Glucocorticoid inhibition of human SP-A1 promoter activity in NCI-H441 cells

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Glucocorticoids have complex effects on human surfactant protein (SP) *SP-A1* and *SP-A2* gene expression that occur at both transcriptional and post-transcriptional levels. In the lung adenocarcinoma cell line NCI-H441, dexamethasone causes a dose-dependent decrease in total SP-A mRNA levels and inhibits *SP-A* gene transcription. In this study, a deletional analysis of the *SP-A1* promoter was performed in order to identify *cis*-acting elements that mediate dexamethasone responsiveness in NCI-H441 cells. The region $-32/+63$ relative to the start of *SP-A1* transcription mediated both basal promoter activity and dexamethasone repression of transcription. Removal of the region $+18/+63$ abolished dexamethasone responsiveness, indicating that sequences within this region are necessary for the inhibitory effect. Furthermore, the region $-32/+63$ formed a sequence-specific DNA–protein complex with NCI-H441 nuclear extract. This DNA–protein complex was induced by dexamethasone exposure and its formation was mediated partially by sequences within the region $+26/+63$.

Key words: electrophoretic mobility-shift assay, gene expression, surfactant protein, transcription, transfection.

INTRODUCTION

Pulmonary surfactant is secreted by alveolar type-II cells and lies at the alveolar air–liquid interface where it prevents alveolar collapse by lowering surface-tension forces (reviewed in [1]). Surfactant protein A (SP-A) is the most abundant protein of pulmonary surfactant and is involved in surfactant physiology, structure and metabolism. SP-A also has a major role in lung host defence and inflammatory processes. A number of studies have shown that SP-A is involved in the activation of alveolar macrophages, proliferation of lymphocytes and the production of inflammatory cytokines (reviewed in [2]). SP-A-deficient mice do not exhibit respiratory problems in normal situations but have difficulty in clearing bacteria from their lungs [3].

Human SP-A is encoded by two very similar but non-identical genes, *SP-A1* [4] and *SP-A2* [5]. The two genes are 94 % identical at the nucleotide level and encode proteins with about 96 % identity. The human *SP-A* locus has been mapped to 10q22–23 [6,7] and also contains a non-functional pseudogene [8]. The *SP-A* pseudogene lies between the two human *SP-A* genes, which are approx. 35–40 kb apart [9].

Glucocorticoids have complex effects on human *SP-A* gene expression. In human fetal lung explants, the synthetic glucocorticoid dexamethasone increases SP-A mRNA levels at low concentrations (< 10 nM), but decreases SP-A mRNA and protein levels at higher concentrations (100 nM) [10–15]. Moreover, the degree of inhibition of SP-A1 and SP-A2 expression varies in explant cultures [16–19]. In contrast to the biphasic effects seen in lung explant culture, dexamethasone has only an inhibitory effect on SP-A1 and SP-A2 expression in the lung adenocarcinoma cell line NCI-H441 ([18,20,21] and R. R. Hoover, K. H. Thomas and J. Floros, unpublished work), which occurs partly at a transcriptional level. At 10 nM and after 18 h of exposure, dexamethasone inhibits *SP-A* gene transcription in NCI-H441 cells to about 60 % of control [21].

In order to identify potential *cis*-acting elements involved in the glucocorticoid inhibition of human *SP-A* gene transcription, a deletional analysis of the *SP-A1* promoter was performed in NCI-H441 cells. The region $-32/+63$ relative to the *SP-A1* transcription start site was found to be sufficient for both basal transcription and dexamethasone repression. Removal of the region $+18/+63$ from the *SP-A1* promoter significantly reduced the ability of dexamethasone to inhibit transcription. Furthermore, $-32/+63$ formed a sequence-specific DNA–protein complex with NCI-H441 nuclear extracts that was induced by dexamethasone exposure and partially mediated by sequences within the region $+26/+63$.

EXPERIMENTAL

Cell culture

The lung adenocarcinoma cell line NCI-H441 is presumably of Clara-cell origin, grows as an attatched monolayer with epithelial-cell characteristics, and expresses SP-A and SP-B in a regulated manner [20]. NCI-H441 cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.) and were grown in RPMI 1640 + 10 % heat-inactivated fetal bovine serum (FBS; Summit Biotechnology, Ft. Collins, CO, U.S.A.) and 1 × antimycotic/antibiotic solution (Sigma, St. Louis, MO). Cells were grown in 10-cm² cell-culture dishes in a humidified at-

Abbreviations used: EMSA, electrophoretic mobility-shift assay; DIC, dexamethasone-induced complex; SP, surfactant protein; FBS, fetal bovine serum; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; PAC, P1 artificial chromosome; AP-1, activator protein 1; CRE, cAMP-response element; VIP, vasoactive intestinal polypeptide; GR, glucocorticoid receptor; GRE, GR-binding site; nGRE, negative GRE; NF-κB, nuclear factor κB; TTF-1, thyroid transcription factor 1; TBE1, TTF-1-binding site 1.

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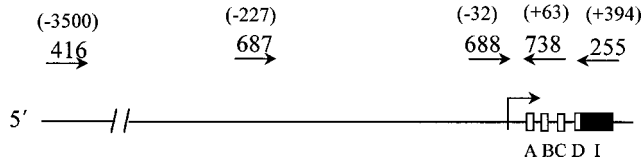

Figure 1 Primers used in the construction of *SP-A1* promoter constructs

The relative position and orientation of primers (arrows) used in generation of *SP-A1* promoter fragments are shown. Positions of primers are relative to the transcriptional start site (bent arrow) and are indicated in parentheses. 5′-Untranslated exons (A–D) are labelled and shown as open boxes; coding exon I is shown as a black box. The Figure is not to scale.

mosphere containing 5% CO_2 and were fed biweekly and passaged weekly.

SP-A1 promoter constructs

Reporter constructs in which expression of chloramphenicol acetyltransferase (CAT) was under the direction of the *SP-A1* promoter were generated by subcloning PCR products representing various fragments of the *SP-A1* 5′-flanking region [22] into the *Sal*I site of the pCATenhancer vector (Promega, Madison, WI, U.S.A.). Unless otherwise noted, nucleotide positions are relative to the SP-A1 transcription-initiation site [23].

