Cigarette Smoke Induces MUC5AC Protein Expression through the Activation of Sp1*

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Background: Cigarette smokers have increased mucus secretion and MUC5AC gene expression.

Results: Cigarette smoke increases Sp1 protein expression and activates Sp1 binding to a smoke-responsive promoter region of the MUC5AC.

Conclusion: Sp1 is the key regulator of cigarette smoke-induced MUC5AC mRNA transcription in lung epithelial cells.

Significance: Sp1 may be a putative target to treat mucus hypersecretion in COPD patients.

Cigarette smoke (CS) exposure is associated with increased mucus production and chronic obstructive pulmonary disease (COPD). MUC5AC is the major inducible mucus gene in the airway. The purpose of this investigation was to elucidate the mechanisms of CS-induced activation of MUC5AC gene transcription. We observed that the region −3724/−3224 of the MUC5AC promoter is critical for CS-induced gene transcriptional activity and that this region contains two Sp1 binding sites. Using a lung-relevant model, we observed that CS increased nuclear Sp1 protein expression. Consequently, CS exposure resulted in enhanced Sp1-DNA binding activity and Sp1 trans-activation. Co-transfection of the MUC5AC-luc reporter with Sp1 expression plasmids resulted in significantly increased MUC5AC-luc activity, whereas co-treatment with mithramycin A, a Sp1 inhibitor, abolished CS-induced MUC5AC promoter activity. Using mobility shift assay and chromatin immunoprecipitation, we demonstrated that two Sp1 binding sites in the MUC5AC promoter are functional and responsive to CS exposure. A mutation of either Sp1 binding site in the MUC5AC promoter significantly decreased CS-induced promoter activity. Together, these data indicate that CS induces MUC5AC gene transcription predominantly through increased Sp1 nuclear protein levels and increased Sp1 binding to its promoter region.

Excessive airway mucus is a hallmark feature of several chronic airway diseases including chronic obstructive pulmonary disease (COPD), a disease predominantly caused by cigarette smoke (CS) exposure. This pathophysiologic expression of airway mucus results in acute exacerbations of disease, accelerated loss of lung function, and increased risk of respiratory tract infections (1, 2). Mucus metaplasia, mucus gland hypertrophy, and mucus hypersecretion have all been proposed to be primary mechanisms leading to excessive airway mucus (3–5). Efforts to understand the mechanisms that lead to excessive airway mucus potentially will reveal novel therapeutic targets to improve care for patients with COPD.

Although the link between CS exposure and COPD has been widely accepted, the molecular mechanisms underlying CS-associated alterations in mucociliary clearance and mucus production are not well characterized. Cigarette smoke is a complex mixture of >4,000 chemicals that contains both volatile (acrolein, naphthalene, various aldehydes) and nonvolatile (afloxin B1, benz[a]pyrene, phorbol ester analogs) components (6–8). Therefore, CS likely has pleiotrophic effects on the respiratory tract epithelium. Because current smokers and COPD patients express significantly higher levels of MUC5AC in their respiratory epithelium (9, 10), we postulated that CS-induced mucus hypersecretion of MUC5AC was an important contributor to disease pathogenesis or COPD exacerbations.

Mucins, a subset of glycoproteins, are the major secretory components of mucus produced by respiratory tract epithelia. To date, at least 20 primary human mucin genes (MUCs) have been identified, which are structurally classified as membrane-bound or secreted (11–13). Two gel-forming secreted mucins, MUC5B and MUC5AC, are the most abundant mucins expressed in the respiratory tract; MUC5B is constitutively expressed in the airway whereas MUC5AC is highly inducible (14). Because it is highly inducible, exquisitely responsive to environmental challenges, and is the most sensitive to pathophysiologic changes in the lung, MUC5AC is considered to be the critical mucin in the development of pulmonary disease (15, 16). For these reasons, we evaluated the mechanisms responsible for CS-induced overexpression of MUC5AC. Using various promoter analysis techniques, we were able to identify a specific CS-responsive region on the MUC5AC promoter and the essential transcription factor sites that are responsible for CS-inducible MUC5AC expression.

EXPERIMENTAL PROCEDURES

Sources of Human Airway Tissues and Cells—Human lung tissue was obtained from the University of Pittsburgh Medical Center with consent. The Human Subject Institutional Review
Board of the University of Pittsburgh approved all procedures involved in tissue procurement. Excised tissues were transported to the laboratory in ice-cold minimal essential medium (Sigma). Primary human bronchial epithelial (HBE) cells were isolated from these tissues by a protease-dissociation procedure and cultured in serum-free hormone-supplemented medium as described previously (17).

