Molecular Basis of Cell-specific Endothelial Nitric-oxide Synthase Expression in Airway Epithelium*

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Nitric oxide (NO) plays an important role in airway function, and endothelial NO synthase (eNOS) is expressed in airway epithelium. To determine the basis of cell-specific eNOS expression in airway epithelium, studies were performed in NCI-H441 human bronchiolar epithelial cells transfected with the human eNOS promoter fused to luciferase. Transfection with 1624 base pairs of sequence 5′ to the initiation ATG (position −1624) yielded a 19-fold increase in promoter activity versus vector alone. No activity was found in lung fibroblasts, which do not express eNOS. 5′ deletions from −1624 to −279 had modest effects on promoter activity in H441 cells. Further deletion to −248 reduced activity by 65%, and activity was lost with deletion to −79. Point mutations revealed that the GATA binding motif at −254 is mandatory for promoter activity and that the positive regulatory element between −248 and −79 is the Sp1 binding motif at −125. Electrophoretic mobility shift assays yielded two complexes with the GATA site and three with the Sp1 site. Immunodepletion with antiserum to GATA-2 prevented formation of the slowest migrating GATA complex, and antiserum to Sp1 super-shifted the slowest migrating Sp1 complex. An electrophoretic mobility shift assay with H441 versus fibroblast nuclei revealed that the slowest migrating GATA complex is unique to airway epithelium. Thus, cell-specific eNOS expression in airway epithelium is dependent on the interaction of GATA-2 with the core eNOS promoter, and the proximal Sp1 binding site is also an important positive regulatory element.

There is increasing evidence that nitric oxide (NO), produced by the enzyme nitric-oxide synthase (NOS), plays an important role in physiologic and pathologic processes in the airway (1–3). NO is present in expired gas (4), and studies in animal models as well as in humans suggest that the principle source of expired NO is the airway rather than the pulmonary vasculature (5, 6). The functions of NO in the mature airway include smooth muscle relaxation, neurotransmission, and bacteriostasis, as well as the modulation of ciliary motility, mucin secretion, and plasma exudation (1, 2). Studies in the perinatal period indicate that airway NO is also critically involved in the regulation of lung liquid production and tissue resistance (7–10).

One of the key cellular sources of airway NO is the epithelium (2), and it has been previously demonstrated that the endothelial isoform of NOS (eNOS) is constitutively expressed in the airway epithelium of humans and a variety of animal species (11–13). There is also evidence that airway epithelial NOS expression is attenuated during inflammatory conditions, potentially contributing to airway dysfunction, but the NOS isoform(s) involved has not been identified (14). In addition, studies suggest that there are dramatic changes in whole lung eNOS expression during normal development and with prolonged changes in oxygenation, but the cell specificity of these alterations has not been elucidated (15). As such, the mechanisms regulating eNOS expression in the pulmonary epithelium have an important impact on lung function.

The transcriptional regulation of eNOS in endothelial cells is partially understood. The 1600-base pair regulatory region upstream of the eNOS transcription initiation site has lengthy conserved regions in mice, cows, and humans (16). Contained within this region are response elements to Sp1 and GATA, and these sites are critical to basal transcription of eNOS in aortic endothelial cells (17). The eNOS promoter also contains two regulatory regions that bind to a variety of transcription factors, including Els and Sp family members, MAZ and YY1, and these domains may be important in regulating eNOS transcription as well (18). In addition, recent evidence suggests that eNOS is expressed in selective microvascular beds, and this selectivity may be related to a platelet-derived growth factor response element in the eNOS promoter (19). Thus, a considerable amount of work has identified regulatory elements within the eNOS promoter and the transcription factors that interact with them to regulate eNOS expression in endothelial cells. However, eNOS is also expressed in a variety of nonepithelial cell types, and the cell-specific regulation of eNOS is not understood. In particular, the mechanisms regulating eNOS expression in airway epithelial cells have not been characterized.