PCR was first used to specifically amplify a portion of the *SP-A1* gene in addition to 5′-flanking region using the *SP-A1*-specific primer pair 416/255 (see Figure 1) and genomic DNA from NCI-H441 cells as template. The 416/255 PCR product from the NCI-H441 cell line represents the $6A^4$ allele [23,24]. At the present time we do not have a comparable *SP-A2*-specific primer pair (i.e. that could be used to specifically amplify much of the 5′-flanking region from genomic DNA). Therefore, in order to generate a CAT reporter construct that contained the *SP-A2* promoter, DNA isolated from PAC (P1 artificial chromosome) 307-L19 [9], which contains the *SP-A2* $1A^1$ allele [23,24], was used as template in PCR.

PCR fragments representing various parts of the *SP-A*-flanking region were generated by using primer pairs (Figure 1) 687/255, 688/255, 687/738, 688/738, 688/762 (primer 762 is at position +17 relative to the *SP-A1* transcription start site and is not shown in Figure 1) and the $6A^4$ 416/255 PCR product or PAC 307-L19 ($1A^1$ allele) as template. The sequences of the primers were as follows (5′–3′): 416, CTCTTCCAGGCTTCCATCTTGTCAT; 255, CTCCAAGAAATCAGCGACCTGAG; 687, GGGGGATTTCTCTTCTTACA; 688, ATCTATAAATGCTGCGTCTA; 738, AGGGGATGGGGCTAAACT; and 762, GGGTCTCTGCCTCCAAGT. PCR conditions were dependent on the primer pair used, but consisted typically of 28–30 cycles of a 10 s denaturation at 94 °C, a 30 s annealing at 56–60 °C, and a 30 s–2 min extension at 72 °C. In all PCR reactions a mixture of 1.5 units of AmpliTaq (Applied Biosystems, Foster City, CA, U.S.A.) and 0.5 units of *Pfu* (Stratagene, La Jolla, CA, U.S.A.) DNA polymerases was used.

The PCR products representing *SP-A* promoter fragments were subcloned into the *Sal*I site of pCATenhancer. Prior to ligation, the *Sal*I site of pCATenhancer was repaired using *Pfu* DNA polymerase according to supplier's specifications. Ligations were carried out using standard procedures and were transformed into *Escherichia coli* (XL1 Blue). Colonies containing recombinant plasmid were identified via Southern-blot hybridization using probes specific for the *SP-A* 5′-flanking region. All recombinant plasmids were sequenced to ensure identity and proper insert orientation.

Transient transfections

DNA suitable for transfection was prepared using the Qiagen Plasmid Maxiprep kit (Qiagen, Hilden, Germany) according to manufacturer's specifications. NCI-H441 cells at approx. 80% confluency were transfected with CAT reporter constructs using the calcium phosphate method. Prior to transfection (4 h), the RPMI 1640 + 10% FBS was replaced with Dulbecco's modified Eagle's medium (DMEM) + 10% FBS. CAT reporter construct (10 μg) was combined with 10 μg of the β-galactosidase expression vector, pCMVSPORT–βgal (Gibco Life Technologies, Gaithersberg, MD, U.S.A.). To this mixture, 50 μl of 2.5 M $CaCl_2$ and H_2O for a final volume of 0.5 ml were added. The $CaCl_2$/DNA solution was added dropwise to 0.5 ml of 2 × Hepes-buffered saline (0.28 M NaCl/0.05 M Hepes/1.5 mM Na_2HPO_4) with constant agitation. The resulting 1 ml of precipitate was overlaid on to the NCI-H441 cells. Transfections were carried out overnight in DMEM + 10% FBS, after which time the DMEM was removed and the cells were washed twice with cold PBS. Transfected NCI-H441 cells were incubated in serum-free RPMI 1640 for 8–10 h prior to being treated with either 0.01% ethanol (control) or 100 nM dexamethasone for 18 h.

CAT and β-galactosidase assays

After treatment, transfected cells were washed with and scraped into cold PBS and then centrifuged at 1000 ***g*** for 1 min. Cell pellets were resuspended in about 200 μl of 0.25 M Tris/HCl, pH 8.0. Cells were lysed by three cycles of freeze–thawing. Cell debris was removed by centrifugation and the cell lysates were then assayed for CAT and β-galactosidase activities.

CAT activity was measured using the phase-extraction method [25]. Cell extract (50 μl) was heated at 65 °C for 10 min. To this was added 20 μl of 0.25 M Tris/HCl (pH 8.0), 0.5 μl of 50 mCi/mmol [^{3}H]chloramphenicol (NEN, Boston, MA, U.S.A.) and 5 μl of 5 mg/ml n-butyryl CoA (Sigma). Reactions were incubated at 37 °C for 6–16 h and then terminated by the addition of 300 μl of mixed xylenes (Sigma). Reactions were vortexed for 1 min and centrifuged at 16000 ***g*** for 1 min. The upper (xylene) phase was extracted with 100 μl of 0.25 M Tris/HCl, pH 8.0. The upper (xylene) phase (200 μl) was removed and added to 10 ml of scintillation fluid. CAT activity was determined using a scintillation counter.

In order to correct for transfection efficiency, all CAT reporter constructs were co-transfected with pCMVSPORT-βgal. Thus CAT activity is expressed as a ratio of CAT/β-galactosidase activity. β-Galactosidase activity was determined using a colorimetric assay [26]. Cellular extract (50–100 μl; not heated) was added to 0.5 ml of reagent buffer (60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgCl_2$/50 mM β-mercaptoethanol) and 0.1 ml of *o*-nitrophenyl β-D-galactopyranoside (dissolved in 60 mM Na_2HPO_4/40 mM NaH_2PO_4). Reactions were incubated at 37 °C for approx. 1 h and were terminated by the addition of 0.5 ml of 1 M Na_2CO_3. β-Galactosidase activity was quantified by spectrophotometry at a wavelength of 420 nm.

