Arsenite Exposure of Cultured Airway Epithelial Cells Activates \( \kappa \)-B-dependent Interleukin-8 Gene Expression in the Absence of Nuclear Factor-\( \kappa \)-B Nuclear Translocation

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Airway epithelial cells respond to certain environmental stresses by mounting a proinflammatory response, which is characterized by enhanced synthesis and release of the neutrophil chemotactic and activating factor interleukin-8 (IL-8). IL-8 expression is regulated at the transcriptional level in part by the transcription factor nuclear factor \( \kappa \)-B. We compared intracellular signaling mediating IL-8 gene expression in bronchial epithelial cells cultured in vitro and exposed to two inducers of cellular stress, sodium arsenite (As\( ^{III} \)), and vanadyl sulfate (V\( ^{IV} \)). Unstimulated bronchial epithelial cells expressed IL-8, and exposure to both metal compounds significantly enhanced IL-8 expression. Overexpression of a dominant negative inhibitor of NF-\( \kappa \)-B depressed both basal and metal-induced IL-8 expression. Low levels of nuclear NF-\( \kappa \)-B were constitutively present in unstimulated cultures. These levels were augmented by exposure to V\( ^{IV} \), but not As\( ^{III} \). Accordingly, V\( ^{IV} \) induced IxB\( \beta \)-breakdown and nuclear NF-\( \kappa \)-B translocation, whereas As\( ^{III} \) did not. However, both As\( ^{III} \) and V\( ^{IV} \) enhanced \( \kappa \)-B-dependent transcription. In addition, As\( ^{III} \) activation of an IL-8 promoter-reporter construct was partially \( \kappa \)-B-dependent. These data suggested that As\( ^{III} \) enhanced IL-8 gene transcription independently of IxB\( \beta \)-breakdown and nuclear translocation of NF-\( \kappa \)-B in part by enhancing transcription mediated by low levels of constitutive nuclear NF-\( \kappa \)-B.

Nuclear factor-\( \kappa \)-B (NF-\( \kappa \)-B)\(^{1} \) was originally described as a constitutive nuclear transcription activator in mature B lymphocytes that bind a specific DNA sequence in the intronic enhancer of the immunoglobulin \( \kappa \)-light chain (Igx) gene and mediated constitutive Igx expression. (1). However, numerous subsequent studies have shown that NF-\( \kappa \)-B is polymorphic. It is composed of homo- or heterodimers of at least five structurally related mammalian proteins that have a broad tissue distribution. Likewise, NF-\( \kappa \)-B modulates the expression of a large number of genes whose products participate in immune, inflammatory, and environmental stress responses (2, 3).

In many tissues NF-\( \kappa \)-B mediates transient changes in gene expression in response to humoral and environmental stimuli. In this case, NF-\( \kappa \)-B is held inactive in the cytoplasm by IxBs, a family of inhibitor proteins that mask its nuclear translocation signal. The activation of NF-\( \kappa \)-B is mediated in part by the inactivation of IxBs through stimulus-specific posttranslational modifications of IxBs. To date, the most commonly observed mechanism of IxB inactivation involves phosphorylation of two N-terminal serine residues by IxB kinase, a large multimeric complex that receives input from a variety of signal transduction pathways (4, 5). Phosphorylation of IxBs by the IxB kinase complex targets IxBs for ubiquitination and proteolytic degradation. Upon its release from IxB, NF-\( \kappa \)-B translocates into the nucleus and binds to \( \kappa \)-B response elements (RE) in the enhancer regions of target genes.

A number of pharmacological interventions that inhibit inducible \( \kappa \)-B-dependent transcription, however, do not inhibit the translocation of cytoplasmic NF-\( \kappa \)-B into the nucleus or its DNA binding activity (6–10). So an increase in nuclear NF-\( \kappa \)-B alone is not sufficient for the maximal activation of \( \kappa \)-B-dependent transcription. Conversely, enhanced \( \kappa \)-B-dependent transcription has been observed in the absence of an increase in nuclear NF-\( \kappa \)-B in cells that have low levels of constitutive nuclear NF-\( \kappa \)-B (11). This indicates that the mobilization of cytoplasmic NF-\( \kappa \)-B is not invariably necessary for the activation of transcription. Thus \( \kappa \)-B-dependent transcription is dependent upon both the abundance of nuclear NF-\( \kappa \)-B and additional cooperative factors and regulatory processes that influence the transcription activating (transactivating) potential of NF-\( \kappa \)-B.