To begin to better understand the regulation of lung epithelial NO production and eNOS expression, the present studies were designed to determine the molecular basis of cell-specific eNOS expression in the airway epithelium. In this investigation, the cis DNA sequences required for basal eNOS transcription in airway epithelium have been identified. In addition, since studies employing immunohistochemistry or in situ hybridization have revealed that pulmonary eNOS expression is limited solely to the vascular endothelium and airway epithelium (11–13), these mechanisms have been compared in the epithelium and endothelium.
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Experimental Procedures

Cell Culture—Airway epithelial cell experiments were principally performed with NCI-H441 human bronchial epithelial cells, which are of Clara cell lineage (20–22). We have previously demonstrated that these cells exclusively express the eNOS isoform in a constitutive manner and that the abundance of eNOS is approximately 2% of that found in pulmonary endothelial cells (23). The use of this continuous cell line allows for the specific examination of airway epithelial cell eNOS regulation without contamination by resident macrophages or endothelial cells that may be present in primary cell cultures. In addition, eNOS expression in this cell line is not altered after multiple passages in culture. The cells were propagated in RPMI medium containing 10% fetal bovine serum, 1% l-glutamine, 1% antibiotic/antimycotic mixture, 0.5% ampicillin, 0.15% nystatin, 0.15% gentamycin, and 0.10% tylosin in a humidified incubator with 5% CO₂ in air at 37 °C. The cells were studied in six-well tissue culture plates (Corning Costar Corp., Cambridge, MA) at subconfluence.

In order to compare eNOS expression in different pulmonary cell types, additional experiments were performed in primary ovine intrapulmonary artery endothelial cells known to express the enzyme (24) and also in CCD-Lu human lung fibroblasts (ATCC, Manassas, VA), which do not express the enzyme. The primary pulmonary artery endothelial cells were obtained and propagated as described previously and studied at passage 4–6 (24), and the CCD-Lu cells were maintained in the RPMI medium described above. Studies were also done using BEAS-2B (ATCC) and NHBE cell lines (Cletonics Corp., San Diego, CA) to evaluate eNOS expression in airway epithelium that is not of Clara cell origin. BEAS-2B and NHBE cells were propagated in BEGM medium (Clonetics).

Construction of Reporter Plasmids and Mutagenesis—A fragment of DNA containing the 5′-flanking region of the human eNOS gene was amplified using the polymerase chain reaction (PCR). The template DNA for the PCR was a BlueScript plasmid (Stratagene) containing 6 kilobase pairs of genomic human DNA, including 1.6 kilobase pairs upstream from the human eNOS gene (25). This was kindly provided by Thomas Jefferson University (Philadelphia, PA) and inserted into the pGL2 vector. The primers for PCR were designed to amplify DNA from 1.6 kilobase pairs upstream of the eNOS AUG to the transcriptional start site of eNOS were 5′-GGAGCTCACATCATGATCGTCTGGTCGTA-3′ (forward) and 5′-CGCCAGACTTGGTTACGTCTC-3′ (reverse). The 1.6-kilobase pair PCR product was inserted into Kpn1/HindIII sites of the luciferase reporter gene plasmid, pGL2 (Promega Corp., Madison, WI) to yield the full-length promoter-reporter plasmid denoted as −1624eNOS-Luc. The nucleotide sequence was determined to confirm that no errors had been introduced by PCR amplification.

To generate progressive 5′ deletion mutants, a series of forward primers were used in combination with the noted reverse primer in a PCR with the full-length promoter as the template. The forward primers were designed to introduce a Kpn1/HindIII site (underlined) into the 5′-GGCCGTCACATCCTGGGAGCCATGTC-3′, 5′-GGCCCTACACCTCCCTGGACAGATGCGC-3′, 5′-GGCCGTAAGAGGTACCGCAGGAGGAGG-3′, and 5′-GGCCGAAGGCTCATCTCACACAGCGGAAC-3′ (upper strand), and the mutant sequences were inserted by PCR using the appropriate forward primer from upstream or downstream of the mutation. The resulting fragments were gel-purified, digested, and subcloned into the Kpn1/HindIII sites of pGL2. All constructs were verified by sequencing the inserts and flanking regions of the plasmid.

