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Transcriptional Activation of the Interleukin-8 Gene by Respiratory Syncytial Virus Infection in Alveolar Epithelial Cells: Nuclear Translocation of the RelA Transcription Factor as a Mechanism Producing Airway Mucosal Inflammation

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The most common cause of epidemic pediatric respiratory disease, respiratory syncytial virus (RSV), stimulates interleukin-8 (IL-8) synthesis upon infecting airway epithelium, an event necessary for the development of mucosal inflammation. We investigated the mechanism for enhanced IL-8 production in human A549 type II pulmonary epithelial cells. Infection with sucrose-purified RSV (pRSV) produced a time-dependent increase in the transcriptional initiation rate of the IL-8 gene. Transient transfection of the human IL-8 promoter mutated in the binding site for nuclear factor-κB (NF-κB) demonstrated that this sequence was essential for pRSV-activated transcription. Gel mobility shift assays demonstrated pRSV induction of sequence-specific binding complexes; these complexes were supershifted only by antibodies directed to the potent NF-κB transactivating subunit RelA. Both Western immunoblot and indirect immunofluorescence assays showed that cytoplasmic RelA in uninfected cells became localized to the nucleus after pRSV infection. RelA activation requires replicating RSV, because neither conditioned medium nor UV-inactivated pRSV was able to stimulate its translocation. We conclude that RelA undergoes changes in subcellular distribution in airway epithelial cells upon pRSV infection. The ability of replicating RSV to activate RelA translocation may play an important role in activating IL-8 and other inflammatory gene products necessary for airway mucosal inflammation seen in RSV disease.

Respiratory syncytial virus (RSV), an enveloped single-stranded negative-sense RNA-containing virus, is the major cause of serious lower respiratory tract disease in children (reviewed in reference 27). It is estimated that 40 to 50% of children hospitalized with bronchiolitis and 25% of children with pneumonia are infected with RSV, resulting in 100,000 hospital admissions annually in the United States alone (23). Indeed, substantial morbidity and mortality occur in infants with underlying heart and lung diseases (24, 36). In addition to the acute morbidity of RSV infection in infancy, there are long-term consequences: RSV has been shown to predispose to the development of hyperreactive airway disease (26), and recurrent episodes of wheezing are often precipitated by subsequent RSV infection (52).

RSV infection results either from inhalation or from self-inoculation of the virus into the nasal mucosa, followed by infection of the local respiratory epithelium. RSV spreads along the respiratory tract mainly by cell-to-cell transfer through intracytoplasmic bridges (25). In RSV-infected infants, the severity of respiratory symptoms correlates with the presence and concentrations of inflammatory mediators in nasopharyngeal secretions (19, 21, 58). Histopathological studies of fatal RSV-induced respiratory disease have identified necrosis of the small airway epithelium, peribronchial mononuclear cell infiltration, and plugging of the bronchial lumen by sloughed epithelium and mucus (1). Moreover, investigations of bronchial lavage specimens obtained from infants with RSV bronchiolitis have demonstrated a striking predominance of neutrophils (17). These findings suggest that the inflammatory response triggered by the infection of respiratory epithelial cells is an essential pathogenic component of RSV disease.

The airway epithelium plays a primary role in pulmonary clearance of inhaled particulates, antimicrobial defense and maintenance of alveolar integrity. In addition, respiratory epithelial cells are involved in the induction and modulation of airway inflammatory responses through the synthesis of a variety of proinflammatory mediators as well as cytokines, including interleukin-8 (IL-8) (40, 53). IL-8 is known to be a potent chemoattractant and activator for neutrophils, T cells, basophils, and primed eosinophils (53) which express functional IL-8 receptors (31). High concentrations of IL-8 have been detected in nasal lavage fluids of children with RSV upper respiratory infection (42) and in middle ear effusions of children with viral otitis media (14). It is therefore likely that this cytokine plays a major role in the recruitment of leukocytes to the lung after infection by RSV.

In vitro studies have shown a dramatic production of IL-8 by airway epithelial cells upon RSV infection through an incompletely characterized mechanism involving enhanced gene expression (2, 30, 41). Using the model of well-differentiated lung type II alveolar epithelial cells (A549) to study the initial events in virus-induced regulation of gene expression, we investigated the mechanism for IL-8 induction by RSV infection. After RSV infection, IL-8 protein and mRNA were enhanced primarily through enhanced transcription (as demonstrated by nuclear run-on transcription assay). Transient transfections of the IL-8 promoter reproduced the magnitude and kinetics of

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endogenous gene activation. Site-directed mutations of the NF-κB binding sequence completely abolished RSV-inducible reporter activity. Finally, we demonstrate that RelA, the potent transcriptional activator subunit of the NF-κB family, is induced to bind the IL-8 promoter in response to RSV infection through cytoplasmic-nuclear translocation.

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MATERIALS AND METHODS

RSV preparation. The human Long strain of RSV (A2) was grown in Hep-2 cells and purified by centrifugation on discontinuous sucrose gradients as described elsewhere (56). The virus titer of the sucrose-purified RSV (pRSV) pools was 7.5 to 8.5 log PFU/ml according to a methylcellulose plaque assay (32). To inactivate replicating virus, pRSV was diluted in 1 ml of minimal essential medium (MEM) containing 2% fetal bovine serum (FBS) and exposed for 3 min to a 254-nm UV light source at a 10-cm distance on ice. No contaminating cytokines, including IL-1, tumor necrosis factor alpha, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, and interferon were found in these sucrose-purified viral preparations (44). Lipopolysaccharide, assayed with the limulus hemocyanin agglutination assay, was not detected. RSV-conditioned medium was prepared by infecting monolayers of A549 cells with pRSV at a multiplicity of infection (MOI) of 1 in MEM with 2% FBS and then incubated at 37°C in 5% CO_2. At 12 h, when NF-κB DNA binding activity was measured, the supernatant was collected, centrifuged at 3,000 × *g*, and exposed to a 254-nm UV light source to inactivate replicating virus. Virus pools and conditioned medium were aliquoted, quick-frozen on dry ice-alcohol, and stored at −70°C until used.