Of the five known mammalian NF-\( \kappa \)-B family members, p65 (RelA), RelB, c-Rel, p50/p105, and p52/p100, only three, p65, RelB, and c-Rel, are capable of transcriptional activation (2). The transactivation potential of the p65 subunit of NF-\( \kappa \)-B has been shown to depend upon specific p65 protein domains. NF-\( \kappa \)-B family members share a conserved 300-amino acid, N-terminal Rel homology domain that mediates dimerization, nuclear localization, and DNA binding. Phosphorylation of a cAMP-dependent protein kinase site in the Rel homology domain of p65 strongly increases \( \kappa \)-B-dependent transcription and requires IxB\( \beta \)-degradation (6). In contrast, studies using constitutive nuclear chimeric transcription factors have suggested that the transactivation potential of p65 can also be regulated by nuclear processes that are independent of IxB\( \beta \)-degradation and nuclear translocation of p65. Transcriptional regulation by
NF-κB in Arsenite-induced IL-8 Gene Transcription

The mitogen-activated protein (MAP) kinase signal transduction cascades (13–15) have been implicated as upstream regulatory pathways that mediate the activation of NF-κB-dependent transcription by processes that are independent of IκBα degradation and NF-κB nuclear translocation (7, 9, 11, 12). We have recently demonstrated that two metal compounds, sodium arsenite and vanadyl sulfate, activate MAP kinases in airway epithelial cells in vitro (16). These metals also evoke a proinflammatory response as indicated by enhanced production of interleukin-8 (IL-8), an α-chemokine that is a neutrophil chemotactant and stimulant (17, 18). IL-8 gene transcription is induced by phorbol esters and the proinflammatory cytokines tumor necrosis factor-α and interleukin-1β (IL-1β). This induction depends upon an enhancer region of the IL-8 gene located upstream of the transcription start site (base pairs −126 to −72), which includes activator protein-1, C/EBP, and NF-κB response elements. All three of these cis-acting elements are necessary for maximal transcriptional activation, although there are tissue-specific differences in this dependence. The activator protein-1 and C/EBP elements are employed in a tissue-specific fashion, whereas the NF-κB element is necessary in all tissues examined (19–22).

In this study, we investigated the role of NF-κB mobilization in NF-κB-dependent gene transcription following treatment with AsIII and VIVO. Our results suggest that in cultured airway epithelial cells both AsIII and VIVO activated NF-κB-dependent transcription; however, VIVO mobilized cytoplasmic NF-κB, whereas AsIII did not.

Experimental Procedures

Cell Culture and in Vitro Exposure—Primary normal human bronchial epithelial (NHBE) cells were obtained from healthy, nonsmoking adult volunteers. Epithelial specimens were obtained by cytologic brushing at bronchoscopy and subsequently expanded in culture as described previously (23). The human BEAS-2B bronchopulmonary cell line was cultured as described previously (24). Vanadyl sulfate or sodium arsenite (both from Sigma) were diluted in BEGM (NHBE) or KGM (MAS) before addition to the cell culture.

Analysis of IL-8 Expression by RT-PCR and Enzyme-linked Immunosorbent Assay—Extraction of RNA, first-strand cDNA synthesis, and DNA amplification were performed as described previously (23) using the following oligonucleotide primers: GAPDH, sense, CCAAGAGAGCTGAGG, and antisense, CAAAGTGGTCTCAGGATTAC; IL-8, sense, GCTGTCGTCTGTAGAGGTCCT; and antisense, AACCCTTGGACACCATTTCCCT; and c-Jun, sense, CAGCGCTGGCGCCCTGATAAT, and antisense, GCGTGTTCTGGCTGTGCAGTT. Following amplification, products were analyzed by alkaline gel electrophoresis through 2% agarose gels in 1× Tris/borate/EDTA buffer. The gel was stained using 1 μg/ml ethidium bromide and photographed under UV illumination with Polaroid type 55 P/N film (Polaroid, Cambridge, MA). The specific bands were quantified using the Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY), and optical densities for IL-8 mRNA bands were normalized to GAPDH RNA by use of Image Analysis Software (Eastman Kodak Company, Rochester, NY).

Analysis of Nuclear Factor-KB Transactivation—For nuclear transactivation, the human BEAS cells grown to 40–80% confluence in 24-well tissue culture plate were transiently cotransfected with p1.5IL8wt-luc and pCMV3. The NF-κB and C/EBP response elements in p1.5IL8wt-luc were disrupted by site-directed mutagenesis using PCR and uracil-containing oligonucleotides as described (24, 25). The NF-κB response element was mutated from 5′-GTTGGAATTTCT-3′ to 5′-GGAaATTTCT-3′ (27), generating p1.5IL8mut-luc. The C/EBP response element was mutated from 5′-GTTGCAAATCT-3′ to 5′-GcAGcGcAGcGc-3′ (21), generating p1.5IL8C/EBPmut-luc. Mutations were confirmed by sequencing (University of North Carolina Automated DNA Sequencing Facility, Chapel Hill, NC).