Cell Transfection—Cell transfection was performed using methods modified from those previously reported (26, 27). Cells grown to 50–60% confluency in 6-well plates were preincubated in Opti-MEM medium (Life Technologies, Inc.) for 30 min at 37 °C. −1624eNOS-Luc or 5′ deletion or site-directed mutant constructs (1 μg) and a plasmid containing SV40-driven β-galactosidase (pSV-β-Gal; Promega Corp.), to normalize for transfection efficiency, were mixed with LipofectAMINE (Life Technologies; 10 μg/well) and incubated in a total volume of 200 μl for 30 min at room temperature. The lipid-coated DNA and 800 μl of Opti-MEM were then added to each well of cells. After 5 hr, 1 ml of growth medium containing 20% iron-supplemented calf serum and 20% lamb serum was added to each well, and the following day the medium was replaced with growth medium. 72 h following transfection, the cells were lysed, and the extracts were centrifuged at 10,000 × g to remove unbroken cells and debris. Luciferase activity was measured using a luciferase assay kit (Analytical Luminescence Laboratory, Ann Arbor, MI) and β-galactosidase activity was measured spectrophotometrically (at 420 nm) by the generation of o-nitrophenol from the substrate, o-nitrophenyl-β-D-galactopyranoside (29). The results are normalized as relative luciferase light units/β-galactosidase activity. In selected wells, the cells were transfected with pGL2-Control Vector (Promega Corp.) containing an SV40 promoter and enhancer to serve as a positive control for luciferase expression.

Nuclear Extract Preparation—Cells were rinsed twice with ice-cold phosphate-buffered saline, scraped, and pelleted at 500 × g for 5 min. Cell pellets were lysed in buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40 and incubated at 4 °C for 5 min. The lysate was then transferred into an ice-cold Dounce homogenizer and sonicated with 35 strokes of a pestle, and centrifuged at 500 × g for 5 min at 4 °C. The nuclear pellet was suspended in buffer containing 20 mM Tris-HCl (pH 8.1), 75 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 50 mM glycerol, 1 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride. The nuclear extracts were sonicated and stored at −80 °C.

Electrophoretic Mobility Shift Assays—The oligonucleotide probes were prepared by annealing complementary DNA strands at 95 °C for 5 min. Probes were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Life Technologies), and unlabeled oligonucleotide was removed using P6 columns (Bio-Rad). Typically, specific activities were 300,000 cpm/ng DNA. Nuclear extracts (8 μg) were incubated in buffer containing 25 mM HEPES (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 5% serum (1 μg of poly(I) and poly(C) (8 μg) and 5 μg of poly(A) (5 μg) for 10 min. The 32P-labeled oligonucleotide probe (approximately 2 × 10⁶ cpm) was then added for an additional 20 min in a total reaction volume of 30 μl. In competition studies, excess wild-type, mutant, or unrelated oligonucleotides was added in a 2–200-fold molar excess prior to the addition of the 32P-labeled probe. The wild-type GATA probe was 5′-GGGGCTGCTTCATCATACGGCCTGAGTT-3′ (upper strand), and the mutant GATA probe was 5′-GGGGCTGCTTCATCATACGGCCTGAGTT-3′ (upper strand), and the mutant probe was 5′-GGGGCTGCTTCATCATACGGCCTGAGTT-3′ (upper strand). To identify the nuclear proteins that bound to the GATA and Sp1 domains, antibody depletion or supershifting of the DNA-protein complex was performed. For GATA, 2 μl of antiserum to GATA-1 or GATA-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or unrelated antiserum was added to the nuclear extract for 30 min prior to the addition of oligonucleotide probe. For Sp1, 2 μl of antiserum to Sp1 (Santa Cruz Biotechnology) or unrelated antiserum was added to the DNA-nuclear protein complexes for 45 min at room temperature prior to electrophoresis. All nuclear protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gels containing 1× Tris borate/EDTA buffer. Dried gels were exposed to Kodak XAR film for autoradiography.

