotan and Clifford, 1991].

The present studies were initiated to identify DNA sequences of regulatory function involved in the glucocorticoid and retinoic acid receptor-mediated regulation of type I procollagen gene expression. Through the use of CAT constructs in stably transfected mouse fibroblasts, we demonstrate that glucocorticoids down-regulate and all-trans-retinoic acid upregulates α2(I) procollagen gene expression in these cells. Three GREs, one true RARE, and a modified RARE were identified in the 5′ flanking region of the α2(I) collagen gene. Deletion experiments show that only one of the three GREs are required for glucocorticoid activity and that the true RARE and a modified RARE may be responsible for all-trans-retinoic acid–mediated up-regulation of collagen gene expression.

METHODS

Cell Culture

The cell line used in these studies was the TIB80 embryonic mouse fibroblasts purchased from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's Modified Eagles Medium (Gibco/BRL, Grand Island, NY) supplemented with 10% heat-inactivated and charcoal-stripped fetal bovine serum and 1% (w/v) penicillin-streptomycin (10,000U/ml, 10,000 μg/ml) at 12.5% CO_2. Cells were supplemented daily with ascorbate at 2×10^{-4} M. The cells were passed at 95% confluency at a ratio of 1:10 to 1:15. In all experiments, TIB80 cells were used at passages 3–7.

Dexamethasone and All-Trans-Retinoic Acid Treatments

The dexamethasone solution was prepared by homogenizing the corticosteroid in whole media and incubating this suspension at 37°C for an additional 30 min. The suspension was then filter-sterilized and diluted with whole media to the proper concentration before adding to cell

culture flasks. All-trans-retinoic acid (Sigma, St. Louis, MO) was solubilized in DMSO and PBS (without Ca^{2+} and Mg^{2+}) at 37°C. This solution was directly added to the cell cultures at the appropriate concentrations.

Plasmid Preparation

A single colony from an overnight plate was grown in L-broth (1% (w/v) bactotryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 0.16% (w/v) dextrose) in the presence of the appropriate antibiotic (50 mg/ml) at 37°C. The cells were allowed to reach late log growth and the whole culture was inoculated into 1 liter of L-broth and allowed to grow for 12–16 h. The cells were pelleted and the plasmids were prepared by an alkaline lysis procedure and purified by use of CsCl gradients as described by Maniatis et al. [1982].

Preparation of Plasmid DNA for Transfection

The preparation of plasmid DNA for transfection was a modified large scale plasmid preparation. The plasmid DNA was banded through two CsCl gradients prior to extraction with T.E. buffer saturated with 1-butanol. The plasmid DNA was then ethanol precipitated, washed with 70% (v/v) ethanol, and dried under vacuum. The dried pellet was then resuspended in T.E. buffer, extracted with PCI and chloroform/isoamyl alcohol. Sodium chloride was added to a final concentration of 0.25 M prior to reprecipitating the plasmid with ethanol. The precipitated plasmid was resuspended in T.E. buffer and the concentration was determined by absorbance.

Transfection of Plasmid DNA in Eukaryotic Cells

For transfection studies the calcium phosphate precipitation method of Chen and Okayama [1987] was used. Transient and stable transfection cells were prepared. For stable transfections, the concentration of G418 used for selection was 500 μg/ml.

Preparation of Cell Lysates and CAT Activity

At the end of the treatment period with dexamethasone and all-trans-retinoic acid, the cells were washed with TNE buffer. The cells were harvested in TNE buffer and cell lysates were prepared as described [Gorman et al., 1982]. CAT activity was determined by the method of Gorman et al. [1982]. The same amount of protein was added to the reaction medium (0.25 M Tris HCl, pH 7.9) in a final volume of 41.5 μl. One microliter of the substrate, chloramphenicol, D-threo-[dichloroacetyl-1,2-^{14}C] (60 mCi/mM; NEN, Boston, MA), and 7 μl of the cofactor acetyl-coenzyme A (10 mg/ml) purchased from Pharmacia LKB Biotechnology (Piscataway, NJ) were then added. The reaction was incubated at 37°C and more acetyl-coenzyme A was added after 4 h of incubation. CAT activity was assayed within the linear range of enzyme activity. At the completion of the reaction, the acetylated chloramphenicol products were extracted with 10 volumes of ethyl acetate and the solvent was allowed to evaporate overnight. Once dry, the products were resuspended in ethyl acetate for spotting on a thin-layer chromatography plate. The percent control data were calculated from the mean CAT activity of duplicate cell cultures of duplicate experiments at two different saturating doses of dexamethasone. The mean range of CAT activity values for the appropriate figures is given in the legend. CAT activity data are presented as percent of control since the percent acetylation and the CPM/μg protein for controls varied from experiment to experiment and since the figures represent the data from multiple experiments. The percent of acetylation was determined by dividing the 1- and 3-acetylated chloramphenicol products by the total [^{14}C] chloramphenicol.

