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Glucocorticoid Coordinate Regulation of Type I Procollagen Gene Expression and Procollagen DNA-Binding Proteins in Chick Skin Fibroblasts†

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ABSTRACT: Nuclei were isolated from control and dexamethasone-treated (2 h) embryonic chick skin fibroblasts and transcribed in vitro. Nuclei isolated from dexamethasone-treated fibroblasts transcribed less proα1(I) and proα2(I) mRNAs but not β-actin mRNA. Fibroblasts receiving dexamethasone and [5,6-^{3}H]uridine also demonstrated decreased synthesis of nuclear type I procollagen mRNAs but not β-actin mRNA. In fibroblasts treated with cycloheximide the newly synthesized nuclear type I procollagen mRNA species were markedly decreased. An enhanced inhibitory effect was observed when fibroblasts were treated with cycloheximide plus dexamethasone. Since the studies above demonstrate that active protein synthesis is required to maintain the constitutive expression of the type I procollagen genes, we determined if glucocorticoids regulate DNA-binding proteins with sequence specificity for the α2(I) procollagen gene. Nuclear protein blots were probed with the ^{32}P-end-labeled pBR322 vector DNA and ^{32}P-end-labeled α2(I) procollagen promoter containing DNA. Nonhistone proteins remained bound to labeled DNA at stringency washes of 0.05 and 0.1 M NaCl. As the ionic strength was increased to 0.2 and 0.3 M NaCl, the nonhistone-protein DNA binding was preferentially lost. Only the low molecular weight proteins remained bound to labeled DNA at the highest ionic strength, indicating nonspecific binding of these nuclear proteins. Dexamethasone treatment resulted in an increase of binding of nonhistone proteins to vector- and promoter-labeled DNAs over that observed in control fibroblasts at stringency washes of 0.05 and 0.1 M NaCl and to a lesser extent at 0.2 M NaCl. The binding specificities of nonhistone proteins for the α2(I) procollagen promoter containing DNA were calculated. Three nonhistone DNA-binding proteins of M_r 90 000, 50 000, and 30 000 had altered specificities following dexamethasone treatment.

Corticosteroids have an antianabolic effect on procollagen metabolism by selectively decreasing procollagen synthesis in vivo and in fibroblast cell cultures [for review see Cutroneo et al. (1986)]. The inhibitory effect of glucocorticoids on procollagen synthesis is associated with a decrease in total cellular type I procollagen mRNAs in vivo and in fibroblasts [for review see Cutroneo et al. (1986)]. In a previous study we demonstrated that dexamethasone decreased the total cellular, nuclear, cytoplasmic, and polysomal steady-state levels of proα1(I) and proα2(I) mRNAs in embryonic chick skin fibroblasts (Cockayne et al., 1986). Dexamethasone treatment of these fibroblasts also decreased the synthesis of total nuclear type I procollagen mRNAs. The molecular mechanism(s) by which the steady-state levels and synthesis of type I procollagen mRNAs are decreased in fibroblasts is (are) not completely understood. Corticosteroids down regulate type I procollagen gene expression.

The selective expression of messenger RNA in eucaryotic cells results largely from the regulation of the rate of transcription of structural gene sequences. Although the mecha-

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nism(s) of transcriptional regulation is (are) as yet not fully understood, the interaction of nuclear proteins with specific DNA sequences in the regulatory regions of genes appears to be a key step in this process. The interaction of regulatory regions such as promoters and enhancers with sequence-specific DNA-binding proteins has been demonstrated in numerous eucaryotic systems (Jack et al., 1982; Lassar et al., 1983; Miskimins et al., 1985; Wu, 1985; Cote et al., 1985; Singh et al., 1986; Shimada et al., 1986; Keegan et al., 1986; Hatamochi et al., 1986; Hill et al., 1986). This highly specific mode of gene regulation enables the cell to activate, repress, or constitutively express specialized genes. In addition, such mechanisms can also coordinately regulate genes located on different chromosomes provided they contain similar regulatory DNA sequences that are recognized by a common sequence specific protein(s). The dependence of the constitutive level of type I procollagen mRNA synthesis on active protein synthesis demonstrated in the present study suggests the involvement of nuclear proteins in procollagen gene expression.

We have used a protein blotting technique to identify sequence-specific DNA-binding proteins to a promoter-containing fragment of the α2(I) procollagen gene. This technique was first described by Bowen et al. (1980) and has been used to identify sequence-specific DNA-binding proteins to the promoter region of a drosophila heat shock gene (Jack et al., 1982). Cote et al. (1985) have recently improved this technique by using end-labeled gene probes for identifying the α-fetoprotein gene DNA-binding proteins in rat liver and Morris hepatoma 7777. In the present studies nuclear extracts from control and dexamethasone-treated embryonic chick skin fibroblasts were fractionated on sodium dodecyl sulfate–polyacrylamide gels, and DNA-binding proteins were identified by using a 400-bp fragment of chicken genomic α2(I) procollagen DNA containing promoter-specific sequences.

We have demonstrated by pulse-labeling fibroblasts with [^{3}H]uridine and transcription of isolated nuclei in vitro that corticosteroids decrease the steady-state levels of type I procollagen mRNAs by regulating gene expression. This decrease of type I procollagen gene transcription is coordinate with glucocorticoid-mediated regulation of specific nuclear DNA-binding proteins. Corticosteroid regulation of type I procollagen gene expression may involve the interaction of these sequence-specific nuclear proteins and regulatory regions of the type I procollagen α2 genes.

Experimental Procedures

Egg Maintenance. Fertilized white leghorn chick eggs were obtained from Oliver, Merrill and Sons, Londonderry, NH, and were maintained as previously described (Sterling et al., 1983). Primary embryonic chick skin fibroblasts were prepared from 12-day-old embryos as described (Hunter, 1979). Cells were cultured and maintained for up to 5 days with the daily addition of 2×10^{-4} M ascorbate (Sterling et al., 1983).

Recombinant DNA. The recombinant plasmids pCg54 and pCg45, containing cDNAs to the chicken proα1(I) and proα2(I) mRNAs, respectively, were obtained from Dr. Helga Boedtker, Department of Biochemistry and Molecular Biology, Harvard University, Boston, MA. These recombinant plasmids have been previously characterized (Lehrach et al., 1978, 1979). The recombinant plasmid pA1 contains a chicken β-actin cDNA and was obtained from Dr. Don W. Cleveland, Department of Biochemistry and Biophysics, University of California, San Francisco, CA. The recombinant genomic plasmid pCα2PRO-3 was obtained from Dr. Benoit deCrombrugghe, Genetics Department, MD Anderson Hospital and Tumor Institute, Houston, TX (Merlino et al., 1981). Bacteria were amplified and the plasmids were isolated as described (Maniatis et al., 1982). Host contamination of the recombinant plasmids was monitored by restriction enzyme digestion and agarose gel electrophoresis.

