Dexamethasone-Mediated Repression of *MUC5AC* Gene Expression in Human Lung Epithelial Cells

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Glucocorticoids regulate gene expression via binding of the ligand-activated glucocorticoid receptor (GR) to glucocorticoid-responsive elements (GRE) in target gene promoters. The *MUC5AC* gene, which encodes the protein backbone of an abundant secreted airway mucin, has several putative GRE *cis*-elements in its 5' sequence. Mechanism(s) whereby glucocorticoids regulate mucin genes have not previously been described. In this study, the glucocorticoid dexamethasone (Dex) decreased *MUC5AC* mRNA abundance in A549 and NCI-H292 cell lines and primary differentiated normal bronchial epithelial cells by 50–80%, suggesting a common mechanism of *MUC5AC* gene repression in human lung epithelial cells. Kinetic analyses showed that *MUC5AC* mRNA was not significantly decreased until 6 h after Dex exposure, and that nuclear translocation of GR was biphasic, suggesting that Dex-mediated *cis*-repression of *MUC5AC* gene expression was a delayed response of GR translocation. Transfection analyses demonstrated that Dex transcriptionally repressed the *MUC5AC* promoter. Electrophoretic mobility shift assays with wild-type and mutant oligonucleotide probes showed that GR bound to two GRE *cis*-sites (nucleotides –930 to –912 and –369 to –351) in the *MUC5AC* promoter. Analyses of mutated *MUC5AC* promoter constructs demonstrated that NF- κ B *cis*-sites were not involved in Dex-mediated repression of *MUC5AC*. Dex did not alter mRNA stability of *MUC5AC* transcripts. Taken together, the data indicate that Dex transcriptionally mediates repression of *MUC5AC* gene expression in human lung epithelial cells at quiescent states after binding of GR to one or more GRE *cis*-elements in the *MUC5AC* promoter.

Keywords: *MUC5AC* mucin gene; gene repression; dexamethasone; lung cells; glucocorticoids

Glucocorticoids are small, hydrophobic molecules that are produced by the adrenal glands and endogenously mediate physiologic processes such as cortisol suppression, cellular trafficking, and gene regulation (1). Synthetic glucocorticoids, a major class of pharmacologic agents with antiinflammatory and immunosuppressive properties, are used clinically to treat lung, inflammatory bowel and autoimmune diseases, and leukemia, as well as used in organ transplantation. The antiinflammatory effects of synthetic glucocorticoids are typically mediated by (1) up-regulation of genes that encode antiinflammatory proteins, such as lipocortin-1, interleukin (IL)-10, IL-1 receptor antagonist, secretory leukocyte inhibitory protein, Clara cell protein, inhibitory kappaB- α , and neutral endopeptidase and/or (2) by down-regulation/repression

of genes that encode proinflammatory cytokines, enzymes, receptors, and/or adhesion molecules activated during inflammatory responses (reviewed in Refs. 2, 3).

The mechanisms by which glucocorticoids regulate gene expression are varied and complex, but all require a ligand-activated glucocorticoid receptor (GR) (4, 5). After glucocorticoid binding, activated GR translocates to the nucleus, homodimerizes, and binds to glucocorticoid-responsive elements (GREs) in the 5'-upstream flanking sequences of target genes to affect gene expression by *cis*-activation or *cis*-repression (3, 5, 6). Glucocorticoids repress expression of inflammatory genes, which lack GRE *cis*-elements or functional GRE *cis*-elements (7) in their promoter region, by GR *trans*-repression of activated NF- κ B or activator protein (AP)-1 transcription factors in the cytoplasm or nucleus (reviewed in Refs. 3, 5, 6, 8).

The GRE *cis*-element to which activated GR binds has been extensively studied in target genes (4, 5, 9–12). The consensus sequence, GGTACAnnnTGTTCT, is a pentadecameric, imperfect palindrome with two half-site hexamers separated by n, any three nucleotides (9). In genes that are up-regulated by glucocorticoids, the 3' half of the GRE palindrome is typically well conserved, whereas the 5' half exhibits more nucleotide divergence. GRs also bind to GRE *cis*-elements of genes expressed in the endocrine system, bone, and lungs, and during angiogenesis or lactation (5, 6), resulting in *cis*-repression of at least 20 mammalian genes, including osteocalcin (13), IL-1 β (14), vasoactive intestinal peptide receptor (15), collagen (16), and five keratin genes (17). The GRE sequences in glucocorticoid-repressed genes are termed negative GRE (nGRE) elements (9), although there is not complete agreement on whether nGRE elements have a unique consensus sequence (3). Often only one conserved half-palindrome sequence is implicated in glucocorticoid-induced *cis*-repression (reviewed in Refs. 4, 6, 9).

Glucocorticoids are used clinically to decrease lung inflammation in diseases like asthma, cystic fibrosis (CF), and bronchitis (18, 19). Therefore, glucocorticoids may alleviate clinical problems associated with mucus hypersecretion/overproduction because inflammatory/immune-response mediators up-regulate mucin gene expression *in vitro* (reviewed in Refs. 20–22) and likely contribute to mucin overproduction *in vivo*. In one study, a glucocorticoid, beclomethasone dipropionate, has been shown to significantly decrease inflammation and mucin levels in patients with CF (19). However, glucocorticoids may also directly repress mucin gene expression, because dexamethasone (Dex) decreases steady-state mRNA abundance of secretory mucin genes in airway epithelial cells *in vitro* (23, 24). Glucocorticoids repress expression of some genes in lung epithelial cells at the transcriptional level (25, 26) and other genes at the post-transcriptional level (26, 27). However, mechanisms whereby glucocorticoids repress mucin gene expression in airway epithelial cells have not been reported. The *MUC5AC* gene, which encodes the protein backbone of the *MUC5AC* mucin glycoprotein (28), a well-expressed mucin in the airway secretions of healthy individuals and patients with asthma (29), has several

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candidate GRE *cis*-elements in its 5'-upstream flanking sequences (30). An understanding of how glucocorticoids repress *MUC5AC* gene expression may result in the development of better pharmacologic agents to block mucin overproduction. Thus, the effects of Dex on *MUC5AC* gene expression in human airway epithelial cells were investigated.

MATERIALS AND METHODS

Cell Culture and Dex Exposure

A549 and NCI-H292 cells from the American Type Culture Collection (ATCC; Manassas, VA) were grown as previously described and maintained as cultures at 37°C in a humidified 5% CO₂ atmosphere (31). For Dex exposure experiments, A549 (1 × 10⁶ cells/well) and H292 (2 × 10⁶ cells/well) cells were seeded into six-well plates (Costar, Corning, NY) and grown overnight in complete media. Cells were then washed and maintained in serum-free media (SFM) overnight before incubation with vehicle (PBS) or Dex (Sigma Biochemicals, St. Louis, MO) at the concentrations and times indicated in the figure legends.