**Cell Line and Cell Culture**—Human A549 cells, a lung epithelial cell line derived from human lung carcinoma with characteristics of type II alveolar epithelial cells, were obtained from ATCC (ATCC®CCL-185). A549 cells were propagated and maintained in F12 medium supplemented with 2 mM glutamine, adjusted to contain 1.5 g/liter sodium bicarbonate and 10% fetal bovine serum.

**Reagents**—The chemical inhibitor mithramycin A was purchased from Tocris (Ellisville, MO), cycloheximide and actinomycin D were obtained from Sigma, and all restriction enzymes were purchased from Promega (Madison, WI). Synthetic oligonucleotides were purchased from IDT (Coralville, IA). Anti-Sp1 antibody was obtained from Millipore (Billerica, MA), and anti-GAPDH antibody was purchased from Novus Biologicals (Littleton, CO). All other common chemical were purchased from Sigma.

**Cigarette Smoke Extract (18) and in Vitro Cigarette Smoke Exposure**—Research-grade 2R1 cigarettes without filters were obtained from the University of Kentucky Cigarette Research and Development Center (Lexington, KY). To obtain consistent smoke exposure for all experiments, cigarette smoke extract (CSE) was freshly prepared by bubbling one cigarette through 5 ml of serum-free F12 medium. This “100%” CSE solution was adjusted to pH 7.4 and filtered through a 0.22-μm filter prior to treatment of cells. Cells were grown in either 12.5-cm² (for transfections) or 25-cm² (for protein and RNA isolation) Falcon flasks (BD Biosciences) to 80% confluence, serum-starved for 16 h, and then exposed to various concentrations of CSE and incubated at 37 °C with 5% CO₂ for various time periods. In experiments utilizing chemical inhibitors, cells were pretreated with desired concentrations of chemical inhibitors for 2 h prior to CSE exposure.

**RNA Extraction and Real-time RT-PCR**—Cells were harvested for total RNA isolation using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Real-time reverse transcription (RT) quantitative PCR was performed on an ABI PRISM® 7700 Sequence Detection System (Foster City, CA) with MUC5AC-specific oligonucleotide primers and probe. TaqMan probes were labeled with carboxyfluorescein (FAM) at the 5’-end and with the quencher carboxytetramethylrhodamine (TAMRA) at the 3’-end. Validation experiments were performed to confirm equivalent PCR efficiencies for the targeted gene, and all reactions were performed in triplicate. Relative quantity of MUC5AC mRNA was measured using a comparative cycle of threshold method, and results were normalized against glucuronidase-β (Assays on Demand; Applied Biosystems) for endogenous control and expressed as relative -fold compared with control. The primers and probe for MUC5AC were as follows: forward, 5’-CGTGTGTTCACCGAGAACGT-3’; reverse, 5’-ATCTTGTGCGCTTTGAGCA-3’; and probe, 5’-CTGCCACCACAGGGACCA-3’.

**Protein Extraction**—Nuclear and cytoplasmic protein extraction from cells were performed using a NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce) according to the user’s manual. Briefly, cells were washed twice with cold phosphate-buffered saline (PBS), and cell pellets were collected by centrifugation at 500 × g for 3 min. 200 μl of ice-cold reagent, CER-I, was added to cell pellets and vigorously vortexed for 15 s. After incubation on ice for 10 min, 11 μl of ice-cold CER-II was added to the reaction and incubated on ice for 1 min. After centrifugation at >16,000 × g for 5 min, the supernatant was collected for cytoplasmic protein. The insoluble pellet, which contained the nuclei, was resuspended in 100 μl of ice-cold nuclear extraction reagent (NER) and incubated on ice for 40 min with repeated vortexing for 15 s every 10 min. The vortexed solution was centrifuged at ~16,000 × g, and the supernatant was collected for nuclear protein.