Preparation of Internal Deletion DNA Constructs, pINT.2, pINT.3, and pINT.4

The internal deletion constructs were generated from the parent construct pR40. pINT.3 and pINT.4 were made by making two separate enzyme cuts with AccI/XbaI and XbaI/BglII, respectively, and the double-cut constructs were isolated on 0.8% agarose gels. The staggered ends were end-filled using Klenow large fragment polymerase and the constructs were blunt-end ligated using T4 DNA ligase at 20°C for 8 h. pINT.2 was made by cutting pR40 with AccI and utilizing a partial digestion with Hind III. The digested fragments were separated on a 0.8% agarose gel and the desired fragment was isolated. This fragment was also end-filled using Klenow polymerase and blunt-end ligated with T4 DNA ligase at 20°C for 8 h.

Transformation of Competent *E. coli* Cells

Competent *E. coli* DH5α cells (GIBCO/BRL, Grand Island, NY), were transfected by the

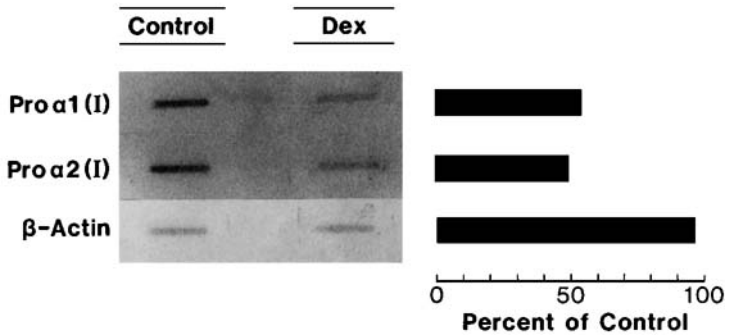

Fig. 1. The effect of dexamethasone treatment on the steady state levels of type I procollagen mRNAs in embryonic mouse fibroblasts. Late log phase cells were treated with and without dexamethasone (2.5×10^{-5} M). At 12 h after glucocorticoid treatment the cells were collected and total cellular RNA was isolated for dot blot analysis.

method of Hanahan [1985]. Five colonies from each plate were selected and grown up for screening of the desired mutants.

Construction of pGEM 1009

The 5′ flanking region of the pAZ1009 plasmid was excised with the enzyme Hind III and subsequently isolated from a 0.8% agarose gel. The sample was melted and the DNA was extracted from agarose [Maniatas et al., 1982]. The vector was prepared by Hind III and alkaline phosphatase digestion. Ligation of the construct was accomplished by mixing the fragments at a 1:1 ratio and ligating with T4 DNA ligase at 14°C for 8 h. Verification of the construct was done by restriction mapping and sequencing. All enzymes were purchased from Gibco/BRL (Grand Island, NY).

Sequencing of Plasmid DNA

Plasmid DNA was alkali denatured for sequencing by the method of Zhang et al. [1988]. The Sequenase 1.0 System, a dideoxy-chain termination method USB (Cleveland, OH) based on the approach of Sanger et al. [1980], was used. The sequencing gels used to separate the DNA sequence ladder were 8% (w/v) polyacrylamide (38:2), 50% (w/v) urea, 1× TBE run in a 1× TBE buffer system at 1,600–2,000 V (60 W). At the completion of the electrophoretic separation, the gel was blotted onto 3 MM chromatography paper (Whatman International Ltd., Maidstone, England), dried under vacuum, and autoradiographed for 12–24 h. The sequence was verified by repeating the sequencing using the ABI automated sequencing system (Model 370A) (Foster City, CA).

