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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

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Abstract Glucocorticoids have previously been shown to decrease Type I collagen synthesis in vivo and in fibroblast cell culture. Several studies have demonstrated that glucocorticoids decrease Type I procollagen gene expression. These latter studies have included uridine incorporation into proα1(I) and proα2(I) mRNAs and nuclear run-off experiments. Using the ColCat 3.6 plasmid, which contains part of the 5′ flanking region of the proα1(I) collagen gene and the reporter gene, chloramphenicol acetyltransferase, the present studies demonstrate by stable transfection of fetal rat skin fibroblasts that dexamethasone down regulates the promoter activity of the proα1(I) collagen gene. The glucocorticoid-mediated down-regulation of procollagen gene expression was demonstrated using the ColCat 3.6, 2.4, 1.7, or 0.9 plasmid. In addition, competitive oligonucleotide transfection experiments and site specific mutation of the glucocorticoid response element (GRE) in the whole ColCat 3.6 plasmid did not eliminate the effect. The possibility existed that another cis-element in the 5′ flanking region of the proα1(I) collagen gene was also required for the glucocorticoid-mediated down-regulation of procollagen gene expression, since TGF-β has been shown to stimulate collagen proα1(I) and proα2(I) gene activities. Dexamethasone treatment of non-transfected skin fibroblasts did result in a decrease of transforming growth factor-β (TGF-β) secretion into the media. In addition, CAT activity was decreased by dexamethasone and increased by TGF-β. The decrease of CAT activity by dexamethasone was brought back to control value by the addition of exogenous TGF-β to the culture media. Gel mobility studies demonstrated that glucocorticoid treatment of rat skin fibroblasts decreased glucocorticoid receptor binding to the GRE and TGF-β activator protein to the TGF-β element which were brought back to control values by coordinate exogenous TGF-β treatment. Thus the interaction of these TGF-β molecules with cellular membrane receptors and subsequent transduction is dramatically decreased resulting in less signals to regulate collagen gene expression. These data indicate that glucocorticoids coordinately regulate procollagen gene expression through both the GRE and TGF-β elements. Depression of procollagen gene expression by glucocorticoids through the TGF-β element is mediated by decreased TGF-β secretion, possibly involving a secondary effect on regulatory protein(s) encoded by noncollagenous protein gene(s). The present studies provide the basis for a novel mechanism of glucocorticoid-mediated regulation of eukaryotic genes containing the TGF-β element. © 1995 Wiley-Liss, Inc.

Key words: collagen synthesis, glucocorticoids, transforming growth factor, eukaryotic genes, oligonucleotide, transfection, glucocorticoid regulation

Glucocorticoids have an anti-anabolic effect on skin collagen synthesis. In rat skin, collagen synthesis is decreased to a greater extent than total noncollagen protein synthesis [Newman and Cutroneo, 1979], yet fibronectin synthesis is increased [Cutroneo et al., 1986]. Initial studies determined that glucocorticoids decrease the amounts of functioning procollagen mRNAs [Rokowski et al., 1981; Oikarinen et al., 1983].

Abbreviations: CAT, chloramphenicol acetyl transferase; GRE, glucocorticoid response element; TGF-β, transforming growth factor beta; TBE, 89 mM Tris borate, 89 mM boric acid, 2 mM EDTA (pH 8.0).

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Later studies demonstrated that procollagen mRNAs were decreased by corticosteroids [Sterling et al., 1983a,b; Hamalainen et al., 1985; Raghow et al., 1986; Walsh et al., 1987; Weiner et al., 1987].

There is accumulating evidence indicating that glucocorticoids down-regulate procollagen gene expression. Nuclear type I procollagen mRNA synthesis was selectively decreased in glucocorticoid-treated chick skin fibroblasts which were pulse labeled with radioactive uridine [Cockayne et al., 1986]. In an in vitro run-off system, nuclei isolated from glucocorticoid-treated skin fibroblasts synthesized significantly less type I procollagen mRNAs as compared to control nuclei [Cockayne and Cutroneo, 1988]. These studies indicate that glucocorticoid-mediated down-regulation of procollagen synthesis is at the level of gene transcription. This mechanism of regulation is also invoked by nuclear run-off data from rat small intestine [Walsh et al., 1987] and in rat hepatocytes [Weiner et al., 1987].

DNA binding protein interactions within the 5′ flanking region of the type I collagen genes have been observed by de Crombrugghe and colleagues, where mutations in the upstream elements inhibit the activity of the mouse collagen promoter [de Crombrugghe et al., 1991]. Studies with transgenic mice have identified a region upstream of the α1(I) promoter which regulates expression of the proα1(I) collagen gene in a tissue specific manner [Slack et al., 1991]. Positive and negative regulation through *cis* and *trans* acting elements have been demonstrated in osteoblastic cells [Pavlin et al., 1992], and in type I collagen producing mouse fibroblasts [Ravazzolo et al., 1991; Karsenty and de Crombrugghe, 1990, 1991; Boast et al., 1990].

The studies in this paper indicate the dependence of the glucocorticoid-mediated decrease in proα1(I) collagen gene expression on the interaction of the glucocorticoid receptor with the consensus GRE and the interaction of the transforming growth factor β activator protein with the TGF-β element. Using oligodeoxyribonucleotide competitive transfection experiments and the mutated GRE in the whole plasmid we examined responsiveness of the promoter activity of the proα1(I) collagen gene to dexamethasone. We determined the binding of nuclear proteins isolated from dexamethasone-treated, TGF-β-treated, and TGF-β- and dexamethasone-treated cell cultures to both the GRE and TGF-β elements using the gel mobility shift assay.

MATERIALS AND METHODS

Cell Culture Methods

Fetal rat skin fibroblasts (FRS) were purchased from the American Type Culture Collection (CRL 1213, batch F-9707), Rockville, MD. FRS cells were grown in Dulbecco's modified Eagle's medium, 90% (v/v) (Whittaker M.A. Bioproducts, Walkersville, MD); heat inactivated fetal bovine serum, 10% (v/v) (Hyclone Laboratories, Inc., Logan, UT); 10 U/ml penicillin; and 10 μg/ml streptomycin. Prior to exposure of cells to dexamethasone the medium was changed. Dexamethasone treatments were performed in medium made 10% (v/v) with fetal bovine serum which had been charcoal-stripped. When cell cultures were treated with TGF-β1 (R & D Systems, Minneapolis, MN), the cells were washed twice with AIM V (GibcoBRL, Grand Island, NY), a synthetic medium. The cells were treated in the presence and absence of TGF-β1 in AIM V medium.

Dexamethasone Treatment

Dexamethasone (Steraloids, Inc., Wilton, NH) was added to cultures as a suspension in charcoal-stripped serum containing medium. Dexamethasone was homogenized in medium and incubated at 37°C for 30 min. The suspension was filter-sterilized and diluted to the appropriate concentrations prior to addition to the cell cultures. When cell cultures were treated with TGF-β and dexamethasone, dexamethasone was prepared as above except AIM V medium was substituted for charcoal-stripped serum containing medium.