Normal human bronchial epithelial (NHBE) primary cells and culture media were obtained from Clonetics Cell Systems (Cambrex, East Rutherford, NJ) and established as differentiated cell cultures, essentially as described by Krunkosky and colleagues (32), except for an increased amount of bovine pituitary extract. NHBE cells were plated on type I collagen-coated, semipermeable Transwell membrane (Corning Inc., Corning, NY) inserts at a density of 1 × 10⁵ cells/24-mm well in a 1:1 (vol/vol) mixture of bronchial epithelial cell growth medium (Clonetics, San Diego, CA) and Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with human recombinant epidermal growth factor (0.5 ng/ml), hydrocortisone (0.5 µg/ml), transferrin (10 µg/ml), epinephrine (0.5 µg/ml), triiodothyronine (6.5 ng/ml), insulin (5 µg/ml), gentamicin (50 µg/ml), amphotericin B (50 ng/ml), bovine pituitary extract (130 µg/ml), all-*trans* retinoic acid (5 × 10⁻⁸ M), and BSA (1.5 µg/ml). NHBE cells were grown completely submerged for a week; basal (1.5 ml) and apical (1.0 ml) culture media were changed every other day. The apical media was removed on Day 8 and cells were grown at an air-liquid interface with culture media in the basal compartment changed daily. Under these biphasic culture conditions, NHBE cells differentiate to form a mucociliary epithelium, which histologically mimics human airway epithelium, by 2 wk after establishment of air-liquid interface (32–34). Differentiated NHBE cells were grown in hydrocortisone-free media for 3 d before the addition of Dex or vehicle to the apical and basal media.

RNA Isolation and Northern Blot Hybridization

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Gel electrophoresis was performed using 10 µg of total RNA per sample in a 1% agarose-formaldehyde gel. RNA was transferred to GeneScreen Plus nylon membranes (NEN Life Science Products, Boston, MA) using capillary blotting and cross-linked at 1,200 µJ × 100 in a Stratilinker 1800 (Stratagene, La Jolla, CA). Membranes were prehybridized in a sodium phosphate (0.5 M) pH 7.6 buffer containing SDS (7.5%), EDTA (1 mM), BSA (0.5%), and denatured salmon sperm DNA (100 µg/ml) for a minimum of 3 h at 63°C, then hybridized for a minimum of 16 h at 63°C in the same buffer to which random, primed, labeled cDNA probes (2 × 10⁹ cpm/µg) had been added. The 541-bp EcoRI/BamHI fragment of the NP3a cDNA clone that encodes the 3' end of *MUC5AC* (28) and the 1,500-bp cyclophilin insert probe (ATCC) were labeled with [α -³²P]deoxycytidine triphosphate (NEN Life Science Products) using a random primer labeling kit (Stratagene). Membranes were washed and developed as previously described (31). Band densities were quantified by densitometry using SCION Image (Scion Corp., Frederick, MD).

Real-Time PCR Analysis of Gene Expression

RNA was extracted from differentiated NHBE cell lysates using TRIzol reagent. cDNA was generated from 5 µg of total RNA using Superscript II RT (Invitrogen) and oligo(dT) primers. Real-time PCR was performed on the generated cDNA products in the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using Taqman primers, universal PCR Master mix, and reagents from Applied

Biosystems (Branchburg, NJ). The probe of real-time PCR was labeled with carboxyfluorescein (FAM) at the 5' end and with the quencher carboxytetramethylrhodamine (TAMRA) at the 3' end. The following sequences for *MUC5AC* mRNA analyses were used (35): forward 5'-CGTGTGTCACCGAGAACGT-3' and reverse 5'-ATCTTGATGGCCTTGAGCA-3' primers and fluorescence probe FAM-CTGCGGCACCACAGGGACCA-TAMRA. GAPDH was unchanged by Dex exposure and was used as an internal control for normalizing *MUC5AC* mRNA levels in control and experimental samples. Sequences for GAPDH primers and fluorescent probe were as described by Applied Biosystems. Dilution curves confirmed the linear dependence of the threshold cycles on the concentration of template RNA samples. Relative quantification of *MUC5AC* mRNA in control and experimental samples was obtained using the standard curve method.

Reporter Assay of *MUC5AC* Promoter and Deletion Constructs

A549 (2 × 10⁵) or NCI-H292 (2.2 × 10⁵) cells were seeded onto 12-well plates (Costar) and grown overnight in complete media. At 60% confluency, cells were rinsed with 2 ml of SFM and transfected using 1 µl of Lipofectamine (Invitrogen) in 100 µl Opti-MEM 1 (Invitrogen) media per well combined with 0.33 µg of *MUC5AC* promoter-Luciferase (*Luc*) plasmid DNA (T. Nickola, M. Vesely, and M. C. Rose, unpublished manuscript) or pGL3-basic plasmid (Promega, Madison, WI) in 100 µl of Opti-MEM 1 media per well, with a final volume of 0.5 ml/well. After 6 h, cells were washed and incubated with complete media overnight, then washed and maintained in SFM for 12 h before exposure to Dex or vehicle. Cell lysates were prepared, and reporter gene activity was determined using the Luciferase Assay Kit (Promega) and a single-photon monitor (Beckman Instruments, Fullerton, CA). Samples were analyzed for total protein concentration using the BCA protein analysis (Pierce Chemicals, Rockford, IL) to normalize for harvesting efficiency. Each sample was analyzed in triplicate. Each experiment was performed on at least three separate occasions.

Preparation of Nuclear Extracts

A549 cells exposed to Dex or vehicle for 24 h were washed twice with cold PBS and centrifuged at 1,000 rpm at 4°C for 5 min. Nuclear extracts were prepared as previously described (36) and were dialyzed for 3 h at 4°C in 10,000 molecular weight cutoff Slide-A-Lyzer against Immuno-Pure immobilized avidin (Pierce) to remove any potential trace amounts of biotin. Total nuclear protein (NP) content was determined using the BCA Protein Assay Reagent (Pierce). Extracts were snap-frozen and stored at -80°C in single-use aliquots.

Western Blot Analyses

Ten micrograms of NP were electrophoretically separated on precast SDS/polyacrylamide (4–12%) gels (Invitrogen). Proteins were transferred to nitrocellulose membranes, and immunoblotted with polyclonal anti-GR (1:1,000) or monoclonal anti-actin (1:1,000) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were detected using a secondary goat anti-rabbit or rabbit anti-mouse horseradish peroxidase-conjugated antibody. Detection was performed with SuperSignal West Dura Extended Duration Substrate kit (Pierce) according to the manufacturer's instructions.

Electrophoretic Mobility Shift and Supershift Assays

Single-stranded oligonucleotides (Table 1) were synthesized and HPLC-purified by Integrated DNA Technologies (IDT; Coralville, IA). An oligonucleotide probe with a wild-type (wt) GRE consensus sequence (Santa Cruz Biotechnology) was biotin-labeled (5' and 3') by IDT. Double-stranded oligonucleotide probes were prepared by annealing complementary unlabeled oligonucleotide to the biotinylated probe as per the manufacturer's guidelines (IDT). DNA/protein binding reactions were performed using 50 pg of biotin-labeled probe and 3.5 µg of nuclear extract protein in 10 mM Tris pH 7.5, 1 mM EDTA, 4 mM DTT, 5% glycerol, 1 mM MgCl₂, 50 mM NaCl, 5 mM NaF, 0.1% NP40, 1 µg poly dI:dC, and 10 µg BSA at room temperature for 30 min and then on ice for 15 min. In competition reactions, proteins were incubated with a 100-fold molar excess of unlabeled oligonucleotides before the addition of biotin-labeled probe. In supershift assays, nuclear extracts were incubated with 2 µg of anti-GR antibody for 15 min at room temperature and then on ice for 15 min before addition

TABLE 1. SEQUENCES OF GRE OLIGONUCLEOTIDES USED IN ELECTROPHORETIC MOBILITY SHIFT ASSAYS

Oligonucleotide	Sequence [†]	Strand [‡]	Nucleotide No. ^{**}
GRE*	GGTACA nnn TGTCT		
wt GRE [†]	agaggatc TGTACA GGA TGTCTagat		
mut GRE [‡]	agaggatc TCAACA GGA TCATCTagat		
GRE1	gc TGGGCC CTT TGTCTgctg	–	–1215 to –1197
GRE2	tg GGACCT TTC TGTGCTgg	+	–975 to –957
GRE3 [§]	cc TGTCCA GAG*GGTACTga	+	–930 to –912
mut1GRE3	cc TCACCA GAG GCAACTga		
mut2GRE3	cc TCACGT GAG GGTACTga		
GRE4	ag GAGTCC ACC TGTGCTtc	–	–837 to –819
GRE5	ctggc TGGGCC CCC TGTCTgctg	+	–369 to –351
mut1GRE5	ctgggcTGGGCC CCC TCACCTgctg		
mut2GRE5	ctgggcTCCGAC CCC TGTCTgctg		

Definition of abbreviations: GRE, glucocorticoid-responsive element; mut, mutated; wt, wild-type.