Western Blot Analysis—Protein samples (50 μg) were separated by SDS-polyacrylamide gel electrophoresis on 14% Tris-glycine gels, followed by immunoblotting using specific rabbit antibodies to IκBα or p65 (both 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Antigen-antibody complexes were stained with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:4000, Bio-Rad) and enhanced chemiluminescence (ECL) reagent and ECL film (both from Amersham Pharmacia Biotech). Immunoblot films were denatured at 95 °C for 10 min, and stored at –70 °C for immunoblot analysis. Protein content of a small aliquot of the cytoplasmic fraction was determined using the DC Bradford assay (Bio-Rad). The nuclei were washed with CEB and centrifuged again at 15,000 × g for 30 s. The supernatant was aspirated, and the nuclei were incubated for 10 min on ice with 10 μg/ml of the protease inhibitors, 1 mM pepstatin, 40 μg/ml leupeptin, 50 μg/ml chymostatin, 100 μg/ml aprotinin, 1 mM sodium arsenite, 1 mM vanadyl sulfate, and 1 mM sodium selenite for 30 min. Nuclei were pelleted by centrifugation at 15,000 × g for 30 s. The supernatant containing the cytoplasmic fraction was mixed with 1% SDS, 0.7 M mercaptoethanol, 0.05% bromophenol blue, and stored at −80 °C until analysis by SDS-polyacrylamide gel electrophoresis and immunoblotting.
digested, and the optical densities of specific antigen-antibody complexes were quantified as described above (see RT-PCR methods).

Indirect Immunofluorescent Localization of Hemagglutinin-tagged IκBα(S32A,S36A)—BEAS-2B cultures that had been infected with Ad5IκBα (see above) or Ad5Luc2 24 h earlier were fixed for 5 min with 4% paraformaldehyde in CEBr at room temperature, lysed for 2 min on ice with 0.2% Nonidet P-40 in CEB, washed once with CEB, fixed again for 20 min on ice, and finally blocked by incubation in 2% BSA/PBS on ice for 1 h. The hemagglutinin-tagged IκBα(S32A,S36A) was localized by incubation overnight in 1 μg/ml mouse anti-hemagglutinin monoclonal antibody (Santa Cruz Biotechnology) diluted in 0.2% BSA/PBS followed by a 45-min incubation in a 1:1000 dilution of ALEXA 488 goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) diluted in 0.2% BSA/PBS. Samples were washed with 2% BSA/PBS and photographed on a Zeiss Axiovert 10 fluorescence microscope using a standard fluorescence excitation and emission filter set.

RESULTS

Exposure to Sodium Arsenite or Vanadyl Sulfate Enhanced IL-8 Gene Expression in NHBE Cells—Exposure of primary human airway epithelial cells to nontoxic concentrations of sodium arsenite or vanadyl sulfate in vitro has been shown to enhance IL-8 expression (16, 23). These observations were confirmed and extended by estimating the concentration thresholds for AsIII and VIV induced IL-8 expression. Levels of IL-8 protein in supernatants of NHBE cells cultured in the absence or presence of various concentrations of AsIII and VIV for 24 h are shown in Fig. 1. NHBE cultures constitutively expressed IL-8, and this expression was augmented in a dose-dependent fashion by challenge with the metals. The threshold concentration for metal-induced IL-8 production was lower for VIV (12.5 μM) than for AsIII (25 μM). Likewise, VIV induced greater increases in IL-8 production compared with AsIII when the metals were used at the same concentrations. These data indicated that VIV was a stronger stimulant than AsIII. The same doses of iron, nickel, and copper sulfate did not evoke IL-8 expression (not shown). Thus the response to AsIII and VIV was independent of colloidal properties of metal salts and dependent upon metal species-specific interactions with cellular constituents.

The AsIII and VIV-induced IL-8 production by NHBE cultures was preceded by an increase in steady-state levels of IL-8 mRNA. As shown in Fig. 2A, both AsIII and VIV elevated IL-8 mRNA levels above the basal level within 2 h. Quantitative estimates of IL-8 mRNA abundance using GAPDH mRNA levels to normalize between samples showed that arsenite induced approximately a 2.4-fold increase and vanadium a 5-fold increase in steady-state IL-8 mRNA abundance (Fig. 2B). As in the case of IL-8 protein production, VIV showed greater potency than AsIII in inducing a response.