In Vitro Transcription and Isolation of RNA. Nuclei were isolated from late log phase embryonic chick skin fibroblasts by a modification of the procedures of Mulvihill and Palmiter (1977) and McKnight et al. (1980). Nuclei were released from cells by vortexing in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 2.5 mM $MgAc_2$, 0.5 mM dithiothreitol (DDT), 0.25% (v/v) Triton X-100, and 2.4 M sucrose. The nuclei were recovered by centrifugation at 110000g for 60 min at 4 °C. The absence of cytoplasmic contamination was determined by light microscopy. The nuclear pellet was suspended in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 8.0), 5 mM $MgAc_2$, 40% (v/v) glycerol, and 0.5 mM DTT at a concentration of 1×10^6 nuclei/mL and used immediately for in vitro transcription assays. The in vitro transcription assay contained 1×10^4 nuclei in a 100-μL reaction mixture with 20 mM Hepes (pH 7.9), 20% (v/v) glycerol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 4 mM $MgCl_2$, 3 mM $MnCl_2$, 150 mM KCl, 4 mM DTT, 0.4 mM ATP, CTP, and GTP, 0.5 unit/mL RNAsin (Promega Biotec, Madison, WI), and 100 μCi of [α-^{32}P]UTP (600 Ci/mM, ICN, Irvine, CA). The reaction was incubated at 20 °C for 30 min. Aliquots of the transcription assay mixture were spotted onto Whatman 3MM filters, and the Cl_3CCOOH-precipitable counts were determined. Purification of the RNA products for hybridization analysis was carried out essentially as described by McKnight and Palmiter (1979). Fifty micrograms of tRNA was used as carrier RNA during the RNA isolation. Specific radioactivities were determined for all purified radiolabeled transcripts before hybridization.

Whole Cell RNA Synthesis and Isolation of Nuclear RNA. Late log phase embryonic chick skin fibroblasts were pretreated in either the presence or absence of 1×10^{-6} M cycloheximide. Fresh media was added to all cultures, and the cells were incubated with 125 μCi/mL of [[5,6-^{3}H]uridine (39 Ci/mmol), ICN, Irvine, CA] in the presence of 2.5×10^{-5} M dexamethasone, 1×10^{-6} M cycloheximide, or both. Total nuclear RNA was isolated by a modification of the method of Roop et al. (1978) as described by Cockayne et al. (1986) for hybridization analysis.

Hybridization to Immobilized cDNAs on Nitrocellulose Filters. Four micrograms of linearized plasmids containing cDNA inserts were bound to nitrocellulose and hybridized with 5,6-^{3}H-labeled RNA or α-^{32}P-labeled RNA as described by Lyons and Schwarz (1984). For determining nonspecific hybridization 4 μg of linearized pBR322 DNA or 4 μg of sheared *Escherichia coli* DNA was simultaneously hybridized to radiolabeled RNA. To measure the hybridization efficiency of our assay [^{3}H]poly(A+) β-globin mRNA was hybridized to 4 μg of a linearized plasmid containing a β-globin cDNA insert. Nonspecific hybridization of radiolabeled β-globin mRNA was determined by using linearized pBR322 DNA. Quantification of radioactive hybrids was determined by liquid scintillation spectroscopy of the hybridized nitrocellulose filters. This method of hybridization using nitrocellulose for quantitation of labeled RNA was characterized. All hybridizations of RNA were carried out in DNA excess. Second, we demonstrated a linear relationship of radioactive RNA binding when different amounts of the RNA were hybridized. Finally, when the efficiency of hybridization was determined by using radioactive β-globin mRNA and β-globin cDNA, the hy-

bridization efficiency was always approximately 38%.

Preparation of Nuclear Extracts. Nuclei were isolated as previously described except with the addition of 3 mM phenylmethanesulfonyl fluoride (PMSF) in the homogenizing buffer. The nuclear pellet was washed once and resuspended at $100A_{260}$/mL in DNase I digestion buffer [10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 10% (v/v) glycerol, 0.5 mM DTT, 0.5 mM PMSF] as described by Cote et al. (1985). DNA was digested by the addition of 50 μg/mL Macaloid-treated DNase I (Sigma, St. Louis, MO). The sample was incubated on ice for 2 h. Following DNase I digestion the nuclear extract was made 63 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) glycerol, and 5% (v/v) β-mercaptoethanol. Total nuclear protein was determined by the BCA assay (Pierce Chemical Co., Rockford, IL).

Gel Electrophoresis of Nuclear Extract and Transfer to Nitrocellulose. SDS–polyacrylamide gel electrophoresis (PAGE) was performed according to the procedure of Laemmli (1970). Chick skin fibroblast nuclear extract was applied to a 3% (w/v) stacking gel using a 12-cm preparative gel comb. We routinely electrophoresed 0.5 mg of nuclear extract in one 12-cm preparative well. The nuclear proteins were separated on a 5–15% (w/v) gradient gel at constant voltage. Bromophenol blue (0.05%) (w/v) was used as a tracking dye, and BRL prestained high molecular weight markers (BRL, Gaithersburg, MD) were used to estimate molecular weight. Coomassie brilliant blue (J. T. Baker Chemical Co., Phillipsburg, NJ) was used to stain the fractionated nuclear proteins.

Following PAGE electrophoresis, the nuclear proteins were transferred to nitrocellulose paper according to the procedure of Towbin et al. (1979). Transfer was routinely carried out in 24 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% (v/v) methanol for 250 V h in a trans-blot cell apparatus (Bio-Rad, Rockville Centre, NY).

DNA-Binding Assay. The DNA-binding assay for nuclear proteins was carried out essentially as described by Cote et al. (1985). Following electrophoretic transfer, the nitrocellulose was sliced into uniform 1-cm strips and a representative section stained with 0.1% (w/v) amido black (Eastman Kodak Co., Rochester, NY), 2% (v/v) acetic acid, and 43% (v/v) methanol to determine the efficiency of transfer. This approach eliminated the possibility of variation in band intensities that could result from slight differences in electrophoretic profiles and transfer efficiencies. Nitrocellulose strips were assayed for DNA binding in a chambered hybridization tray. Preincubation was carried out in 3 mL of binding buffer [10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, and 5× Denhardt's reagent [(0.1% (w/v) Ficoll, 0.1% (w/v) bovine serum albumin, 0.1% (w/v) poly(vinylpyrrolidone)]] for 1 h at room temperature. Following preincubation the buffer was removed, and fresh binding buffer was added along with a 500-fold excess (150 μg) of sheared *E. coli* DNA (United States Biochemical Corp., Cleveland, OH) and ^{32}P-end-labeled DNA (300 ng) containing pBR322 vector labeled DNA fragments and α2(I) procollagen promoter containing DNA labeled fragments to a final volume of 3 mL.

pBR322 plasmid pCα2PRO-3 was end-labeled essentially as described (Maniatis et al., 1982). Procollagen DNA-binding proteins were identified by probing nitrocellulose blots with *Hin*dIII-digested end-labeled plasmid pCα2PRO-3. [^{32}P]TTP (3000 Ci/mM, ICN Pharmaceuticals, Inc., Irvine, CA) was added to *Hin*dIII-digested pCα2PRO-3 in the presence of the Klenow fragment of DNA polymerase I (BRL, Gaithersburg, MD) with excess unlabeled dATP, dCTP, and dGTP. The reaction was incubated from 10 min at room temperature and the total labeled DNA including ^{32}P-end-labeled α2(I) procollagen promoter containing DNA fragments and ^{32}P-end-labeled pBR322 vector DNA fragments were purified by using the spun minicolumn procedure (Maniatis et al., 1982). The specific activity of DNA labeled by this technique was greater than or equal to 3×10^6 cpm/μg. Total labeled DNA was allowed to bind to nitrocellulose filters at a concentration of 100 ng/mL for 1 h at room temperature. Following DNA binding the filters were washed 4× in buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1× Denhardt's reagent, and NaCl. DNA binding was detected by autoradiography using Kodak XAR X-ray film.