* Consensus sequence for GRE elements (9).

† Consensus wt GRE sequence (Santa Cruz Biotechnology).

‡ Mut GRE sequence (Santa Cruz Biotechnology).

§ The GRE3 sequence is bisected at *, as indicated in the 1.0-kbp construct (Figure 4).

^{||} 5'→3', sense strand; n is any nucleotide. GRE *cis*-sequence sites in the 15-bp palindrome are capitalized; flanking sequences are in lower case; and mut bases are in boldface type.

[‡] The plus signs refer to the sense strand and the minus signs to the complementary strand of DNA.

^{**} Numbers refer to the nt position of putative GRE *cis*-sites identified in the *MUC5AC* promoter by MatInspector, as indicated in Figure 4. Flanking nucleotides are from the *MUC5AC* promoter sequence (30).

of probe. Ultraviolet cross-linking was used to stabilize DNA/protein complexes, which were then separated by electrophoresis (200 V, 4°C, 3 h) in a 6% polyacrylamide gel containing 0.5× Tris-borate-EDTA buffer with buffer recirculation. Complexes were electroblotted (Owl Scientific, Portsmouth, NH) onto the positively charged Brightstar nylon membrane (Ambion, Austin, TX), and ultraviolet cross-linked on ice at 600,000 μJ, per the manufacturer's suggestions. Chemiluminescent detection of gel shift analysis was performed using BrightStar BioDetect (Ambion) and XOMAT LS film (Eastman Kodak, Rochester, NY).

RNA Stability Assays

Cells were plated on six-well plates as described above, grown overnight, washed in SFM and incubated in SFM with or without Dex (100 nM) for 24 h. Transcription was stopped by addition of 5 μg/ml of actinomycin D (Sigma) for 0.5, 1, 2, 4, 6, 8, and 24 h. Total cellular RNA was extracted and Northern blot analysis was performed as described above.

Statistics

All assays/experiments were performed on multiple occasions with triplicate samples unless otherwise indicated. Transfection experiments were typically expressed as percent control of the wt 1.4-kb *MUC5AC-Luc* plasmid normalized for total protein content and statistically analyzed using one-way analysis of variance to allow for multiple comparisons. The effects of mutating putative NF-κB sites within the *MUC5AC* promoter on the ability of Dex to regulate *MUC5AC* expression were statistically compared with wt + Dex exposure using one-way analysis of variance. Western blot and electrophoretic mobility shift assay (EMSA) analyses were performed using Quantity One software and ChemiDoc Imaging system (BioRad, Hercules, CA) and normalized to internal controls. Statistics for RT-PCR results were performed using GraphPad Prism software (San Diego, CA).

RESULTS

Dex Decreases MUC5AC mRNA Abundance in Airway Epithelial Cells

The effects of Dex on MUC5AC mRNA abundance were evaluated in two respiratory tract-derived epithelial cancer cell lines (A549 and NCI-H292) and in primary differentiated NHBE epithelial cells over a range of Dex concentrations (10^{−9} to 10^{−6} M) that approximate glucocorticoid levels in the microenvironment of the airway epithelium after oral delivery of glucocorticoids

(37). Steady-state analyses showed that Dex, in a concentration-dependent manner, decreased the abundance of MUC5AC mRNA within 24 h in A549 cells. MUC5AC mRNA levels decreased with increasing Dex concentration over a range of 1 to 100 nM, significantly decreased at 10 and 100 nM (Figure 1A), and were nondetectable at 1,000 nM by Northern blot analyses (data not shown). Dex also decreased the abundance of MUC5AC mRNA in NCI-H292 cells in a concentration-dependent manner, with a significant decrease at 100 nM Dex (data not shown). Thus, Dex decreased MUC5AC mRNA abundance in two cancer cell lines typically used for mucin gene expression analyses (21), suggesting that glucocorticoids can directly repress *MUC5AC* gene expression in airway cells in the absence of inflammation and at concentrations similar to or lower than those used for treating patients with asthma.

To verify the biological significance of these findings, the effect of Dex on MUC5AC mRNA abundance was also evaluated in primary differentiated NHBE cells, which when maintained at an air-liquid interface histologically mimic airway epithelium with ciliated, goblet, and basal cells (reviewed in Ref. 33). A concentration-effect study demonstrated a significant decrease in MUC5AC mRNA abundance evaluated by quantitative RT-PCR at 100 and 1,000 nM Dex (Figure 1B). The ability of Dex to significantly decrease MUC5AC mRNA abundance occurred at similar Dex concentrations in primary differentiated NHBE cells and in airway epithelial cancer cell lines, suggesting a common mechanism for Dex-regulated *MUC5AC* gene expression in human lung epithelial cells.

Analyses of the temporal effects of Dex on *MUC5AC* gene expression were performed to determine parameters wherein Dex induced repression of this mucin gene. Temporal analyses of A549 cells exposed to vehicle for 0.5 to 24 h showed that MUC5AC mRNA abundance increased in control cells beginning at 4 h and continuing for 24 h (Figure 2). This reflects an increase in A549 cell numbers over time; therefore, mRNA levels in cells exposed to Dex were compared at each time point with control (e.g., vehicle-exposed) cells. Furthermore, Dex exposure did not significantly alter cell numbers; therefore, comparisons were corrected by normalization to cyclophilin. When compared

Figure 1. Dexamethasone (Dex) decreases MUC5AC mRNA abundance in airway epithelial cells. (A) A549 cells were exposed to Dex (1, 10, or 100 nM) or vehicle for 24 h. MUC5AC and cyclophilin mRNA levels were quantified by Northern blot analyses as described in MATERIALS AND METHODS. Results are expressed as mean $\pm$ SE. Each experiment was performed in duplicate on three separate occasions. Statistically significant differences are indicated by asterisks: * P < 0.05, ** P < 0.01. (B) Primary differentiated NHBE cells established at an air-liquid interface were exposed to Dex (10, 100, 1,000 nM) or vehicle for 24 h. MUC5AC and GAPDH mRNA were evaluated in experimental and control samples using real-time PCR as described in MATERIALS AND METHODS. RNA from triplicate plates was assayed in triplicate. Results are expressed as mean $\pm$ SE. Statistically significant differences are indicated by asterisks: ** P < 0.01, *** P < 0.001.

with control cells, MUC5AC mRNA abundance was not significantly decreased until 6 h after Dex exposure and the Dex-induced decrease was maintained for 24 h (Figure 2). These data indicate that down-regulation of MUC5AC expression is a delayed response to Dex exposure.