The Sodium Arsenite- and Vanadyl Sulfate-induced IL-8 Expression in Airway Epithelial Cells Was NF-κB-dependent— The enhanced levels of IL-8 mRNA induced by AsIII and VIV could be mediated by enhanced IL-8 gene transcription. Because extracellular stimulus-dependent IL-8 gene transcription has been shown to be regulated in part by the transcription factor NF-κB (19–22), the role of NF-κB in the AsIII- and VIV-induced IL-8 expression was investigated. NF-κB activity was suppressed in NHBE cultures by overexpression of a dominant negative IκBα mutant (IκBα(S32A,S36A)) in which serines 32 and 36 had been substituted with alanines. Overexpression of this mutant IκBα can sequester NF-κB into IκBα(S32A,S36A)-NF-κB complexes that are unresponsive to numerous stimuli that mobilize NF-κB by activating IκBα kinases that specifically phosphorylate serines 32 and 36. NHBE cultures were infected with Ad5IκBα, an adenoviral expression vector encoding hemagglutinin-tagged IκBα(S32A,S36A) (28) or with a nonrecombinant control vector (Ad5CMV3). Analysis of IκBα levels following infection by immunoblotting confirmed overexpression of IκBα (data not shown). As expected, stimulation with AsIII or VIV up-regulated steady-state IL-8 mRNA levels in the control infected cultures (Fig. 3, Ad5CMV3). In marked contrast, overexpression of the dominant negative IκBα depressed both the AsIII- and VIV-induced increases in steady-state IL-8 mRNA abundance to levels below those observed in Ad5CMV3-infected, unstimulated cultures (Fig. 3, Ad5-IκBα). Basal IL-8 mRNA levels were also suppressed, suggesting that NF-κB may regulate basal IL-8 expression in NHBE cultures. Arsenite also induced an increase in c-Jun mRNA levels, but this response was not affected (Fig. 3A, c-jun), indicating that IκBα(S32A,S36A) overexpression selec-
FIG. 3. Basal levels and arsenite- or vanadium-induced increases in steady-state IL-8 mRNA levels in NHBE cells were suppressed by overexpression of a dominant negative IκBα mutant. NHBE cultures were infected with a nonrecombinant adenovirus (Ad5-CMV3) or an adenoviral expression vector encoding a dominant negative IκBα mutant (Ad5-IκBα) at a multiplicity of infection of 100 plaque-forming units/cell for 3 h and subsequently left untreated (C) or stimulated with 50 μM sodium arsenite (As) or vanadyl sulfate (V) 24 h post-infection. After 2 h stimulation, total RNA was isolated and analyzed for mRNA levels by RT-PCR. A, representative ethidium bromide-stained amplification products of IL-8 (top), GAPDH (middle), and c-Jun (bottom) mRNAs analyzed by alkaline agarose gel electrophoresis are shown. B, densitometric analysis of IL-8 and GAPDH amplification products from at least three independent experiments are shown. The data are expressed as the mean fold increase over levels in unchallenged, Ad5CMV3-infected control cultures ± S.E.

FIG. 4. Vanadyl, but not arsenite, induced IκBα breakdown in NHBE cells. NHBE cultures were left untreated (Control) or were treated with 50 μM sodium arsenite (As) or vanadyl sulfate (V) for 30 or 60 min. Cytoplasmic extracts were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted using a specific anti-IκBα antibodies (see “Experimental Procedures”). A and C, representative immunoblots are shown. B, densitometric analysis of the optical densities of the anti-IκBα immunoreactive bands from at least three independent experiments are shown. The data are expressed as the mean IκBα levels ± S.E.
The mean increase in p65 levels relative to unchallenged controls three independent experiments are shown. The data are expressed as optical densities of the anti-p65 immunoreactive bands from at least were analyzed by immunoblotting using a specific anti-p65 antibody. NHBE cultures separated by SDS-polyacrylamide gel electrophoresis for 30 or 60 min. or were treated with 50 μM arsenite (As) or vanadyl sulfate (V).

AsIII and VIV enhanced transcription above its basal level (Fig. 6A, compare 50 μM As/100 plaque-forming units/cell for 3 h. 48 h post-transfection, cultures were challenged with 50 μM arsenate or vanadyl sulfate for 1 h and harvested 7 or 3 h later, respectively. Specific luciferase activity in culture lysates was determined using β-galactosidase activity as a normalizing factor. The data are expressed as mean specific luciferase activity ± S.E., n = 5. A, both arsenite and vanadyl treatment enhanced kB-dependent transcription. The inducible as well as the basal activity was inhibited by overexpression of IκBα(S32A,S36A). B, indirect immunofluorescent localization of the hemagglutinin-tagged IκBα(S32A,S36A) transgene product showed that it was present in both the cytoplasm and nucleus of Ad5IκBα-infected BEAS-2B cells. Nuclear IκBα(S32A,S36A) could explain the inhibition of the basal and arsenite-induced activity which were independent of NF-κB mobilization. C, hemagglutinin immunoreactivity was not detected in cultures infected with Ad5lacZ, an expression vector encoding untagged β-galactosidase. Bar, 25 μm.