Elution of DNA from Nitrocellulose and Analysis of the Bound [^{32}P]DNA Fragments. Following autoradiography the radiolabeled bands were localized to their respective positions on the nitrocellulose filters, and the bound [^{32}P]DNA fragments were analyzed as described by Cote et al. (1985). Bands were excised from the nitrocellulose strips and gently agitated for 1 h in 0.75 mL of 0.1% (w/v) SDS and 1 mM EDTA. The filters were removed, and the radiolabeled DNA was lyophilized to dryness. The DNA samples were reconstituted in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA and electrophoresed on 1% (w/v) agarose gels by using 7% (w/v) sucrose and 0.05% (w/v) bromophenol blue as tracking dye. The gels were air-dried and autoradiographed. The elution of DNA from the nitrocellulose strips was carried out for a time at which no more radiolabeled DNA could be eluted. The amount of DNA eluted did not vary with the DNA fragments since the specificity ratios of certain nuclear proteins differed.

Preparation of Standard Curves and Quantification of Autoradiograms by Densitometric Scanning. The electrophoretically separated [^{32}P]DNA fragments were autoradiographed, and the band intensities of the 4.3-kb pBR322 DNA fragment and the 0.4-kb α2(I) procollagen promoter containing DNA fragment were quantitated by densitometric scanning of the area under the peaks using a Shimadzu dual-wavelength TLC scanner, Model CS-930 (Shimadzu Corp., Kyoto, Japan). Standard curves were generated for each DNA-binding assay in order to determine the limits of linearity between cpm of [^{32}P]DNA bound to nitrocellulose and the densitometric units obtained by scanning the area under the peaks. Standard curves were prepared by spotting serial dilutions of ^{32}P-end-labeled DNA onto nitrocellulose and autoradiographing these filters alongside the corresponding DNA-binding assay filters. The cpm spotted onto the nitrocellulose were plotted as a function of the densitometric units obtained when the area under the peaks was determined for each serial dilution. In the linear range of the standard curves, arbitrary densitometric units were a quantitative measure of the cpm of radiolabeled DNA fragment bound to nitrocellulose.

The binding specificity of each radiolabeled protein band was determined by using the area under the peak obtained by scanning the respective vector- and promoter-containing DNA bands. Binding specificities were calculated as follows, and the resulting values are referred to as specificity ratios.

$$\text{specificity ratio} = \frac{\text{band intensity (area under peak) pBR322 DNA fragment}}{\text{band intensity (area under peak) promoter DNA fragment}}$$

On the basis of this analysis, the smaller the specificity ratio, the greater the binding specificity for the α2(I) procollagen promoter containing DNA fragment. As the specificity ratio approaches (or exceeds) a value of 1.0, little or no preference

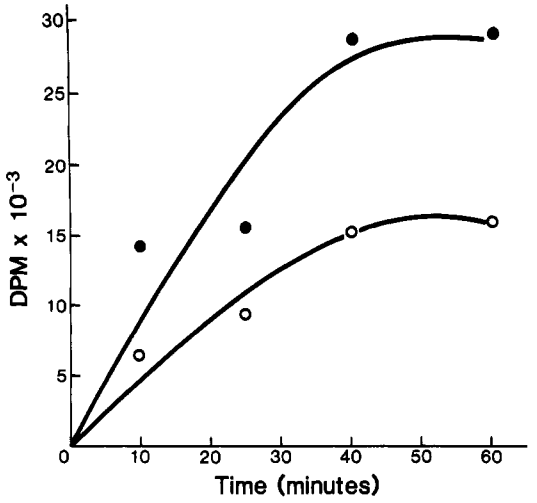

FIGURE 1: Effect of α-amanitin on RNA synthesis by nuclei in vitro. Nuclei were isolated from embryonic chick skin fibroblasts. For each time point indicated 1 × 10^4 nuclei were assayed in triplicate. Transcription assays were carried out under standard assay conditions as described under Experimental Procedures except for the presence (○) or absence (●) of α-amanitin (1 μg/mL). Aliquots (10 μL) were withdrawn at the time intervals indicated, and the Cl_3CCOOH-precipitable counts were determined as described under Experimental Procedures.

is demonstrated for binding the α2(I) procollagen promoter containing DNA fragment. There are limitations with calculating the specificity ratio. The vector DNA and the procollagen gene fragment must be equally eluted from nitrocellulose as discussed above. Second, if only minute amounts of vector DNA and the procollagen gene fragment are bound to a nuclear protein, during the elution of DNA and subsequent agarose chromatography enough DNA may be lost so that these DNAs are not detectable by densiometric analysis.

RESULTS

Transcription of Embryonic Chick Skin Fibroblast Nuclei in Vitro. Nuclei were isolated from late log phase embryonic chick skin fibroblasts and transcribed in vitro by using [α-^{32}P]UTP. Nuclei ranging from 5 × 10^3 to 5 × 10^5 were assayed at 20 °C, the temperature at which total nuclear RNA synthesis was optimal (data not shown). Total nuclear RNA synthesis was linearly dependent on the concentration of nuclei. RNA synthesis was linear at 20 °C for at least 30 min of incubation (data of shown). RNA synthesis was optimal at 150 mM KCl, 4 mM $MgCl_2$, and 3 mM $MnCl_2$ (data not shown).

α-Amanitin Sensitivity of Total Nuclear RNA Synthesis in Vitro. To determine if mRNA synthesis occurred in this in vitro nuclear runoff system, nuclei were assayed in the absence and presence of α-amanitin (Figure 1). RNA polymerase II is specifically inhibited by α-amanitin (Roeder et al., 1970). Low concentrations of α-amanitin have been used in numerous eucaryotic systems to demonstrate the dependence of mRNA synthesis on RNA polymerase II activity (Wu, 1978; Marzluff et al., 1973; Gross & Ringler, 1979). When nuclei were isolated from late log phase embryonic chick skin fibroblasts and transcribed in the presence of 1 μg/mL α-amanitin, incorporation of [α-^{32}P]UTP into total nuclear RNA was significantly inhibited.

Total RNA Synthesis by Nuclei Isolated from Dexamethasone-Treated Embryonic Chick Skin Fibroblasts. To determine the effect of dexamethasone on [α-^{32}P]UTP incorporation into total nuclear RNA in vitro, late log phase embryonic chick skin fibroblasts were treated with 2.5 × 10^{-5} M dexamethasone. Nuclei were isolated and transcribed in vitro. Nuclei derived from dexamethasone-treated cultures incorporated radioactivity into Cl_3CCOOH-precipitable RNA to a slightly decreased though nonsignificant extent as compared to nuclei isolated from control cultures (Figure 2).

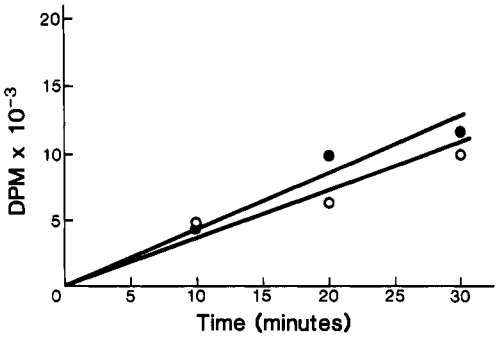

FIGURE 2: In vitro synthesis of total nuclear RNA transcripts by nuclei isolated from control and dexamethasone-treated embryonic chick skin fibroblasts. Late log phase embryonic chick skin fibroblasts were treated either in the presence (○) or absence (●) of 2.5 × 10^{-5} M dexamethasone for 2 h. Nuclei were isolated, and 1 × 10^4 nuclei were assayed in triplicate under standard assay conditions. Aliquots (10 μL) were withdrawn at the time intervals indicated, and the Cl_3CCOOH-precipitable counts were determined as described under Experimental Procedures.