Dex Increases Nuclear GR Translocation in a Biphasic Manner

Ligand-activated GR can directly activate or repress target genes with GRE *cis*-elements after translocation to the nucleus and homodimerization. Alternatively, ligand-activated GR can indirectly *trans*-repress target genes by binding to transcription factors in the cytoplasm, thereby preventing nuclear translocation of GR and/or activated transcription factors (reviewed in Refs. 3, 5). To determine whether exposure of A549 cells to Dex resulted in increased nuclear translocation of GR, the abundance of GR protein in A549 cell nuclear lysates after exposure to Dex or vehicle was evaluated by Western blot analyses. Data showed that the GR level was increased in A549 nuclear lysates after Dex (100 nM) exposure for 24 h (Figure 3A), indicating that Dex induced GR nuclear translocation. Temporal analyses of GR nuclear abundance were then performed to determine whether the Dex-induced GR nuclear translocation in A549 cells correlated with the delayed response of Dex-induced repression of MUC5AC mRNA. Translocation of GR to the nucleus was rapid and occurred within 30 min after Dex exposure (Figure 3B).

Figure 2. Kinetic effects of Dex on MUC5AC mRNA levels. A549 cells were incubated with 100 nM Dex or vehicle for 0, 0.5, 1, 2, 4, 6, 8, and 24 h. Northern blot analyses of MUC5AC and cyclophilin mRNA were quantified as described in MATERIALS AND METHODS at each time point for control (solid bars) or Dex-exposed (open bars) cells. Results are expressed as mean $\pm$ SE. Each experiment was performed in duplicate on four separate occasions. Statistically significant differences are indicated by asterisks: *** P < 0.001.

Expression of GR was significantly decreased at 1 h relative to the 0.5-h time point and was then followed by a second increase in GR levels over 2 to 6 h, at which time the abundance of GR NP was comparable to that observed after 30 min of Dex exposure. Levels of GR NP were decreased by 24 h, but still remained high (e.g., $\geq$ 3-fold above baseline levels). The second induction of GR translocation to the nucleus corresponded temporally to the Dex-induced repression of MUC5AC observed at 6 h (Figure 2). These results suggested that MUC5AC gene repression by Dex is not mediated by a block in nuclear translocation of GR but rather by a more direct role of nuclear GR on MUC5AC expression, which was subsequently evaluated.

Figure 3. Dex increases the abundance of nuclear GR. Western blot analysis using a polyclonal anti-GR antibody were performed on nuclear extracts of A549 cells. (A) Cells were exposed to vehicle (–) or 100 nM Dex (+) for 24 h; 7 or 14 μ g of nuclear protein (NP) were analyzed. (B) Cells were exposed to 100 nM Dex for the times indicated. Ten micrograms of NP were evaluated for GR and actin protein for each time point. Results are shown as the relative fold induction of Dex-induced nuclear GR levels normalized to actin levels, expressed as the mean $\pm$ SE. Experiments were performed in triplicate on three separate occasions. * P < 0.05.

Figure 4. Selective putative transcription factor binding sites in the 1.4-kbp *MUC5AC* promoter and deletion constructs. Putative GRE *cis*-elements, indicated above the rectangle depicting the 1.4-kbp *MUC5AC* promoter, were identified using MatInspector (Genomatix) at anchor positions: nt -1206, -965, -921, -829, and -361. Deletion constructs are indicated by dotted lines and are ~1.0, 0.5, 0.2, and 0.1 kbp in length (T. Nickola, M. Vesely, and M. C. Rose, unpublished manuscript). The TATA box and the CACCC epithelial box are indicated; the NF- κ B *cis*-elements at nt -973, -957, and -225 are indicated below the rectangle.

Dex Transcriptionally Down-Regulates *MUC5AC* Gene Expression

Analysis of transcription factor binding sites within the reported 1.4 kbp of the 5' flanking region of the *MUC5AC* gene (30) using MatInspector (version 6.2.1; Genomatix, Munich, Germany) identified five potential high-affinity GRE *cis*-sequences/GRE binding sites in the *MUC5AC* promoter (Figure 4). To determine whether the aforementioned decreases in *MUC5AC* mRNA levels after Dex exposure were due in part to Dex-mediated transcriptional regulation of the *MUC5AC* gene, promoter analysis studies were performed. Toward that end, activity of the *MUC5AC* promoter was evaluated, after transfection of the 1.4-kbp *MUC5AC* promoter-*Luc* construct or of the pGL3 *Luc* construct into lung cells followed by exposure to various concentrations of Dex for 1 or 24 h. After 1 h of exposure to the highest concentration (100 nM) of Dex used, there was no significant decrease in *MUC5AC* promoter activity (data not shown), in keeping with our earlier observation that Dex did not alter *MUC5AC* mRNA levels before 6 h of exposure. However, after 24 h of Dex exposure, there was a concentration-dependent decrease in transcriptional activity of the 1.4-kbp *MUC5AC* promoter construct in two airway epithelial cell lines (Figure 5). This decrease was significant at 10 and 100 nM Dex in A549 cells (Figure 5B) and at 100 nM Dex in H292 cells (Figure 5A). It accounted for an approximately 40% decrease in promoter activity at 100 nM Dex in both cell lines. These results show that Dex can alter the activity of an exogenous *MUC5AC* promoter in airway epithelial cells. Moreover, the Dex concentrations required to significantly repress expression from the transfected *MUC5AC* promoter were similar to those that decreased *MUC5AC* mRNA expression from the genomic locus (Figure 1), although the repression was not as great at the same Dex concentration (e.g., 66% decrease in steady-state abundance versus 40% decrease in promoter activity). Nevertheless, these dose response results validate the use of the *MUC5AC* promoter construct in these studies in airway epithelial cell lines.

Evaluation of GRE *cis*-Elements in the *MUC5AC* Promoter

To determine which *MUC5AC* upstream sequences were responsive to Dex, airway epithelial cells were transiently transfected with deletion constructs (1.0, 0.5, 0.2, 0.1 kbp) of the 1.4-kbp *MUC5AC* promoter-*Luc* construct (T. Nickola, M. Vesely, and M. C. Rose, unpublished manuscript). After transfection, cells were exposed to vehicle or Dex (100 nM) for 24 h. As expected, promoter activity of the 0.2- and 0.1-kbp constructs, which lack

predicted GRE *cis*-elements (Figure 4), were not altered by Dex. However, Dex decreased *MUC5AC* promoter activity of the 0.5-kbp construct in both cell lines (Figures 6A and 6B), indicating that the 0.5-kbp construct contained a target *cis*-element that contributes to Dex-mediated repression of *MUC5AC* expression. Consistent with this possibility, the putative GRE5 binding site resides in the 0.5-kbp construct (Figure 4). Interestingly, Dex did not decrease *MUC5AC* promoter activity of the 1.0-kbp construct in H292 cells, and increased promoter activity of this construct in