For all experiments, transfections were performed in duplicate, as were the CAT and β-galactosidase assays. CAT activity was expressed as CAT/β-galactosidase ratios and, for dexamethasone-treated cells, is presented as percentage of control cells. Data from transfections that differed in their duplicate CAT/β-galactosidase ratios by more than 10% were not used.

Preparation of nuclear extracts

Nuclear extracts used in electrophoretic mobility-shift assays (EMSAs) were prepared according to established methods [27], with minor modifications. NCI-H441 cells that had been treated with either 0.01 % ethanol (control) or 100 nM dexamethasone for 18 h were washed with and scraped into cold PBS. We chose 18 h of dexamethasone exposure because this time point lies within the steepest part of a 100 nM dexamethasone time course, where presumably mechanisms responsible for the decrease in SP-A mRNA levels are ongoing. Moreover, the dexamethasone-induced complex (DIC; see results below) formed at all time points tested; 4, 10, 18 and 24 h. Cells were centrifuged at 1000 ***g*** for 2 min at 4 °C and the supernatant was discarded. The cell pellet was resuspended in 500 μl of hypotonic buffer [10 mM Hepes (pH 7.9 at 4 °C)/1.5 mM $MgCl_2$/10 mM KCl/0.2 mM PMSF/0.5 mM dithiothreitol (DTT)] and incubated on ice for 10 min. Cells were then vortexed vigorously for approximately 3 min and then centrifuged at 16000 ***g*** for 10 min at 4 °C. After centrifugation, the supernatant was discarded and the lysed cellular pellet (nuclei) was resuspended on ice in 100 μl of low-salt buffer [20 mM Hepes (pH 7.9 at 4 °C)/1.5 mM $MgCl_2$/0.02 M KCl/25 % glycerol/0.2 mM PMSF/0.5 mM DTT]. An equal volume of high-salt buffer (as low-salt buffer but with 1.0 M KCl) was then added dropwise with constant agitation. Nuclei were extracted by vortexing for 20 min at 4 °C and then centrifuged at 16000 ***g*** for 20 min at 4 °C. Supernatant was aliquoted and immediately frozen in liquid nitrogen. Protein concentrations were determined using the BioRad Bradford assay (BioRad, Hercules, CA, U.S.A.). Nuclear extracts were kept at −85 °C until use.

EMSA

Double-stranded DNA used as probes in EMSA was radiolabelled on 5′ ends with [γ-^{32}P]ATP using T4 polynucleotide kinase as per the specifications of the manufacturer (New England Biolabs, Beverly, MA, U.S.A.). Probes were either PCR products or annealed complementary oligonucleotides. The sequence of the sense oligonucleotide that contained two consensus activator protein 1 (AP-1) binding sites is 5′-GATCCTTCGTGACTC-AGCGGGATCCTTCGTGACTCAGCGG-3′. The sequence of the sense oligonucleotide that contained a consensus glucocorticoid receptor (GR) binding site is 5′-GTTTATGGTTAC-AAACTGTTCTAAAACAAGA-3′. About 25000–50000 c.p.m. (0.2 ng) of radiolabelled DNA probe was incubated with 5.0 μg of nuclear extract in 20 μl of 10 mM Hepes (pH 7.9)/50 mM KCl/0.2 mM DTT/10 % glycerol/0.05 % Nonidet P-40/1 μg of poly(dI-dC) (Sigma). The DNA/protein mixtures were incubated at room temperature for 20 min. DNA–protein complexes were resolved on a 5 % non-denatured polyacrylamide gel. For EMSA competition experiments, competitors were added at 200 molar excess to the reaction mixtures before the addition of DNA probe. For supershift experiments, EMSA was allowed to proceed as before; however, after 20 min of incubation 5 μl (2000 μg/ml) of antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was added to the reaction. Reactions were incubated an additional 15 min at room temperature. For UV-crosslinking experiments, EMSA was performed as before and the reactions were then subjected to 254-nm UV radiation (120000 μJ) for 15 min using a Stratalinker (Stratagene). After exposure to UV light, reactions were digested with 1 unit each of DNase I and micrococcal nuclease for 30 min at 37 °C. Reactions were subjected to electrophoresis through SDS/PAGE (8 % gel) and dried under vacuum.

RESULTS

Dexamethasone inhibition of *SP-A1* promoter activity

Dexamethasone has been shown to decrease total *SP-A* transcription in both human fetal lung explants [13] and NCI-H441 cells [21]. In order to identify *cis*-acting elements that may mediate the transcriptional effects of dexamethasone, PCR fragments representing various deletions of the *SP-A1* (6A^4 allele) promoter were cloned into the *Sal*I site of the pCATenhancer vector. Transient transfection of these constructs into NCI-H441 cells and subsequent exposure of transfected cells to 100 nM dexamethasone for 16–18 h revealed that dexamethasone decreases *SP-A1* promoter activity to about 60–65 % of control (Figure 2). This occurred for all the *SP-A1* promoter constructs shown in Figure 2 (constructs 1–5), but not for a construct in which CAT expression was driven by the simian virus 40 promoter. An *SP-A2* promoter construct, with sequences −287/+394 relative to the *SP-A2* transcription start site, mediated a similar reduction in CAT activity in response to dexamethasone (results not shown).

The results shown in Figure 2 indicate that the region −32/+63 relative to the *SP-A1* transcription start site (shown by arrows in Figure 2) was sufficient to mediate a decrease in CAT activity. The non-recombinant pCATenhancer vector (Figure 2, construct 7) did not exhibit transcriptional activity when transfected into NCI-H441 cells. Thus the sequence −32/+63 of the *SP-A1* promoter was sufficient for both basal transcription and dexamethasone-mediated inhibition of transcription.