Infection of epithelial cells with RSV. A549 human alveolar type II-like epithelial cells (American Type Culture Collection, Rockville, Md.) were grown to confluence in MEM containing 10% (vol/vol) FBS, 10 mM glutamine, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. Cell monolayers were infected with RSV at an MOI of 1 as described previously (30). An equivalent amount of a 20% sucrose solution was added to control A549 cell cultures which received no RSV. At designated time points, the supernatants were removed, centrifuged at 200 × *g*, and stored at −70°C until assayed.

IL-8 ELISA. Immunoreactive IL-8 was quantitated by a double-antibody enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, Minn.) by following the manufacturer's protocol.

RT-PCR. Total RNA was extracted from uninfected or infected A549 cell monolayers by the acid guanidium thiocyanate-phenol chloroform method (Trisolv; Biotecx, Houston, Tex.). One microgram of total RNA was used to perform the reverse transcription (RT) and PCR under conditions specified by the manufacturer (Perkin-Elmer). In the PCR step, 25 pmol each of sense and antisense primers for human IL-8 (hIL-8) or G3PDH (Clontech, Palo Alto, Calif.) was used. For quantitation, internal control MIMIC cDNA (Clontech) was included in the IL-8 reaction mixtures. The PCR amplification conditions were hot start at 95°C for 2 min, followed by 95°C for 45 s, 60°C for 30 s, and 72°C for 1 min for 33 cycles with a final amplification at 72°C for 10 min. PCR products were electrophoresed alongside ϕX-174-*Hae*III DNA markers (Sigma, St. Louis, Mo.) in a 6% polyacrylamide gel. The DNA was visualized by ethidium bromide staining, and the band density was determined with a ScanJet II optical densitometer.

Nuclear run-on transcriptional rate assay. A nuclear run-on transcriptional rate assay was performed with 50 μl of nuclear suspension from uninfected or RSV-infected A549 cells (corresponding to 10^7 cells) as previously described (37). Briefly, after trichloroacetic acid and ethanol precipitations of ^{32}P-labeled RNA, equal numbers of cpm (2.5×10^6 to 5×10^6 cpm/ml) were hybridized in separate bags to nitrocellulose membrane strips containing immobilized plasmid DNAs (containing hIL-8 and actin cDNAs as indicated) in Denhardt's solution (1% [vol/vol] polyvinylpyrrolidone, 1% Ficoll, and 1% bovine serum albumin)–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM Na_2HPO_4, and 1 mM EDTA [pH 7.7])–0.1% sodium dodecyl sulfate (SDS), and 100 μg of herring sperm DNA. After 48 h of hybridization at 42°C, the membrane strips were washed twice with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate [pH 7.0]) and 0.1% SDS for 15 min each at 22°C, followed by being washed once with 0.25× SSC and 0.1% SDS at 65°C for 15 min. The membrane strips were then finally exposed to Kodak X-ray film in the presence of an intensifying screen for 2 to 6 days at −70°C or to a Molecular Dynamics PhosphorImager screen for image quantification.

Plasmid construction. The −162 hIL-8 gene promoter was amplified from a 1.4-kb IL-8 LUC plasmid (kindly provided by N. Mukaida, Kanazawa University) by PCR with 5′ AACTTTGGATCCACTCCGTATTTGATAAGG 3′ (upstream primer) and an antisense primer to the luciferase cDNA from positions +86 to +55 (downstream primer) (7). The PCR product was digested with the endonucleases *Bam*HI and *Hin*dIII and cloned into the promoterless luciferase plasmid pOLUC (9). Site-directed mutation in the NF-κB site was introduced into the −162 IL-8/Luc promoter by PCR (28) with 5′-TTCATTATGTCAGATTA AATTAAACGATTT-3′ and 5′-TTGCAAATCGTTTAATTTAATCTGA CATA-3′ used as mutagenic primers. The final product was ligated into pOLUC with *Bam*HI and *Hin*dIII. Promoter constructs were sequenced in their entirety prior to transfection by the dideoxy chain termination method with the Sequenase version 2.0 kit (Amersham International).

Cell transfection. Logarithmically growing A549 cells were transfected at 50 to 60% confluency in triplicate 60-mm-diameter petri dishes by incubation overnight in 1.8 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered Dulbecco's MEM (10 mM HEPES [pH 7.4]) and 60 μl of 60 mg of DEAE-dextran (Pharmacia) per ml premixed with 5 μg of IL-8/Luc reporter plasmid and 1 μg of cytomegalovirus β-galactosidase internal control plasmid. After 6 h, the medium was removed, and 0.5 ml of 10% (vol/vol) dimethyl sulfoxide in phosphate-buffered saline (PBS) was added to the cells for 2 min. The cells were then washed with PBS and cultured overnight in 2% FBS–Dulbecco's MEM. The next morning, cells were infected with RSV. At the indicated times, cells were lysed to independently measure luciferase and β-galactosidase reporter activity exactly as described previously (35). Luciferase was then normalized to the internal control β-galactosidase activity.