Statistical Analysis—Data for promoter activity were analyzed by analysis of variance and Neuman-Keuls post hoc testing (30). Results are expressed as mean ± S.E. All stated differences achieved statistical significance at the 0.05 level of probability or less.

Results

eNOS Promoter Activity in Pulmonary Cell Types—Since pulmonary eNOS expression is limited solely to the vascular endothelium and airway epithelium (11–13), we first compared basal promoter activity in the epithelium and endothelium and in lung fibroblasts, which do not express eNOS, serving as a negative control. Transfection of H441 airway epithelial cells with −1624eNOS-Luc resulted in a 19-fold increase in promoter activity compared with transfection with vector alone (Fig. 1). In parallel but to a greater extent, transfection of pulmonary artery endothelial cells with −1624eNOS-Luc yielded 35-fold more activity than vector alone. However, there was no detectable eNOS promoter activity in the lung fibro-

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blasts. Basal promoter activity was then compared in different airway epithelial cell types, using the H441 cells, which are of Clara cell lineage, and BEAS-2B and NHBE cells, which are not of Clara cell origin (Fig. 2). Transfection with −1624eNOS-Luc resulted in approximately 6-fold greater promoter activity in the H441 Clara cells compared with the other epithelial cell types, which displayed 3–4-fold more activity than vector alone.

Promoter Elements Required for Airway Epithelial Expression—To determine the elements in the eNOS promoter required for airway epithelial expression of the gene, the activities of progressive 5’ deletion mutants were evaluated in the H441 cells. Deletion from −1624 to −994 resulted in a modest decline in promoter activity of 21% (Fig. 3A). However, further deletion to −318 caused a return to activity levels that were comparable with those with −1624eNOS-Luc. Deletion from −318 to −279 yielded a modest increase in promoter activity of 27% (Fig. 3B). Further deletion from −279 to −248 resulted in a 65% decline in promoter activity. In addition, deletion from −248 to −79 caused a complete loss of basal promoter activity.

Inspection of the proximal eNOS promoter reveals that there is a potential GATA binding site at −254 and a potential Sp1 regulatory element at −125 (25). Because a dramatic decrease in promoter activity in the H441 airway epithelial cells was observed with progressive 5’ deletion of these domains, site-directed mutants of either the GATA or Sp1 site within −1624eNOS-Luc were tested (Fig. 4, upper panel). Mutation of the GATA motif caused a marked decrease in basal promoter activity to levels comparable with the background observed with vector alone. Mutation of the Sp1 site resulted in a 74% decline in promoter activity. Since these binding domains have also been found to be critical to basal transcription of eNOS in endothelial cells (27), parallel studies were performed in that cell type (Fig. 4, lower panel). Similar to the findings in airway epithelium, mutation of the GATA motif caused a complete loss of basal promoter activity in the pulmonary endothelial cells, and mutation of the Sp1 site caused an 82% decrease in basal promoter activity.

Evaluation of Relevant Nuclear Proteins—To evaluate the nuclear proteins in airway epithelial cells involved in binding to the GATA and Sp1 domains of the eNOS promoter, electrophoretic mobility shift assays were performed. Incubation of nuclear extracts from the H441 airway epithelial cells with a double-stranded oligonucleotide probe encompassing the putative GATA site at −254 resulted in the appearance of two major DNA-protein complexes that were diminished by a 200-fold molar excess of unlabeled probe (Fig. 5A). The slower migrating complex was not formed with mutated GATA site probe, but the faster migrating complex was formed. The use of the mutated oligonucleotide as a competitor did not prevent the formation of either complex by the wild-type probe. In addition, competition with an unrelated oligonucleotide did not prevent complex formation by the wild-type probe (data not shown). Incubation of H441 nuclear extracts with a probe containing the putative Sp1 site at −125 resulted in the appearance of three major DNA-protein complexes and one minor DNA-protein complex that were prevented by a 200-fold molar excess of unlabeled probe (Fig. 5B). The three major Sp1-nuclear protein complexes were not formed with mutated Sp1 DNA probe, and the mutated oligonucleotide did not prevent the formation of the major complexes by the wild-type probe. Unrelated oligonucleotide also did not prevent complex formation by the wild-type probe (data not shown).