Description of DNA Constructs

The proα2(I) gene constructs were a generous gift from Dr. Benoit de Crombrugghe [Rossi and de Crombrugghe, 1987]. The pAZ1003 plasmid contains the 5′ flanking region of the proα2(I) collagen gene (−2048 to +54) and the CAT gene cassette. Plasmid pR40 is an augmented version of pAZ1003 containing the native collagen enhancer. pR40 contains the intron fragment of the proα2(I) collagen gene (+418 to +1524). The plasmid pR36 was derived from the construct pA10CAT2 which contains the SV40 early promoter and the CAT gene cassette. Inserted upstream of this cassette is the enhancer containing intronic fragment (+418 to +1524) of the proα2(I) collagen gene.

Determination of Type I Procollagen mRNA Steady State Levels in Embryonic Mouse Fibroblasts

Total cellular RNA was isolated and dot blot hybridization was performed as previously described [Cockayne et al., 1986] using ^{32}P labeled recombinant rat cDNA probes [Genovese et al., 1984].

RESULTS

Effects of Dexamethasone on Gene Expression

We determined the ability of glucocorticoids to down-regulate gene expression in embryonic mouse fibroblasts as a mechanism for the glucocorticoid-mediated decrease of steady state levels of type I procollagen mRNAs (Fig. 1). To eliminate any effect of the steroid on the transfection process, stable transfected embryonic mouse fibroblasts were established. As a model system for glucocorticoid-induced down-regula-

tion of proα2(I) collagen gene we chose the plasmid pR40 in which the SV40 enhancer sequence is replaced with a segment of the first intron (+418 to +1524) containing a native enhancer of the proα2(I) collagen gene. We used the pR40 plasmid in our studies instead of pAZ1003 since pR40 had a higher basal level of CAT expression and demonstrated a greater decrease of expression with dexamethasone treatment. In stably transfected embryonic mouse fibroblasts with the pR40 DNA construct (pR40A and pR40B cell lines) dexamethasone caused an approximately 50% decrease of CAT activity at 10^{-5} M (Fig. 2). These percent decreases of CAT activity observed in stable transfected mouse fibroblasts agree well with the percent decreases of the steady state levels of type I procollagen mRNAs (Fig. 1). To verify that the observed results in stable cell lines were not the result of the integration site in chromatin, multiple cell lines were established and assayed for dexamethasone-mediated activity.

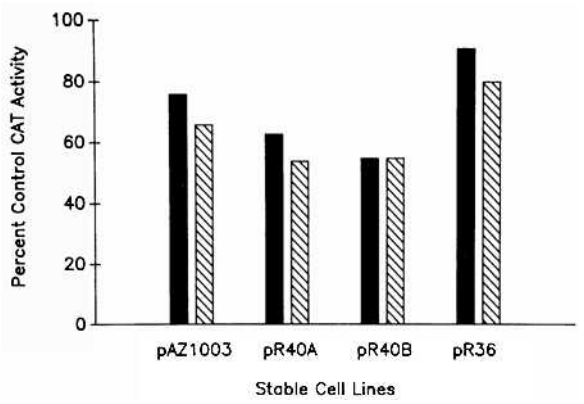

Fig. 2. Glucocorticoid alteration of CAT activity of plasmids pAZ1003, pR40A, pR40B, and pR36 in stable transfected mouse fibroblasts. The stably transfected fibroblasts were treated within dexamethasone at 10^{-7} M (solid bars) and 10^{-5} M (hatched bars) for 48 h. The cell lysates were assayed for CAT activity as described in the text. The mean range of CAT activity values was ± 10.3%.

In an attempt to localize this effect in the 5′ flanking region or in the first intron of pR40 plasmid, the pR36 DNA construct was stably transfected into mouse fibroblasts. The pR36 plasmid contains the intronic segment from +418 to +1524 of the pR40 plasmid cloned upstream of the SV40 early promoter and the CAT sequence (Fig. 2). The level of expression of pR36 when treated with the glucocorticoid was only insignificantly decreased, 10% at 10^{-7} M and 20% at 10^{-5} M. Alternately, stably transfected pR40A and pR40B CAT activity was significantly decreased by dexamethasone treatment. We conclude that the response to the steroid treatment was mediated through the 5′ flanking region and not part of the first intron of pR40. However, the intronic fragment may cooperatively work to decrease gene expression through the 5′ flanking region.