Transcriptional activity of the *SP-A1* promoter was previously shown to be reduced significantly upon the removal of the region −227/−31 bp relative to the start of transcription [28–30]. Deletion of this region eliminates the CRE (cAMP-response element)$_{SP\text{-}A}$ site, GT box and the thyroid transcription factor 1 (TTF-1)-binding site (TBE1) site previously identified as having important roles in basal *SP-A2* gene transcription [28–30]. Because removal of these sequences did not effect dexamethasone-mediated repression of *SP-A1* promoter activity (compare the dexamethasone responses of the −227/+394 and −32/+394 constructs in Figure 2), it is unlikely that CRE$_{SP\text{-}A}$, the GC box or TBE1 are involved (or play a major role) in the dexamethasone inhibition of *SP-A1* transcription. Furthermore, it was found by EMSA analysis that dexamethasone had no effect on the binding activity of proteins to these elements (results not shown).

Dexamethasone induction of a protein(s) that binds to −32/+63 of the *SP-A1* promoter

Regulation of transcription is often a result of interactions between *trans*-acting factors and *cis*-acting elements. To determine whether dexamethasone alters the binding of *trans*-acting factors to −32/+63 of the SP-A1 promoter, EMSA was performed using radiolabelled −32/+63 as a probe with nuclear extracts from control and 100-nM-dexamethasone-treated NCI-H441 cells. Figure 3 shows that a number of non-distinct DNA–protein complexes form with nuclear extracts from control cells (Figure 3, lanes 2 and 4). On the contrary, incubation of nuclear extracts from dexamethasone-treated cells (Figure 3, lanes 3 and 5) with −32/+63 forms a single, larger DNA–protein complex. This DIC forms with nuclear extracts from NCI-H441 cells treated with dexamethasone for either 18 or 24 h (Figure 3, lanes 3 and 5, respectively). The results in Figure 3 are representative of EMSA using at least four different nuclear extract preparations. Formation of a complex between proteins induced

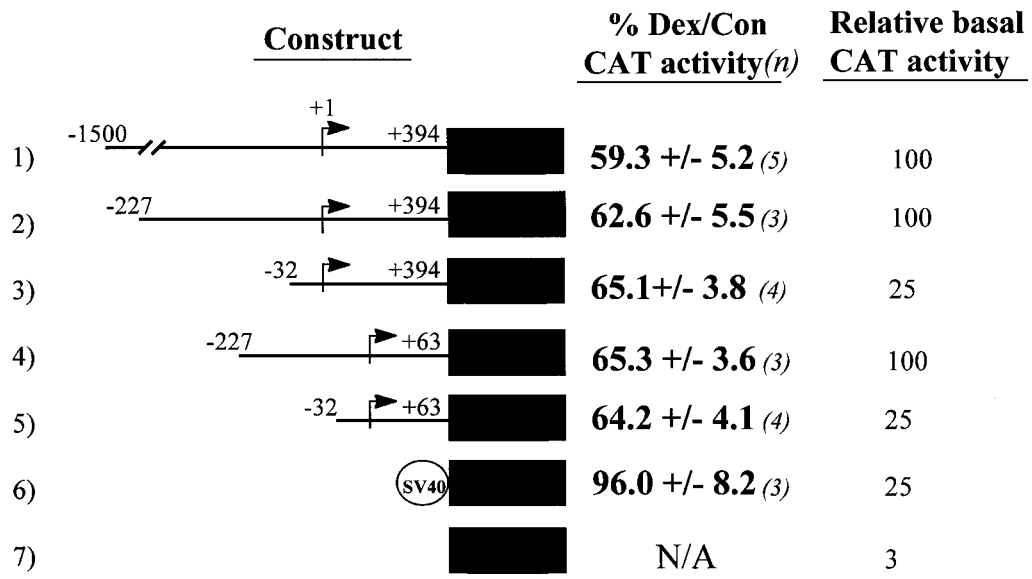

Figure 2 The *SP-A1* promoter mediates a decrease in CAT activity in response to 100 nM dexamethasone (Dex)

NCI-H441 cells were transiently transfected in duplicate with CAT reporter contructs under the direction of various fragments of the *SP-A1* promoter; black box represents CAT CDNA. Cells were co-transfected with a β-galactosidase expression vector in order to correct for transfection efficiency. CAT activity is expressed as a CAT/β-galactosidase ratio. CAT activity of cells treated with 100 nM dexamethasone for 18 h is presented as percentage of that found in control (Con) cells (treated with 0.01% ethanol). Deletional analysis of the *SP-A1* 5′-flanking region indicates that a *cis*-acting element(s) within the region $-32/+63$ relative to the start of transcription (bent arrow) is able to mediate transcriptional repression by dexamethasone. The transcriptional activity of the simian virus 40 (SV40) promoter was not affected by dexamethasone (construct 6). The relative basal CAT activity of the constructs is also shown, and reduction of transcription is seen with the removal of $-227/-33$. The promoter-less pCATenhancer vector (construct 7) had little transcriptional activity. N/A, not applicable (basal CAT levels not significantly above background). Values are presented as means $\pm$ S.E.M., with sample sizes, *n*, shown in parentheses.

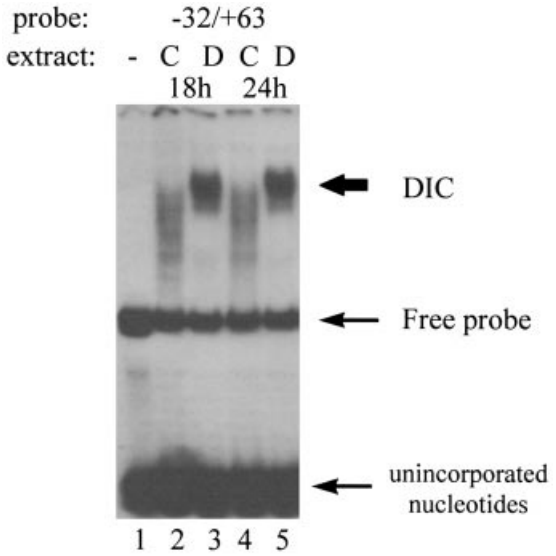

Figure 3 EMSA with $-32/+63$ of *SP-A1* as probe and nuclear extracts from control and dexamethasone-treated NCI-H441 cells