Transfection of FRS Fibroblasts and Stable Selection

Cells were transfected using the calcium phosphate coprecipitation method [Chen and Okayama, 1987]. Plasmids were purified by double CsCl banding according to standard methods. ColCat 3.6 (kindly supplied by D. Rowe and A. Lichtler, Depts. of Pediatrics and Medicine, Univ. Conn. Health Science Center, Farmington, CT) or constructs containing deletions or mutations of the proα1(I) collagen promoter, and pSV2neo were cotransfected into FRS cells

for stable cell selection [Lichtler et al., 1989; Ritzenthaler et al., 1991]. Stable transfections [Southern and Berg, 1982; Gorman et al., 1982] were used since reproducible results were not obtained using transient transfections. Cells were selected using G418 (200 μg/ml).

We observed a discrepancy from the published ColCat 3.6 sequence. It was necessary to sequence the nonmutagenized ColCat 3.6 plasmid for purposes of comparison to the mutagenized plasmid. This resulted in a sequence different by two bases from that previously published in the GenBank database. The bases 5′-AG-3′ at −1616 and −1615 are actually erroneous in the previous publication [Lichtler et al., 1989] and the restriction enzyme site recognized by Sty I in this region does not exist.

Preparation of Cell Lysates and Assay of CAT Activity

At the appropriate time following dexamethasone treatment, cells were placed on ice and washed 3× with cold phosphate buffered saline (GibcoBRL, Grand Island, NY). Cells were harvested in 1 ml of 40 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA at 4°C as previously described [Gorman et al., 1982]. CAT activity was determined [Neumann et al., 1987]. The same amount of protein was added to each reaction mixture containing 100 mM Tris-HCl, pH 7.8, in a volume of 50 μl. Two hundred microliters of 1.25 mM chloramphenicol, 100 mM Tris-HCl, pH 7.8, was then added to each sample. Finally, 0.5 μCi [^{3}H]acetyl-CoA (200 mCi/mmol, DuPont NEN, Boston, MA) and 22.5 μl of 1 mM cold acetyl-CoA were added and the entire reaction mixture overlaid with liquid scintillation fluid (Econofluor-2, DuPont NEN, Boston, MA). This assay method allows multiple time points to be determined on the same assay tube by liquid scintillation counting at various times. The acetylated chloramphenicol product is miscible in the aqueous-immiscible scintillation cocktail and the data are collected continuously. 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 are reported for all samples.

Protein Assay

Protein concentrations of cell lysates were determined as described [Lowry et al., 1951].

Competitive Oligonucleotide (d_sGRE)Transfections Assays

Synthetic oligomers corresponding to the canonical hexamer sequence, TGTTCT, were annealed with the complementary hexamers. The double-stranded hexamers were transiently transfected into stable FRS cells containing the proα1(I) collagen promoter construct (FRS/ColCat 3.6) using the calcium-phosphate coprecipitation method [Gorman et al., 1982]. Cells were glycerol shocked in 15% (v/v) serumless medium 4 h after transfection. Twenty-four hours after, the transfected cells were treated with dexamethasone as described.

Site-Specific Mutagenesis of the GRE in the Rat Proα1(I) Collagen Promoter

PCR mutagenesis of ColCat 3.6 was performed as previously described [Nelson and Long, 1989]. Figure 1 shows the appropriate plasmid and the restriction map. The position of the GRE is indicated in Figure 1A. Figure 1B indicates the position of the TGF-β element (α-TAE).

DNA Sequencing of Mutagenized (GRE) ColCat 3.6

Sequence-grade DNA was prepared from a 3 ml overnight culture of cells picked from positive colonies and purified using Qiagen Tip-20 columns (Qiagen Inc., Chatsworth, CA) according to the procedure provided by the manufacturer. Sequencing was performed on an automated ABI Model 373A DNA Sequencer (Foster City, CA) using primers E and F (Table I) for the region encompassing the GRE. For each sequencing reaction 1 μg of dsDNA template, 3.2 pmol forward sequence primer (each primer), 9.5 μl reaction primer (5 × TACS buffer, dNTP mix, DyeDeoxy™ A, T, G, and C terminator and AmpliTaq DNA polymerase) were used in the ABI cycle sequencing protocol supplied with the cycle sequencing kit (ABI). Final reaction volumes were 20 μl. Cycle conditions consisted of placing the tubes in a thermal cycler preheated to 96°C and 25 cycles were then performed at 96°C for 30 min, 50°C for 15 min, and 60°C for 4 min.

TGF-β Assay

TGF-β protein was assayed in acid-activated media as described [Kelley et al., 1993]. All

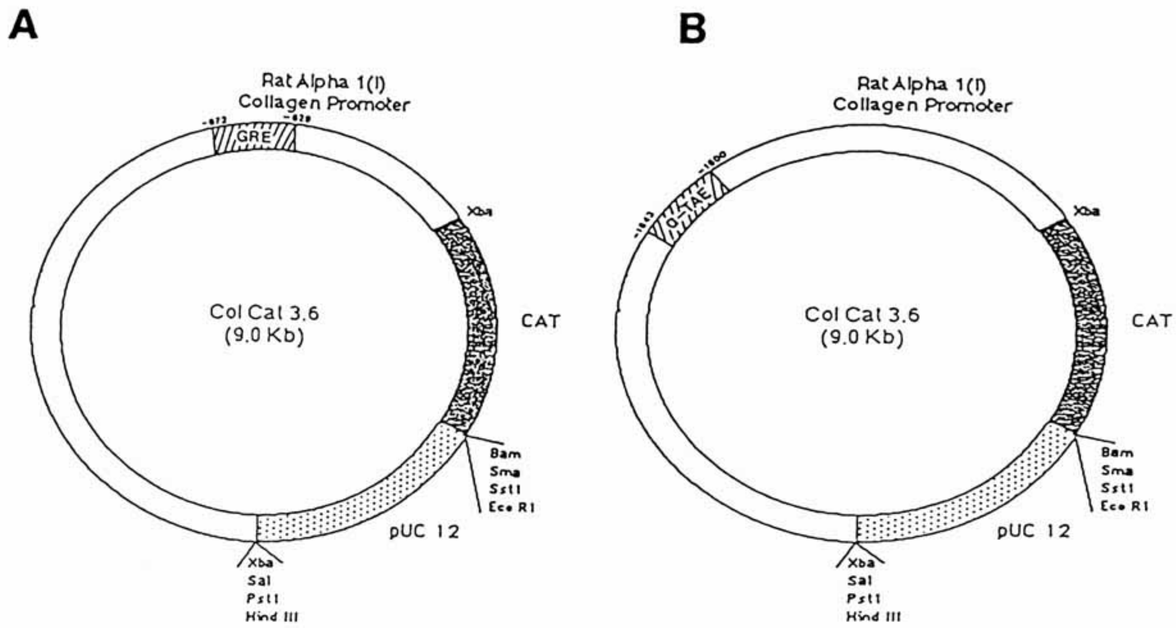

Fig. 1. Plasmid containing the GRE and α-TAE elements in the 5′-flanking region of the proα1(I) collagen gene. **A:** The structure of ColCat 3.6 plasmid. The wild-type GRE region is indicated by the striped bars (from −672 to −629) containing the GRE consensus sequence. **B:** The wild-type TGF-β activating element (α-TAE) region is indicated by the striped bars (from −1643 to −1600). [Lichtler et al., 1989].