Overexpression of IκBα(S32A,S36A) inhibited both the basal and metal-induced luciferase activity (Fig. 6A, compare Media−/− to Media+/−, 50 μM As+/− to 50 μM As+/− and 50 μM V+/− to 50 μM V+/−), whereas infection with the nonrecombinant adenovirus (Ad5CMV3) did not affect either activity (Fig. 6A, compare Media−/− to Media+/−, 50 μM As+/− to 50 μM As+/− and 50 μM V+/− to 50 μM V+/−).

Inhibition of the VIV−/−-induced kB-dependent activity was expected, because IκBα(S32A,S36A), a cytoplasmic inhibitor, would be expected to prevent NF-κB mobilization, and anti-p65 immunoblotting had shown that it prevented the VIV−/−-induced increase in p65 (not shown). The inhibition of basal and arsenite-induced kB-dependent activity was unexpected, because they appeared to be independent of NF-κB mobilization. However, indirect immunofluorescent localization of the hemagglutinin-tagged IκBα(S32A,S36A) transgene product demonstrated that it was present in both the nucleus and cytoplasm (Fig. 6B). The presence of nuclear IκBα(S32A,S36A) and inhibition of kB-dependent transcription was in accordance with reports that IκBα when uncharged with NF-κB is imported into the nucleus where it can extract NF-κB from transcription initiation complexes and inhibit kB-dependent transcription (30–33). Thus, IκBα(S32A,S36A) overexpres-
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sion suggested that airway epithelial cell cultures supported a basal level of NF-κB-dependent transcription that was augmented by exposure to either AsO₃⁻ or V⁷⁺.

Sodium Arsenite Induced κB-dependent IL-8 Promoter-Reporter Activity in Airway Epithelium—The IκBα(S32A,S36A)-mediated inhibition of IL-8 mRNA levels (Fig. 3B) suggests that arsenite may be stimulating κB-dependent IL-8 gene transcription. To investigate this possibility, the influence of arsenite on the activity of an IL-8 promoter-luciferase construct was examined. The IL-8 promoter-reporter construct was active in unchallenged cultures (Fig. 7, Media →), consistent with the observed basal expression of IL-8 mRNA and protein in cultures (Figs. 1–3). Moreover, basal transcriptional activity was suppressed by overexpression of IκBα(S32A,S36A) (Fig. 7, WT, compare Media → to Media⁻/⁻), consistent with the observed decrease in basal IL-8 mRNA levels following infection with Ad5IκBα (Fig. 3). Arsenite induced a significant increase in the transcriptional activity (Fig. 7, WT, compare 50 μM As⁻/⁻ to Media⁻/⁻), whereas overexpression of IκBα(S32A,S36A) inhibited this response (Fig. 7, WT, compare 50 μM As⁻/⁻ to 50 μM As⁺/⁻). There was, however, a residual difference in the activity of IL-8 promoter-reporter construct in unstimulated and arsenite-challenged cultures that had been infected with Ad5IκBα (Fig. 7, WT, compare Media⁻/⁻ to 50 μM As⁻/⁻). This suggested that only a portion of the arsenite-induced IL-8 promoter-reporter activity was κB-dependent. Infection with the nonrecombinant adenovirus did not affect the basal or inducible transcriptional activity (Fig. 7, WT, compare Media⁻/⁻ to Media⁺/⁺ and 50 μM As⁻/⁻ to 50 μM As⁺/⁻). In addition, expression of IκBα did not affect the activity of the promoterless parent luciferase vector of the IL-8 promoter-reporter construct or that of a constitutively active SV40 promoter-luciferase construct (not shown). The specificity of the inhibition mediated by IκBα(S32A,S36A) overexpression was investigated by determining its effect on the activity of an IL-8 promoter reporter construct in which the NF-κB response element had been inactivated by mutation. As expected, the dominant negative IκBα did not affect the κB-independent activity of the mutant IL-8 promoter (NF-κB⁻ 50 μM As⁺), indicating that IκBα(S32A,S36A) selectively inhibited κB-dependent transcription. These data supported the notion that the arsenite-induced increase in IL-8 expression was partially dependent upon enhanced, κB-dependent IL-8 gene transcription.