**Electrophoretic Mobility Shift Assays (EMSAs)**—Cells were exposed to CS, nuclear extracts were prepared, and EMSAs were performed using biotin-labeled double-stranded consensus probes (see Table 1). Detection of protein/oligonucleotide complexes was performed using an EMSA Gel-Shift kit (Pamomics, Redwood City, CA). Briefly, nuclear protein (5 μg) was incubated with 10 ng/μl of biotin-labeled oligonucleotide for 30 min at room temperature in 5× binding buffer and 100 ng of poly(dI-dC). The specificity of the DNA/protein binding was determined in competition reactions in which a 50-fold molar excess of unlabeled oligonucleotide was added to the binding reaction. Binding reaction products were resolved by electrophoresis on a 6% polyacrylamide gel using 0.5× Tris borate-EDTA buffer followed by electrophoresis to a nylon membrane (Schleicher & Schuell, Keene, NH). After incubation in blocking buffer for 15 min at room temperature, membranes were incubated with streptavidin-horseradish peroxidase conjugate for 30 min at room temperature. After membranes were washed again, peroxidase activity was visualized using SuperSignal chemiluminescence reagent (Pierce).

**Immunoblot and Immunofluorescence Analyses**—Cells were exposed to CS (18) for various time points and washed three times with chilled PBS containing 1 mM Na₂VO₃. Cytoplasmic and nuclear protein was prepared using NE-PER Nuclear and Cytoplasmic Extraction kit as described above. Equal protein amounts (40 μg) were resolved by 12% discontinuous SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) at room temperature for 1 h followed by incubation with anti-Sp1 (rabbit) polyclonal antibody overnight at 4 °C. Membranes were then washed three times with TBST before probing with horseradish peroxidase-conjugated (anti-rabbit) secondary antibodies for 1 h at room temperature. Blots were visualized with SuperSig-

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**TABLE 1**

| EMSA probes and nucleotide sequence for each probe | Probe name | Sequence (5’-3’) |
|--------------------------------------------------|------------|-----------------|
| Sp1                                              | ATTCCATCCGCGGCAGG |                 |
| MUC5AC-Sp1A                                      | CTGTTGCGCGGCAAACTC |                 |
| MUC5AC-Sp1B                                      | CTGGGAATCCAGGAGACCCCTT |             |
| MUC5AC-Sp1B                                      | CTGGAGGCGCTAAGAGGCCGCTT |             |
| MUC5AC-Sp1B                                      | CTGTTATGTCATGACATGCCTT |             |

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nal chemiluminescence reagent. To ensure equal protein loading, membranes were stripped and probed with anti-GAPDH antibody.

For immunofluorescence analyses, A549 cells were seeded and covered slips in T25 flask. After overnight serum-starved conditions, cells were directly exposed to 3% CSE for 4 h. Cells were washed with PBS, fixed with −20 °C methanol for 10 min, washed in PBS (3 × 5 min), and blocked with 10% normal goat serum/1% BSA in PBS for 1 h at room temperature. After one wash with PBS, cells were incubated with anti-Sp1 (rabbit) polyclonal antibody overnight at 4 °C. The coverslips were then washed and incubated with fluorescently labeled goat anti-rabbit secondary antibody. Nuclei were stained with DAPI to identify individual cells.

Plasmid Constructs—Plasmid cytomegalovirus (pCMV)-Sp1 expression vector was generously provided by Horowitz (19, 20). The MUC5AC promoter luciferase construct, containing 3.7 kb of the 5′-flanking region of the human MUC5AC gene, was a kind gift from Basbaum (21, 22). Deletion constructs were obtained by combining restriction digestion with PCR using gene-specific primers. The MUC5AC-3.3 construct was created by cloning the MUC5AC promoter/enhancer sequence from −3.7 kb to −3.3 kb upstream of the MUC5AC transcription start site into the pTAL luciferase vector via two NheI and BglII restriction enzyme sites. We created several point mutations in the MUC5AC promoter-reporter construct using the QuickChange II Site-Directed Mutagenesis kit (Agilent, Santa Clara, CA) according to the manufacturer’s protocol. The primers used to introduce these mutations are shown in Table 2. We generated tandem repeat consensus (3×Sp1-Luc) or mutant (3×mtSp1-Luc) Sp1 promoter-luciferase reporter constructs using annealed DNA oligonucleotides (see Table 2) inserted into the pTAL luciferase vector digested by NheI and BglII restriction enzymes. All constructs were verified by DNA sequencing.

Transfection and Luciferase Reporter Assays—DNA transfections were performed using FuGENE 6 reagent according to the manufacturer’s protocol (Roche Applied Science). Briefly, cells were transiently transfected with 500 ng of promoter-reporter constructs, followed with smoke exposure with 3% CSE for 4 h. Luciferase activities were measured at the end of 4 h using a steady-glow luciferase kit (Promega). Cells were co-transfected with constructs, followed with smoke exposure with 3% CSE for 4 h.