Glucocorticoid Regulation of Type I Procollagen Gene Expression. Previous studies in our laboratory demonstrated that treatment of embryonic chick skin fibroblasts with dexamethasone results in a marked decrease in the incorporation of [5,6-^{3}H]uridine into nuclear proα1(I) and proα2(I) mRNAs (Cockayne et al., 1986). To determine if this decrease in type I procollagen mRNA synthesis results from a decrease in procollagen gene expression, nuclei were isolated from control and dexamethasone-treated embryonic chick skin fibroblasts and transcribed in vitro. Proα1(I)-, proα2(I)-, and β-actin-specific mRNA sequences were quantified by isolating [α-^{32}P]RNA and hybridizing 1.5 × 10^5 cpm of radiolabeled RNA transcripts to linearized nitrocellulose-bound recombinant plasmids. The dpm hybridized to the proα1(I), proα2(I), and β-actin cDNA probes were corrected for background hybridization to pBR322 DNA and for hybridization efficiency. In two separate experiments the transcription of proα1(I) and proα2(I) mRNAs directed by nuclei isolated from dexamethasone-treated fibroblasts was significantly decreased (Table I). In contrast, the transcription of β-actin mRNA sequences was not altered.

In a separate series of in vitro transcription experiments, proα1(I)-, proα2(I)-, and β-actin-specific mRNA sequences were quantified by hybridizing 15 μg of isolated RNA containing radioactive transcripts and carrier tRNA to linearized nitrocellulose-bound recombinant plasmids (Figure 3). The dpm hybridized were corrected for background hybridization to *E. coli* DNA, and the data were normalized to dpm hybridized/μg of RNA. As seen in Figure 3, dexamethasone significantly decreased the transcription of proα1(I) and proα2(I) mRNAs by 42% and 50%, respectively, while the transcription of β-actin mRNA remained unaffected. This alternative hybridization strategy produced results similar to those presented in Table I.

Effect of Cycloheximide Pretreatment on the Dexamethasone-Mediated Decrease of Nuclear Type I Procollagen mRNA Synthesis. To gain a more complete understanding of the molecular mechanism(s) by which glucocorticoids decrease type I procollagen gene expression in primary embryonic chick skin fibroblasts, cells were pulse-labeled with [5,6-^{3}H]uridine in the presence or absence of dexamethasone. Total nuclear RNA was isolated, and 15 μg of RNA containing the

Table I: Transcription of Proα1(I), Proα2(I), and β-Actin mRNAs Expressed as dpm Hybridized in Nuclei Isolated from Control and Dexamethasone-Treated Embryonic Chick Skin Fibroblasts[a]

	total input [^{32}P]RNA (dpm)	transcripts hybridized (dpm)		
		proα1(I)	proα2(I)	β-actin
expt I				
control	1.5×10^5	1008	374	366
dexamethasone	1.5×10^5	489	226	374
decrease (%)		51	40	0
expt II				
control	1.5×10^5	1021	724	413
dexamethasone	1.5×10^5	674	297	416
decrease (%)		34	59	0

[a] Late log phase embryonic chick skin fibroblasts were treated in the presence or absence of 2.5×10^{-5} M dexamethasone for 2 h. Nuclei were isolated from control and glucocorticoid-treated chick skin fibroblasts, and 1×10^4 nuclei were transcribed in vitro as described under Experimental Procedures. Purified radiolabeled RNA transcripts (1.5×10^5 cpm) were hybridized to 4 μg of the linearized recombinant plasmids pCg54, pCg45, and pA1 under conditions of DNA excess. For determining nonspecific background, 4 μg of pBR322 DNA was simultaneously hybridized to the same amount of radiolabeled RNA transcripts. Transcripts hybridized were calculated by subtracting the nonspecific hybridization to pBR322 DNA (42.0 dpm). The values were corrected for the respective length of the cDNA probes and hybridization efficiency. The hybridization efficiency of our assay was 38% as determined by using ^{3}H-labeled β-globin mRNA and a plasmid containing a β-globin cDNA insert.

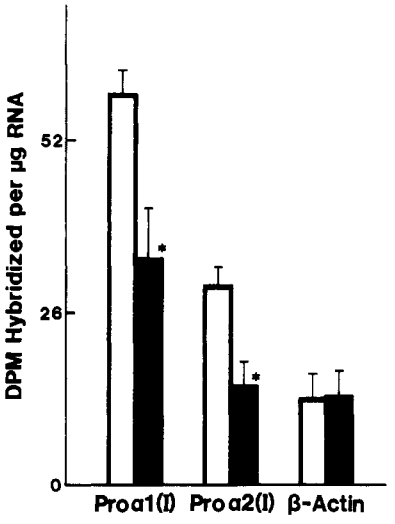

FIGURE 3: Transcription of proα1(I), proα2(I), and β-actin mRNAs expressed as dpm hybridized per microgram of RNA isolated from control and dexamethasone-treated embryonic chick skin fibroblast nuclei. Late log phase embryonic chick skin fibroblasts were treated in the presence (shaded bars) or absence (open bars) of 2.5×10^{-5} M dexamethasone for 2 h. Nuclei were isolated from control and glucocorticoid-treated skin fibroblasts, and 1×10^4 nuclei were transcribed in vitro as described under Experimental Procedures. Total RNA was isolated as described in the text, and 15 μg of this RNA containing the radiolabeled RNA transcripts was hybridized to 4 μg of the linearized recombinant plasmids pCg54, pCg45, and pA1 under conditions of DNA excess. For determining nonspecific background, 4 μg of *E. coli* DNA was simultaneously hybridized to 15 μg of RNA. Nonspecific hybridization (60.0 dpm) to *E. coli* DNA was subtracted from the dpm hybridized to 15 μg of RNA. These values were corrected for the respective lengths of the cDNA probes and the hybridization efficiency. The hybridization efficiency was 38% as determined by using radiolabeled β-globin mRNA and a plasmid containing a β-globin cDNA insert. The corrected dpm hybridized for 15 μg of RNA was normalized to 1 μg of RNA. The data represent the means ± SE of three separate in vitro transcription experiments. (*) Significantly different from control at $P \leq 0.05$.

radioactive transcripts was hybridized to linearized nitrocellulose-bound recombinant plasmids. As seen in Figure 4, dexamethasone significantly decreased type I procollagen