The Basal and Arsenite-induced IL-8 Promoter-Reporter Activity Required the Compound C/EBP/NF-κB Response Element of the IL-8 Promoter—The κB dependence of the basal and arsenite-induced activity of the IL-8 promoter-reporter construct suggested by the suppressive effects of IκBα(S32A,S36A) overexpression (Fig. 7) was confirmed by mutational analysis of the IL-8 promoter. Several studies have indicated that inducible κB-dependent IL-8 gene transcription requires a compound C/EBP (nuclear factor-IL-6)/NF-κB response element located upstream (base pairs −94 to −72) of the transcription start site in the IL-8 gene (20–22, 34), although the C/EBP element may be dispensable in some instances (19). Consequently, the C/EBP and NF-κB elements of the compound RE in the IL-8 promoter-reporter construct were independently disrupted by site-directed mutagenesis, and the phenotype of these mutations was characterized by transient transfection of BEAS-2B cultures. The basal activity of the κB⁻ construct was reduced about 20-fold compared with the wild type construct (Fig. 8, compare lanes C at both WT and NF-κB⁻). Reversion of the κB⁻ construct to wild type restored basal activity to wild type levels (not shown), indicating that the reduction in basal activity was due solely to disruption of the NF-κB response element. Disruption of the C/EBP RE also significantly reduced the basal IL-8 promoter-reporter activity (Fig. 8, compare lanes C at both WT and C/EBP⁻), although to a lesser degree than mutation of the NF-κB RE. Thus, the basal activity of the IL-8 promoter-reporter construct was dependent upon both the NF-κB and C/EBP elements of the IL-8 promoter. Moreover, the full basal activity of the wild type construct was approximately 2.7 times greater than the sum of the activities κB⁻ and C/EBP⁻ constructs, suggesting that IL-8 promoter activity in unstimulated airway epithelium depends upon synergistic interactions between nuclear factors that bind to the C/EBP/NF-κB compound RE of the IL-8 promoter.

Even though the overall activity of the κB⁻ construct was greatly reduced, it retained some arsenite responsiveness (Fig. 8, compare lane C at NF-κB⁻ to lane As at NF-κB⁻). Arsenite induced a 3.8 ± 1-fold increase of the activity of the wild type construct, whereas it induced a significantly smaller 2.4 ±
0.5-fold increase in the activity of the \( \kappa B \) construct. This suggested that transcription factors in addition to NF-\( \kappa B \) dominated responsiveness of the IL-8 promoter to arsenite exposure. Mutation of the C/EBP RE suppressed the arsenite inducibility of the IL-8 promoter-reporter construct (Fig. 8, compare lane C at C/EBP to lane As at C/EBP\( ^{-} \)). Thus the C/EBP RE had a greater influence on the arsenite-inducible activity than the NF-\( \kappa B \) RE had. As in the case of basal activity, the activity of the wild type construct was approximately 6.3 times greater than the sum of activities of the \( \kappa B \) and C/EBP\( ^{-} \) constructs, suggesting synergistic interactions between transcription factors.

Because the basal and arsenite induced IL-8 promoter-reporter activity was dependent upon the compound C/EBP/NF-\( \kappa B \) response element of the IL-8 promoter (Fig. 8), nuclear extracts of NHBE cultures were analyzed by EMSA for DNA binding activities specific for this sequence. There were detectable levels of a single DNA binding activity in the nuclei of unchallenged cultures that were enhanced following treatment with arsenite for 1 h (Fig. 9A, arrow). The increases were transient, returning to control levels after 4 h of exposure. The activity was specific for the sequence of the compound C/EBP/NF-\( \kappa B \) RE, because competition with 100-fold molar excess of unlabeled probe inhibited radiolabeled complex formation (Fig. 9B). Mutation of either half of the response element resulted in a significant reduction in DNA binding (Fig. 9C, compare \( wt \) with \( mNF-\kappa B \) and \( mC/EBP \)). This basal and enhanced DNA binding activity for the C/EBP/NF-\( \kappa B \) compound response element and its sensitivity to mutation correlated with the observed basal and arsenite-induced activation of the IL-8 promoter-reporter construct (Figs. 7 and 8) and its inhibition by disruption of the compound C/EBP/NF-\( \kappa B \) response element (Fig. 8).

Nuclear extracts were also examined using a radiolabeled probe corresponding to the solitary C/EBP response element of the IL-6 gene (Table I). A DNA binding activity was observed in unstimulated cultures, and this activity was enhanced following exposure to arsenite (Fig. 9D, arrow, lanes C and As). These data demonstrated the presence of a constitutive nuclear factor in airway epithelium that binds the C/EBP response element and whose activity was increased by arsenite exposure.