EMSA. Nuclear extract was prepared from uninfected and infected A549 cells for the indicated times by our previously published protocols (30, 35). Duplex oligonucleotides used in the electrophoretic mobility shift assay (EMSA) corresponded to the −96 to −69 nucleotides of the IL-8 gene promoter (mutations in the known NF-κB contact sites are underlined)

IL-8 wild type (WT): GATCCATCAGTTGCAAATCGTGGAATTTCCTCTA
GTAGTCAACGTTTAGCACCTTAAAGGAGATCTAG

IL-8Δκ: GATCCATCAGTTGCAAATCGTTTAATTTAATCTA
GTAGTCAACGTTTAGCAAATTAAATTAGATCTAG

DNA-binding reaction mixtures contained 15 μg of total protein, 1 μg of poly(dA-dT), and 20,000 cpm of α-^{32}P-labeled double-stranded IL-8 WT in a total volume of 30 μl. The binding reaction mixtures were incubated for 15 min at room temperature and then fractionated by 6% nondenaturing polyacrylamide gel electrophoresis (PAGE) in TBE buffer (22 mM Tris-HCl, 22 mM boric acid, 0.25 mM EDTA [pH 8]). In competition assays, 0.5 and 2 pmol of unlabeled IL-8 WT or IL-8Δκ competitors were added at the same time the probe was added. For the supershift assay, 1 μl of either preimmune, anti-NF-κB1, anti-NF-κB2, anti-c-Rel, anti-RelA NH_2-terminal (amino acids 3 to 19), or anti-RelA COOH-terminal antibody (Santa Cruz Biotech) was added to the binding reaction mixtures, and the reactions continued for an additional hour on ice prior to fractionation by 5% PAGE. For the EMSAs with recombinant RelA, 100 ng of affinity-purified RelA [p65(12-317)] was added in the same incubation buffer (29). After electrophoretic separation, gels were dried and exposed for autoradiography with Kodak XAR film at −70°C with intensifying screens.

Western immunoblot. Plates of uninfected and pRSV-infected A549 cells were mechanically harvested in PBS, and cells were collected in microcentrifuge tubes by centrifugation (1 min at 500 × *g*). The cytoplasmic and soluble nuclear extracts were prepared by nonionic detergent lysis and high-salt extraction as previously described (35), and protein content was measured relative to bovine serum albumin (Protein Reagent; Bio-Rad, Hercules, Calif.). Two hundred micrograms of cytoplasmic or nuclear protein was then fractionated by SDS-PAGE (10% polyacrylamide) and transferred onto polyvinylidene difluoride membranes by electroblotting at 50 V in 1× CAPS buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11), 10% (vol/vol) methanol] at room temperature for 1 h. The membrane was then washed, blocked, and probed with a 1:1,000 dilution of NH_2-terminal anti-RelA antibody (Santa Cruz Biotech), followed by washing and secondary detection with horseradish peroxidase-coupled goat anti-rabbit immunoglobulin G according to the manufacturer's recommendations (Amersham Life Sciences). No signal was observed with preimmune serum in place of the primary antibody.

Immunofluorescence staining. A549 cells grown in eight-well chamber slides were treated with sucrose or infected with pRSV for 24 h. Cells were washed in PBS, fixed with 4% paraformaldehyde in PBS for 20 min, washed with PBS, and incubated with 50 mM NH_4Cl for 10 min. The cells were then permeabilized with 0.2% (vol/vol) Triton X-100 for 15 min and washed with 2% bovine serum albumin–PBS. The cells were incubated with the primary antibodies (preimmune rabbit serum or anti-RelA antibody diluted 1/100) for 1 h at room temperature. The slides were then washed with cold 0.05% Tween 20–PBS three times and incubated with secondary antibodies (fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G diluted 1/200) for 1 h. After being washed with cold 0.05% Tween 20–PBS, the cells were counterstained with 1/100-diluted Evans-Blue and examined with a fluorescence microscope (Nikon OPTIPHOT) equipped with a photomicrographic attachment (Nikon HFX-DX).

RESULTS

Production of IL-8 protein and mRNA by RSV-infected airway epithelial cells. The A549 cell line, derived from an alveolar cell carcinoma of the lung, retains features of type II alveolar epithelial cells and is susceptible to RSV infection (44). Monolayers of A549 cells were exposed to pRSV at an

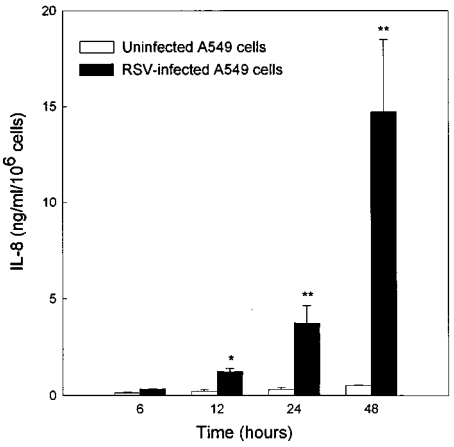

FIG. 1. Kinetics of IL-8 production in A549 culture supernatants upon RSV infection. Human A549 type II pulmonary epithelial cells were infected for various times with pRSV (at an MOI of 1). Concentrations of IL-8 in culture supernatant were determined by ELISA at the indicated times after infection. Data are expressed as the mean ± standard deviation of five experiments. *, $P < 0.01$; **, $P < 0.001$ (compared with uninfected controls [Student's t test]).

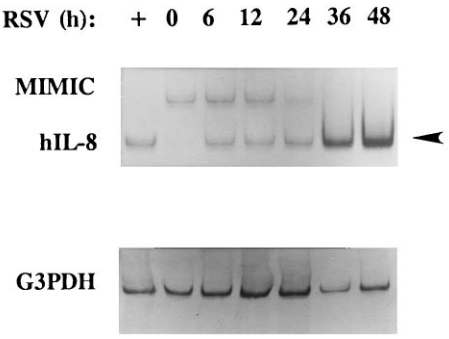

FIG. 2. Time course of changes in IL-8 mRNA abundance in response to RSV infection by competitive RT-PCR. Total cellular RNA from A549 cells infected with pRSV for the indicated times was harvested and used for competitive RT-PCR as described in Materials and Methods. (Top) Photograph of ethidium bromide-stained PAGE of PCR products with IL-8 cDNA plasmid as a template (+) or cDNA isolated from A549 cells at the indicated times (in hours) after pRSV infection. PCR mixtures contained A549 cDNA, IL-8 mRNA-specific primers, and internal control MIMIC DNA (for PCR efficiency). (Bottom) Products from the same RNA samples with G3PDH-specific primers in the PCR mixture. Relative to uninfected controls, IL-8 products increased upon pRSV infection at 6 h and continued to increase until 48 h, whereas G3PDH transcripts remained constant.