Nuclear extracts from NCI-H441 cells that were treated with 100 nM dexamethasone (D) or 0.01% ethanol (control, C) were used in EMSA. A single distinct DNA–protein complex forms upon incubation of radiolabelled $-32/+62$ with 5 μg of nuclear extract from NCI-H441 cells treated with 100 nM dexamethasone for 18 h (lane 3) or 24 h (lane 5). DNA–protein complexes were resolved on a 5% non-denaturing polyacrylamide gel. The DIC is indicated by a thick arrow. Free probe and un-incorporated nucleotides are indicated by thin arrows.

by dexamethasone and $-32/+62$ of *SP-A* supports the speculation that this region is involved in the dexamethasone inhibition of *SP-A* gene expression at a transcriptional level. The interaction between sequences within $-32/+63$ and proteins induced by dexamethasone treatment may inhibit *SP-A1* promoter activity by interfering with transcription initiation or elongation.

Identification of sequences necessary for the dexamethasone induced DNA–protein complex

EMSA competition analysis was performed to better define the sequences necessary for DIC formation. The DIC represents a protein (or proteins) bound specifically to $-32/+63$, since its formation was abolished by the presence of excess unlabelled $-32/+63$ as competitor (Figure 4, lane 4). On the contrary, the presence of non-specific competitor (an oligonucleotide that contains two AP-1 consensus binding sites) did not have any effect on DIC formation (Figure 4, lane 5). Competition with an oligonucleotide that contained a consensus binding site for the GR also did not have any affect on DIC formation (Figure 4, lane 6), suggesting that the GR does not directly bind to $-32/+63$. Furthermore, addition of an antibody against GR to the EMSA reaction did not cause a supershift or affect DIC formation (Figure 4, lane 8), indicating that GR is not a component of the DIC.

Competition with excess oligonucleotides spanning the region $-32/+62$ revealed that sequences within $+26/+63$ of *SP-A1* were necessary for DIC formation, because the presence of $+26/+63$ as competitor abolished complex formation (Figure 4, lane 7). Moreover, there was no competition with an oligonucleotide that spanned the region $+2/+34$ (results not shown), suggesting that sequences $+35/+63$ of *SP-A1* are necessary for DIC formation or that the protein-binding site(s) is not completely included within $+2/+34$ (see below).

No DIC was seen when $-32/+17$ was used as probe in EMSA (Figure 5, lanes 5 and 6), indicating that sequences

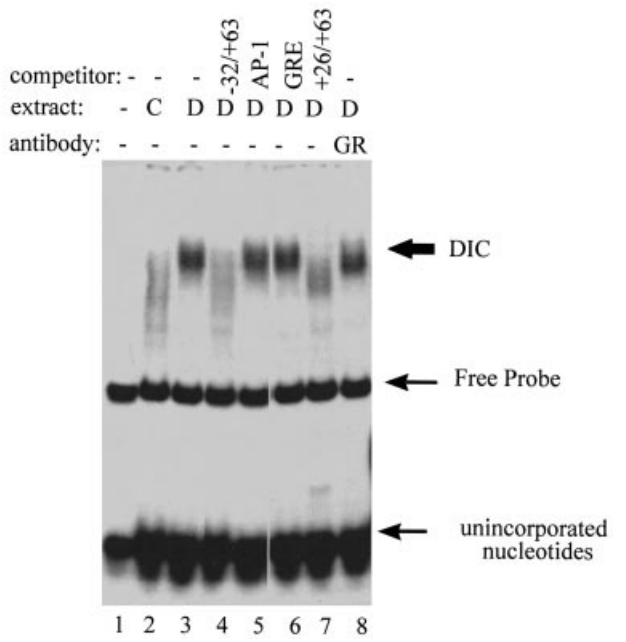

Figure 4 EMSA competition and supershift analyses of the DIC

EMSA was performed with probe −32/+63 in the presence of a 200 molar excess of unlabelled competitors that contained consensus transcription-factor-binding sites or spanned various regions of the *SP-A1* promoter. In some cases 5 μl of 2000 μg/ml antibody to the GR was included in the reaction. DIC (thick arrow) formation was only abolished with unlabelled −32/+63 (lane 4) or an unlabelled oligonucleotide spanning +26/+63 (lane 7). An oligonucleotide containing two consensus AP-1-binding sites or a consensus GR-binding site (GRE) did not abolish DIC formation (lanes 5 and 6, respectively), indicating that GR does not directly bind to the area −32/+63. Oligonucleotides that span −32/+2, −8/+23 or +2/+34 also did not abolish DIC formation (results not shown). Antibody to GR did not affect the mobility of the DIC (lane 8), indicating that GR is not a component of the DIC. C, nuclear extracts from control untreated cells; D, extracts from dexamethasone-treated cells.

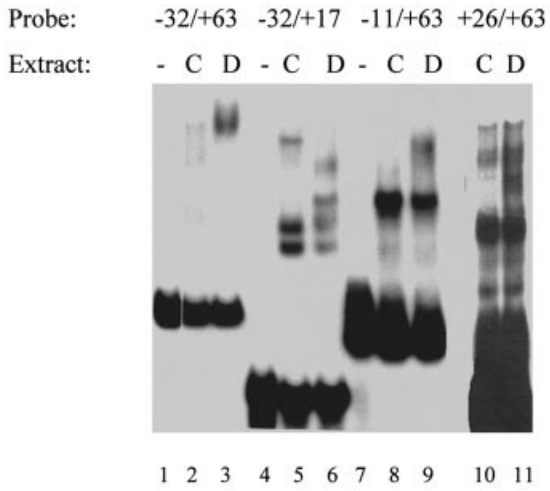

Figure 5 EMSA analysis using overlapping segments spanning −32/+63 as probes

DIC formation was only seen with the *SP-A1* −32/+63 probe, and not with +26/+63 (lanes 10 and 11), although this region abolished DIC formation when used as competitor. No readily obvious DIC was formed with probes −32/+17 (lanes 5 and 6) or −11/+63 (lanes 8 and 9), although differences in protein binding to these probes between control (C) and dexamethasone (D) nuclear extracts appear to exist.