**DISCUSSION**

In this study we investigated As\( ^{III} \)- and V\( ^{IV} \)-induced NF-\( \kappa B \) activation pathways, which culminate in IL-8 gene expression in airway epithelial cells. Both the As\( ^{III} \)- and V\( ^{IV} \)-induced IL-8 expression were NF-\( \kappa B \)-dependent; however, V\( ^{IV} \) induced IkB degradation and NF-\( \kappa B \) translocation, whereas exposure to As\( ^{III} \) failed to do so. Thus, despite the \( \kappa B \) dependence of arsenite-induced gene expression, there was no detectable mobilization of cytoplasmic NF-\( \kappa B \), suggesting that the response to arsenite was mediated by low levels of constitutive nuclear NF-\( \kappa B \) that were detected in the airway epithelial cell cultures.

The presence of low levels of constitutive nuclear NF-\( \kappa B \) was suggested by several pieces of evidence: (i) Nuclear p65 was detected by immunoblotting of nuclear extracts of unchallenged cultures (Fig. 5); (ii) EMSA of nuclear extracts using a radiolabeled probe corresponding to the solitary C/EBP response element of the IL-8 gene revealed basal nuclear levels of a \( \kappa B \)-dependent DNA binding activity (Fig. 9B); (iii) unstimulated cultures supported \( \kappa B \)-dependent transcription from both 5xNF-\( \kappa B \)-reporter (Fig. 6) and IL-8 promoter-reporter constructs (Figs. 7 and 8); and (iv) basal expression of IL-8 mRNA was \( \kappa B \)-dependent (Fig. 3).

The \( \kappa B \) dependence of basal IL-8 mRNA expression and basal activities of the promoter-reporter constructs was suggested by their suppression following global inhibition of NF-\( \kappa B \) function by overexpression of a dominant negative IkB\( a \) mutant (Figs. 3, 6, and 7). The mutant IkBo was present not only in the cytoplasm but also in the nucleus (Fig. 6B). This is in accordance with observations that IkB\( a \) is imported into the nucleus when uncharged with NF-\( \kappa B \) (30), a likely situation when IkBo is overexpressed. Nuclear IkBo inhibits \( \kappa B \)-dependent transcription (31–33), which was also observed here. The
specificity of the inhibition for NF-κB-dependent processes was suggested by a number of observations. Overexpression of IκBα(S32A,S36A) did not inhibit the AsIII-induced increase in c-Jun message (Fig. 3) or the B-independent activity of the IL-8 promoter (Fig. 7). Additional studies indicate that overexpression of the mutant IκBα does not inhibit basal or phorbol myristate acetate-induced activator protein-1-dependent transcription but does inhibit phorbol myristate acetate-induced B-dependent transcription. Thus, it is clear that IκBα(S32A,S36A) overexpression did not inhibit transcription in a nonspecific fashion. The data consistently supported the notion that there were low levels of constitutive nuclear NF-κB and basal IL-8 expression in airway epithelial cell cultures.

The origin of the low levels of constitutive nuclear NF-κB and IL-8 expression is not clear. Environmental stresses because of artificial cell culture conditions have been shown to elicit IL-8 from cultured peripheral blood mononuclear cells, whereas freshly isolated (naïve) peripheral blood mononuclear cells do not express IL-8 (35). Thus, it is possible that stresses because of artificial culture conditions in addition to AsIII are acting on freshly isolated (naïve) peripheral blood mononuclear cells, whereas artificial cell culture conditions have been shown to elicit IL-8 from cultured peripheral blood mononuclear cells, whereas dysfunctions in the low levels of constitutive nuclear NF-κB and basal IL-8 expression in airway epithelial cell cultures.

Constitutive nuclear NF-κB has been observed previously in mature B cells (46), activated monocytes and macrophages (47), neurons (48), vascular endothelial cells (49), and fibroblasts (11). These studies suggest that the proportion of NF-κB that is constitutively nuclear and that the subunit composition of nuclear NF-κB varies with tissue type as well as with cellular differentiation and activation state. How constitutive nuclear levels of NF-κB are set remains unclear. In neurons it may be determined by an autocrine activation loop (48). B lymphocyte maturation is associated with decreased stability of IκBα as well as de novo expression of nuclear RelB-p52/p100 heterodimers, which are not efficiently inhibited by IκBα (50, 51). In certain tumor cell lines, elevated constitutive nuclear NF-κB is associated with decreased stability of IκBα (52, 53), whereas dysfunctional IκBα mutants have been observed in other tumor cells (54). Generalizing these observations leads to the suggestion that levels of constitutive nuclear NF-κB may also vary as a consequence of genetic polymorphisms in NF-κB or in the macromolecules that regulate NF-κB expression or activity. The findings presented here may provide a framework for anticipating variation in the effects of arsenic exposure depending on tissue type, cellular differentiation or activation state, and possibly individual susceptibility.