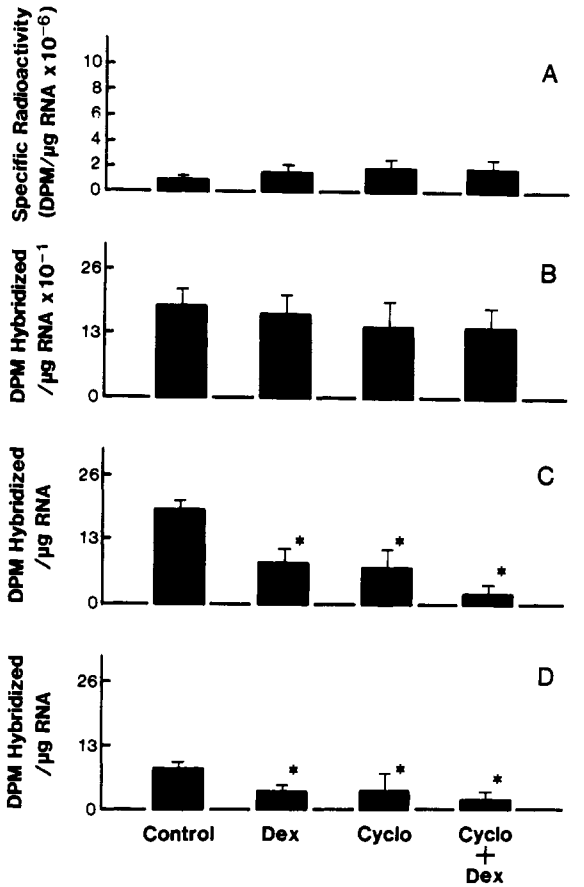

FIGURE 4: Effect of dexamethasone and cycloheximide on the synthesis of proα1(I), proα2(I), and β-actin mRNAs in embryonic chick skin fibroblasts. Late log phase embryonic chick skin fibroblasts were incubated in either the presence or absence of 1×10^{-6} M cycloheximide for 30 min. Following pretreatment with cycloheximide, the culture media was removed, and the skin fibroblasts were labeled with [5,6-^{3}H]uridine for 1 h in the presence or absence of 2.5×10^{-5} M dexamethasone and 1×10^{-6} M cycloheximide as indicated in the figure. Total nuclear RNA was isolated as described under Experimental Procedures. The specific radioactivity of the total nuclear RNA was determined. Type I procollagen and β-actin-specific mRNA sequences were quantified by hybridizing 15 μg of purified nuclear RNA containing the radiolabeled RNA species to 4 μg of the linearized nitrocellulose-bound recombinant plasmids pCg54, pCg45, and pA1 under conditions of DNA excess. For determining nonspecific background 4 μg of *E. coli* DNA was simultaneously hybridized to 15 μg of purified nuclear RNA. Nonspecific hybridization to *E. coli* DNA (37.0 dpm) was subtracted. These values were corrected for the respective lengths of the cDNA probes and hybridization efficiency. The hybridization efficiency was 38% as determined by using ^{3}H-labeled β-globin mRNA and a plasmid containing a β-globin cDNA insert. The corrected dpm hybridized for 15 μg of total nuclear RNA was normalized to 1 μg of RNA, and the data were expressed as dpm hybridized per microgram of RNA. (A) Total nuclear RNA; (B) β-actin mRNA; (C) proα1(I) mRNA; (D) proα2(I) mRNA. Data represent the mean ± SE of three individual experiments. (*) Significantly different from control at $P \leq 0.05$.

mRNA synthesis. Proα1(I) and proα2(I) mRNA sequences were coordinately decreased by 47% and 52%, respectively. In control embryonic chick skin fibroblasts newly synthesized proα1(I) and proα2(I) nuclear mRNA sequences were present in a ratio of 1.8. In dexamethasone-treated cells the ratio was 1.9. Thus, dexamethasone did not alter the ratio of newly synthesized type I procollagen mRNAs.

To investigate the role of active protein synthesis in the glucocorticoid-mediated decrease of nuclear type I procollagen mRNA synthesis, primary embryonic chick skin fibroblasts were pretreated with 10^{-6} M cycloheximide. Protein synthesis was inhibited by 80% at this dose as determined by

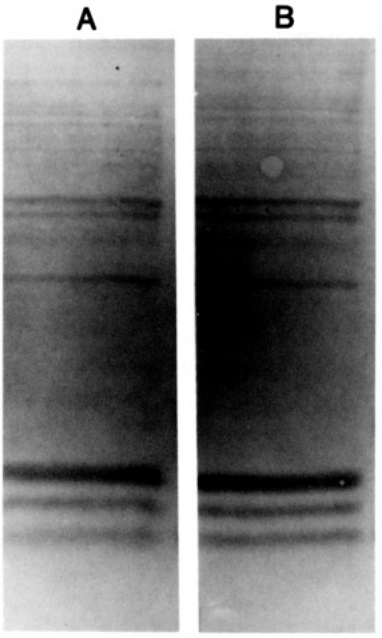

FIGURE 5: Nuclear protein patterns after transfer of control and dexamethasone-treated nuclear extracts to nitrocellulose. After transfer to nitrocellulose of nuclear proteins (0.5 mg) from 5–15% Laemmli gels under reducing conditions, the proteins were stained with amido black. (A) Control nuclear extract; (B) dexamethasone-treated nuclear extract.

Cl_3CCOOH-precipitable counts (data not shown). Following pretreatment with cycloheximide for 30 min, the media was removed, and the cultures were pulse-labeled with [5,6-^{3}H]-uridine in the presence of 10^{-6} M cycloheximide alone or dexamethasone plus cycloheximide. Total nuclear RNA was isolated, and 15 μg of total nuclear RNA was hybridized to linearized nitrocellulose-bound recombinant plasmids. As observed in Figure 4, cycloheximide treatment given alone mimicked the effect of dexamethasone on type I procollagen mRNA synthesis by decreasing nuclear proα1(I) and proα2(I) mRNAs by 58% and 45%, respectively. When embryonic chick skin fibroblast cultures were treated with both dexamethasone and cycloheximide, the synthesis of type I procollagen mRNAs was inhibited to a greater extent than in the presence of either drug alone. In cultures treated with both dexamethasone and cycloheximide, the synthesis of nuclear proα1(I) and proα2(I) mRNA sequences was decreased by 83% and 76%, respectively. Neither dexamethasone nor cycloheximide given alone or together significantly altered β-actin mRNA synthesis or the synthesis of total nuclear RNA. The inability of cycloheximide to significantly decrease total nuclear RNA synthesis and β-actin mRNA synthesis indicates that this inhibitor of protein synthesis does not impair general cellular RNA synthesis.

Effect of Ionic Strength on Nuclear Protein–DNA Binding. Since the constitutive levels of transcription of proα1(I) and proα2(I) mRNAs were dependent on active protein synthesis, a protein blotting technique was employed to detect the binding of nuclear proteins to a α2(I) procollagen promoter containing DNA fragment. Nuclear extracts were isolated from embryonic chick skin fibroblasts and were fractionated by using SDS–PAGE gradient gels and electrophoretically transferred to nitrocellulose paper. Electrophoretic transfer was monitored for equivalent blotting of control and dexamethasone-treated samples. The nuclear protein patterns of control and dexamethasone-treated samples after transfer to nitrocellulose are presented in Figure 5. These data show no differences between control and dexamethasone-treated samples.

The effect of various concentrations of NaCl on the binding of radiolabeled vector DNA and promoter DNA to nuclear proteins obtained from control and dexamethasone-treated embryonic chick skin fibroblasts was assessed (Figure 6). The effect of increasing ionic strength was studied by varying the NaCl concentration in the stringency wash buffer. The binding assays in this experiment were carried out in the presence of a 500-fold excess of *E. coli* DNA. As the ionic strength of the stringency wash buffer was increased, the binding of total labeled DNA to nonhistone proteins was preferentially lost while the low molecular weight proteins (≤27 000) remained bound to total labeled DNA. When the ionic strength of the wash buffer was increased to 0.3 M NaCl, only the low molecular weight protein binding was detected, indicating the nonspecificity of binding of these proteins to DNA.