To further localize the dexamethasone response, a series of constructs containing internal deletions in the 5′ flanking region were prepared and stably transfected into mouse fibroblasts (Fig. 3). The regions removed in the constructs pINT.2 (−2048 to −981) and pINT.4 (−506 to −351) were both required for the dexamethasone-mediated down-regulation (Fig. 4). The region removed in pINT.3 (−981 to −506) appears not to be required. Doses of either 10^{-7}

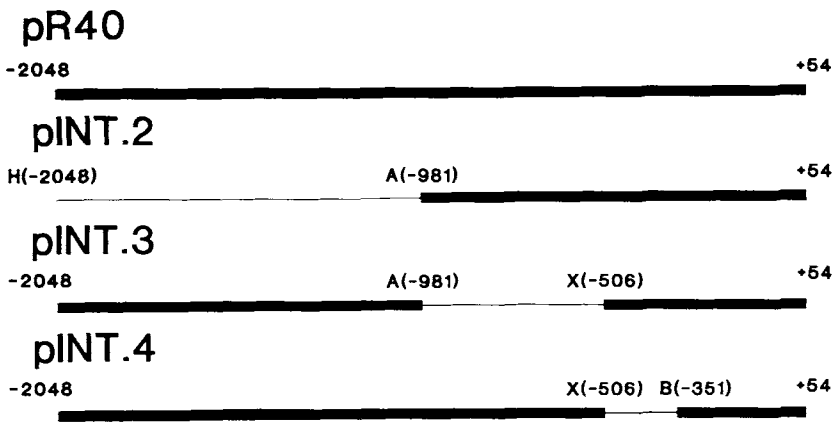

Fig. 3. Internal restriction deletion series of the pR40 5′ flanking region of the α2(I) procollagen gene. The internal deletions were prepared as described in the text using H, Hind III; A, AccI; X, XbaI; B, BgI II.

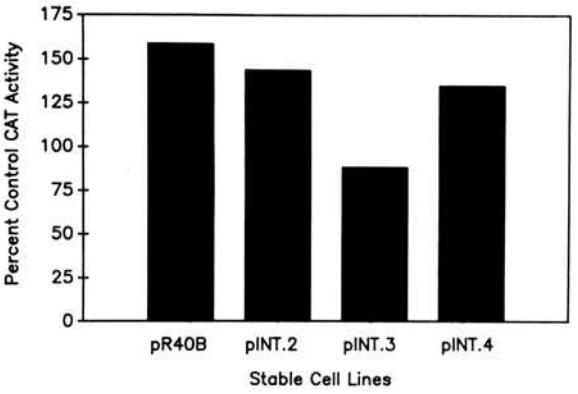

Fig. 4. The effect of dexamethasone on CAT activity of pR40 DNA CAT deletion constructs. Stable cell lines were established incorporating the deletion constructs in Fig. 3. These cell lines were treated with 10^{-7} M (solid bars) or 10^{-5} M (hatched bars) dexamethasone for 48 h. The cell lysates were prepared and CAT activity was determined. The mean range of CAT activity values was ± 10.4.

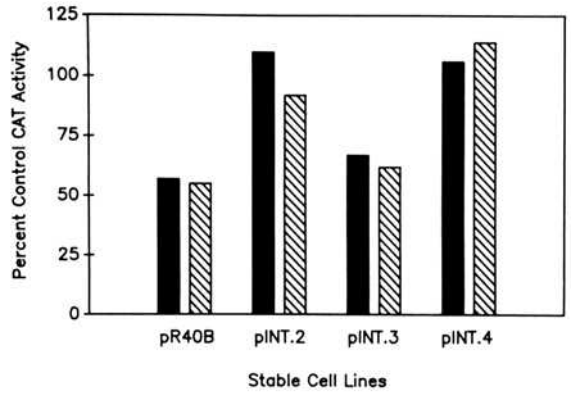

Fig. 5. Modulation of CAT activity by trans-retinoic acid in stable transfected mouse fibroblasts. pR40 plasmid and restriction deletion constructs (Fig. 3) were transfected. The cells were treated with 10^{-6} M trans-retinoic acid for 60 h. The cells were lysed and the CAT activity was determined as described in the text. The mean range of CAT activity values was ± 9.0%.