MOI of 1. Productive infection was confirmed by observation of viral cytopathic effects and by positive staining of the cells with a monoclonal antibody directed against the RSV F glycoprotein. By immunofluorescence microscopy, RSV antigen could be observed in the cytoplasm starting 8 h after infection. Multiple foci of typical syncytia could be detected by light microscopy observation at 18 to 24 h. Extensive cytopathic effect in the monolayers was appreciable at 48 h, when the A549 cells started to die and fall off the plastic surface of the culture vessels. Therefore, as previously observed (44), the sensitivity of A549 cells for the growth of RSV appears to be similar to that of human heteroploid cell lines, such as the HEp-2 cell line (49). The presence of immunoreactive IL-8 was determined in the cell-free supernatant by a specific ELISA (Fig. 1). Uninfected A549 cells produced <500 pg of IL-8 per ml at all time points tested. On the other hand, by 12 h postinfection, progressively increasing concentrations of IL-8 were measured in RSV-infected A549 cell supernatant until 48 h, when almost 15 ng/10^6 cells was released in the infected supernatant. The production of IL-8 was dependent on the replication of RSV because exposure of the pRSV to UV light almost completely abrogated its release (0.43 ± 0.07 ng/10^6 cells at 48 h postinfection), indicating a requirement for viral replication in IL-8 induction.

To investigate the mechanism for enhanced IL-8 production, changes in the abundance of polyadenylated IL-8 mRNA were investigated by competitive RT-PCR. A specific 302-bp IL-8 band was detectable in the presence of IL-8 gene-specific primers; this product comigrates with a PCR product produced with the plasmid containing IL-8 cDNA as a template (Fig. 2). RNA from uninfected A549 cells contained barely detectable IL-8 transcripts yet abundantly detectable internal control MIMIC PCR products. In RSV-infected cells, levels of IL-8 mRNA increased at 6 h and continued to increase until 48 h, a time at which cells lose viability. Therefore, the RSV-induced generation of IL-8 protein was paralleled by an increase in the levels of IL-8 mRNA, suggesting that the IL-8 induction was mediated through enhanced mRNA production.

Transcriptional induction of IL-8 by RSV infection. To determine the transcriptional component for endogenous IL-8 gene expression, nuclear run-on transcription assays were performed with RSV-infected A549 cells at the indicated times after infection (Fig. 3). In uninfected cells, hybridization signals corresponding to the IL-8 transcripts were low relative to the hybridization signals of the internal control β-actin and cyclophilin genes. Upon RSV infection, IL-8 hybridization signals increased, resulting in an increased transcription rate of fivefold at 12 and 24 h. We noted that IL-8 mRNA induction is temporally related and similar in magnitude to increases in IL-8 transcription, suggesting that in RSV-infected lung epithelial cells, IL-8 protein secretion was primarily transcriptionally mediated.

The requirement of the NF-κB binding sequence for RSV-induced IL-8 gene transcription. To determine *cis* regulatory elements mediating the induction of the hIL-8 promoter, transient transfections of hIL-8 promoter linked to the sensitive luciferase reporter gene were performed. In preliminary experiments, the reporter activities in the absence and presence of RSV infection of a series of 5′ deletions from −1.4 kbp to −0.162 kbp (position numbering according to nucleotide location 5′ of the transcriptional start site) of the hIL-8 promoter were tested and were shown to be equally inducible (not shown). The −162 hIL-8/Luc reporter, then, reproduced the basal and inducible activity of the longer IL-8 promoter fragments. Figure 4A shows the strategy for the transient transfection assay of wild-type or NF-κB-mutated hIL-8 promoter fragments linked to the luciferase reporter gene. Batches of A549 cells were transfected simultaneously (at time zero). Subsets of transfectants were then infected with pRSV for the indicated times (from 0 to 36 h) prior to harvest (all cells were harvested at 48 h). This strategy controls for the effects of pRSV infection and cell number on transfection efficiency and, because of the rapid turnover of the luciferase reporter gene (7), allows determination of the temporal profile of hIL-8 transcriptional activity at various times after pRSV infection. This temporal profile of −162 to +44 hIL-8/Luc reporter activity after RSV infection is shown in Fig. 4B. Luciferase activity was induced transiently with statistically significant enhancements of fivefold and fourfold at 12 and 24 h postinfection (relative to uninfected A549 transfectants). These results showed a close temporal relationship to the kinetics of endogenous IL-8 gene

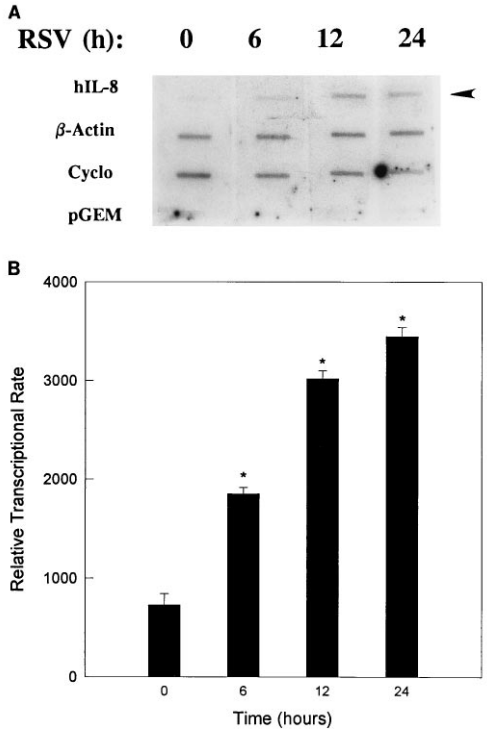

FIG. 3. Nuclear run-on assays of IL-8 gene transcription in RSV-infected A549 cells. (A) Autoradiogram of pRSV-infected A549 cell nuclear RNA labeled with [α-^{32}P]UTP and hybridized to excess single-stranded plasmid immobilized on nitrocellulose membranes. Time of infection is shown at the top. At the left are the plasmids immobilized: IL-8 (hIL-8), β-actin, cyclophilin (Cyclo), and pGEM (control plasmid). Shown is an individual result, reproduced in five experiments. (B) Quantitation of relative changes in the IL-8 transcription rate. Hybridization signals were quantified by PhosphorImager, and changes in IL-8 incorporation relative to actin as an internal control were calculated for each time. The normalized IL-8 transcription rate increases fivefold at 12 and 24 h postinfection. All values are significantly different from those of the control at $P < 0.002$ (Student's t test).

induction (Fig. 3) and indicate that transcription of the hIL-8 promoter is transient in response to pRSV infection.