Lung Epithelial Cells—Lung epithelial A549 cells were serum-starved overnight and then exposed to 5% CSE at various time points. The cells were fixed in 1% paraformaldehyde at 37 °C for 10 min. Cells were washed twice with ice-cold PBS containing protease inhibitor mixture, and cell pellets were scraped and collected by centrifugation at 4 °C. Cells were resuspended in protein lysis buffer and incubated for 10 min on ice and sonicated to shear DNA. The supernatant was collected by centrifugation at 13,000 rpm for 30 min at 4 °C and then incubated with (rabbit) anti-Sp1 antibody overnight at 4 °C with rotation. The immunoprecipitant was collected on protein A/G-agarose and washed sequentially with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.1, and 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.1, and 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1), and finally 1× Tris/EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The immunocomplex was eluted with elution buffer (1% SDS, 0.1 mM NaHCO3, and 200 mM NaCl), and cross-links were reversed by heating at 65 °C for 4 h. After the reaction, the samples were incubated with 40 μg/ml proteinase K at 45 °C for 1 h. DNA was recovered using a GENECLEAN III kit and subjected to PCR amplification using the specific primers for the Sp1A and Sp1B binding sites within the −3.7 kb to −3.3 kb promoter region of MUC5AC. β-Actin primers used were for negative input control. The primers for Sp1A and Sp1B are as follows: Sp1A forward, 5′-CGTGCTAGCAGCCGGCCCTGGTG-3′ and reverse, 5′-CAGTCCATACGGTGACTCTCT-3′; Sp1B forward, 5′-GCCGAAGTCACGAGGCCTGGTT-3′ and reverse, 5′-TCAGTGGGCGCATAGGGAGG-3′.

Statistical Analyses—Results are expressed as mean ± S.E. Changes observed in treated groups compared with controls were analyzed using one-way ANOVA followed by Bonferroni’s post hoc t test or Student’s t test (for Fig. 1A only). p values < 0.05 were considered significant.

RESULTS

Cigarette Smoke Stimulates MUC5AC Synthesis in Human Lung Epithelial Cells—MUC5AC mRNA levels are known to be significantly elevated in the lungs of human smokers (9, 21). However, the mechanisms by which this occurs are unknown. To evaluate whether MUC5AC is increased in COPD patients, we first compared the expression of MUC5AC in human lung tissues between nonsmokers and COPD patients by TaqMan-based quantitative real time PCR. We that found MUC5AC mRNA levels were elevated in the lungs of human COPD patients compared with nonsmokers (Fig. 1A). To determine

| Primer name | Sequence (5’–3’)|
|-------------|----------------|
| NheI-MUC5AC-3.3-Fwd | GGCCGCACTCTGGTGGGTCCT |
| BglII-MUC5AC-3.3-Rev | TGGTCCTTGCTTCAGTCGTC |
| MUC5AC-mtSp1A | GGAAGAAACCTGTTATAGTTATCATC |
| MUC5AC-mtSp1B | TACGGCTGAGGGGACTTGGCACG |
| 3×Sp1 | CAGTTGCTGCTGGCCTGAGGT |
| 3×mtSp1 | TGTTTTTTGCTGAGGTTG |

Regulation of Smoke-induced MUC5AC by Sp1

TABLE 2
Primer pairs used for mutation studies

Chromatin Immunoprecipitation Assay—ChIP (24) was performed as described previously (25) with modifications. A549 cells were serum-starved overnight and then exposed to 5% CSE at various times. The cells were fixed in 1% paraformaldehyde at 37 °C for 10 min. Cells were washed twice with ice-cold PBS containing protease inhibitor mixture, and cell pellets were scraped and collected by centrifugation at 4 °C. Cells were resuspended in protein lysis buffer and incubated for 10 min on ice and sonicated to shear DNA. The supernatant was collected by centrifugation at 13,000 rpm for 30 min at 4 °C and then incubated with (rabbit) anti-Sp1 antibody overnight at 4 °C with rotation. The immunoprecipitant was collected on protein A/G-agarose and washed sequentially with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.1, and 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.1, and 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1), and finally 1× Tris/EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The immunocomplex was eluted with elution buffer (1% SDS, 0.1 mM NaHCO3, and 200 mM NaCl), and cross-links were reversed by heating at 65 °C for 4 h. After the reaction, the samples were incubated with 40 μg/ml proteinase K at 45 °C for 1 h. DNA was recovered using a GENECLEAN III kit and subjected to PCR amplification using the specific primers for the Sp1A and Sp1B binding sites within the −3.7 kb to −3.3 kb promoter region of MUC5AC. β-Actin primers used were for negative input control. The primers for Sp1A and Sp1B are as follows: Sp1A forward, 5′-CGTGCTAGCAGCCGGCCCTGGTG-3′ and reverse, 5′-CAGTCCATACGGTGACTCTCT-3′; Sp1B forward, 5′-GCCGAAGTCACGAGGCCTGGTT-3′ and reverse, 5′-TCAGTGGGCGCATAGGGAGG-3′.