Arsenite activated B-dependent transcriptional activity of a 5xNF-κB-reporter and IL-8 promoter-reporter construct in airway epithelium without increasing the low levels of constitutive nuclear NF-κB. This finding is consistent with a recent study in which enhanced B-dependent transcription induced by Ras or Raf transformation of NIH 3T3 fibroblasts was mediated by constitutive nuclear NF-κB (11). In this case, transformation was shown to activate a chimeric transcription factor composed of the DNA-binding domain of the yeast Gal4 transcription factor and the C-terminal TA1 transactivation domain of p65. This suggested that transcriptional activation was due to functional activation of p65. Using the same strategy, the transcription promoting activity of the TA1 and TA2 transactivation domains of p65 have also been shown to be responsive to tumor necrosis factor-α (12). Both of these studies suggested that activation does not depend upon translocation of the hybrid transcription factor into the nucleus but rather is a consequence of nuclear processes that are dependent upon activation of p38 and/or ERK1/2 MAP kinases. Arsenite has been shown to activate ERK1/2, JNK/SAPK, and p38 MAP kinases in a variety of tissues (55–60) including airway epithelial cells (16). Taken together, these studies present a plausible mechanism by which arsenite may activate B-dependent gene transcription in the presence of low levels of constitutive nuclear NF-κB. Currently, we are addressing the role of MAP kinase activation in arsenite-induced B-dependent transcription in airway epithelium.

The arsenite-induced activation of the IL-8 promoter-reporter construct was shown to depend upon the compound C/EBP/NF-κB response element of the IL-8 gene (Fig. 8). Previously, this compound RE was shown to be essential for the activation of IL-8 promoter-reporter constructs by phorbol esters, tumor necrosis factor-α, and IL-1β (20–22). Both p65 and the β isoform of C/EBP (C/EBPβ) family of transcription factors (61) have been shown to bind this RE in vitro (21, 27) and synergistically activate transcription dependent upon the C/EBP/NF-κB RE (21, 34). However, it is likely that other combinations of C/EBP and NF-κB isoforms may cooperate at this site, because numerous synergistic combinations of the six known isoforms of C/EBP with NF-κB isoforms have been suggested (61). In this study, analysis of nuclear extracts by EMSA

| Gene       | Binding site | Sequence (5' to 3') |
|------------|-------------|---------------------|
| IL-8       | C/EBP/NF-κB | GTCATCAGTTGCAAATCCTG|
| IL-8       | C/EBP/NF-κB | TGGCGATCAGTTGCAAATCCTG|
| IL-8       | mC/EBP/NF-κB| TAGCCCATCAGCTGAGTG|
| MHC class II| NF-κB      | GGCGTTGGATCCCTCACCT |
| IL-6       | C/EBP       | TGGCGATCAGCTGAGTG |

2 W. Reed, unpublished observations.
indicated the presence of a DNA binding activity for the compound RE in unstimulated cultures whose activity was transiently enhanced by arsenite exposure (Fig. 9A). This activity correlated with the basal and arsenite-enhanced activity of the IL-8 promoter-reporter construct (Fig. 8). Additional analysis of nuclear extracts by EMSA indicated that a nuclear factor that binds a solitary C/EBP RE is present in unstimulated cultures and its activity is enhanced by arsenite exposure (Fig. 9D). C/EBP isoforms can be phosphorylated on independent regulatory sites by cAMP-dependent protein kinase, calcium/calcmodulin-dependent protein kinase, protein kinase C, and MAP kinase, and these modifications influence nuclear translocation and DNA binding activity (61, 62). Based upon our data, we would predict that a C/EBP-like factor complexed with constitutive nuclear p65, both in functionally activated states, explain the arsenite-induced DNA binding activity for the C/EBP/NF-kB RE, as well as the dependence of arsenite-induced IL-8 promoter-reporter activity upon the compound RE. Further studies are necessary to identify the factor or factors binding to the C/EBP/NF-kB RE of the IL-8 promoter as well as their individual transactivation potentials in unstimulated and arsenite-treated airway epithelial cells.

In conclusion, the study presented here describes a distinct mechanism of enhanced kB-dependent gene expression in airway epithelium. In the absence of iNOS breakdown and mobilization of cytoplasmic NF-kB, exposure to arsenite increased kB-dependent transcription and gene expression, implying a potential role for basal levels of nuclear NF-kB in inducible gene transcription.

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