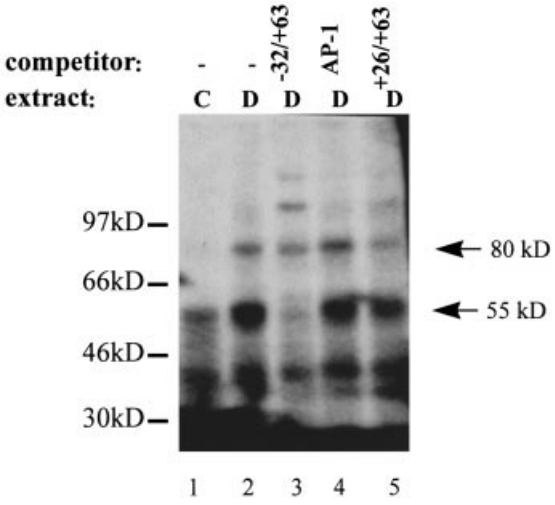

Figure 6 UV crosslinking of control (C) and dexamethasone (D)-induced nuclear proteins to −32/+63

After EMSA, reactions were subjected to exposure to 120 000 μJ of UV radiation (254 nm) for 15 min, then digested with 1 unit each of DNase I and micrococcal nuclease. The UV-crosslinked proteins were fractionated by SDS/PAGE (8% gel). Two proteins with approximate sizes of 55 and 80 kDa (arrows) appeared with increased intensity on dexamethasone (D) treatment (lane 2). The intensity of both of these complexes was reduced by competition with a 200 molar excess of unlabelled −32/+63 (lane 3), but not with an oligonucleotide containing two AP-1 consensus binding sites (lane 4). Only the 80 kDa complex was diminished by competition with a 200 molar excess of unlabelled +26/+63 (lane 5). A complex of lower molecular mass, which is also competed by excess −32/+63 was also seen. C, control nuclear extract.

further downstream of the transcription start site were involved in DIC formation. Surprisingly, when EMSA was performed using radiolabelled +26/+63 as a probe, there was no difference in complex formation between nuclear extracts from control and dexamethasone-treated NCI-H441 cells (Figure 5, lanes 10 and 11).

UV crosslinking of the proteins in nuclear extracts from control and dexamethasone-treated NCI-H441 cells to the radiolabelled probe −32/+63 was performed following EMSA. Fractionation of the resulting DNA–protein complexes through an SDS/PAGE gel revealed that two complexes, with apparent molecular masses of approx. 55 and 80 kDa (Figure 6), had increased intensity when using nuclear extracts from dexamethasone-treated NCI-H441 cells. A smaller complex with an apparent molecular mass of approx. 35 kDa was also seen, but had about the same intensity in nuclear extracts from both control and dexamethasone-treated NCI-H441 cells. The intensities of both the 55- and 80-kDa complexes were reduced when excess unlabelled −32/+63 was used as competitor (Figure 6, lane 3). The intensity of only the higher-molecular-mass complex was reduced when +26/+63 was used as competitor (Figure 6, lane 5). Competition with an oligonucleotide that contained two AP-1 consensus binding sites did not affect the formation of either of the 35-, 55- or 80-kDa complexes (Figure 6, lane 4). The size of the smallest complex was consistent with that of the TATAA-box-binding protein (32 kDa); however, the uppermost band appeared to be too small to be the GR homodimer (95 kDa).

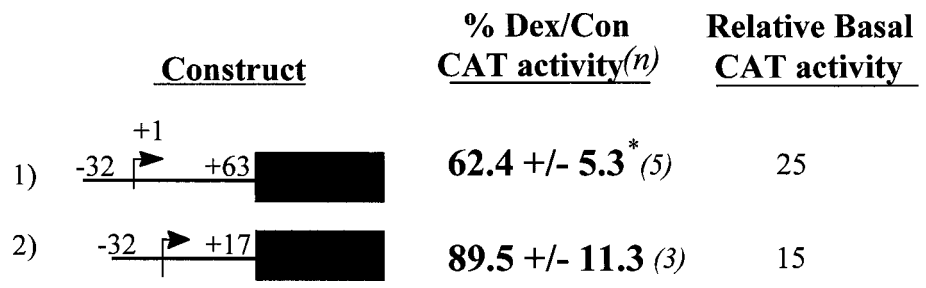

Figure 7 Reduction of dexamethasone response for an *SP-A1* promoter that lacks +18/+63

Transfected NCI-H441 cells were treated with 0.01% ethanol (control, Con) or 100 nM dexamethasone (Dex). Removal of the region +18/+63 from the human *SP-A1* construct diminishes the dexamethasone-mediated repression of transcription; black box represents CAT cDNA. The arrows indicate the transcriptional start site. $^*P < 0.05$ (by analysis of variance), when compared with human −32/+17 (construct 2).

Deletion of *SP-A1* sequence +18/+63 abolishes dexamethasone-mediated transcriptional repression

To determine what area of −33/+63 was necessary for the dexamethasone-mediated inhibition of *SP-A1* transcription, a PCR fragment that spanned −32/+17 of the *SP-A1* promoter was cloned into the *Sal*I site of pCATenhancer and transiently transfected into NCI-H441 cells. Figure 7 shows that there is no significant dexamethasone inhibition of CAT activity when transcription is driven by the −32/+17 human *SP-A1* promoter, indicating that sequences within +18/+63 of *SP-A1* are necessary for transcriptional repression by dexamethasone. These data are consistent with the fact that a DIC does not form when we used the −32/+17 as an EMSA probe (Figure 5), and supports the speculation that DIC formation is necessary for transcriptional repression. Removal of the sequence +18/+63 of *SP-A1* also decreased the basal CAT activity (Figure 7).