Statistical Analyses—Results are expressed as mean ± S.E. Changes observed in treated groups compared with controls were analyzed using one-way ANOVA followed by Bonferroni’s post hoc t test or Student’s t test (for Fig. 1A only). p values < 0.05 were considered significant.
whether CS increased MUC5AC through a direct effect on respiratory tract epithelium, we established primary HBE cell cultures that were maintained in an air-liquid interface culture system to obtain a mucociliary differentiated epithelial cell phenotype. We treated well differentiated HBE cells with various concentrations of cigarette smoke extract (18) and examined the expression of MUC5AC at 6 h and 16 h after CSE treatment. We found MUC5AC mRNA levels to be significantly increased in CSE-treated human primary epithelial cells (Fig. 1B). Similarly, a dose-dependent increase in MUC5AC message was observed in A549 cells treated with varying doses of CSE for 2 h (Fig. 1C). As shown, MUC5AC was induced by 1% CSE and reached peak induction with 3% CSE. Also, 3% CSE induced MUC5AC gene expression in a time-dependent manner, with peak expression observed at 2–4 h after CSE treatment (Fig. 1D). Cellular toxicity, as measured by lactate dehydrogenase assays, was not observed for any dose or time period used in these experiments (data not shown).

These data suggested that CSE-induced mucin hypersecretion and MUC5AC overexpression are because of direct effects on transcriptional regulation. To examine this possibility, we transfected various MUC5AC promoter-luciferase reporter deletion constructs into human lung A549 cells and exposed transiently transfected cells to 3% CSE for 4 h. With the full-length construct (−3752), we observed an ~4-fold increase in induced luciferase activity after exposure to CSE compared with basal MUC5AC promoter activity (Fig. 2A). Luciferase activity was significantly reduced in the remaining constructs, which suggested that the promoter region between −3752 and −3337 contains the critical CSE-responsive cis-elements to regulate transcription of MUC5AC.

To identify the CSE-responsive cis-elements contained within −3752 to −3337 of the MUC5AC promoter, potential transcription factor binding sites in this region were identified using a web-based program, Transcription Element Search System (26). Referenced to a standard data base (data base versions: TRANSFAC 4.0), the Transcription Element Search System revealed several major putative transcription factor binding sites in this region including two Sp1 sites (Fig. 2B). Sp1 is involved in a number of regulatory processes of gene transcription (27) but has not been demonstrated.
previously to mediate CSE-induced mucin gene expression. This prompted us to evaluate further the potential role of Sp1 in regulating CSE-induced MUC5AC expression.

**CSE Induces Sp1 Expression and Sp1 Nuclear Localization**

We first examined the effects of CSE exposure on Sp1 expression and localization. After 4-h CSE exposure, we observed a significant increase in nuclear Sp1 protein expression by Western blotting (Fig. 3A). This increase was likely because of either augmented Sp1 translation or increased nuclear import of Sp1 protein. Pretreatment of cells with cycloheximide (10 μg/ml) 1 h prior to CSE exposure decreased CSE-induced increases in nuclear Sp1 levels (Fig. 3A), which suggested that novel protein translation was required for CSE-induced nuclear localization of Sp1. This increased expression correlated with increased nuclear localization of Sp1 as shown by immunofluorescent staining (Fig. 3B). In contrast, novel mRNA transcription does not appear to be an important component of increased nuclear localization of Sp1. As shown, 1-h pretreatment with actinomycin D did not affect Sp1 mRNA levels up to 3 h after CSE exposure (Fig. 3C).