Figure 5. Dex-mediated down-regulation of *MUC5AC* promoter activity in airway epithelial cells. Plasmid DNA from the 1.4-kbp *MUC5AC* promoter-*Luc* or the pGL3-*Luc* construct was transfected into (A) NCI-H292 or (B) A549 cells as described MATERIALS AND METHODS. Cells were exposed to Dex (1, 10, 100 nM) or vehicle for 24 h and lysed. Luciferase activity (cpm) and protein concentration (μ g) were determined for each transfection; results were normalized for *MUC5AC* promoter activity (*MUC5AC* promoter-*Luc*/pGL3-*Luc*) and expressed as mean $\pm$ SE. Experiments were performed in triplicate on four separate occasions. Statistically significant differences are indicated by asterisks: * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 6. The effects of Dex on deletion constructs of the 1.4-kbp *MUC5AC* promoter-*Luc*. Plasmid DNA from the 1.4-, 1.0-, 0.5-, 0.2-, or 0.1-kbp *MUC5AC* promoter-*Luc* or the pGL3-*Luc* construct was transfected into (A) NCI-H292 or (B) A549 cells. Cell lysates were analyzed for luciferase activity and total protein content for each transfection after a 24-h incubation with or without 100 nM Dex. Results were normalized for *MUC5AC* promoter activity (*MUC5AC* promoter-*Luc*/pGL3-*Luc*). The percentage of these values (mean $\pm$ SE) for control/Dex-exposed cell lysates is plotted on the y axis for each promoter construct, with 100% being the normalized value for controls. Experiments were performed in triplicate on three separate occasions. Statistically significant differences are indicated by asterisks: ** $P < 0.01$, *** $P < 0.001$.

A549 cells. The 1.0-kbp construct lacks the GRE1 or GRE2 *cis*-elements but contains the GRE4 and GRE5 *cis*-elements, as well as the 3' half of GRE3. This implicated GRE3 in Dex-mediated inhibition of *MUC5AC* expression, because the GRE3 palindromic site is bisected in the 1.0-kbp construct (Table 1). In addition, it suggested that elements between nucleotide (nt) -919 and nt -457 in the *MUC5AC* promoter might overcome Dex-mediated repression of the 0.5-kbp *MUC5AC* promoter construct in A549 cells. Taken together, these data implicated GRE3 and GRE5 *cis*-elements in Dex-mediated *MUC5AC* gene repression in airway epithelial cells.

To determine whether GR binds to GRE3 and GRE5 *cis*-elements in the 5' upstream sequence flanking the *MUC5AC* gene, EMSA and supershift assays were performed using a biotinylated wt GRE consensus sequence (Table 1) as probe. EMSA analyses demonstrated that the biotinylated wt GRE probe was bound to a high molecular mass band in nuclear extracts from control and Dex-exposed A549 cells (Figures 7A and 7B, lane 1). Preincubation of lysates with either unlabeled wt consensus sequence GRE oligonucleotide (Figures 7A and 7B, lane 2) or unlabeled *MUC5AC* GRE5 or GRE3 oligonucleotides (Figures 7A and 7B, lanes 3 and 4) decreased complex formation, indicating that these oligonucleotides effectively competed with the biotin-labeled wt probe in the protein/DNA complex. Supershift assays showed GR was present in the protein/DNA complexes, because the protein bound to the labeled GRE probe shifted to a higher molecular mass

after preincubation of nuclear extracts with an anti-GR antibody (Figures 7A and 7B, lane 5). These results show that GR binds to the GRE3 and GRE5 *cis*-elements in the *MUC5AC* promoter, consistent with the promoter analysis data (Figure 6).

Experiments to evaluate the binding hierarchy of ligand-activated GR to *MUC5AC* GRE *cis*-elements were also performed using oligonucleotides specific to the five putative GRE sequences in the *MUC5AC* promoter (Table 1). Data showed that unlabeled GRE3 and GRE5 oligonucleotides, in contrast to GRE1, GRE2, and GRE4 oligonucleotides, competed with the labeled wt GRE probe in nuclear extracts from Dex-exposed cells (Figure 7C). Thus, GRE, *MUC5AC* GRE3, and *MUC5AC* GRE5 oligonucleotides with GT/CA mutations in their 5' and/or 3' half-palindromic sequences (Table 1) were evaluated by EMSA. As expected, the mutated GRE oligonucleotide, unlike the wt GRE, did not inhibit binding of the wt GRE biotinylated probe to GR (data not shown), verifying the specificity of the GR interaction. Mutant *MUC5AC* GRE3 oligonucleotides with GT/CA mutations in both the 5' and 3' half-palindrome sequences (mut1) or mutations only in the 5' half-palindrome sequence (mut2) did not inhibit binding of the wt GRE biotinylated probe to GR, implicating the 5' half-palindrome sequence and/or the full palindrome of GRE3 in GR binding. Oligonucleotides with GT/AC mutations in the 3' (mut1) or 5' (mut2) half of the GRE5 palindrome sequence (Figure 7D) also did not inhibit binding to GR, suggesting that both half-palindromes are needed for GR binding. These data demonstrated that GRE3 and GRE5 *cis*-elements in the *MUC5AC* promoter were bound *in vitro* by GR and predictably mediated Dex repression of *MUC5AC* gene expression.

NF- κ B *cis*-Elements Do Not Contribute to Dex-Mediated Repression of *MUC5AC* Gene Expression

Dex *trans*-represses expression of the *IL8* gene, even though it has a putative GRE *cis*-element in its promoter, by interfering with NF- κ B binding to the NF- κ B *cis*-element (7). The *MUC5AC* gene has several putative GRE *cis*-elements, as well as several putative NF- κ B *cis*-elements, in the first 1.4 kbp of the *MUC5AC* promoter. Constructs in which NF- κ B sites were mutated have been generated (T. Nickola, M. Vesely, and M. C. Rose, unpublished manuscript) and were used to evaluate whether Dex-induced repression of *MUC5AC* expression involved NF- κ B *cis*-elements in the *MUC5AC* promoter. The effects of Dex on *MUC5AC* expression after transfection of *MUC5AC*-*Luc* promoter constructs with mutated NF- κ B *cis*-elements at nt -973, -957, -973/957, or -225 were evaluated. Data demonstrated that Dex decreased *MUC5AC* promoter activity in the mutant NF- κ B constructs similar to its effect on the wt 1.4-kbp construct (Figure 8), indicating that these NF- κ B *cis*-elements were not targets for Dex-mediated repression of *MUC5AC* gene expression in A549 cells that have not been stimulated by inflammatory mediators.

MUC5AC mRNA Stability Is Similar in Dex-Exposed and in Vehicle-Exposed A549 Cells

The *MUC5AC* gene is regulated at the post-transcriptional level, as well as at the transcriptional level, by specific mediators (reviewed in Ref. 22). Likewise, glucocorticoids, which typically regulate genes at the transcriptional level, have been shown to regulate genes at the post-transcriptional level (28, 38). Thus, the effect of Dex on the stability of *MUC5AC* transcripts was evaluated. A549 cells were exposed to vehicle or to Dex (100 nM) for 24 h; the decay in *MUC5AC* transcript levels was monitored after exposure to actinomycin D, an RNA polymerase II inhibitor, for 0.5, 1, 2, 4, 6, 8, and 24 h. When *MUC5AC*