Previous studies have identified the requirement of the NF-κB binding site located between −91 and −71 (relative to the cap site) in the tumor necrosis factor-mediated induction of IL-8 transcription in HeLa cells (59). Therefore, the effect of a site-directed mutation of the NF-κB binding site in the −162 hIL-8/Luc promoter (−162 hIL-8Δκ) was measured in response to RSV infection (Fig. 4A). This site mutation corresponds to the known guanosine contact points for the NF-κB transcription factor and abolishes NF-κB binding (33, 50, 54) (see Fig. 6). In contrast to the wild-type promoter, the reporter activity of −162 hIL-8Δκ/Luc was uninducible. These data indicate that the NF-κB binding site is absolutely essential for RSV activation of IL-8 gene transcription.

Effect of RSV infection on NF-κB DNA-binding activity. Gel mobility shift assays were used to determine whether RSV infection produced changes in the abundance of DNA-binding proteins that recognized the inducible IL-8 enhancer, a duplex oligonucleotide spanning −96 to −69 (IL-8 WT). Figure 6A (left panel) shows that a single nucleoprotein complex (C3) is formed from nuclear extracts of uninfected A549 cells (C1 and C2 are only faintly detectable). In contrast, the faint binding of C1 and C2 was markedly and consistently increased in nuclear extracts prepared from cells at 12 and 24 h after pRSV infection. Sequence specificity of the C1-C2 complexes is shown by

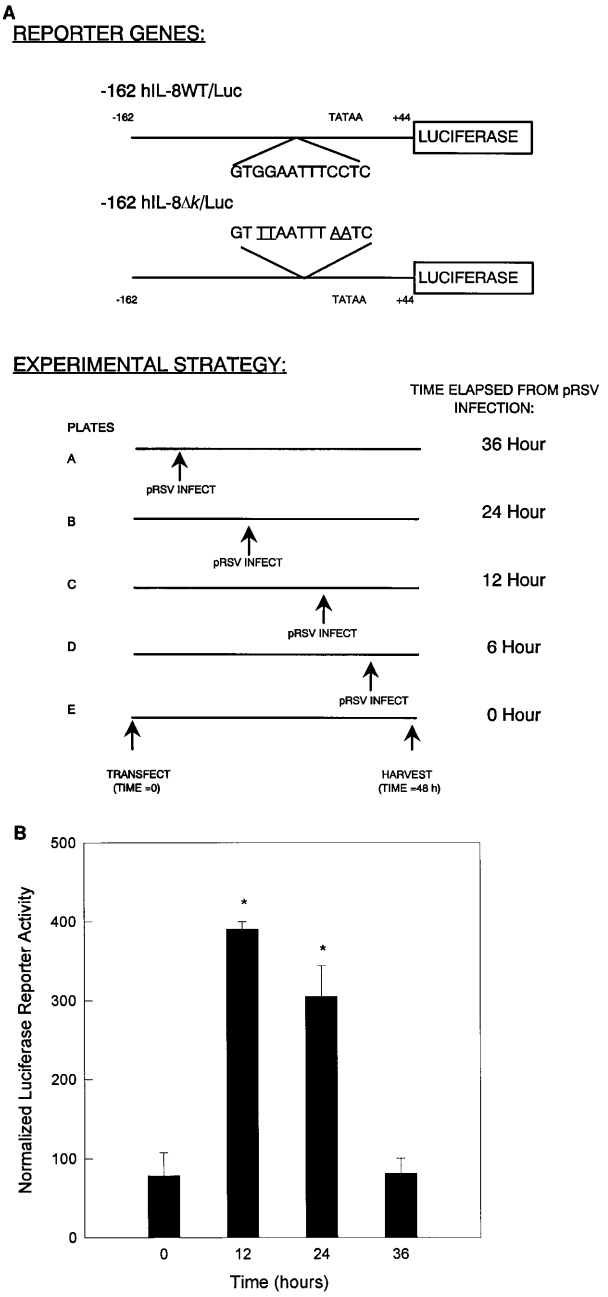

FIG. 4. Transient transfection of hIL-8/Luc reporter genes. (A) (Top) sequence of hIL-8 gene promoter and site mutation. The −162 to +44 hIL-8 gene promoter was ligated as a *Bam*HI-*Hin*dIII fragment into the promoterless luciferase vector pOLUC (9). Mutation of the NF-κB site in the context of the −162 hIL-8 promoter was generated by the PCR strategy of gene splicing by overlap extension (28). Purine-to-noncomplementary-pyrimidine substitutions of all known NF-κB contact sites were used as indicated (50). (Bottom) Experimental strategy for determining the time course of IL-8 transcription. All cells were transfected and harvested simultaneously (at 48 h). Selected triplicate plates were infected with pRSV for 36, 24, 12, 6, and 0 h (uninfected) prior to the 48-h harvest. (B) RSV infection transiently increases −162 hIL-8/Luc reporter activity. A549 cells were transfected with −162 hIL-8/Luc and internal control cytomegalovirus β-galactosidase plasmid with DEAE-dextran (Materials and Methods). Luciferase activity normalized to β-galactosidase is plotted for each time point. ∗, $P < 0.05$ versus control in six experiments (Student's t test).