TABLE I. Oligonucleotide Primers Used for PCR Mutagenesis of GRE in Rat Alpha I(1) Collagen Promoter and DNA Sequencing

Primers	Sense[a]	Sequence correspondence[b]	Sequence[c]
			* * * *
A	Forward	−672−−633	5′-GGAGCCACATTCATGGCGGGCCC GATAGCCACCCCACTGC-3′
B	Inverse	−397–332	5′-GGTGTACTAGTAACCTAGGCCTTC TGGGACCTCAAATCAGAAGGA-3′
C	Forward	−2,377−−2,358	5′-GAGCTGGGTACCCGCCAATC-3′
D	Inverse	None	5′-GGTGTACTAGTAACCTAGG-3′
E	Forward	−958−−934	5′-GGCTGTAGCCACAGCTGACACAAGA-3′
F	Forward	−675−−657	5′-CATGGAGCCACATTCATGG-3′

[a]Primer sense corresponds to the rat alpha I(1) collagen promoter coding strand.
[b]Numbering convention according to the sequence of the ColCat 3.6 plasmid [Lichtler et al., 1989].
[c]Asterisk indicates the positions of the directed mutations and underlined bases that compose the *ApaI* recognition sequence.

values were corrected for possible dexamethasone inhibition of the bioassay used to measure TGF-β.

DNA Mobility Shift Assays

Fetal rat skin fibroblasts (FRS) were grown to late log phase. The cells were washed twice with AIM V medium. The cells were then treated with either dexamethasone (final concentration 10^{-6} M), TGF-β1 (5.0 ng/ml of media), or a mixture of 10^{-6} M dexamethasone and 5.0 ng/ml TGF-β1 in AIM V medium. Control cells and cells treated with dexamethasone alone received the vehicle (4 mM HCl with 1 mg/ml bovine serum albumin) used to dissolve TGF-β. Nuclear protein extracts were prepared by the method of Andrews and Faller [1991] and the protein concentration was determined [Lowry et al., 1951].

Single-stranded oligonucleotides containing either the GRE sequence (5′-AGAACA) or the TGF-β element sequence (5′-TGC CCACG GCCAG) were synthesized (Integrated DNA

Technologies, Inc., Coralville, IA). In further purification, 20 μg of the single-stranded oligonucleotides were combined with an equal volume of 100% formamide, heated to 90°C for 3 min, and run on a 20% polyacrylamide/8M urea gel in 1 × TBE buffer for 2 h at 500 V. The DNA was visualized by UV shadowing. The gel strips containing the DNA were cut from the gel and placed in oligo elution buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 2 mM EDTA). After overnight incubation, the gel particles were removed by centrifugation and the supernatants containing the oligonucleotides were purified on Sep-Pak C28 Cartridges (Millipore Corp., Milford, MA). Complementary oligonucleotides in 200 mM NaCl were annealed by heating to 95°C for 7 min and then slowly cooled to 4°C. The double-stranded oligonucleotides were stored at −20°C.

The oligonucleotides were labeled with ^{32}P using the 5′ DNA Terminus Labeling System (GibcoBRL, Grand Island, NY). Gel shift binding reactions (20 μl) contained ^{32}P-end labeled double-stranded oligonucleotide having either the GRE or TGF-β element sequences (approximately 8×10^5–1×10^6 cpm/pm); 10 μg of nuclear protein extract; 1.5 μg of poly d(I-C) (Pharmacia, Piscataway, NJ); 90 mM KCl; 1 mM EDTA; 1 mM DTT and 5% glycerol. Reaction mixtures were incubated for 30 min at room temperature and separated on a 6% polycrylamide gel (19:1 acrylamide to bisacrylamide) in 0.25 × TBE buffer 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 densiometric analysis using a Shimadzu dual-wavelength TLC scanner, Model CS-930 (Shimadzu Corp., Kyoto, Japan). Using the Model GS-250 Molecular Imager System (BIO-RAD, Hercules, CA), the total radioactivity in each lane (free probe plus bound protein-DNA complex) was also measured to insure that there was equal loading of each sample onto the gel. The total radioactivity in each lane of each gel did not vary significantly: ±6.8% and ±7.0% for the TGF-β element and for the GRE, respectively.

RESULTS

Effect of Dexamethasone on Stable FRS Fibroblast Cell Lines

Four stable cell lines transfected with the chimeric construct ColCat 3.6 were established. Exposure to 10^{-5} M dexamethasone for 48 h caused a significant decrease in pro α1(I) collagen promoter activity in all four cell lines (Fig. 2). Although the cell lines displayed a variety of activities, with Neo A demonstrating 7.4× the activity observed in Neo B, all of the stable cell lines responded to dexamethasone by decreasing pro α1(I) collagen promoter activity. The degree of decrease induced by dexamethasone in promoter activity varied with each cell line, as seen in Figure 2. Neo A demonstrated a 39% decrease with dexamethasone, Neo B was decreased 56%, Neo C was decreased 51%, and Neo D was decreased 63% at 10^{-5} M dexamethasone.

The effect of dexamethasone is selective for procollagen gene expression. A previous study from our laboratory demonstrated that although dexamethasone decreased the steady-state level and synthesis of proα1(I) collagen mRNA, this synthetic glucocorticoid did not effect β-actin mRNA.

Time Course of Response to Dexamethasone

Two stable cell lines, Neo B and Neo D, were examined for pro α1(I) collagen promoter activity responsiveness to 10^{-5} M dexamethasone with time. Neither cell line responded significantly within 24 h (Fig. 3). After 48 h both cell lines demonstrated a substantial decrease in pro α1(I) collagen promoter activity. Neo D was decreased by 44% and Neo B was decreased by 66%. The decrease in promoter activity after a single dose of dexamethasone was maintained over the entire time course studied. Neo D was maximally decreased by 57% after 5 days of

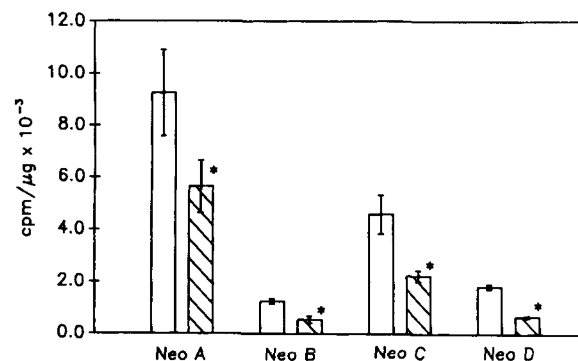

Fig. 2. Dexamethasone decreases proα1(I) collagen promoter activity in stable FRS fibroblast cell lines. Four different stable lines (Neo A, B, C, D) of FRS fibroblasts transfected with ColCat 3.6 were evaluated for responsiveness to dexamethasone. Cells were grown to late log phase and then exposed to 10^{-5} M dexamethasone for 48 h. Cell lysates were assayed for CAT activity which is expressed as cpm per μg protein. □ = control cell cultures; ▧ = dexamethasone exposed cell cultures. *Significantly different from control values at $P \leq 0.05$. Values represent the mean of 4 separate dishes ± S.D.

exposure to dexamethasone. Neo B demonstrated a dramatic decrease of 87% in pro α1 (I) collagen promoter activity after 5 days of dexamethasone.