To identify the epithelial nuclear proteins involved in complex formation with the GATA and Sp1 eNOS promoter elements, immunodepletion and supershift analyses were done. Preincubation of H441 nuclear extracts with an antiserum directed against GATA-2, but not an antiserum directed against GATA-1 or an unrelated antiserum, caused diminished formation of the slower migrating DNA-protein complexes involving the GATA site (Fig. 6A). Antiserum to Sp1 shifted the slowest migrating Sp1 probe-nuclear protein complex, whereas an unrelated antiserum did not do so (Fig. 6B).

To reveal which DNA-nuclear protein complexes are involved in cell-specific eNOS expression, electrophoretic mobility shift assays were compared using nuclear extracts from H441 epithelial cells, which express eNOS, and from CCD-18Lu lung fibroblasts, which do not express eNOS. As observed previously (Fig. 5), two primary DNA-protein complexes were formed when oligonucleotide probe encompassing the GATA site was incubated with H441 nuclear extract, and complex formation was prevented by unlabeled probe (Fig. 7A). However, whereas the faster migrating DNA-protein complex was present when fibroblast nuclear extract was employed, the
slower migrating complex was completely absent. In contrast, in the experiment using the Sp1 DNA probe (Fig. 7B), identical multiple DNA-protein complexes were obtained with both H441 epithelial and lung fibroblast nuclear extracts. Thus, the slower migrating GATA site-protein complex is unique to the epithelial cells, which express eNOS.

**DISCUSSION**

In the present study, we have determined the molecular basis of cell-specific eNOS expression in the airway epithelium. In transient transfection experiments with 1624 base pairs of the eNOS gene promoter activity in different airway epithelial cell types. The eNOS promoter-reporter gene construct, −1624eNOS-Luc, or vector alone, −Luc, were cotransfected with SV40-driven β-galactosidase plasmid (β-gal), and relative activities (Luc/β-gal) were determined in cell lysates 72 h later. Studies were performed in H441 cells of Clara cell origin and the non-Clara cell lines BEAS-2B and NHBE. Values are mean ± S.E. (n = 3), and the results are representative of the findings of three independent experiments.

![Graph showing eNOS gene promoter activity in different airway epithelial cell types.](image)

**FIG. 2.** eNOS gene promoter activity in different airway epithelial cell types. The eNOS promoter-reporter gene construct, −1624eNOS-Luc, or vector alone, −Luc, were cotransfected with SV40-driven β-galactosidase plasmid (β-gal), and relative activities (Luc/β-gal) were determined in cell lysates 72 h later. Studies were performed in H441 cells of Clara cell origin and the non-Clara cell lines BEAS-2B and NHBE. Values are mean ± S.E. (n = 3), and the results are representative of the findings of three independent experiments.

**FIG. 3.** Activity of 5′ deletion mutants of the eNOS gene promoter in H441 airway epithelial cells. A, the eNOS promoter-reporter gene constructs, −1624eNOS-Luc, −994eNOS-Luc, or −318eNOS-Luc, were cotransfected with SV40-driven β-galactosidase plasmid (β-gal), and relative activities (Luc/β-gal) were determined in cell lysates 72 h later. The activity with vector alone was less than 5% of that obtained with the eNOS promoter constructs (not shown). B, similar studies were performed with −318eNOS-Luc, −279eNOS-Luc, −248eNOS-Luc, or −79eNOS-Luc. Values are mean ± S.E. (n = 3), and the results are representative of the findings of three independent experiments.