**CSE Increases Sp1-DNA Binding**

We next explored the DNA binding activity and transcriptional activation of Sp1 to determine whether this protein was critical for CSE-induced expression of MUC5AC. We first performed EMSA with consensus Sp1 oligonucleotides to determine whether CSE-induced Sp1 proteins were capable of enhanced DNA binding activity. CSE exposure resulted in a significant increase in Sp1-DNA complex formation compared with no treatment (Fig. 3C).
Regulation of Smoke-induced MUC5AC by Sp1

Next, we examined whether or not Sp1 binds to the MUC5AC promoter in vivo using the chromatin immunoprecipitation (24) assay. As shown in Fig. 4D, ChIP confirmed that CSE exposure resulted in increased Sp1 binding to in vivo chromosomal DNA at the Sp1A and Sp1B binding sites of the MUC5AC promoter. Consistent with the EMSA results, in vivo DNA binding of Sp1 protein to the Sp1B site occurred earlier compared with the Sp1A site. This suggested that higher transcriptional activation occurs through the Sp1B site. Together, these results suggest strongly that CSE-induced Sp1 protein binding to the −3865 to −3337 bp MUC5AC promoter region is critical for transactivation of this gene.

CSE-induced Sp1 Is Transcriptionally Active—To determine whether Sp1 protein-DNA binding resulted in increased transcriptional activation of the MUC5AC promoter, we performed a series of transfection experiments using various promoter-luciferase reporter constructs. We initially utilized a generic Sp1 promoter-luciferase reporter construct, which consisted of three consensus binding sites for the Sp1 transcription factor fused to the pTAL luciferase vector (3×Sp1). In support of the notion that CSE-induced Sp1 expression is transcriptionally active, CSE exposure significantly enhanced Sp1-dependent luciferase activity in transiently transfected A549 cells (Fig. 5A). This effect was abrogated when cells were transfected with promoter-reporter constructs that contained mutated Sp1 sites (3×mtSp1). To determine whether increased Sp1 protein expression contributes directly to enhanced MUC5AC expression, a Sp1 expression vector was co-transfected with the MUC5AC-3752 full-length luciferase promoter construct into A549 cells. Similar to CSE exposure, increased expression of Sp1 protein significantly enhanced MUC5AC promoter activity (Fig. 5B). Pretreatment of transfected cells with 100 nM mithramycin A, an aureolic antibiotic that selectively inhibits gene expression by blocking Sp1 binding to GC-rich recognition sequences (28), had no effect on base-line MUC5AC transcription activity but completely abolished CSE-induced MUC5AC transcription (Fig. 6A). Based on cell morphology and lactate dehydrogenase release, mithramycin A did not incur cytotoxicity at the doses we used for these experiments (data not shown).

To clarify that Sp1-mediated transactivation of MUC5AC occurs through the “smoke-responsive” region, we cloned the −3752/−3337 promoter region of MUC5AC into the pTAL luciferase vector and exposed it to CSE without or with mithramycin A pretreatment. As shown, CSE increased luciferase activity ~4-fold, and this activity was significantly attenuated by pretreatment of cells with mithramycin A (Fig. 6B). These results provided further evidence that CSE-induced transcription of MUC5AC is under the control of Sp1 and occurs primarily through the CSE-sensitive MUC5AC promoter region.

To confirm the specific contribution to transcription of each of the two Sp1 sites in the MUC5AC promoter, we utilized several MUC5AC promoter-reporter constructs. Each construct contained a specific mutation of either the Sp1A or Sp1B cis-element contained within the CSE-responsive (−3752 and −3337 bp) region of the MUC5AC promoter (Fig. 7A). Vector-mediated augmentation of Sp1 protein expression considerably enhanced MUC5AC promoter activity, which was significantly

![Figure 3](https://example.com/figure3.png)

**FIGURE 3. Expression and nuclear localization of Sp1 is induced by CSE.**

A, A549 cells grown in submerged cell culture conditions were treated with or without 3% CSE for 4 h followed by harvest of cytoplasmic and nuclear protein for Western blots using anti-Sp1 antibody. A portion of cells was treated with cycloheximide (10 μg/ml) for 1 h prior to CSE treatment. Membranes were stripped and reprobed with anti-GAPDH antibody to ensure equal protein loading. B, A549 cells were treated with or without 3% CSE for 1 h followed by fixation and immunohistochemical staining of cells for Sp1 protein. DAPI counterstain was used to identify cell nuclei. Arrows indicate significantly increased nuclear staining of Sp1 that could only be detected in green fluorescence channel. Original magnification, ×200. C, A549 cells grown in submerged cell culture conditions were treated with or without 3% CSE at various time points (0–3 h) followed by harvest of RNA for quantitative real-time PCR. A portion of cells was treated with actinomycin D (10 μg/ml) for 1 h prior to CSE treatment. Data represent mean ± S.E. of triplicate samples of three independent experiments. No significant difference was observed for CSE-treated groups with or without actinomycin D treatment.