Effect of Varying Concentrations of Dexamethasone

At 10^{-6} M, dexamethasone there was a 73% decrease in CAT activity (Fig. 4). A typical dose response curve was observed, with activity increasing as the dexamethasone concentration decreased. At 10^{-9} M dexamethasone CAT activity was decreased 52%. Finally, at 10^{-10} M dexamethasone, the cell line did not demonstrate a statistically significant decrease in pro α1(I) collagen promoter activity.

The Effect of Dexamethasone on Internal Deletions of the Pro α1(I) Collagen 3.6 ColCat Promoter

Comparison of reporter gene activity amongst different gene constructs was always made to the reporter activity of the control plasmid. Internal deletion constructs stably transfected into FRS fibroblasts were examined for responsiveness to 10^{-6} M dexamethasone after 48 h exposure. Deletions resulting in promoter lengths of 2.4 and 1.7 Kb demonstrated decreases similar to that observed with the intact 3.6 Kb rat pro α1(I) collagen promoter (Fig. 5). Two separate cell stable lines were examined for each deletion construct. The 3.6 Kb promoter length responded to 10^{-6} M dexamethasone by a 52–50% decrease in promoter activity. The 2.4 Kb promoter length demonstrated a 52–55% decrease in activity in the presence of dexamethasone and the 1.7 Kb promoter length decreased activity to 52–59% of control values (Fig. 5). Contrasted with the longer deletion constructs, the 0.9 Kb length promoter construct demonstrated a dramatic 84–90% decrease in activity in the presence of 10^{-6} M dexamethasone. In the 0.9 Kb construct the GRE may be either more efficiently accessible to transacting factors resulting in the almost complete abolishment of promoter activity in the presence of dexamethasone, or the greater inhibition may be due to the low level of basal CAT activity of the 0.9 Kb construct. Examination of the sequence of the rat pro α1(I) collagen promoter reveals a consensus GRE from −655 to −650 (Figure 1A), which would react with the glucocorticoid receptor. The presence of the GRE between −655 and −650 has previously been determined [Lichtler et al., 1989]. We did not assay chimeric promoter deletion constructs shorter than −900 bp since their basal activity was too low, which made glucocorticoid-mediated depression of collagen gene expression difficult to measure.

Competition of Synthetic dsGRE for the Pro α1(I) Collagen Promoter

The dexamethasone-induced decrease in pro α1(I) collagen promoter activity of the whole

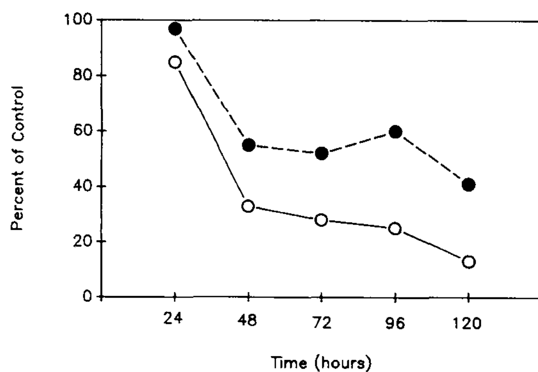

Fig. 3. Time course response of proα1(I) collagen promoter activity in stable FRS fibroblasts to dexamethasone. Two stable lines of FRS fibroblasts transfected with ColCat 3.6 were examined for dexamethasone responsiveness after varying exposure times. Cells were grown to mid-late log phase and then exposed to 10^{-5} M dexamethasone. Control cells and dexamethasone treated cells were collected after 24, 48, 72, 96, and 120 h. Cell lysates were assayed for CAT activity. Values expressed as percent of control values are all the mean of at least 3 separate dishes. ○, Neo B; ●, Neo D.

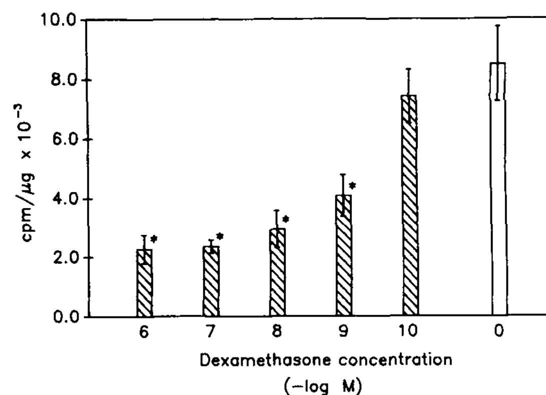

Fig. 4. Dose-response relationship of pro α1(I) collagen promoter activity to dexamethasone concentration in stable FRS fibroblasts. Stable FRS fibroblasts transfected with ColCat 3.6 were examined for dose dependence to dexamethasone. Cells were grown to mid-late log phase then exposed to 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M dexamethasone for 48 h. Cell lysates were assayed for CAT activity which is expressed as cpm per μg protein. *Significantly different from control values at $P \leq 0.05$. Values represent the mean of 3–4 separate dishes ± S.D.

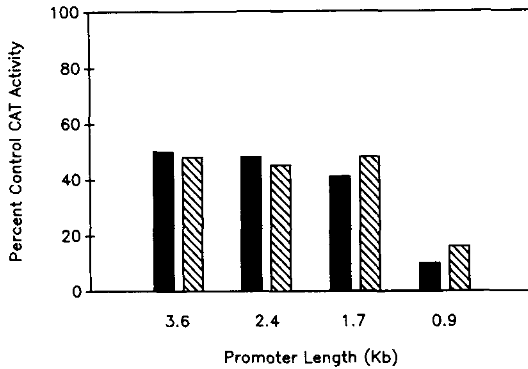

Fig. 5. Effect of dexamethasone on pro α1(I) collagen promoter activity and deletion mutants in stable FRS fibroblasts. Stable FRS fibroblast lines were established for cells transfected with CAT constructs containing varying lengths of the pro α1(I) collagen promoter. Cells were grown to mid-late log phase and then exposed to 10^{-6} M dexamethasone for 48 h. Cell lysates were assayed for CAT activity. Values represent the average of 2–4 separate dishes. ■ and ▧ represent different stable cell lines transfected with the same construct.

plasmid was challenged with competitive oligonucleotides comprising the canonical GRE sequence (AGAACA) (Fig. 6). These experiments were performed at two different concentrations of oligonucleotide, 10 μg/dish and 25 μg/dish, and at two different concentrations of dexamethasone, 10^{-6} and 10^{-7} M, for 48 h of exposure. Similar results were observed for both sets of conditions. When 10 μg/dish dsGRE were used to compete with ColCat 3.6 for the 10^{-6} M dexamethasone effect, the decrease in pro α1(I) collagen promoter activity was observed to be 80% compared to the control (no dexamethasone but containing competing dsGRE) (Fig. 6A). Similarly, in Figure 6B, when 25 μg/dish of dsGRE was used to compete in cells exposed to 10^{-7} M dexamethasone, a decrease in pro α1(I) collagen promoter activity of 64% was observed compared to the dsGRE competed controls. These results indicate that competition for the GRE may not be sufficient to interfere with the dexamethasone-induced decrease in pro α1(I) collagen promoter activity of the whole ColCat 3.6 plasmid.