An interesting observation was the dramatic increase in the binding of nonhistone proteins in nuclear extracts of dexa-

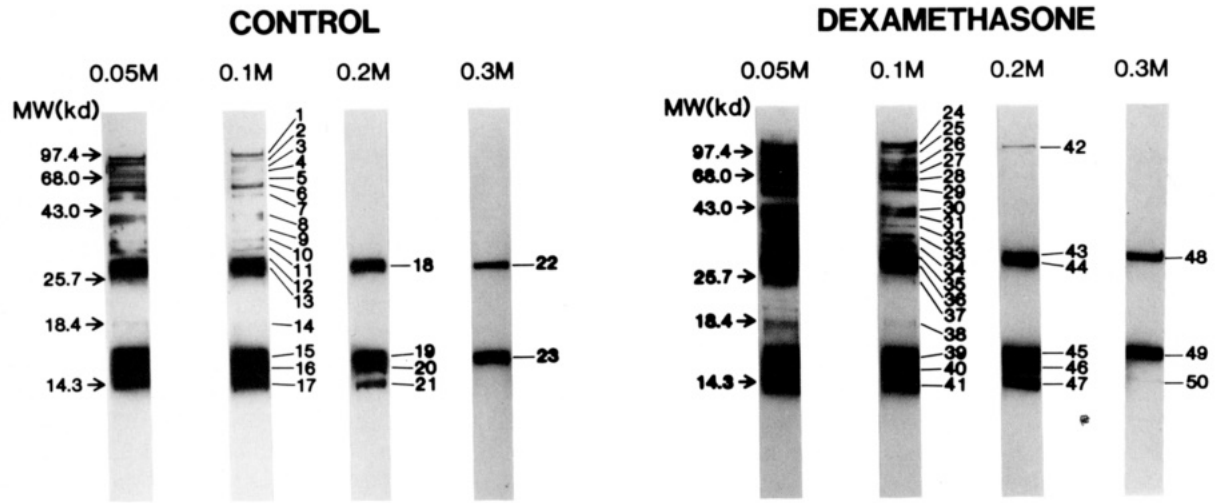

FIGURE 6: Effect of ionic strength on the binding of labeled pBR322 vector DNA fragment and α2(I) procollagen promoter containing DNA fragment to nuclear proteins. Nuclear extracts were prepared from control and dexamethasone-treated (2 h) embryonic chick skin fibroblasts as described under Experimental Procedures. The nuclear extracts were electrophoresed (0.5 mg/12 cm preparative well) on a 5–15% (w/v) SDS–PAGE gradient gel under reducing conditions. The proteins were transferred to nitrocellulose. Nitrocellulose strips were assayed for DNA binding as described under Experimental Procedures. Blots were probed with 100 ng/mL of the ^{32}P-end-labeled α2(I) procollagen promoter containing DNA fragment and ^{32}P-end-labeled pBR322 vector DNA fragment in the presence of a 500-fold excess of unlabeled *E. coli* competitor DNA. Nitrocellulose filters were stringency washed in the presence of 0.05, 0.1, 0.2, or 0.3 M NaCl. Autoradiography was carried out for 10 days.

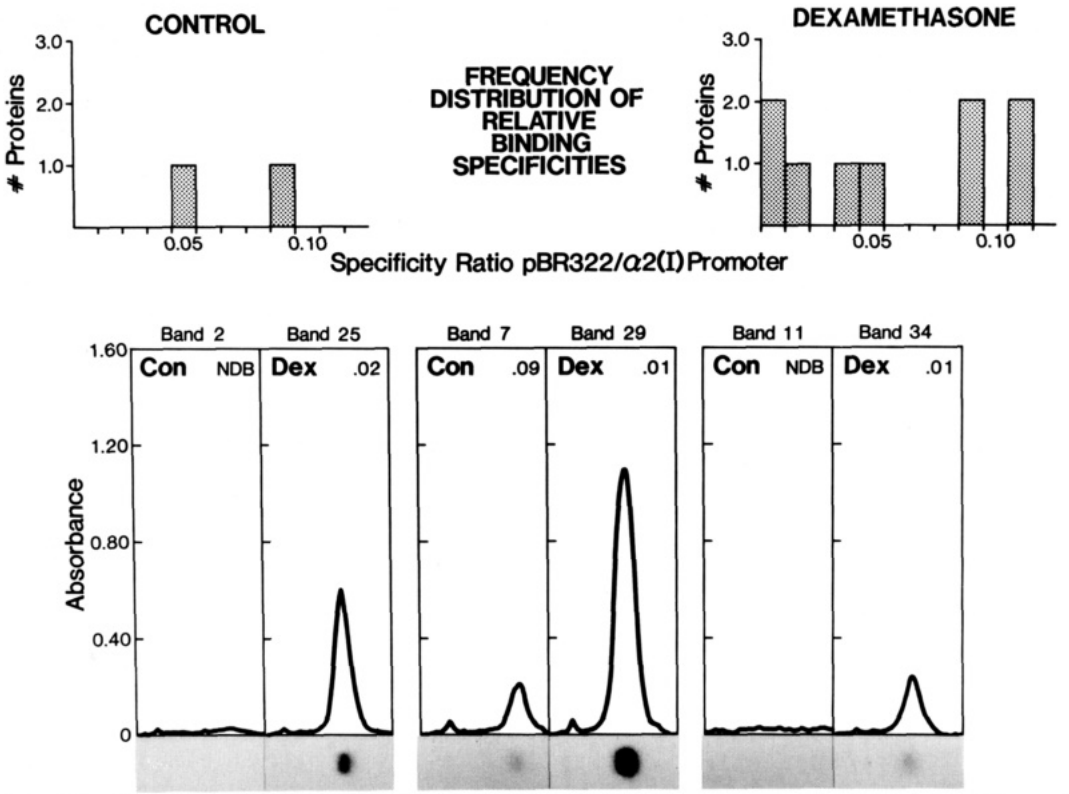

FIGURE 7: Analysis of the abundance and binding specificities of nonhistone DNA-binding proteins present in control and dexamethasone-treated embryonic chick skin fibroblasts. Analysis was restricted to the 24 nonhistone DNA-binding proteins in nuclear extracts of control and dexamethasone-treated fibroblasts in Figure 6 following a stringency wash of 0.1 M NaCl. DNA was eluted from the corresponding numbered bands and electrophoresed on a 1% (w/v) agarose gel. Autoradiography was carried out for 10 days. In the upper panel, the specificity ratios of this subset of nonhistone proteins are represented in histogram form to illustrate the frequency with which DNA-binding proteins of a given specificity ratio occur in control and dexamethasone-treated nuclear extracts. The lower panel analyzes in detail the binding characteristics of the three nonhistone DNA-binding proteins present in nuclear extracts of dexamethasone-treated fibroblasts with specificity ratios ≤0.02. The densitometric scans and specificity ratios of these latter proteins are compared to those of proteins in control nuclear extracts with comparable electrophoretic mobilities. Within each scan the peak to the left represents the 4.3-kb pBR322 vector DNA fragment, while the peak to the right represents the 0.4-kb α2(I) procollagen promoter containing DNA fragment. Below each scan is the corresponding DNA autoradiogram. The band number above each scan corresponds to the numbered band in Figure 6. (NDB) no detectable binding at the indicated exposure time.

methasone-treated skin fibroblasts to a ^{32}P-end-labeled pBR322 vector DNA fragment and a [^{32}P]procollagen promoter containing DNA fragment (Figure 6). The quantitative difference in the 0.05 M lanes results from increased binding of DNA, both specific and nonspecific, to nuclear proteins of the dexamethasone-treated sample. As seen in Figure 5, essentially no qualitative or quantitative differences are seen in nuclear extract proteins after transfer to nitrocellulose. When the stringency wash was carried out in the presence of 0.1 M NaCl, the nonhistone proteins in nuclear extracts of dexamethasone-treated fibroblasts exhibited enhanced binding of total radiolabeled DNA as compared to the nonhistone proteins of control nuclear extracts. Specific binding was taken as that DNA which bound at 0.1 M NaCl, a salt concentration close to physiological conditions. When the ionic strength conditions were increased to 0.2 M NaCl, the binding to the majority of these proteins was no longer detectable. Only one protein at an ionic strength of 0.2 M NaCl remained bound to labeled DNA. This protein, with an apparent molecular weight of 90 000, was detected in nuclear extracts of dexamethasone-treated fibroblasts but not in the nuclear extracts of control fibroblasts.