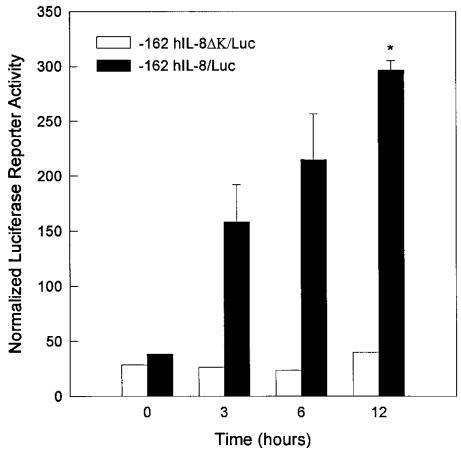

FIG. 5. NF-κB binding site is essential for RSV induction of hIL-8 promoter activity. Wild-type (−162 hIL-8/Luc) and NF-κB mutant (−162 hIL-8Δκ/Luc) promoters were transiently transfected into A549 cells according to the experimental strategy outlined in Fig. 4A (bottom panel). Normalized luciferase reporter activity is plotted for each construct at the indicated time points.

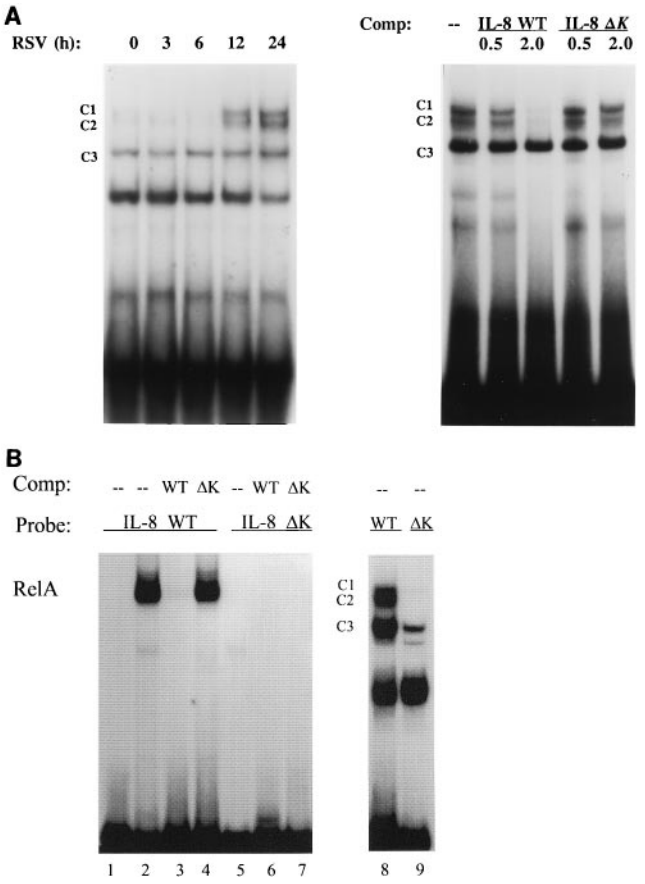

FIG. 6. (A) EMSA of hIL-8 NF-κB binding complexes in response to RSV infection. (Left) Autoradiogram of time course. Nuclear extracts were prepared from control or RSV-infected cells at the indicated times and used for EMSA (with double-stranded IL-8 WT probe). C1 and C2 are RSV-inducible complexes; C3 is constitutive. (Right) competition analysis. Nuclear extracts from A459 cells infected for 24 h were used to bind to the IL-8 WT probe; 0.5 and 2.0 pmol unlabeled competitors (Comp.) were included in the binding reaction mixture as indicated at the top. C1 and C2 compete with high affinity with the WT but not the mutant oligonucleotide. (B) Recombinant RelA binds IL-8 WT in a sequence-specific fashion. Autoradiogram of EMSA with 100 ng of recombinant purified RelA DNA-binding domain (lanes 1 to 7) or 24-h pRSV-infected A549 nuclear extract (lanes 8 and 9) either in the presence of radiolabeled IL-8 WT (lanes 1 to 4 and 8) or IL-8Δκ probes. Unlabeled competitors were included in the binding reaction mixture as indicated at the top.

competition with unlabeled oligonucleotides in the EMSA. Competition assays with either unlabeled oligonucleotide IL-8 WT or IL-8Δκ, an oligonucleotide containing a mutation of the NF-κB contact points that correspond to the transcriptionally inactive promoter (tested as shown in Fig. 5), were added together with the radiolabeled IL-8 WT probe. As shown in Fig. 6A (right panel), the IL-8 WT probe was able to compete for the binding of the RSV-inducible complexes C1 and C2, but IL-8Δκ failed to compete. These data indicate that the RSV-inducible C1 and C2 complexes bind to the −96 to −69 IL-8 promoter fragment with NF-κB binding specificity. The ability of the purified recombinant NF-κB RelA subunit [p65(12-317) (29)] was next tested for its ability to bind to the IL-8 WT and IL-8Δκ duplex oligonucleotides. In the assay shown in Fig. 6B (left panel), p65(12-317) was used to bind either to duplex radiolabeled IL-8 WT or IL-8Δκ probes in the presence or absence of unlabeled competitor. p65(12-317) produces a single complex on the IL-8 WT probe that competes specifically with unlabeled IL-8 WT, but not the IL-8Δκ oligonucleotides. Similarly, p65(12-317) does not directly bind radiolabeled IL-8Δκ probe. In Fig. 6B (right panel), the C1 and C2 complexes were tested for their ability to form on the radiolabeled IL-8Δκ probe. Complexes C1, C2, and, to a lesser extent, C3 did not form on the IL-8Δκ probe. These data indicate that IL-8 WT, but not the mutant, binds to RelA and the C1 and C2 complexes indistinguishably.