Effect of Dexamethasone on Pro α1(I) Collagen Promoter Containing a Mutated GRE

Since the competitive oligonucleotide may not have gotten to the correct nuclear location and these experiments gave negative results, we next performed a more direct experiment and mutated the GRE in the whole ColCat 3.6 plasmid. Site-specific mutagenesis of the pro α1(I) collagen promoter was affected by replacement of the sequence AGAACA (−655 to −650) with the hexamer GGGCCC. The mutagenized construct was then co-transfected with pSV2neo into FRS cells and selected as described in Materials and Methods. Two cell lines harboring the mutant plasmid were selected (Fig. 7). Both of the cell lines, mutants-Neo A and -Neo B, were examined for the effect of exposure to dexamethasone. At 10^{-6} M dexamethasone for 48 h, in both cell lines the proα1(I) collagen promoter responded similarly, demonstrating decreased promoter activity compared to control values of 61% for Neo A and 68% for Neo B (Fig. 7), which

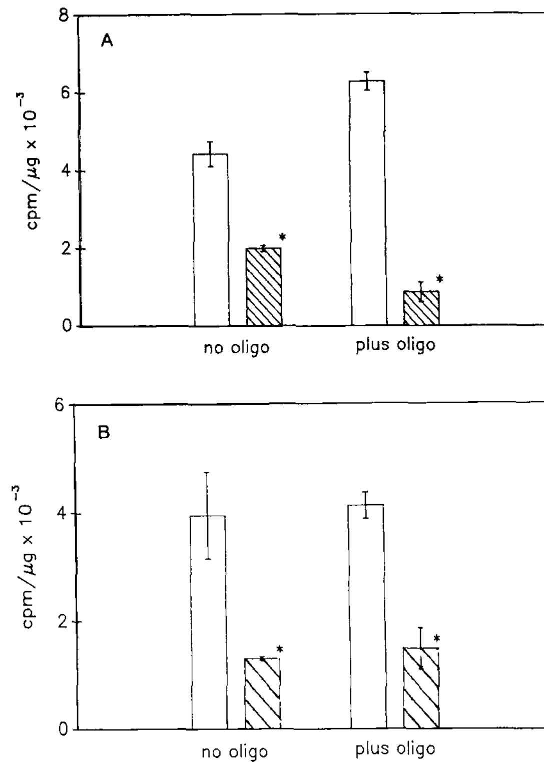

Fig. 6. Competitive dsGRE (hexamer) does not alter the dexamethasone-induced decrease in pro α1(I) collagen promoter activity. Stable FRS fibroblasts were transfected with dsGRE oligomers (plus oligo, on the right side of the figure). In **A**, cells were transfected with 10 μg/dish dsGRE and exposed to 10^{-6} M dexamethasone for 48 h. In **B**, cells were transfected with 25 μg/dish and exposed to 10^{-7} M dexamethasone for 48 h. Cell lysates were assayed for CAT activity, which is normalized as cpm per μg protein. no oligo = no added dsGRE; plus oligo = added dsGRE, □ = control cell cultures, ▧ = dexamethasone exposed cell cultures. *Significantly different from respective control values at $P \leq 0.01$. Values represent the mean of 3–5 separate dishes ± S.D.

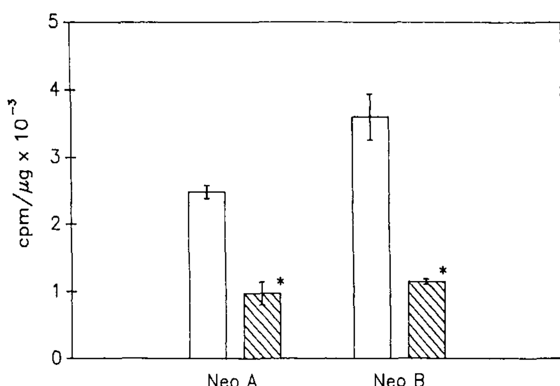

Fig. 7. Site-specific mutation in the GRE alone does not alter the dexamethasone-induced decrease in pro α1(I) collagen promoter activity. Stable FRS fibroblast lines were established for cells harboring CAT constructs containing specific mutations in the GRE sequence (from AGAACA to GGCCCC) of the pro α1(I) collagen promoter. After stable selection, two separate cell lines, Neo A and Neo B, were grown to late log phase and then exposed to 10^{-6} M dexamethasone for 48 h. The cell lysates were assayed for CAT activity which was normalized as cpm per μg protein. □ = control cell cultures; ▧ = dexamethasone exposed cell cultures. *Significantly different from control values at $P \leq 0.01$. Values represent the mean of 4 separate dishes ± S.D.

confirms the results of the competitive GRE oligonucleotide experiments.

The Effect of Dexamethasone on TGF-β

The negative results obtained in the competitive oligonucleotide transfection experiments in Figure 6 and the GRE mutagenesis studies in Figure 7 may be explained by a glucocorticoid mediated down regulated element preceding the GRE at −650 to −655. Since Ritzenthaler et al. [1991] demonstrated that TGF-β treatment of fibroblasts transiently transfected with the 3.6 ColCat plasmid resulted in an increase of CAT activity, we focused our attention on the TGF-β element (α-TAE) located at −1,628 bp upstream from the start site of transcription. The TGF-β element is required for CAT activity of FRS fibroblasts when transfected with the 3.6 ColCat plasmid. When cells were transfected with the 3.6 ColCat plasmid containing the mutated TGF-β element, basal CAT activity was decreased in the mutated plasmid by tenfold (data not shown). When both the GRE and TGF-β elements were mutated in the same plasmid, basal CAT activity was also tenfold lower. However, dexamethasone treatment significantly lowered CAT activity in the mutated plasmids (data not shown). These results may have been obtained since we are looking at a down regulation of gene expression.

Dexamethasone treatment of nontransfected skin fibroblasts resulted in a decrease of TGF-β secreted into the media (Fig. 8), although the dose curve of reduction of TGF-β secretion does not correlate with the inhibition of CAT activity. This may be because a saturating effect may have been obtained, since the glucocorticoid dose response study was done on stably transfected FRS fibroblasts, whereas the glucocorticoid dose response on TGF-β secretion was done on nontransfected FRS fibroblasts to more closely mimic what would occur in vivo. The amount of TGF-β in the media is relatively low. This is because the FRS fibroblasts were grown up in serum containing media, washed twice with DMEM and dosed with glucocorticoid in 0.1% BSA/DMEM.