M or 10^{-5} M dexamethasone significantly inhibited CAT activity.

Effects of All-Trans-Retinoic Acid on Gene Expression

Because all-trans-retinoic acid directly affects collagen synthesis and also antagonizes glucocorticoid-induced down-regulation of collagen synthesis in many model systems, we evaluated it for its effects on stably transfected pR40B fibroblasts. When these cells were treated with 10^{-6} M trans-retinoic acid, CAT activity was elevated 60% over control (Fig. 5). Fibroblasts which had been stably transfected with the internal deletion constructs pINT.2, pINT.3, and pINT.4 were treated with all-trans-retinoic acid. Removal of the segments (−2048 to −981) or (−506 to −351) resulted in a significant induction of CAT activity by retinoic acid. However, removal of the segment between −981 to −506 totally eliminated the induction of CAT activity by all-trans-retinoic acid (Fig. 5).

Sequence Analysis of the proα2(I) Collagen Gene

To further understand the molecular mechanism of regulation of the proα2(I) collagen gene expression we sequenced part of the 5′ flanking region (Fig. 6). The remaining sequence was obtained from the literature [Schmidt et al., 1986]. Analysis of this sequence directed for common consensus sequences was accomplished with the aid of the GCG versions 7.0 (©1991) sequence analysis software package. Three GRE sequences [Beato et al., 1987] were found: a singlet, TGTTCT, at −1023 to −1018 and a direct repeat of the motif, TGATCT at −873 to −856. A true RARE, GTTCAC, was identified at positions −879 to −874, directly adjacent to the GRE doublet [Sucov et al., 1990]. A modified RARE was observed at −867 to −862, six bases from the true RARE.

The sequence of the proα2(I) first intron segment from +418 to +1524 has previously been published [Rossi and de Crombrugghe, 1987] and was also searched for the relative consensus sequences, GREs, or RAREs. Within this region of the first intron there were no additional RARE sites. However, one GRE consensus sequence was located at +1268 to +1273.

DISCUSSION

We have established a model for studying glucocorticoid and retinoid regulation of procollagen gene expression. This was achieved by stably transfecting embryonic mouse fibroblasts with the pR40 construct. The pR40 construct contains the 5′ flanking region of the proα2(I) gene (−2048 to +54), the CAT gene, and the native downstream enhancer of this gene contained in the first intron fragment (+418 to +1524). Two stable transfection cell cultures pR40A and pR40B, were established and both of these cell lines demonstrate a significant decrease in the level of CAT expression when treated with 10^{-7} to 10^{-5} M dexamethasone. This decrease is in agreement with the decrease of steady state levels of type I procollagen mRNAs. pR40 was used in this transfection