Confirming the increased DNA binding capacity of CSE-induced Sp1. The specificity of binding was confirmed by loss of labeled binding in the presence of excess (50×) unlabeled Sp1 oligonucleotide (Fig. 4A).

To determine whether CSE-induced Sp1 bound to the native MUC5AC promoter, we designed two oligonucleotides (MUC5AC-SP1A and MUC5AC-SP1B) that corresponded to the two Sp1 binding sites identified in the smoke response region (−3752 to −3337) of the MUC5AC promoter (see Fig. 2B). Consistent with our initial observations, CSE increased Sp1 binding activity to both MUC5AC Sp1 sites by EMSA (Fig. 4, B and C). These results suggested that Sp1 preferentially binds to the MUC5AC-SP1B site compared with the MUC5AC-SP1A site. The addition of excess unlabeled probe or mutant probe abrogated labeled DNA-protein binding, which confirmed Sp1 specificity.

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diminished when either of the two Sp1 binding sites was mutated (Fig. 7B). Similarly, treatment of transiently transfected A549 cells with or without 3% CSE treatment for 4 h resulted in enhanced luciferase activity only in cells expressing the intact Sp1 sites (Fig. 7C). Concurrent mutations in both Sp1A and Sp1B sites resulted in the largest decrease in luciferase activity compared with single mutations alone. Together, these findings suggest that both sites are essential for CSE-induced MUC5AC transcription.

**DISCUSSION**

In this study, we examined the regulatory mechanism responsible for induction of MUC5AC by cigarette smoke exposure. Our results indicate that CSE exposure increases MUC5AC mRNA expression in both human primary lung epithelial cells and alveolar type II-like A549 epithelial cells. In addition, CSE significantly increased MUC5AC gene expression in a time- and dose-dependent manner. Using various MUC5AC promoter-luciferase promoter constructs, we (1)
verified that CSE-induced MUC5AC expression occurs through transcriptional regulation (2), identified the key CSE-responsive region on the MUC5AC promoter, and (3) identified the Sp1 transcription factor as the major regulator of the MUC5AC promoter region, acting through two Sp1 cis-elements. To the best of our knowledge, this is the first published report that demonstrates Sp1-regulated MUC5AC induction after CSE exposure.

Sp1 is a ubiquitously expressed C_{2}H_{2}-type zinc-finger DNA-binding protein that was first identified based on its ability to interact with GC-rich motifs (29). Because of that initial description, Sp1 has been implicated in a wide range of cellular events and is associated with the transactivation of numerous genes (30, 31). The mechanisms responsible for Sp1-mediated gene regulation are similarly varied. These include increased expression of Sp1 protein or signaling mechanisms that lead to one of several possible posttranslational modifications (32, 33). Our results suggest that modulation of protein expression is a predominant mechanism responsible for CSE-induced, Sp1-mediated MUC5AC expression. We observed that Sp1 protein translation was activated as early as 4 h after CSE treatment.

CSE treatment increased nuclear localization of Sp1 by immunohistochemical staining and Western blotting. This increased nuclear localization was inhibited by cycloheximide supporting the need for novel protein transcription (Fig. 3). In addition, overexpression of Sp1 protein resulted in enhanced MUC5AC expression similar to CSE treatment (Fig. 5B). Together, these data support the notion that CSE induces MUC5AC through enhanced Sp1 protein expression.

However, posttranslational modifications of Sp1 may also be essential for regulated expression of MUC5AC. Transcription factors of the Sp family have recently emerged as potent regulators of mucin genes (34–37), and it is likely this regulation occurs through phosphorylation events acting on Sp1. Specifically, Sp1 has been shown to mediate MUC5AC expression by phorbol 12-myristate 13-acetate, TGF, or EGF, mitogens and growth factors that are known to signal through well characterized phosphorylation cascades (35, 38). Similarly, Jonckheere et al. reported previously that TGFβ-induced transcriptional activation of the murine muc5ac promoter occurs through cooperative interactions of Sp1 and Smad4 (38). However, they identified Sp1-specific cis-elements required for TGFβ signaling that were different from the sites we identified for CSE-mediated transcriptional activity. Collectively, these data suggest that Sp1 is a central mediator to regulate MUC5AC in response to multiple environmental stimuli in mice and humans.
Although our work suggests that CSE-mediated MUC5AC expression occurs predominantly through increased Sp1 protein expression, CSE-induced cell signaling events may also contribute to enhanced expression of this gene.