The effect of dexamethasone, TGF-β, and dexamethasone plus TGF-β on CAT activity of rat skin fibroblasts was determined (Fig. 9). TGF-β treatment alone caused a 1.8-fold increase in CAT activity. In a previous study using fibroblasts, TGF-β treatment alone resulted in about a twofold increase in CAT activity [Jimenez et al., 1994]. In addition, TGF-β blocked the

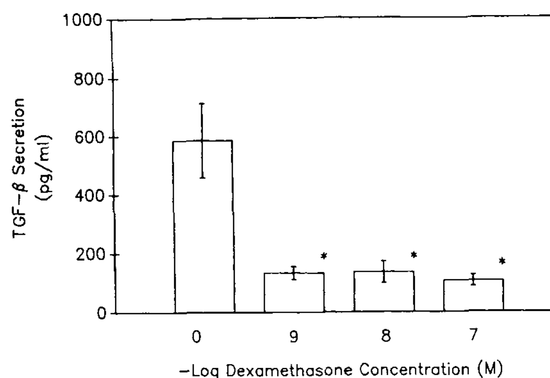

Fig. 8. FRS fibroblasts were tested for the effect of dexamethasone on the TGF-β protein secreted into the media of cell cultures. Cells were grown to mid-late log phase in 100 × 20 mm dishes. The cells were washed twice with Dulbecco's modified Eagle's medium (DMEM). 0.1% BSA/DMEM containing dexamethasone at the final concentrations of 1×10^{-7} M, 1×10^{-8} M, or 1×10^{-9} M was added to the cells for 48 h. The media was removed from the dishes, filter sterilized, and stored at −20°C. TGF-β protein was assayed in acid-activated media by measuring the inhibition of growth of Mv1Lu mink lung epithelial cells [Kelley et al., 1993]. *Significantly different from control values at $P \leq 0.01$. Values represent the mean of 4 separate dishes ± S.D.

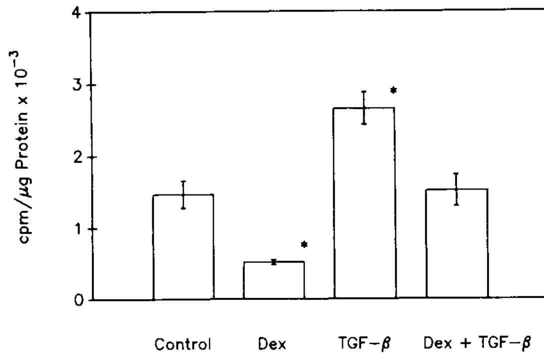

Fig. 9. Effect of dexamethasone, TGF-β and dexamethasone plus TGF-β on the pro α1 (I) collagen promoter activity in stable FRS fibroblasts. Stable FRS fibroblasts transfected with ColCat 3.6 were examined for responsiveness to dexamethasone, TGF-β, and dexamethasone plus TGF-β. Cells were grown to mid-late log phase and washed twice with AIM-V medium. The cells were then treated with either 10^{-6} M dexamethasone, 2.5 ng/ml TGF-β1, or 10^{-6} M dexamethasone plus 2.5 ng/ml TGF-β1 in AIM-V medium for 48 h. Control cells and cells treated with dexamethasone received the vehicle (4 mM HCl with 1 mg/ml bovine serum albumin) used to dissolve TGF-β. Cell lysates were assayed for CAT activity which is expressed as cpm per μg protein. *Significantly different form control values at $P \leq 0.01$. Values represent the mean 3–4 separate dishes ± S.D.

dexamethasone-mediated decrease of CAT activity.

To test our hypothesis concerning glucocorticoid-mediated decreased signal transduction, we used the gel mobility shift assay to study the binding of nuclear proteins, isolated from dexamethasone, TGF-β, and dexamethasone plus TGF-β treated rat skin fibroblasts, to the GRE and TGF-β elements (Figs. 10 and 11). The A and B panels represent two different experiments. As can be seen, dexamethasone decreased the glucocorticoid receptor binding to the GRE *at* 48 h (Fig. 10) and decreased the amount of TGF-β activator protein binding to the TGF-β element (Fig. 11). The amount of TGF-β activator protein bound to the TGF-β element was increased in TGF-β treated cultures and brought back to control value by concomitant dexamethasone treatment. We propose that there is less TGF-β secreted by the dexamethasone-treated cells into the media [Shull et al., 1995] to react with cell membrane receptors resulting in decreased signal transduction and decreased DNA binding proteins to react with TGF-β elements in the 5′ flanking region of the proα1(I) collagen gene. Dexamethasone treatment decreased the amount of glucocorticoid receptor bound to the GRE, while the amount bound was increased in TGF-β treated cultures and brought back to control value in cell cultures treated with dexamethasone plus TGF-β (Fig. 10). Thus, glucocorticoids decrease proα1(I) collagen gene expression through both the GRE and TGF-β element.

DISCUSSION

Since the 1960s glucocorticoids have been shown to decrease collagen synthesis [Cutroneo et al., 1986]. A site of regulation of Type I collagen synthesis by these steroid hormones is the modulation of procollagen gene expression. We previously demonstrated using several stably transfected mouse skin fibroblasts that glucocorticoids decrease proα2(I) procollagen promoter activity [Perez et al., 1992]. Presently we focused on glucocorticoid regulation of promoter activity of the proα1(I) collagen gene and the cis-elements in a segment of the 5′ flanking region required for the glucocorticoid-mediated down regulation of collagen synthesis.

The dependence of constitutive transcription of type I procollagen genes on continued protein synthesis suggests the existence of nuclear regulatory sequence-specific protein factor(s) [Cockayne and Cutroneo, 1988]. Accordingly, the present studies were initiated to identify DNA sequences of regulatory function involved in the dexamethasone receptor-mediated down regulation of proα1(I) collagen gene expression. The classically accepted sequence of events in steroid hormone regulation of gene expression involves association of glucocorticoid with the glucocorticoid receptor. This complex is then activated, localized in the nucleus, and associated with a glucocorticoid response element to elicit an up regulation or a down regulation of gene expression. Using an agonist and antagonists our laboratory has previously demonstrated that the glucocorticoid-mediated down regulation of procollagen synthesis is at least in part a receptor mediated process [Cockayne et al., 1986].