Effect of Dexamethasone on the Specificity of DNA-Binding Proteins for the Promoter-Containing α2(I) Procollagen Gene Fragment. To further in investigate the dexamethasone-mediated increase in binding of the labeled α2(I) procollagen promoter containing DNA fragment to nonhistone proteins, the specificity ratios of the nonhistone proteins detected on nitrocellulose filters after a 0.1 M NaCl stringency wash were determined. DNA was eluted from the bands numbered 1–12 and 24–35 in Figure 6. The DNA was electrophoresed on agarose gels, and the specificity ratios were determined as previously described under Experimental Procedures. The proteins in the lower molecular weight region of the Figure 6 blots were excluded from analysis.

The upper panel of Figure 7 represents a frequency distribution of binding specificities for the nonhistone proteins of interest (i.e., those with low specificity ratios or high binding specificities). This histogram depicts the number of nonhistone proteins, from either control or dexamethasone-treated fibroblasts, possessing a specificity ratio of ≤0.11. Dexamethasone treatment of fibroblasts results in an increase in the number of nonhistone proteins with specificity ratios of ≤0.11. Nuclear extracts of dexamethasone-treated fibroblasts contain three nonhistone DNA-binding proteins with specificity ratios of ≤0.02. This is in contrast to the absence of a nonhistone DNA-binding protein with a specificity ratio ≤0.02 in nuclear extracts of control fibroblasts. These data suggest that dexamethasone treatment of embryonic chick skin fibroblasts results in either an increase in the efficiency of certain nonhistone proteins to preferentially bind the α2(I) procollagen promoter containing DNA fragment or an induction of these DNA-binding proteins.

Densitometric scans of labeled DNA bound to the three nonhistone DNA-binding proteins present in nuclear extracts of dexamethasone-treated fibroblasts with specificity ratios

of ≤0.02 are seen in the lower panel of Figure 7. Comparison was made between these proteins and corresponding proteins in control extracts. The paired densitometric scanning profiles were judged to represent comparable nonhistone proteins of control and dexamethasone-treated extracts on the basis of the electrophoretic mobility and DNA-binding profile of the bands in Figure 6. Bands 2 and 25 represent the previously mentioned DNA-binding protein with an apparent molecular weight of 90 000. In extracts of dexamethasone-treated fibroblasts this protein bound labeled DNA with a specificity ratio of 0.02, while no detectable binding (NDB) to a protein of this molecular weight was observed in the nuclear extracts of control fibroblasts. These data suggest that dexamethasone treatment of embryonic chick skin fibroblasts results in an increase of binding of a 90 000 molecular weight protein to labeled DNA with greater specificity to the α2(I) procollagen promoter containing DNA fragment. Bands 7 and 29 represent a DNA-binding protein with an apparent molecular weight of 50 000. The specificity ratios of this protein in nuclear extracts of control and dexamethasone-treated fibroblasts were 0.09 and 0.01, respectively. These data indicate that following dexamethasone treatment of embryonic chick skin fibroblasts this protein demonstrates a 9-fold greater specificity for the α2(I) procollagen promoter containing DNA as compared to that observed for a comparable nuclear protein in control fibroblasts. Bands 11 and 34 represent a nonhistone DNA-binding protein with an apparent molecular weight of 30 000. Binding of this protein to labeled DNA is increased in dexamethasone-treated fibroblasts as compared to that observed in control fibroblasts. While no detectable binding (NDB) of labeled DNA to this protein was observed in control extracts, a specificity ratio of 0.01 was observed in extracts of dexamethasone-treated fibroblasts.

Discussion

We have assessed the molecular mechanism(s) by which glucocorticoids alter the cellular levels of type I procollagen mRNAs. Both transcriptional and posttranscriptional mechanisms may be involved in the glucocorticoid-mediated decrease of type I procollagen mRNAs. In human skin fibroblasts cortisol has been shown to increase the degradation of type I procollagen mRNAs without decreasing the synthesis of these mRNAs by nuclei in vitro (Hamalainen et al., 1985). Similar results were also reported for the effect of dexamethasone on type I procollagen mRNAs in rat dermal fibroblasts (Raghow et al., 1986). We have previously shown in embryonic chick skin fibroblasts that the dexamethasone-mediated decrease in the steady-state levels of type I procollagen mRNAs results from a decrease in the rate of synthesis of these mRNA species and not from an increase in their rate of degradation (Cockayne et al., 1986). More recently this glucocorticoid-mediated regulation of procollagen gene expression has been demonstrated in intestine (Walsh et al., 1987), in liver (Weiner et al., 1987a), and in hepatocytes (Weiner et al., 1987b).

The purpose of this investigation was to determine if glucocorticoids regulate type I procollagen gene expression in fibroblasts. Using an in vitro nuclear runoff system, we have demonstrated that dexamethasone selectively decreases the rate of transcription of type I procollagen mRNAs, while the rate of transcription of β-actin mRNA is not affected. Glucocorticoids regulate type I procollagen synthesis by coordinately decreasing the synthesis of proα1(I) and proα2(I) mRNAs.

To assess the role of active protein synthesis in the glucocorticoid-mediated decrease of type I procollagen mRNA synthesis, the protein synthesis inhibitor cycloheximide was utilized. Cycloheximide treatment of primary embryonic chick skin fibroblasts resulted in a decrease of nuclear type I procollagen mRNA synthesis that was equivalent to the decrease observed in the presence of dexamethasone alone. Accordingly, the constitutive synthesis of type I procollagen mRNAs appears to be dependent on continued protein synthesis since both cycloheximide and glucocorticoids decrease protein synthesis. These effects were selective since cycloheximide and dexamethasone alone or in combination did not inhibit β-actin mRNA synthesis or total nuclear RNA synthesis. The inhibitory effects of dexamethasone and cycloheximide on type I procollagen mRNA synthesis in embryonic chick skin fibroblast indicate a mechanism of gene regulation involving continued protein synthesis.

Several mechanisms have been proposed for the role of continued protein synthesis in the steroid-hormone regulation of gene expression. In numerous systems the steroid hormone mediated induction of specific mRNAs is inhibited by cycloheximide. Examples include the estrogen and progesterone induction of egg white proteins in the chick oviduct (McKnight, 1978). This phenomenon also occurs in the glucocorticoid induction of tryptophan oxygenase and α_{2u}-globulin in rat hepatocytes (Delap & Feigelson, 1978; Chen & Feigelson, 1979), phosphoenolpyruvate carboxykinase in kidney tubules (Iynedjian & Jacot, 1981), and glycerol-3-phosphate dehydrogenase in the rat C6 glioma cell line (McGinnis & deVellis, 1978). These studies suggest that steroid hormones may initially stimulate the synthesis of regulatory protein(s), which in turn induces the synthesis of specific mRNAs.