**FIG. 4.** Activity of GATA and Sp1 site mutants of the eNOS gene promoter in H441 airway epithelial cells (upper panel) and pulmonary endothelial cells (lower panel). The −1624eNOS-Luc wild-type construct or site-directed mutants (Mut.) of the GATA consensus site at −254 or the Sp1 binding motif at −125 or vector alone (−Luc) were cotransfected with SV40-driven β-galactosidase plasmid (β-gal), and relative activities (Luc/β-gal) were determined in cell lysates 72 h later. Values are mean ± S.E. (n = 3), and the results are representative of the findings of three independent experiments.

H441 epithelial and lung fibroblast nuclear extracts. Thus, the slower migrating GATA site-protein complex is unique to the epithelial cells, which express eNOS.

**FIG. 4.** Activity of GATA and Sp1 site mutants of the eNOS gene promoter in H441 airway epithelial cells (upper panel) and pulmonary endothelial cells (lower panel). The −1624eNOS-Luc wild-type construct or site-directed mutants (Mut.) of the GATA consensus site at −254 or the Sp1 binding motif at −125 or vector alone (−Luc) were cotransfected with SV40-driven β-galactosidase plasmid (β-gal), and relative activities (Luc/β-gal) were determined in cell lysates 72 h later. Values are mean ± S.E. (n = 3), and the results are representative of the findings of three independent experiments.
epithelial cell nuclear extracts. Extracts were incubated with labeled double-stranded oligonucleotide probes containing either the eNOS gene GATA consensus binding site at −254 (A) or the Sp1 binding motif at −125 (B) or their corresponding mutant probes. Competition reactions were performed with either cold, wild-type competitor (comp.) at a 2-, 20-, or 200-fold molar excess or mutant competitor (mut. comp.) at a 200-fold molar excess. The observations were confirmed in at least three independent experiments. Major complexes are identified with solid arrows, and minor complexes are designated by arrowheads.

FIG. 5. Electrophoretic mobility shift assays with H441 airway epithelial cell nuclear extracts. Extracts were incubated with labeled double-stranded oligonucleotide probes containing either the eNOS gene GATA consensus binding site at −254 (A) or the Sp1 binding motif at −125 (B) or their corresponding mutant probes. Competition reactions were performed with either cold, wild-type competitor (comp.) at a 2-, 20-, or 200-fold molar excess. The observations were confirmed in at least three independent experiments. Major complexes are designated by solid arrows, and minor complexes are designated by open arrowheads.

A GATA
- Probe only
- 0x comp.
- 2x comp.
- 20x comp.
- mutant probe
- 200x mut. comp.

B Sp1
- Probe only
- 0x comp.
- 2x comp.
- 20x comp.
- mutant probe
- 200x mut. comp.

the human eNOS promoter sequence 5’ to the initiation ATG fused to a luciferase gene (−1624eNOS-Luc), we have observed that basal transcription is demonstrable in H441 airway epithelial cells as well as in pulmonary endothelial cells but not in lung fibroblasts. These findings are consistent with the previous observation that eNOS protein in the lung is expressed solely in the airway epithelium and vascular endothelium (11–13). In addition, we have found that basal transcription is greater in airway epithelium of Clara cell origin compared with non-Clara cells. Thus, the mechanisms underlying cell-specific eNOS expression in airway epithelium reside in the function of cis DNA sequences of the eNOS gene, and these processes are maximal in certain airway epithelial cell types. Studies of progressive 5’ deletion mutants of the −1624eNOS-Luc construct revealed that there are positive and negative regulatory elements of modest significance between −1624 and −279 and that key positive regulatory elements reside between −279 and −248 and also between −248 and −79. Since there is a potential GATA binding site at −254 and a potential Sp1 binding site at −125 (25), site-directed mutants of either the GATA or Sp1 site within −1624eNOS-Luc were tested in the H441 cells. Mutation of the GATA site at −254 caused complete attenuation of basal promoter activity, and mutation of the Sp1 site at −125 yielded promoter activity that was one-fourth of that with the wild-type promoter. Parallel studies in pulmonary endothelial cells yielded comparable results. These collective findings indicate that both the GATA and Sp1 binding motifs in the core eNOS promoter are critical to the basal transcription of the eNOS gene in the airway epithelium and also the pulmonary vascular endothelium. The present observations contrast with those previously made in aortic endothelium, in which site-directed mutation of the GATA binding motif only yielded a 25–30% decline in promoter activity, whereas mutation of the Sp1 site caused an 85–90% fall (17). This suggests that the GATA binding site may be of particular importance to eNOS gene transcription in the lung, but a comparison of promoter function in epithelium and endothelium from numerous organs would be required to test such a possibility.