Figure 7. EMSA and supershift analyses of putative *MUC5AC* GRE elements. Confluent A549 cells were incubated with Dex (100 nM) or vehicle for 24 h and nuclear extracts were isolated. All EMSAs were performed using biotinylated wt GRE-consensus probe (Santa Cruz Biotechnology). Lysates were preincubated with unlabeled wt GRE or *MUC5AC* GRE1, GRE2, GRE3, GRE 4, or GRE5 oligonucleotides (sequences shown in Table 1) or anti-GR antibody, as indicated. (A) Control cells; (B) cells exposed to Dex. Lanes: 1, no competitor; 2, wt GRE; 3, GRE5; 4, GRE3; 5, anti-GR antibody. Arrow, protein/GRE complex; open arrowhead, anti-GR:GR:GRE complex. (C) Nuclear lysates from A549 cells exposed to Dex were preincubated with unlabeled GRE1, GRE2, GRE3, GRE4, or GRE5 oligonucleotides before EMSA. A representative example of the DNA/protein bands identified by EMSA analyses is shown at the top. Bands from three experiments were quantified by densitometry and expressed as the mean value $\pm$ SE. (D) Nuclear lysates from A549 cells exposed to Dex were preincubated with unlabeled *MUC5AC* GRE3 or GRE5 wt or mut oligonucleotides (Table 1) before EMSA. A representative example of the DNA/protein bands identified by EMSA analyses is shown at the top. Bands from three experiments were quantified by densitometry and expressed as the mean value $\pm$ SE. Statistically significant differences are indicated by an asterisk: * $P < 0.05$ when compared with bands from EMSAs of control lysates without any competing oligonucleotides.

mRNA levels were plotted as a % of the control at each time period, the half-life (defined as the time at which 50% of mRNA remained) was ~ 6 –8 h (Figure 9A), in agreement with that previously reported for *MUC5AC* mRNA in A549 cells (39).

Figure 8. Effect of Dex on *MUC5AC* promoter-*Luc* activity. Plasmid DNA from the 1.4-kbp *MUC5AC* promoter-*Luc* construct, constructs containing mutations at putative NF- κ B sites² (nt -973, -957, -973/957, and -225), or pGL3-*Luc* plasmid were transiently transfected into A549 cells. Cell lysates were analyzed for luciferase activity and total protein content after a 24-h incubation with or without 100 nM Dex. Results are normalized for luciferase activity (*MUC5AC* promoter-*Luc*/pGL3-*Luc*) and protein and expressed as mean $\pm$ SE. Triplicate samples were assayed in three independent experiments. Statistically significant differences are indicated by asterisks: * $P < 0.05$, ** $P < 0.01$.

In cells exposed to Dex for 24 h, *MUC5AC* mRNA levels were, as expected, already markedly decreased by 24 h (Figure 9A). When the *MUC5AC* mRNA levels at the start of actinomycin treatment in each group were normalized to 100%, the rates of *MUC5AC* mRNA degradation after actinomycin exposure were similar in Dex-treated and in control, vehicle-treated cells (Figure 9B). This is in contrast to other mediators (e.g., neutrophil elastase [39] or IL-8 [40]), which markedly increase the stability of *MUC5AC* transcripts. In additional experiments, *MUC5AC* mRNA levels were unchanged in cells exposed to Dex for 1 h, as expected, and the decay patterns in control and Dex-treated cells were undistinguishable (data not shown). Taken together, these data show that the Dex-mediated repression of the *MUC5AC* gene is not regulated at the post-transcriptional level.

DISCUSSION

Glucocorticoids alter mucin gene expression at steady-state equilibrium in mammalian cells and cell lines. Dex increases the mRNA abundance of the *MUC1* gene, which encodes a membrane-tethered mucin, in cancer cell lines (41, 42). In contrast, Dex represses the mRNA abundance of the secretory mucin genes *MUC2* (23) and *MUC5AC* (23, 24; this study) in human respiratory tract cancer cell lines and of *Muc5ac* mRNA in primary rat tracheal cells (24). Each of these *MUC* genes has one or more GRE *cis*-sequences in its promoter region. However, the mechanisms whereby Dex up-regulates or represses mucin gene expression have not been previously reported. This study has now demonstrated that the Dex-induced repression of *MUC5AC* in airway epithelial cells is mediated at the transcriptional, but not at the post-transcriptional, level and used *cis*,

Figure 9. Dex treatment does not alter the stability of MUC5AC mRNA. A549 cells were exposed to vehicle or Dex (100 nM) for 24 h before the addition of actinomycin D (5 μ g/ml; $t = 0$). Cells were harvested at 0.5, 1, 2, 4, 6, 8, and 24 h after exposure to actinomycin D or vehicle. RNA was isolated and analyzed by Northern analysis as described in MATERIALS AND METHODS. (A) MUC5AC mRNA levels plotted at each time period as a percentage of the control, untreated cells. (B) MUC5AC levels in Dex-treated cells and in vehicle-treated cells were normalized to 100% at time 0 (at the start of actinomycin exposure) and replotted. The decay in MUC5AC transcripts was not altered by preexposure of the cells to Dex. Data are represented as the means $\pm$ SE of MUC5AC mRNA levels for three experiments with duplicate samples per experiment.

rather than *trans*, repression. In addition, EMSA analyses with wt or mutant *MUC5AC* GRE oligonucleotides demonstrated that GR bound to two (GRE3 and GRE5) of five putative GRE *cis*-sequences in the *MUC5AC* promoter, thereby validating the promoter transfection analyses studies, which had implicated GRE3 (bisected in the 1.0-kbp deletion construct) and GRE5 *cis*-elements as responding to Dex.

The Dex-induced decrease in MUC5AC mRNA abundance, initially reported in NCI-H292 cells (23), also occurs in A549 cells, another respiratory tract-derived cancer cell line typically used to investigate mucin gene regulation (21). In addition, this study shows Dex-induced repression of MUC5AC mRNA in primary differentiated NHBE cells (Figure 1B), as recently reported in differentiated and undifferentiated rat primary airway epithelial cells (24). The Dex concentrations at which statistically significant repression was observed were slightly higher than those reported by other laboratories for NCI-H292 (23) or A549 (24) cells, likely reflecting interlaboratory variations in cell culture protocols. The 10-fold higher Dex concentration required to statistically repress *MUC5AC* gene expression in primary differentiated NHBE cells compared with cancer cell lines presumably reflects the lower percentage of mucin-producing goblet cells in the heterogeneous NHBE cell cultures. Nevertheless, the concentration range (10^{-8} to 10^{-6} M) over which Dex represses *MUC5AC* mucin gene expression in airway epithelial cells is well within the range estimated for glucocorticoid levels in human lung epithelium after aerosol delivery (37). The similar pharmacologic response in primary cells and in lung cancer cell lines indicated that the latter, which are traditionally used to investigate up-regulation of mucin gene expression, are also useful models for investigating repression of mucin gene expression.

However, the aforementioned studies are in contrast to a study using differentiated normal human nasal epithelial (NHNE) cells, wherein Dex is reported to have no effect on MUC5AC mRNA steady-state expression (43). In that study, NHNE cell cultures established with nasal polyps from one individual exhibited marked variability because only one of seven Transwells expressed MUC5AC mRNA at basal conditions. Consequently, the authors were unable to detect whether or not Dex repressed *MUC5AC* expression. The variable and limited expression of MUC5AC mRNA reported (43) is in contrast to a study wherein

all NHNE and NHBE cell cultures from individuals expressed MUC5AC mRNA under baseline conditions (44).