It is currently believed that regulatory DNA-binding proteins are important in the regulation of eucaryotic gene expression. By binding to specific DNA sequences, regulatory nuclear proteins help to turn specific genes either on or off. These interactions are crucial for maintaining the everyday life of all cells and for guiding the development of complex multicellular organisms. 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). A factor has recently been identified that binds to a segment around −80 in the mouse α2(I) collagen gene promoter (Hatamochi et al., 1986). Accordingly, studies were initiated to identify DNA-binding regulatory proteins involved in the dexamethasone-mediated regulation of type I procollagen gene expression.

We employed a protein blotting technique to examine the binding of a α2(I) procollagen promoter containing DNA fragment to nuclear proteins isolated from control and dexamethasone-treated embryonic chick skin fibroblasts. Nonhistone DNA-binding proteins were observed in nuclear extracts derived from both control and dexamethasone-treated embryonic chick skin fibroblasts. Dexamethasone treatment of embryonic chick skin fibroblasts resulted in a dramatic increase in the binding of a subset of nonhistone chromosomal proteins to total labeled DNA. These nonhistone proteins exhibited glucocorticoid-induced binding to labeled DNA under ionic strength conditions of 0.05 and 0.1 M NaCl. These data suggest that these nonhistone DNA-binding proteins may be operable at physiological ionic strength, i.e., 0.14 M NaCl (Kleinsmith, 1973). As the ionic strength was increased to 0.2 M NaCl, beyond the level that approximates physiological ionic strength, only one protein with an apparent molecular weight of 90 000 remained bound to labeled DNA.

One possible explanation for the increase in the DNA binding to nuclear extract proteins of dexamethasone-treated fibroblast extracts may be a glucocorticoid-mediated increase

in the phosphorylation of this subset of nonhistone proteins. It is believed that hormone-mediated alterations in cellular metabolism may involve phosphorylation/dephosphorylation mechanisms. Recently, Kleine et al. (1986) demonstrated that in rat liver cells dexamethasone stimulates protein kinase C. In addition, studies in which the binding of phosphorylated nonhistone proteins to DNA has been examined (Kleinsmith, 1973; Teng et al., 1971; Patel & Thomas, 1973) suggest that phosphoproteins have a high affinity for DNA under low ionic strength conditions and that the binding decreases as the ionic strength increases. Alternatively, dexamethasone treatment may have also resulted in an increase in the number of nonhistone DNA-binding proteins with sequence specificity for the α2(I) procollagen promoter containing DNA fragment.

When compared to comparable proteins present in control fibroblast nuclear extracts, increased DNA binding to proteins in nuclear extracts of dexamethasone-treated fibroblasts with specificity ratios $\leq$0.02 was observed. Three of these nonhistone DNA-binding proteins have binding specificities that differ from those observed for their protein counterparts in control fibroblast nuclear extracts. For all three proteins there is an increase in the binding specificity for the proα2(I) procollagen promoter containing DNA fragment. At present the function(s) of these DNA-binding proteins, M_r 90 000, 50 000, and 30 000, remain(s) unknown.

We have demonstrated that dexamethasone treatment of embryonic chick skin fibroblasts results in a decrease in type I procollagen gene expression. We have also demonstrated that dexamethasone increases the binding specificity of three nonhistone fibroblast nuclear proteins for α2(I) procollagen promoter containing DNA. Since these two phenomena are temporally related, these three nonhistone DNA-binding nuclear proteins that are regulated by glucocorticoids may be related to the glucocorticoid-mediated decrease of type I procollagen gene expression.

Recently it was found that an enhancer region is located in the first intron of the α2(I) gene (Rossi & deCrombrugghe, 1987). This finding was reported after the completion of our studies. In future studies we will identify possible DNA-binding proteins of sequences within the first intron. We will also determine the effect of glucocorticoids on these DNA-binding protein(s).

The data that were obtained are significant in two respects. First, these data demonstrate that multiple nuclear proteins bind the promoter-containing proα2(I) DNA fragment. The data also demonstrate that glucocorticoids regulate certain proα2(I) DNA-binding proteins.

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Registry No. Dexamethasone, 50-02-2.

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Structure of Azotobactin D, a Siderophore of *Azotobacter vinelandii* Strain D (CCM 289)†

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ABSTRACT: The structure elucidation of azotobactin D, the fluorescent siderophore excreted by *Azotobacter vinelandii* strain D, has been accomplished by using essentially NMR techniques and FAB mass spectrometry. It is a chromopeptide possessing a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline bound to a peptide chain of 10 amino acids constituted with D- and L-serine (2), L-homoserine (3), D-citrulline (1), D-N^{δ}-acetyl-N^{δ}-hydroxyornithine (1), L-aspartic acid (1), D-*threo*-β-hydroxyaspartic acid (1), and glycine (1). The chromophore is located at the N-terminus of the peptide, and one of the homoserines is at its C-terminus. The latter lactonizes readily, yielding a high amount of azotobactin δ, which is in fact the major compound isolated after the purification steps. The chromophore has an *S* chiral center. The structure of this siderophore differs significantly from the structure proposed by Fukasawa et al. [Fukasawa, K., Goto, M., Sasaki, K., Hirata, Y., & Sato, S. (1972) *Tetrahedron 28*, 5359–5365] for the fluorescent peptide excreted similarly by *A. vinelandii* strain O.

Iron is an essential element for all living cells.

In aerobic media, at physiological pH, the availability of iron for the cells is limited by the solubility of ferric hydroxide.

In iron-deficient conditions, most microorganisms synthesize very powerful iron-sequestering molecules of low molecular weight, called siderophores, which solubilize iron and transport it into the cells by a high-affinity iron transport system (Neilands, 1973).

Azotobacter vinelandii is a bacterium that transforms nitrogen into ammonia. It produces nitrogenase and other proteins involved in nitrogen fixation that require high amounts of iron as well as molybdenum.

In iron-deficient conditions, *A. vinelandii* excretes large amounts of a yellow-green water-soluble fluorescent compound, azotobactin, which is a siderophore of this bacterium (Fekete et al., 1983; Page & Huyer, 1984; Knosp et al., 1984).

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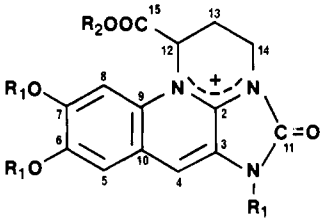
The structure of the pigment excreted by *A. vinelandii* strain O has been reported by Fukasawa and co-workers (Fukasawa et al., 1972). It is a chromopeptide possessing a peptide chain bound to a fluorescent chromophore, **1a**, derived from 2,3-diamino-6,7-dihydroxyquinoline.



1a $R_1 = H$ $R_2 = H$

1b $R_1 = CH_3$ $R_2 = H$

1c $R_1 = H$ $R_2 = CH_3$

The structure of compound **1a** was deduced from X-ray structure determination of its permethylated derivative **1b** (Corbin et al., 1970; Karle & Karle, 1971). It is very similar to the chromophore **2a** common to pyoverdin Pa (Wendenbaum et al., 1983) and pseudobactin (Teintze et al., 1981).

The stereochemistry of **2a** at its asymmetric center is *S* (Teintze et al., 1981; Wendenbaum et al., 1983). No data on the stereochemistry of chromophore **1a** are available since

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