To evaluate the nuclear proteins in airway epithelial cells involved in binding to the GATA domain of the eNOS promoter, electrophoretic mobility shift assays were performed with oligonucleotide probe encompassing the GATA site at −254. Two specific DNA-protein complexes were formed with wild-type probe. When a mutated GATA site probe was used, the slower migrating complex was not formed, but the faster migrating complex was evident. Since the identical mutation of the −1624eNOS-Luc construct yielded a total loss of basal promoter activity, it suggests that the slower migrating complex, and not the faster migrating one, is primarily involved in the regulation of basal eNOS gene expression in the airway epithelium. These findings are consistent with the formation of a single GATA DNA element-nuclear protein complex in previous studies of aortic endothelium (17).

FIG. 6. Immunodepletion and supershift analyses of nuclear protein-eNOS promoter DNA complexes in H441 airway epithelial cells. A, antiserum to GATA-2 or GATA-1 or unrelated antiserum was added to the nuclear extract prior to the addition of oligonucleotide probe containing the GATA consensus binding site at −254. B, antiserum to Sp1 or unrelated antiserum was added to the DNA-nuclear protein complexes formed using oligonucleotide probe containing the Sp1 binding motif at −125 prior to electrophoresis. The observations were confirmed in at least three independent experiments. Major complexes are identified with solid arrows, and minor complexes are designated by solid arrowheads. Supershifted complex is identified with an open arrow.
The identities of the proteins forming the other two complexes with the Sp1 binding motif are yet to be determined. Previous work in aortic endothelial cells has yielded virtually identical findings for immunodepletion and supershift analyses (17), revealing that the GATA-2 and Sp1 nuclear proteins are integrally involved in the expression of eNOS in both the airway epithelium and vascular endothelium.

To reveal which DNA-nuclear protein complexes underlie the cell specificity of eNOS expression in the airway epithelium, electrophoretic mobility shift assays were compared using nuclear extracts from the H441 cells, which express eNOS, and from the CCD-18Lu lung fibroblasts, which do not express eNOS. In contrast to the findings with the GATA site probe and H441 nuclear extract, incubation of GATA probe with fibroblast nuclear extract yielded the faster migrating DNA-protein complex, but the slower migrating complex was completely absent. In experiments using the Sp1 DNA probe, identical multiple complexes were obtained with both H441 endothelial and lung fibroblast nuclear extracts. Thus, the slower migrating GATA-site protein complex is unique to the epithelial cells compared with fibroblasts. Since immunodepletion with antiserum to GATA-2 prevented the formation of this complex in the H441 cells, these findings indicate that GATA-2 protein interaction with the core eNOS promoter is required for cell-specific expression of eNOS in airway epithelium.

In addition to revealing the basis for airway epithelial eNOS expression, the present observations may be relevant to the role of eNOS in nonpulmonary epithelial cells. eNOS expression has been demonstrated in renal epithelium, where it may have a critical role in tubular function (32, 33). In addition, eNOS is expressed in various epithelial cell types of both the male and female reproductive organs, with differential expression during the oestrus cycle in the latter, suggesting that it is involved in modulating reproduction (34, 35). Further studies of eNOS promoter function in the airway epithelium will enhance our basic understanding of the regulation of eNOS gene expression in both pulmonary and nonpulmonary origin.

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