DISCUSSION

Glucocorticoids have powerful anti-inflammatory and lung-maturational properties. Their effects on *SP-A* gene expression are of particular interest because of the roles that SP-A has in lung host defence and surfactant physiology. The synthetic glucocorticoid dexamethasone has complex effects on human *SP-A* gene expression that occur at both transcriptional and post-transcriptional levels. In the human adenocarcinoma cell line NCI-H441, dexamethasone decreases total SP-A mRNA levels in a dose- and time-dependent manner ([18,20,21] and R. R. Hoover, K. H. Thomas and J. Floros, unpublished work). In this report, evidence was presented to indicate the presence of a negative dexamethasone-responsive element(s) within the human *SP-A1* promoter.

Deletional analysis of the *SP-A1* promoter was used to identify *cis*-acting elements involved in the dexamethasone inhibition of human *SP-A* transcription. The region −32/+63 relative to the *SP-A1* transcription start site was shown to be sufficient for both basal transcription and dexamethasone responsiveness. Previous studies [28–30] have shown that three distinct elements within the human/baboon *SP-A* promoter are necessary for basal transcription: a CRE-like site, a GT box and a TTF-1 binding site. In the present study it was shown that basal transcriptional activity was reduced approx. 4-fold upon removal of −227/−31 (which contains the three aforementioned *cis*-acting elements) from the *SP-A1* promoter (Figure 3). The reduction of *SP-A1* promoter activity upon removal of −227/−31 is similar to that seen when either the $CRE_{SP\text{-}A}$ site [24] or the GT box [29] is removed from the *SP-A2* promoter. In the functional analysis of the *SP-A2* $CRE_{SP\text{-}A}$ site and GT box, primary cultures of human alveolar type-II cells were infected with adenoviral human growth hormone reporter constructs, whereas in the present study NCI-H441 cells and CAT reporter constructs were used. The correlation between the studies of Young and Mendelson [28,29] and the present study with regard to a decrease in *SP-A* promoter activity, upon removal of sequences 5′ of the TATAA box, supports the validity of the system used here. Thus it appears that basal *SP-A* transcription is regulated similarly in both primary alveolar type-II and NCI-H441 cells. Furthermore, the conclusions reached in the present study are likely to be independent of the reporter gene (human growth hormone versus CAT), the method of DNA introduction (infection versus transfection), or the vector (adenovirus versus pCATenhancer) used. Although removal of −227/−31 reduces *SP-A1* promoter activity, constructs containing only −32/+63 still remain transcriptionally active.

The region −32/+63 of the *SP-A1* promoter was found to be sufficient for dexamethasone-mediated inhibition of CAT activity. Transient transfection of NCI-H441 cells with CAT reporter constructs containing the region −32/+63 and subsequent exposure to 100 nM dexamethasone for 18 h resulted in a reduction in CAT activity to about 65% of control. A similar result was obtained when using an *SP-A2* promoter construct (−227/+63), suggesting that, at the level of transcription, *SP-A1* and *SP-A2* are not regulated differentially by dexamethasone. This is in spite of the fact that recent studies have shown that *SP-A1* is more responsive to dexamethasone treatment in the NCI-H441 cell line [17,18]. However, whereas the *SP-A1* promoter constructs were derived directly from the NCI-H441 cell line, the *SP-A2* promoter construct was derived from an *SP-A2* genomic clone (allele $1A^1$), described previously [9]. It is possible that sequence differences between the *SP-A2* promoters from NCI-H441 cells and the genomic clone could account for this discrepancy. With regard to the inhibitory effect of dexamethasone, studies using fetal lung explants have shown that *SP-A1* alleles are more responsive to dexamethasone [16], that the *SP-A2* gene is more responsive [19], or that both *SP-A* genes are equally responsive to dexamethasone [18]. Thus apparent discrepancies among these studies may be a result of differential allelic regulation, with certain *SP-A1* or *SP-A2* alleles responding differently.

EMSA analysis showed that the region *SP-A1* −32/+63 formed a complex with a dexamethasone-inducible protein(s), and that the region +26/+63 was necessary for complex formation. Suprisingly, +26/+63 was not sufficient for DIC formation. One would expect that since +26/+63 is able to

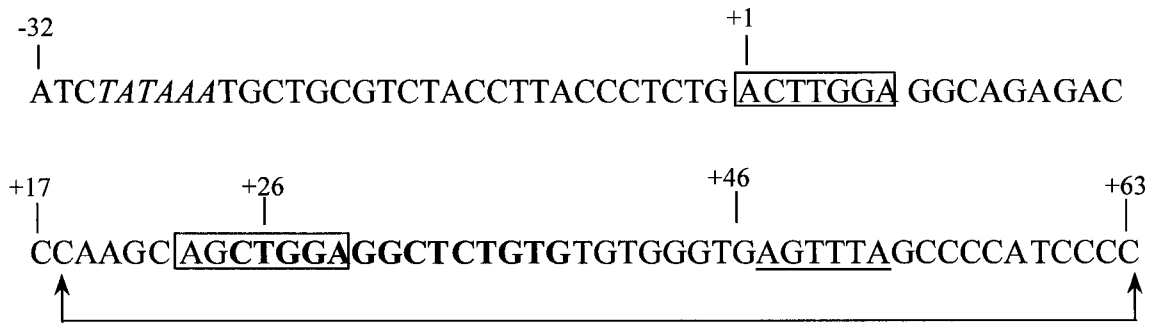

Figure 8 The region of the human *SP-A1* promoter that mediates repression of transcription by dexamethasone in NCI-H441 cells

Nucleotide positions are marked relative to the transcription start site (+1). The TATAA box is shown in italics. Sequences similar to an inverted nGRE found in the promoter of the interleukin-1β and pro-opiomelanocortin genes (AGGTCA) are boxed. Sequence similar to a nGRE found in the rat VIP-receptor promoter (CTGGAGCTTCGCCTC) is shown in bold. A sequence similar to a nGRE in the gonadotropin-releasing-hormone promoter (AGTTTT) is underlined. The sequence that abrogates dexamethasone responsiveness when removed from the *SP-A1* promoter is bracketed by arrows.