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-2048  aagcttggga catttagggg ggttctttca taaatctgtt ttcttaccta
 -1998  cccaagtctt ttgttcagaa acagcacctg tctttcaggt gcctgagaca
 -1948  tagacacaat gacagtctct aaagtcaagc cagcacatcg acagaaataa
 -1898  cttatgctct tattaatgaa gaagaaggag gaaagagaga gagagaaaga
 -1848  aagaaagaaa gaaagaaaga aagaaagaaa gaaagaaaga aagaaagaaa
 -1798  gaaagaaaat cagatgtgca gtcttgaaat gctttctttg ctcagtactt
 -1748  tttcaaaaaa aaaaattccc tccacattat ttaaaaaaca aaacaaacaa
 -1698  acaaaaaacc caaaactctt tctcatctag cctaaaagag acttgaactg
 -1648  ctggggtgag aggaattggg ggaaccggga accgctcaga agaaaagggg
 -1598  ggaggagggg agggattgtg ggaggaggtg atctggaggg aggatttaaa
 -1548  gtgaataagt aagaattaaa ttaaattttt aaaaatgctt gtctctcaaa
 -1498  tcagtgtttt tcacaggatt gaaataacct tattgaaatg tttattctct
 -1448  tctatttcta gcatttttt taatttagaa agagaagcca gttagcaata
 -1398  tccagaacct tttgcttata gcagctgctg atggagctaa cctacttaga
 -1348  aattcttcct caccgggaag tcgaaagggg gaaggtttcc caaggagttc
 -1298  gccagttcct tcttcatgaa cataaaaatg gcctaaagat gctgtgtctg
 -1248  ctgtgacttt ggggacataa tgagagtctg aataggtctc tccagcctca
 -1198  tgatggatgc tcggcaggcc aagaggagcc tgcggcccct gggaacttgg
 -1148  aagactccaa gaaactttgg gaaggagaat ttactatgtc tctgccgctg
 -1098  tcctggacag ccaaagtctc ctactccaat tcttcagagt caaagtctct
 -1048  tcccaagatg gggagagatt gcatcTGTTC Tggaggggac agcttgggat
  -998  gttaaggaag aggttgtata cgccctaggg atgatggaag gtgtgaagat
  -948  gcaggcaggg gtgctggcag tggctgtgga aggggtcata aataggcagc
  -898  aaggccaaga aggctgagtg ttcacTGATC TgttatcTGA TCTaggctcg
  -848  gcatctgagt ctggctggta atccttcatg gggtctgtac atggggctgg
  -798  aaatggggct taaccaaagt aaatgtaata atgcacagtt tatctcttga
  -748  tgcctgctcc cctcatcaac gagagagagg ggggtacaga cagagagagt
  -698  cagagagagg atggaggagg gagggaggaa gggagggagg aagggaggga
  -648  gggagggagg gaggagggag ggagggaggg agggagagac agagagatag
  -598  agagacagag gagagaggag attatggcac acaggccccc ccaaactggg
  -548  gaaattaggg ggcaggggtg gagccaaccc atgagggcag ctctagagct
  -498  cgccacgctg tcaccctcct cccctcacac cccagccccc tccagcctgc
  -448  agatggcccg cagggcacag agtgaagcgg gactggacag ctcctgcctt
  -398  ttcatcttcg gcctccaggt ccctcaacac ccgccccgcc gcccggagat
  -348  ctgtaaagag cccacgtagg tgtcctaaag tgcttccaaa cttggcaagg
  -298  gcgagagagg gcgggtggct ggggagggcg gaggtatgca gacagggagt
  -248  cagagttccc cctcgaaagc cttcaaaagt gtccacgtcc tcaaaaagaa
  -198  tggaaccaat ttaagaagcc ccgtagccac gtccctcccc cctcggctcc
  -148  ctcccctgct cccccgcagt ctcctcccag cactgagtcc cgggccccta
   -98  gccctagccc tcccattggt ggagacgttt ttggaggcac cctccggctg
   -48  gggaaacttt tcccatataa ataaggcagg tctgggcttt attattttAG
    +3  CACCACGGCA GCAGGAGGTT TCGACTAAGT TGGAGGGAAC GGTCCACGAT
   +53  TGCATGCC
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Fig. 6. Sequence of the −2048 to +60 base pairs of the α2(I) procollagen gene. The sequence from −1340 to +60 appeared in the literature. The remaining sequence was determined manually and using an automated sequence system.

model because of its higher basal level of expression and a subsequent greater response to dexamethasone treatment.

To determine the role of the intronic segment (+418 to +1524) in the response to dexamethasone, the plasmid pR36 was stably transfected into mouse fibroblasts, the cells were treated with dexamethasone, and the cell lysates were assayed for CAT activity. The pR36 plasmid contains the proα2(I) intronic segment (+418 to +1524) cloned in upstream of the SV40 early promoter and CAT gene. Treatment of this cell line with dexamethasone for 48 h did not demonstrate a significant decrease of the level of CAT expression. It was concluded that this intronic segment was not directly involved in the dexa-

methasone decrease of the level of CAT expression by the pR40 construct, although the intronic fragment contained a consensus GRE.

To localize the segments of the 5′ flanking region which were involved in the dexamethasone-mediated down-regulation of this gene construct, a series of internal restriction deletion mutants were generated: pINT.2, pINT.3, and pINT.4. Several stable cell transfection lineages were established and these cultures were assayed for changes in the level of CAT expression with dexamethasone treatment. It was observed that the removal of the segment (−981 to −506) was not required for the decrease of CAT expression by dexamethasone. Removal of the segments in pINT.2 (−2048 to −981) and pINT.4 (−506 to −351), however, completely blocked the dexamethasone-mediated down-regulation as seen with the parent construct pR40. Thus both of these segments are required for the dexamethasone-mediated down-regulation of the pR40 gene construct.