One attractive model of down regulation suggests that the binding of the glucocorticoid receptor to a specific binding DNA sequence (GRE) adjacent to hormone responsive regulators is required [Diamond et al., 1990]. This model also involves protein-protein interaction between the glucocorticoid receptor complex and the het-

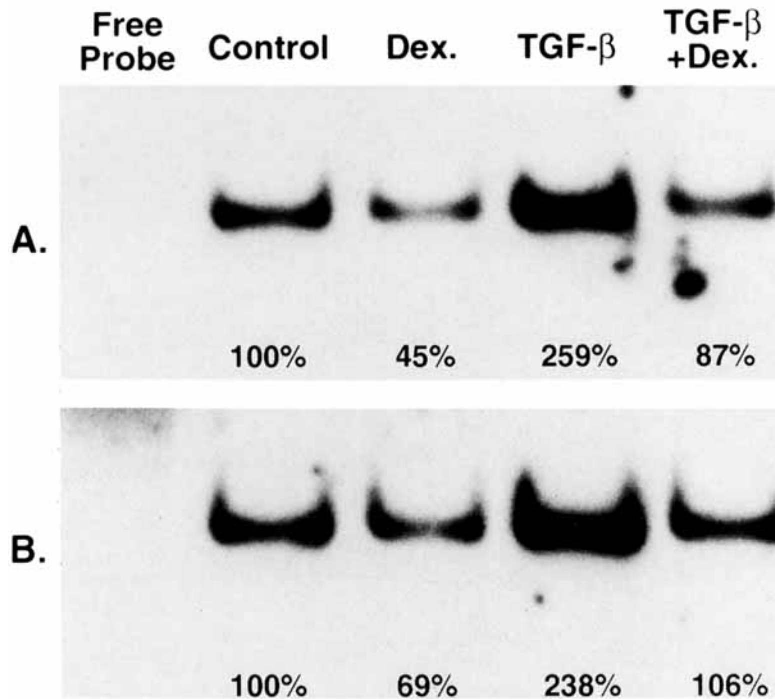

Fig. 10. Effect of dexamethasone, TGF-β, and dexamethasone plus TGF-β on GR bound to the GRE. FRS fibroblasts were treated with either 10^{-6} M dexamethasone, 5.0 ng/ml TGF-β1, or 10^{-6} M dexamethasone plus 5.0 ng/ml TGF-β1 for 48 h. ^{32}P-end labeled double-stranded oligonucleotide (8×10^5–1×10^6 cpm/pm) containing the GRE sequence (5′-AGAACA) 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 total volume of 20 μl. 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 for 4 h at 100 V. Gels were air-dried and exposed to X-ray film. The X-ray film was scanned and the areas under the peaks were quantitated using a Shimadzu dual-wavelength FLC scanner, model CS-930 (Shimadzu Corp., Kyoto, Japan). **A** and **B** represent data from two different experiments.

erodimer c-Fos and C-Jun. Binding of the heterodimer c-Fos and/or c-Jun to the 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 computer sequence analysis of the 3.6 Kb from the start site of transcription in the proα1(I) collagen gene, no AP-1 nor modified AP-1 sites were identified in this 5′ flanking region adjacent to the GRE.

The ability of dexamethasone to decrease the binding of glucocorticoid receptor to the GRE in FRS fibroblasts at 48 h after dexamethasone treatment is compatible with findings in the literature which indicate a down regulation of the glucocorticoid receptor in corticosteroid treated cells and tissues. Oikarinen et al. [1987] demonstrated a down regulation of radiolabeled corticosteroid binding to glucocorticoid receptors in dexamethasone-treated human skin fibroblasts. Glucocortoids have also been shown to decrease collagen synthesis [Russell et al., 1978; Ponec et al., 1979] and type I procollagen mRNAs [Oikarinen et al., 1983; Russell et al., 1989] in human skin fibroblasts. Glucocorticoid treatment has been reported to cause a decrease in glucocorticoid receptor concentration in many tissues and cell lines while adrenalectomy causes an up regulation of glucocorticoid receptor concentration [Okret et al., 1991; Burnstein and Cidlowski, 1992].

In our present studies we have identified a non-GRE containing DNA element, the TGF-β element as functional in the dexamethasone-mediated down regulation of proα1(I) collagen gene expression. We propose that the glucocorticoid-mediated regulation of the proα1(I) collagen gene is directed by DNA binding proteins

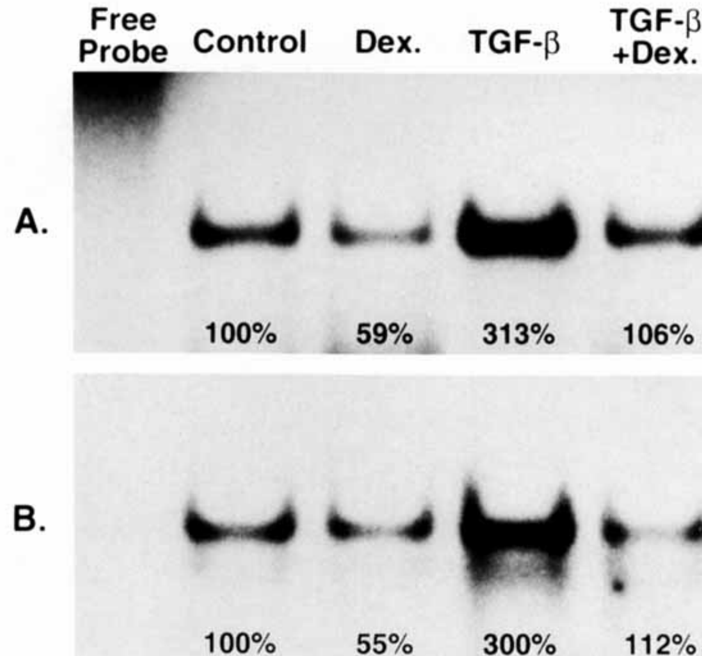

Fig. 11. Effect of dexamethasone, TGF-β, and dexamethasone plus TGF-β on nuclear protein bound to the TGF-β activating element. FRS fibroblasts were treated with either 10^{-6} M dexamethasone, 5.0 ng/ml TGF-β1, or 10^{-6} M dexamethasone plus 5.0 ng/ml TGF-β1 for 48 h. ^{32}P-end labeled double-stranded oligonucleotide (8×10^5–1×10^6 cpm/pm) containing the TGF-β element sequence (5'-TGC CCACG GCCAG) 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 total volume of 20 μl. 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 for 4 h at 100 V. Gels were air-dried and exposed to X-ray film. The X-ray film was scanned and the areas under the peaks were quantitated using a Shimadzu dual-wavelength FLC scanner, model CS-930 (Shimadzu Corp., Kyoto, Japan). **A** and **B** represent two different experiments.

acting through both the GRE and TGF-β regulatory elements. Ritzenthaler et al. [1993] demonstrated that TGF-β treatment of lung fibroblasts transfected with the ColCat 3.6 plasmid increased CAT activity with a coordinated increase of TGF-β activator protein bound to the TGF-β element. The possibility exists that this regulation involves a secondary effect on the synthesis, degradation, and/or modification of these DNA binding proteins. This remains as an unknown in the molecular mechanism of the glucocorticoid-mediated down regulation of proα1(I) collagen gene expression. We postulate that the involvement of the TGF-β response element as well as the GRE in the regulation of the proα1(I) collagen gene elucidates a novel mechanism of glucocorticoid regulation of eukaryotic genes. This finding requires computer sequence analysis for TGF-β elements in these genes. In addition, one should determine the ability of glucocorticoids to decrease the secretion of TGF-β in the glucocorticoid responsive cells.

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REFERENCES

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

Boast S, Su M-W, Ramirez F, Sanchez M, Avvedimento EV (1990): Functional analysis of *cis*-acting DNA sequences controlling transcription of the human type I collagen genes. J Biol Chem 265:13351–13356.

Burnstein KL, Cidlowski JA (1992): At the cutting edge: The down side of glucocorticoid receptor regulation. Mol Cell Endocrinol 83:C1–C8.