The NF-κB subunit RelA is translocated into the nucleus upon RSV infection. NF-κB is a transcription factor superfamily composed of NF-κB1, NF-κB2, RelA, and c-Rel proteins that heterodimerize in cells expressing them, producing complexes with various transcriptional activity and subtle sequence-specific binding preferences (3, 51). We next tested whether the inducible complexes cross-reacted with specific antibodies for various NF-κB subunits in the EMSA. In this assay, the presence of an individual NF-κB family member can be detected by the presence of a supershifted band, representing an antibody-protein-DNA complex. As shown in Fig. 7, the addition of anti-RelA antibodies supershifted the C1 and C2 bands, whereas anti-NF-κB1, -NF-κB2, and -c-Rel antibodies did not produce a supershifted band. We note, however, that the NF-κB1 antibodies reduced slightly the formation of the C2 complex, perhaps indicating its presence in the nucleoprotein complex (see Discussion). Irrespective, these data indicate the presence of RelA in the C1 and C2 complexes.

To determine the mechanism for enhanced RelA binding activity, Western immunoblot was performed with cytoplasmic, nuclear, and whole-cell extracts from control and RSV-infected A549 cells (Fig. 8). An ~65-kDa antigen was detectable with RelA antibodies in cytoplasmic extracts of control cells (and only faintly in the nuclear compartment). However, upon RSV infection, a time-dependent increase in nuclear RelA was observed (Fig. 8, top panel). In Western immunoblots of the cytoplasmic fraction, we observed corresponding depletion of RelA (Fig. 8, middle panel). These changes in cytoplasmic and nuclear RelA abundance occur without significant changes in total RelA abundance as indicated by Western immunoblots of whole-cell extracts (Fig. 8, bottom panel). The time-dependent increase in nuclear RelA by Western immunoblot exactly parallels the changes in DNA binding activity seen in the EMSA. To confirm that RelA was translocated from the cytoplasmic compartment into the nuclear compartment, indirect immuno-

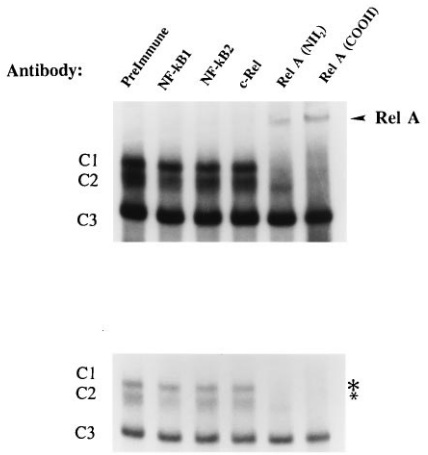

FIG. 7. RelA is the NF-κB subunit present in the RSV-inducible C1 and C2 complexes. Nuclear extract from 24-h-infected A549 cells was used in the EMSA in the presence of preimmune, NF-κB1, NF-κB2, c-Rel, RelA NH_2-terminal, and RelA COOH-terminal antibodies. Two autoradiographic exposures are shown. (Top) Long exposure demonstrating the presence of supershifted RelA bands produced only by the RelA NH_2- and COOH-terminal antibodies. (Bottom) Light exposure demonstrating the selective reduction of the RSV-inducible C1 and C2 complexes (positions are indicated by the large and small asterisks, respectively). Anti-NF-κB1 antibody produces a reduction in the amount of C2 (see Discussion).

fluorescence was performed with control and 24-h pRSV-infected A549 cells (Fig. 9). Little staining was observed in cells treated with preimmune antibody (Fig. 9A and C). In control cells, anti-RelA antibody stained cytoplasmic antigen in a dense perinuclear pattern (Fig. 9B, arrow). In RSV-infected syncytia, the specific RelA immunofluorescence was entirely nuclear (Fig. 9D). These data confirm the Western immunoblot finding that pRSV infection changes the subcellular distribution of RelA.

RSV infection of A549 cells is known to induce the release

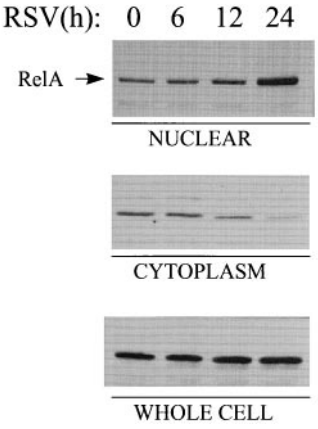

FIG. 8. Western immunoblot for changes in RelA within the nuclear compartment. Results of SDS-PAGE (10% polyacrylamide) of 200 μg of nuclear (top panel), cytoplasmic (middle panel), and whole-cell extracts (bottom panel) from cells infected for various times with pRSV are shown. RelA was detected with an enhanced chemiluminescence assay with anti-RelA (NH_2-terminal) antibodies as the primary antibodies. The ~65-kDa RelA band is not detectable with preimmune antibodies (30); this RelA isoform is predominately cytoplasmic in uninfected A549 cells. In triplicate experiments, nuclear RelA is increased 1.3 ± 0.23-fold (6 h), 2.9 ± 1.1-fold (12 h), and 6.5 ± 2.9-fold (24 h) relative to uninfected controls. The cytoplasmic abundance correspondingly decreases to 0.8 ± 0.05-fold (6 h), 0.6 ± 0.03-fold (12 h), and 0.2 ± 0.07-fold (24 h). There is no consistent change in the abundance of RelA in the whole-cell extract.