Analysis of the sequence of part of the 5′ flanking region of the proα2(I) collagen gene revealed that there were three GREs. A single consensus sequence was found at (−1023 to −1018) and a modified doublet was found at (−873 to −856). Both GREs of the doublet contain an A/T strand switch of the third base pair yielding a repeated TGATCT, six base pairs apart.

The regions required for glucocorticoid-mediated down-regulation of the pR40 construct were the regions removed in pINT.2 (−2048 to −981) and pINT.4 (−506 to −351). Of the known pertinent transcription elements, only the single GRE (−1023) and a long homopurine stretch (−1881 to −1790) were identified in the deleted pINT.2 segment (−2048 to −981). No known regulatory elements were found in the deleted pINT.4 segment (−506 to −351).

Because of the abundant evidence demonstrating that retinoids increase collagen synthesis, we examined the effects of all-trans-retinoic acid on proα2(I) collagen gene expression in the dexamethasone-responsive mouse fibroblast model. The pR40B cells demonstrated an elevation in the level of CAT expression when treated with 10^{-6} M all-trans-retinoic acid. To localize the region of retinoic acid response, the stable restriction deletion mutant cell lineages, pINT.2, pINT.3, and pINT.4, were treated with saturating doses of retinoic acid. Removal of the regions in pINT.2 (−2048 to −981) and pINT.4 (−506 to −351) did not eliminate the retinoic-acid-mediated elevation in CAT expression. Removal of the segment (−981 to −506) in pINT.3 demonstrated a significant effect on the level of CAT expression. By sequence analysis, a true RARE sequence (GTTCAC) was localized from −879 to −874, a modified sequence (GTTATC) from −867 to −862, and an AP-2 site from −552 to −547. The RARE is identical to that in the promoter of the retinoic acid receptor β gene (Sucov et al., 1990). Also contained within this segment was the GRE doublet (−873 to −856). Thus the segments of the 5′ flanking region required for an unequivocal dexamethasone response, (−2048 to −981) and (−506 to −351), are different from the segment required for all-trans-retinoic acid to elevate CAT activity.

There exist many proposed models of glucocorticoid up-regulation of gene expression. However, models of glucocorticoid down-regulation of gene expression are at best scanty. One attractive model of down-regulation suggests that the binding of the glucocorticoid receptor to a specific binding DNA sequence (GRE) close to hormone responsive regulators is required [Diamond et al., 1990]. This model also involves protein-protein interaction between the glucocorticoid receptor complex and the heterodimer, c-Fos and c-Jun. Binding of the heterodimer c-Fos and (or) c-Jun to an AP-1 site adjacent to an active GRE serves as a selector for the positive or negative transcriptional modulation imparted by the binding of the hormone receptor complex. Thus this model of glucocorticoid regulation requires both receptor DNA binding by the glucocorticoid receptor and nonreceptor protein factors. However, in the sequence analysis described above no AP-1 sites were identified in either the 5′ flanking region or the intronic sequence of the α2(I) procollagen gene.

A second model for negative regulation by glucocorticoids involves negative GREs (nGRE) [Sakai et al., 1988]. These are sequences to which the hormone receptor complex also binds but imparts a negative modulation of transcription. None of the published nGREs were found in the 5′ flanking region of the proα2(I) collagen gene sequence.

Any model of glucocorticoid function requires the participation of a GRE and the binding of DNA of the activated glucocorticoid receptor complex. For down-regulation, glucocorticoids may repress the synthesis of their receptor, resulting from transcription or post-transcrip-

tional mechanisms. Our data indicate the localization of one GRE in the 5′ flanking region of the proα2(I) collagen gene which imparts glucocorticoid-mediated down-regulation of procollagen gene expression. Thus, the interaction of the glucocorticoid receptor with this GRE in the 5′ flanking region of the proα2(I) collagen gene may be the determining factor in promoting specific transcriptional repression. Recently we have identified three proteins which bind to proα2(I) gene sequences with increased efficacy when the cells were treated with dexamethasone [Cockayne and Cutroneo, 1988; Sweeney and Cutroneo, 1988]. These DNA binding proteins may interact close to the singlet GRE of the proα2(I) 5′ flanking region to produce a negative transcriptional effect. The interaction of the glucocorticoid receptor with this GRE may act to regulate inhibitory DNA binding proteins to down-modulate procollagen gene expression.

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