Glucocorticoid therapy significantly decreases airway inflammation *in vivo* (2), typically by *trans*-repression of inflammatory genes that lack GRE *cis*-elements (45). However, Dex-mediated *trans*-repression also occurs in the *IL8* inflammatory gene, which has a putative GRE *cis*-element in its promoter region, because activated GR interferes with binding of NF- κ B to its cognate *cis*-element in the *IL8* gene to suppress transcription (7). The *MUC5AC* promoter has several NF- κ B sites, of which two are just upstream of the GRE3 *cis*-element and one is downstream of GRE5. Mutation of three NF- κ B *cis*-elements did not significantly alter Dex repression of *MUC5AC* constructs in transient transfection experiments, indicating that Dex-repression of *MUC5AC* is not mediated by *trans*-repression through these NF- κ B *cis*-elements, at least in quiescent cells (e.g., in the absence of an exogenous inflammatory mediator).

Glucocorticoids classically regulate gene expression through binding to the GR followed by translocation of the ligand-GR complex to the nucleus. However, expression of GR can be autoregulated by glucocorticoids (46) and the glucocorticoid budesonide has been shown to repress GR mRNA levels in a human bronchial epithelial cell line and in bronchial epithelial cells (47). The current study shows that GR nuclear translocation in A549 cells, a cell line used for investigating glucocorticoid-mediated regulation of inflammatory genes (26, 48), is rapid and sustained over a 24-h period. In addition, GR nuclear translocation is temporally regulated in a biphasic manner, with maximal expression at 30 min and again at 4 to 6 h after Dex exposure (Figure 3B). Because MUC5AC mRNA levels did not significantly decrease until A549 cells were exposed to Dex for 6 h, we conclude that the second Dex-induced increase in nuclear GR mediates the observed repression of the *MUC5AC* gene *in vitro*.

Dex is the only pharmacologic agent so far reported to repress expression of secretory airway mucin genes in airway epithelial cells at steady-state equilibrium. As shown here, repression of *MUC5AC* expression at steady-state equilibrium is mediated at the transcriptional, but not the post-transcriptional, level. However, a recent study indicates that Dex may also regulate *Muc5ac* gene expression at the translational level, because the Dex-mediated repression of *Muc5ac* mRNA in rat differentiated and

nondifferentiated airway cells does not decrease Muc5ac protein levels in lysates or secretions but rather enhances the translation and stability of Muc5ac protein (24). Gene transcription does not always result in translation of mRNA into gene products in human lung cells or tissues. For example, (1) α_1 -antitrypsin mRNA is expressed at normal levels in the alveolar macrophages of patients with antiprotease deficiency, but the RNA is not translated so that the gene product is not secreted (49); (2) MUC2 mRNA expression is up-regulated by lipopolysaccharide in bronchial explants of patients with or without CF (50), but MUC2 mucin levels are not increased in the airway secretions of patients with CF (51), who typically are chronically exposed to bacterial byproducts. Thus, studies to determine whether Dex decreases biosynthesis and secretion of MUC5AC mucins in differentiated NHBE cells *in vitro* will be useful in further understanding the effects of glucocorticoids on mucin gene expression, as well as on mucin production and secretion.

Our understanding of the use and efficacy of glucocorticoid therapy in treatment of lung disease is not completely understood, especially with regard to how glucocorticoids repress gene expression. Our data demonstrated that Dex regulates MUC5AC gene expression by *cis*-repression in lung cells in the absence of exposure to inflammatory mediators. Inflammatory mediators, which up-regulate MUC5AC gene expression *in vitro* (20, 21), predictably contribute to the clinical problems associated with mucin overproduction *in vivo*. Thus, an understanding of how glucocorticoids regulate mucin gene expression in cells exposed to inflammatory/immune-response mediators is important because glucocorticoid inhalation therapy is used to treat patients with airway diseases where inflammation and mucin overproduction are characteristic findings. Glucocorticoid therapy, which significantly decreases airway inflammation *in vivo* (2), has been reported to decrease mucin levels monitored by the CA19-9 antibody in the bronchoalveolar lavage fluid of patients with CF (19). This presumably reflects the glucocorticoid-induced decrease of inflammatory mediators, which could decrease mucin production or secretion, but it may also reflect a direct repression by glucocorticoids of MUC5AC gene expression. Studies to determine whether glucocorticoids decrease MUC5AC mucin production and/or secretion in airway secretions of lung patients may be informative. Such studies may culminate in the development of more efficacious synthetic glucocorticoids that better regulate mucin overproduction in airway diseases.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

- Jusko WJ. Pharmacokinetics and receptor-mediated pharmacodynamics of corticosteroids. *Toxicology* 1995;102:189–196.
- Barnes PJ. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci* 1998;94:557–572.
- Pelaia G, Vatrella A, Cuda G, Maselli R, Marsico SA. Molecular mechanisms of corticosteroid actions in chronic inflammatory airway diseases. *Life Sci* 2003;72:1549–1561.
- Truss M, Beato M. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocr Rev* 1993;14:459–479.
- Schoneveld OJLM, Gaemers IC, Lamers WH. Mechanisms of glucocorticoid signalling. *Biochim Biophys Acta* 2004;1680:114–128.
- Dostert A, Heinzel T. Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Curr Pharm Des* 2004;10:2807–2816.
- Mukaida N, Morita M, Ishikawa Y, Rice N, Okamoto K, Kasahara T, Matsushima K. Novel mechanism of glucocorticoid-mediated gene repression: nuclear factor- κ B is target for glucocorticoid-mediated interleukin 8 gene repression. *J Biol Chem* 1994;269:13289–13296.
- Webster JC, Cidlowski JA. Mechanisms of glucocorticoid-receptor-mediated repression of gene expression. *Trends Endocrinol Metab* 1999;10:396–402.
- Beato M, Chalepakis G, Schauer M, Slater EP. DNA regulatory elements for steroid hormones. *J Steroid Biochem* 1989;32:737–748.
- Jantzen HM, Strahle U, Gloss B, Stewart F, Schmid W, Boshart M, Miksicek R, Schutz G. Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. *Cell* 1987;49:29–38.
- Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* 1991;352:497–505.
- Beato M. Gene regulation by steroid hormones. *Cell* 1989;56:335–344.
- Meyer T, Carlsted-Duke J, Starr DB. A weak TATA box is a prerequisite for glucocorticoid-dependent repression of the osteocalcin gene. *J Biol Chem* 1997;272:30709.
- Zhang G, Zhang L, Duff GW. A negative regulatory region containing a glucocorticosteroid response element (nGRE) in the human interleukin-1 β gene. *DNA Cell Biol* 1997;16:145–152.
- Pei L. Identification of a negative glucocorticoid response element in the rat type 1 vasoactive intestinal polypeptide receptor gene. *J Biol Chem* 1996;271:20879–20884.
- Weiner FR, Czaja MJ, Jefferson DM, Giambrone MA, Tur-Kaspa R, Reid LM, Zern MA. The effects of dexamethasone on in vitro collagen gene expression. *J Biol Chem* 1987;262:6955–6958.
- Radoja N, Komine M, Jho SH, Blumenberg M, Tomic-Canic M. Novel mechanism of steroid action in skin through glucocorticoid receptor monomers. *Mol Cell Biol* 2000;20:4328–4339.
- Barnes PJ, Pedersen S, Busse WW. Efficacy and safety of inhaled corticosteroids: new developments. *Am J Respir Crit Care Med* 1998;157:S1–S3.
- Wojtczak HA, Kerby GS, Wagener JS, Copenhaver SC, Gotlin RW, Riches DWH, Accurso FJ. Beclomethasone dipropionate reduced airway inflammation without adrenal suppression in young children with cystic fibrosis: a pilot study. *Pediatr Pulmonol* 2001;32:293–302.
- Basbaum C, Lemjabbar H, Longphre M, Li D, Gensch E, McNamara N. Control of mucin transcription by diverse injury-induced signaling pathways. *Am J Respir Crit Care Med* 1999;160:544–548.
- Rose MC, Piazza FM, Chen YA, Alimam MZ, Bautista MV, Letwin N, Rajput B. Model systems for investigating mucin gene expression in airway diseases. *J Aerosol Med* 2000;13:245–261.
- Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev* 2006;86:245–278.
- Kai H, Yoshitake K, Hisatsune A, Kido T, Isohama Y, Takahama K, Miyata T. Dexamethasone suppresses mucus production and MUC2 and MUC5AC gene expression by NCI-H292 cells. *Am J Physiol* 1996;271:L484–L488.
- Lu W, Lillehoj E, Kim KC. Effects of dexamethasone on Muc5ac mucin production by primary airway goblet cells. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L52–L60.
- Newton R, Kuitert LM, Slater DM, Adcock IM, Barnes PJ. Cytokine induction of cytosolic phospholipase A2 and cyclooxygenase-2 mRNA is suppressed by glucocorticoids in human epithelial cells. *Life Sci* 1997;60:67–78.
- Newton R, Seybold J, Kuitert LM, Bergmann M, Barnes PJ. Repression of cyclooxygenase-2 and prostaglandin E2 release by dexamethasone occurs by transcriptional and post-transcriptional mechanisms involving loss of polyadenylated mRNA. *J Biol Chem* 1998;273:32312–32321.
- Wang Z, Zhu Z, Nolfo R, Elias JA. Dexamethasone regulation of lung epithelial cell and fibroblast interleukin-11 production. *Am J Physiol* 1999;276:L175–L185.
- Meerzaman D, Charles P, Daskal E, Polymeropoulos MH, Martin BM, Rose MC. Cloning and analysis of cDNA encoding a major airway glycoprotein, human tracheobronchial mucin (MUC5). *J Biol Chem* 1994;269:12932–12939.
- Kirkham S, Sheehan JK, Knight D, Richardson PS, Thornton DJ. Heterogeneity of airway mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. *Biochem J* 2002;361:537–546.
- Li D, Gallup M, Fan N, Szymkowski DE, Basbaum CB. Cloning of the amino terminal and 5' flanking region of the human MUC5AC mucin gene and transcriptional upregulation by bacterial exoproducts. *J Biol Chem* 1998;273:6812–6820.
- Berger JT, Voynow JA, Peters KW, Rose MC. Respiratory carcinoma cell lines: MUC genes and glycoconjugates. *Am J Respir Cell Mol Biol* 1999;20:500–510.
- Krunkosky TM, Fischer BM, Martin LD, Jones N, Akley NJ, Adler KB. Effects of TNF- α on expression of ICAM-1 in human airway epithelial cells *in vitro*: signaling pathways controlling surface and gene expression. *Am J Respir Cell Mol Biol* 2000;22:685–692.