completely abolish DIC formation, it would contain the entire protein(s)-binding site. It is possible that, although sequences within +26/+63 are necessary for DIC formation, they are not sufficient; additional flanking sequences (most likely 5′ of position +26) may also be needed. The region −32/+63 contains the core promoter of *SP-A1*, including the TATAA box and the initiator region. Therefore it is possible that interactions between proteins that bind to these areas and a dexamethasone-induced factor that recognizes sequences within +26/+63 are necessary for DIC formation. Indeed, removal of −32/−10, which would include the TATAA box, from the −32/+63 EMSA probe eliminates DIC formation (Figure 5, lanes 8 and 9). It is also possible that a certain-size EMSA probe (such as the 95 bp of the −32/63 probe) is needed for DIC formation. In fact, when DNase-I footprinting studies were performed, almost the entire 95 bp of the region −32/+62 appeared to be protected from DNase by proteins in dexamethasone nuclear extract (results not shown). Interactions between the 55 and 80 kDa protein complexes, identified by UV crosslinking, with components of the basal transcriptional apparatus may be required for complete DIC formation and thus inhibition of *SP-A1* and *SP-A2* transcription.

Removal of +18/+63 from the human *SP-A1* promoter abolished dexamethasone inhibition *in vivo*, indicating a correlation between DIC formation and dexamethasone responsiveness. Interestingly, removal of +18/+63 also reduced the basal activity of the *SP-A1* promoter, suggesting the presence of additional positive elements within this area. However, removal of these sequences would also shorten the 5′-untranslated region of the CAT mRNA, potentially affecting translational efficiency. It is possible that the dexamethasone-induced binding of proteins (possibly the 80-kDa protein identified by UV crosslinking) to +18/+63 affects transcription elongation or initiation by interfering with the interaction of DNA sequences within this region and positive regulators of transcription.

Glucocorticoid induction of gene expression has been relatively well characterized and results from the binding of homodimeric GR to a GR-binding site (GRE) that has the consensus sequence AGAACANNNTGTTCT [31,32]. By contrast, inhibition of gene transcription by glucocorticoids is not understood, but may come about through GR binding to the promoter and interfering with the DNA binding of positive regulating proteins. However, many genes that are negatively regulated by glucocorticoids do not have a consensus GRE within their promoter. Moreover, there does not appear to be a consensus negative GRE (nGRE) for the genes where sequences necessary for glucocorticoid inhibition have been identified. It is of interest to note that there is some similarity between sequences found within human *SP-A1* +18/+63 (Figure 8) and nGREs identified in other genes.

Glucocorticoids decrease the transcriptional activity of the rat type-I vasoactive intestinal polypeptide (VIP)-receptor promoter, and a model has been proposed in which a nGRE is involved in both dexamethasone responsiveness and basal transcription [33]. In this model, the general transcription apparatus interacts with the nGRE, and thus removal of the nGRE also reduces basal transcription (as appears to be the case with SP-A1). Interestingly, a sequence similar to the VIP receptor nGRE (CTGGAGCTTCGCCTC) is found within the region +18/+63 of the *SP-A1* promoter (CTGGAGGCTCTGTG; Figure 8). However, in the case of the rat VIP-receptor promoter, direct binding of activated GR to the nGRE mediates transcriptional repression [33]. This does not appear to be the case for the human *SP-A1* promoter because competition EMSA analysis and supershift analysis indicated that GR does not bind directly to −32/+63 and is not a component of the DIC.

The region +18/+63 also contains a sequence in reverse orientation that is similar to a nGRE found within the promoters of the interleukin-1β [34] and pro-opiomelanocortin [35] genes (AGGTCA). It has been demonstrated that the nGRE of the pro-opiomelanocortin gene promoter binds to a unique complex composed of three GR monomers, one of which binds to the opposite strand of DNA [35]. Glucocorticoid repression of interleukin-1β transcription has been suggested to occur through GR blocking of an AP-2 site found directly next to the nGRE [34]. There is no corresponding AP-2 binding sequence within −32/+63 of *SP-A1*. The region +18/+63 also contains a sequence similar to a nGRE (AGTTTT), identified in the mouse gonadotropin-releasing-hormone gene promoter [36], which does not bind directly to GR (although GR is a component of a multimeric complex that binds to elements within the promoter). The direct involvement of any of these sequences, either individually or co-operatively, in the dexamethasone inhibition of human *SP-A* gene transcription is not known.

Another way in which glucocorticoids decrease transcription is by binding directly to positive regulators of transcription, such as the transcription factors AP-1 [37] and nuclear factor κB (NF-κB) [38], thus preventing their interaction with promoter elements and/or translocation to the nucleus. Glucocorticoids can also

inhibit transcription of genes driven by NF-κB, by increasing the levels and binding of an inhibitor to NF-κB, inhibitory $_{\kappa}$Bα [39]. There is presently no evidence that human *SP-A* gene transcription is regulated by NF-κB. Dexamethasone could possibly affect human *SP-A1* and *SP-A2* gene transcription by preventing the binding of proteins to the $CRE_{SP\text{-}A}$ site, the GT box or the TTF-1-binding site. However, this is unlikely since dexamethasone inhibition of *SP-A1* promoter activity also occurs in constructs that lack these sites.

In summary, the region $-32/+63$ of human *SP-A1* is sufficient to mediate both basal promoter activity and dexamethasone repression of transcription. Removal of the region $+18/+63$ abolished dexamethasone responsiveness, indicating that sequences within this region are necessary for the inhibitory effect. The region $-32/+63$ is also sufficient for the formation of a DIC, and sequences within $+26/+63$ are involved in the complex formation. However, $+26/+63$ alone is not sufficient to form a DIC, suggesting that sequences upstream of $+26$ are also necessary. It is possible that dexamethasone inhibition of *SP-A1* and *SP-A2* transcription comes about through complex interactions among *trans*-acting factors induced by dexamethasone, *cis*-elements within the region $-32/+63$ and the basal transcriptional machinery.

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