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

Cockayne D, Cutroneo KR (1988): Glucocorticoid coordinate regulation of type I procollagen gene expression and procollagen DNA-binding proteins in chick skin fibroblasts. Biochemistry 27:2736–2745.

Cockayne D, Sterling KM Jr, Shull S, Mintz KP, Illeyne S, Cutroneo KR (1986): Glucocorticoids decrease the synthesis of type I procollagen mRNAs. Biochemistry 25:3202–3209.

Cutroneo KR, Sterling KM, Shull S (1986): Steroid hormone regulation of extracellular matrix proteins. In Mechan RP (ed): "Regulation of Matrix Accumulation," Biology of Extracellular Matrix: A Series, Vol 1. New York: Academic Press pp 119–176.

de Crombrugghe B, Vuorio T, Karsenty G, Maity S, Cristy-Rutheshouser E, Goldberg H (1991): Transcriptional control mechanisms for the expression of type I collagen genes. Ann Rheum Dis 50:872–876.

Diamond MI, Miner JN, Yoshinaga SK, Yamamoto KR (1990): Transcription factor interactions: Selectors of positive or negative regulation from a single DNA element. Science 249:1266–1272.

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

Hamalainen L, Oikarinen J, Kivirikko KI (1985): Synthesis and degradation of type I procollagen mRNAs in cultured human skin fibroblasts and the effect of cortisol. J Biol Chem 260:720–725.

Jimenez SA, Varga J, Olsen A, Li L, Diaz A, Herhal J, Koch J (1994): Functional analysis of human α1(I) procollagen gene promoter. J Biol Chem 269(17):12684–12691.

Karsenty G, de Crombrugghe B (1991): Conservation of binding sites for regulatory factors in the coordinately expressed α1(I) and α2(I) collagen promoters. Biochem Biophys Res Commun 177:538–544.

Karsenty G, de Crombrugghe B (1990): Two different negative and one positive regulatory factors interact with a short promoter segment of the α1(I) collagen gene. J Biol Chem 265:9934–9942.

Kelley J, Shull S, Walsh JJ, Cutroneo KR, Absher M (1993): Auto-induction of transforming growth factor-β in human lung fibroblasts. Am J Respir Cell Mol Biol 8:417–424.

Lichtler A, Stover ML, Angilly J, Kream B, Rowe DW (1989): Isolation and characterization of the rat α(1) collagen promoter. J Biol Chem 264:3072–3077.

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

Nelson RM, Long GL (1989): A general method of site-specific mutagenesis using a modification of the *thermus aquaticus* polymerase chain reaction. Anal Biochem 180: 147–151.

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

Newman RA, Cutroneo KR (1978): Glucocorticoids selectively decrease the synthesis of hydroxylated collagen peptides. Mol Pharmacol 14:185–198.

Oikarinen A, Oikarinen H, Meeker CA, Tan EML, Uitto J (1987): Glucocorticoid receptors in cultured human skin fibroblasts: Evidence for down-regulation of receptor by glucocorticoid hormone. Acta Derm Venereol 67:461–468.

Oikarinen J, Pihlajaniemi T, Hamalainen L, Kivirikko KI (1983): Cortisol decreases the cellular concentration of translatable procollagen mRNA species in cultured human skin fibroblasts. Biochem Biophys Acta 741:297–302.

Okret S, Dong Y, Brönnegård M, Gustafsson JÅ (1991): Regulation of glucocorticoid receptor expression. Biochimie 73:51–59.

Pavlin P, Lichtler AC, Dedalov A, Kream BE, Harrison JR, Thomas HF, Gronowicz GA, Clark SH, Woody CO, Rowe DW (1992): Differential utilization of regulatory domains within the α1(I) collagen promoter in osseous and fibroblastic cells. J Cell Biol 116:227–236.

Perez JR, Shull S, Gendimenico GJ, Capetola RJ, Mezick JA, Cutroneo KR (1992): Glucocorticoid and retinoid regulation of α-2 type I procollagen promoter activity. J Cell Biochem 50:26–34.

Ponec M, Kempenaar JA, van der Meulen-van Harskamp GA, Bachra B (1979): Effects of glucocorticosteroids on cultured human skin fibroblasts—IV. Biochem Pharmacol 28:2777–2783.

Raghow R, Gossage D, Kang AH (1986): Pretranslational regulation of type I collagen, fibronectin, and a 50-kilodalton noncollagenous extracellular protein by dexamethasone in rat fibroblasts. J Biol Chem 261:4677–4684.

Ravazzolo R, Karsenty G, de Crombrugghe B (1991): A fibroblast-specific factor binds to an upstream negative control element in the promoter of the mouse α1(I) collagen gene. J Biol Chem 266:7382–7387.

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

Ritzenhaler JD, Goldstein RH, Fine A, Smith BD (1993): Regulation of the α1(I) collagen promoter via a transforming growth factor-β activation element. J Biol Chem 268: 13625–13631.

Rokowski RJ, Sheehy J, Cutroneo KR (1981): Glucocorticoid-mediated selective reduction of functioning collagen messenger ribonucleic acid. Arch Biochem Biophys 210:74–81.

Russell JD, Russell SB, Trupin KM (1978): Differential effects of hydrocortisone on both growth and collagen metabolism of human fibroblasts from normal and keloid tissue. J Cell Physiol 97:221–230.

Russell SB, Trupin JS, Myers JC, Broquist AH, Smith JC, Myles ME, Russell JD (1989): Differential glucocorticoid regulation of collagen mRNAs in human dermal fibroblasts. J Biol Chem 264:13730–13735.

Shull S, Meisler N, Absher M, Phan S, Cutroneo KR (1995): Glucocorticoid-induced down regulation of transforming growth factor-β1 in adult rat lung fibroblasts. Lung 173: 71–78.

Slack JL, Liska DJ, Bornstein P (1991): An upstream regulatory region mediates high-level, tissue-specific expression of the human α1(I) collagen gene in transgenic mice. Mol Cell Biol 11:2066–2074.

Southern PJ, Berg P (1982): 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.

Sterling KM Jr, Harris MJ, Mitchell JJ, Cutroneo KR (1983a): Bleomycin treatment of chick fibroblasts causes an increase of polysomal type I procollagen mRNAs. J Biol Chem 258:14438–14444.

Sterling KM Jr, Harris MJ, Mitchell JJ, DePetrillo TA, Delaney G, Cutroneo KR (1983b): Dexamethasone decreases the amounts of type I procollagen mRNAs in vivo and in fibroblast cell cultures. J Biol Chem 258:7644–7647.

Walsh MJ, Leleiko NS, Sterling KM Jr (1987): Regulation of types I, III, and IV procollagen mRNA synthesis in glucocorticoid-mediated intestinal development. J Biol Chem 262:10814–10818.

Weiner FR, Cazja MJ, Jefferson DM, Giamvrone M-A, Tur-Kaspa R, Reid LM, Zern MA (1987): The effects of dexamethasone on in vitro collagen gene expression. J Biol Chem 262:6955–6958.