33. Wu R, Zhao YH, Chang MMJ. Growth and differentiation of conducting airway epithelial cells in culture. *Eur Respir J* 1997;10:2398–2403.
34. Gray TE, Guzman K, Davis CW, Abdullah LH, Nettesheim P. Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial (NHTBE) cells. *Am J Respir Cell Mol Biol* 1996;14:104–112.
35. Ordonez CL, Khashayaz R, Wong HH, Ferrando R, Wu R, Hyde DM, Hotchkiss JA, Zhang Y, Novikov A, Doglanov G, *et al.* Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am J Respir Crit Care Med* 2001;163:517–523.
36. DiFronzo N, Leung CT, Mammel MK, Georgopoulos K, Taylor BJ, Pham QN. Ikaros, a lymphoid-cell-specific transcription factor, contributes to the leukemogenic phenotype of a mink cell focus-inducing murine leukemia virus. *J Virol* 2002;76:78–87.
37. Dorscheid DR, Wojcik KR, Sun S, Marroquin B, White SR. Apoptosis of airway epithelial cells induced by corticosteroids. *Am J Respir Crit Care Med* 2001;164:1939–1947.
38. Stellato C. Post-transcriptional and nongenomic effects of glucocorticoids. *Proc Am Thorac Soc* 2004;1:255–264.
39. Voynow JA, Young LR, Wang Y, Horger T, Rose MC, Fischer BM. Neutrophil elastase increases MUC5AC mRNA and protein expression in respiratory epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 1999;276:L835–L843.
40. Bautista M, Huang L, Letwin N, Rose MC. Interleukin-8 upregulates MUC5/5AC mucin gene expression in airway epithelial cells [abstract]. *Pediatric Pulmonology* 1999;19:296.
41. Treon SP, Mollick JA, Urashima M, Teoh G, Chauhan D, Ogata A, Raje N, Hilgers JH, Nadler L, Belch AR, *et al.* Muc-1 core protein is expressed on multiple myeloma cells and is induced by dexamethasone. *Blood* 1999;93:1287–1298.
42. Imai M, Hwang HY, Norris JS, Tomlinson S. The effect of dexamethasone on human mucin 1 expression and antibody-dependent complement sensitivity in a prostate cancer cell line in vitro and in vivo. *Immunology* 2004;111:291–297.
43. Ishinaga H, Takeuchi K, Kishioka C, Yagawa M, Majima Y. Effects of dexamethasone on mucin gene expression in cultured human nasal epithelial cells. *Laryngoscope* 2002;112:1436–1440.
44. Bernacki SH, Nelson AL, Abdullah L, Sheehan JK, Harris A, Davis CW, Randell SH. Mucin gene expression during differentiation of human airway epithelia *in vitro*. *Am J Respir Cell Mol Biol* 1999;20:595–604.
45. Ray A, Prefontaine KE. Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc Natl Acad Sci USA* 1994;91:752–756.
46. Burnstein KL, Bellingham DL, Jewell CM, Powell-Oliver FE, Cidlowski JA. Autoregulation of glucocorticoid receptor gene expression. *Steroids* 1991;56:52–58.
47. Korn SH, Wouters EFM, Wesseling G, Arends J-W, Thunnissen FBJM. *In vitro* and *in vivo* modulation of α - and β -glucocorticoid-receptor mRNA in human bronchial epithelium. *Am J Respir Crit Care Med* 1997;155:1117–1122.
48. Ito K, Jazrawi E, Cosio B, Barnes PJ, Adcock IM. p65-activated histone acetyltransferase activity is repressed by glucocorticoids. *J Biol Chem* 2001;276:30208–30215.
49. Mornex J-F, Chytil-Weir A, Martinet Y, Courtney M, Lecocq J-P, Crystal RG. Expression of the alpha-1-antitrypsin gene in mononuclear phagocytes of normal and alpha-1-antitrypsin-deficient individuals. *J Clin Invest* 1986;77:1952–1961.
50. Li J-D, Dohrman AF, Gallup M, Miyata S, Gum JR, Kim YS, Nadel JA, Prince A, Basbaum C. Transcriptional activation of mucin by *Pseudomonas aeruginosa* lipopolysaccharide in the pathogenesis of cystic fibrosis lung disease. *Proc Natl Acad Sci USA* 1997;94:967–972.
51. Davies JR, Svitacheva N, Lannefors L, Kornfalt R, Carlstedt I. Identification of MUC5B, MUC5AC and small amounts of MUC2 mucins in cystic fibrosis airway secretions. *Biochem J* 1999;344:321–330.