Our data suggest that two Sp1 consensus binding sites within the −3.7/−3.3 kb promoter region were necessary and sufficient for CSE-induced MUC5AC transcriptional regulation. Referenced to a standard data base, the Transcription Element Search System revealed several major putative transcription factor binding sites in the −3.7/−3.3 kb MUC5AC promoter region including the two Sp1 sites described here, two AP-1 sites, one retinoid X receptor site, and one NF-κB site. Protein-DNA array screening results suggested that all of these transcription factors bind to their respective consensus binding sequences in response to CS exposure (23). Previously, we and others reported that CS induced NF-κB nuclear translocation and significant NF-κB nuclear activation in human lung epithelial cells and fibroblasts (23, 39). However, specific inactivation of the two Sp1 consensus binding sites within the −3.7/−3.3 kb promoter region led to near complete abrogation of CSE-induced MUC5AC promoter activity, making it unlikely that other cis-elements are functionally significant. In parallel, one

**FIGURE 7. Two Sp1 cis-elements are required to mediate CSE-induced MUC5AC gene promoter trans-activation in A549 cells.** A, schematic representation of the four luciferase reporter vectors driven by the CSE-responsive −3752/−3337 bp promoter region of MUC5AC and various mutant constructs. B, relative luciferase activity in cells transfected with the four luciferase reporter constructs with or without co-transfection of a pCMV-Sp1 expression vector. C, relative luciferase activity in cells transfected with the four luciferase reporter constructs with or without treatment with 3% CSE. All data are presented as mean ± S.E. (error bars) of three independent experiments. *, p < 0.05 relative to control vector co-transfected with the Sp1 expression vector (n = 3). **, p < 0.05 for various MUC5AC3.3 mutants relative to wild-type MUC5AC3.3 promoter activity after co-transfection with the Sp1 expression vector (n = 3). #, p < 0.05 relative to the control vector after CSE treatment (n = 4). ##, p < 0.05 for MUC5AC3.3 mutants relative to intact MUC5AC3.3 promoter activity after CSE treatment (n = 4).
study demonstrated that Sp1 was predominantly responsible for CS-induced transactivation of the matrix metalloproteinase-1 gene. They similarly identified numerous other CS-induced transcription factors and the presence of alternative cis-elements on the matrix metalloproteinase-1 promoter that did not confer functional activity or were inhibitory (40). In contrast, one study identified that AP-1, compared with retinoid X receptor or NF-kB, showed the greatest effects on cigarette smoke-induced MUC5AC promoter activity in BEAS-2B cells (21). Although the reasons for these differences need further delineation, our current data strongly suggest that Sp1 alone is necessary to regulate MUC5AC in response to CSE.

We have demonstrated that CSE induced an increase in MUC5AC mRNA and de novo gene transcription in A549 cells. This occurs predominantly through increased Sp1 expression, enhanced Sp1-DNA binding, and increased Sp1 transcriptional activation through a CSE-sensitive region on the MUC5AC promoter between nucleotides −3752 and −3337 upstream of the transcription start site. In preliminary studies, we observed significantly decreased CS-induced MUC5AC expression and decreased Sp1-DNA binding activity after treatment with EGF receptor (AG1478) or ERK (PD98059 and U0126) signaling inhibitors (data not shown). Therefore, an EGFrceptor-ERK-Sp1-dependent pathway is likely involved in CS-induced MUC5AC expression. Future studies will further examine upstream signaling cascades that additionally contribute to CSE-induced mucus hypersecretion.

We speculate that CSE-induced increases in Sp1 protein are further augmented by CSE-enhanced phosphorylation of Sp1. This likely exacerbates MUC5AC hypersecretion in susceptible individuals but also may increase production of other Sp1-dependent genes affecting growth, apoptosis, or cell transformation to a malignant phenotype. Extension from these studies potentially will identify key molecules that suggest novel therapeutic targets to reduce mucus hypersecretion in COPD patients.

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