TABLE 1. RelA induction requires replicating RSV[a]

Treatment	Fold increase		
	RelA binding at:		IL-8 secretion
	1 h	12 h	
RSV-conditioned medium	0.89	0.62	0.9
UV-inactivated pRSV	0.9	0.96	0.8
pRSV	1.74	3.95	3.8

[a] A549 cells were treated with conditioned medium (from A549 cells pRSV infected for 12 h) after UV treatment to inactivate RSV replication, UV-inactivated pRSV, or pRSV for the indicated times prior to assay of NF-κB binding (by EMSA) and IL-8 secretion (by ELISA). Values represent the fold increase of the either the specific C1 and C2 complexes or IL-8 protein concentrations relative to untreated cells. In contrast to pRSV, both UV-inactivated pRSV and RSV-conditioned medium have no effect on RelA binding.

of cytokines, such as IL-1α and IL-1β (44), which potentially could activate NF-κB DNA binding as a secondary effect of RSV infection (6, 8). To determine whether a stable cytokine (or soluble mediator) was, in fact, the mechanism for NF-κB activation or whether RSV replication directly activated NF-κB in infected cells, NF-κB binding was measured with A549 cells treated with UV-inactivated pRSV or UV-inactivated conditioned medium obtained from A549 cells previously infected with RSV (Table 1). In contrast to cells infected with pRSV, both UV-inactivated pRSV and UV-inactivated conditioned medium had no detectable effect on NF-κB binding or IL-8 secretion. These results indicate that the early induction of NF-κB binding and IL-8 secretion is dependent on viral replication and is not a secondary effect of a stable paracrine factor. However, we cannot exclude the possibility that other factors are activating NF-κB binding or IL-8 gene expression at later time points.

DISCUSSION

Airway epithelial cells represent the principal initiators of pulmonary host defense mechanisms by their ability to synthesize and secrete inflammatory mediators upon injury or infection. The immunomodulatory activity of the airway epithelium is of particular relevance to RSV infection, because RSV produces clinical disease through its restricted replication within the airway mucosa. The mechanisms for the pronounced inflammatory response involve chemokine production by RSV-infected cells. One chemokine in particular, IL-8, is likely to play a major role in the pathogenesis of RSV infection because of the potent chemoattractant and activating properties of IL-8 for neutrophils, T cells, and eosinophils, accounting for the spectrum of cellular infiltration in RSV-infected lungs (1) and the striking predominance of neutrophils in bronchial lavages of patients with RSV infection (17). Moreover, IL-8 is detected in nasal lavage fluids of children with RSV respiratory infection (42). That the production of IL-8 and other proinflammatory cytokines is induced by a variety of infectious agents in well-differentiated and primary airway epithelial cell cultures (2, 13, 20, 34, 38, 40, 41, 53) argues for the relevance of this in vitro model to investigate the pathogenesis of RSV infection. In this study, we examined the mechanisms by which RSV induces the expression of IL-8. We demonstrate that IL-8 protein and mRNA are produced in type II pulmonary airway cells in a defined temporal response to infection with RSV. This event is primarily transcriptional and is dependent on the nuclear translocation of the potent transcriptional activating NF-κB subunit RelA.

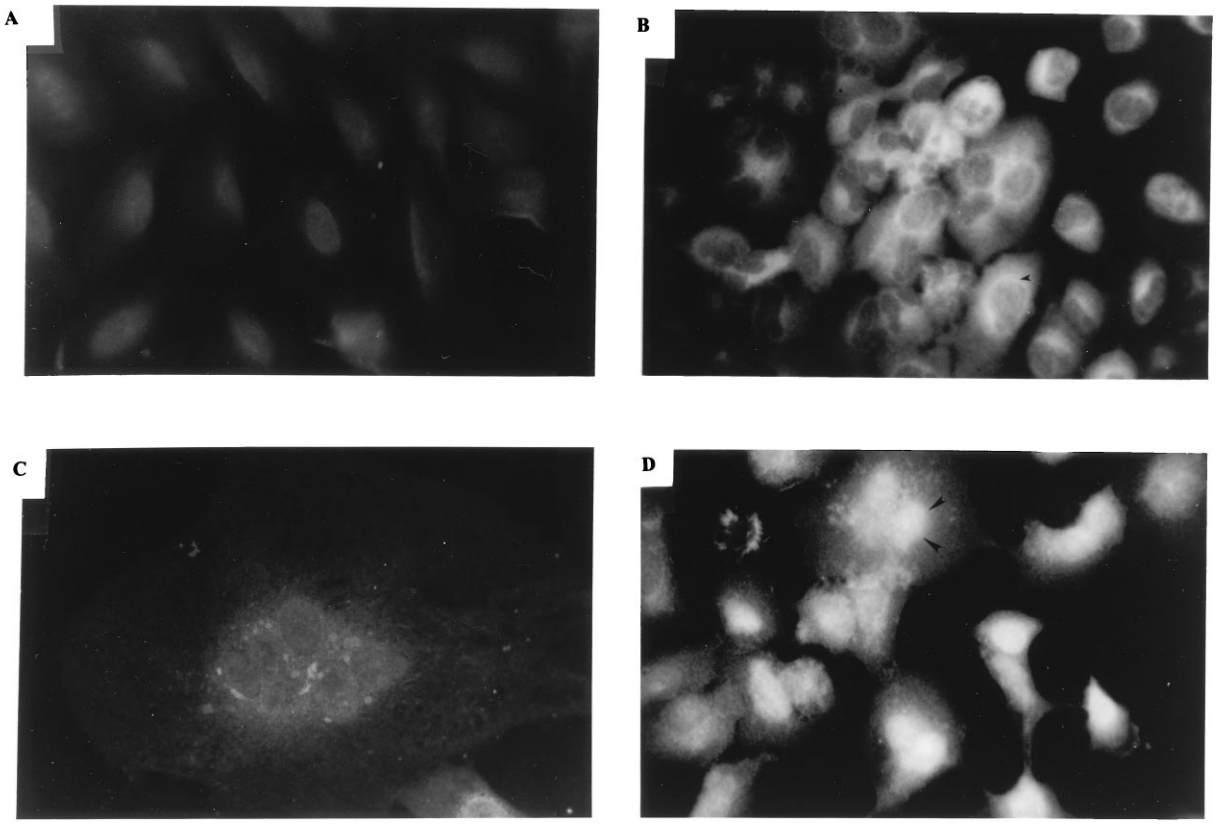

FIG. 9. Changes in subcellular distribution of RelA by indirect immunofluorescence microscopy. Uninfected A549 cells (A and B) or cells infected for 24 h with pRSV (C and D) were stained with preimmune antibody (A and C) or anti-RelA antibody (B and D) and visualized by indirect immunofluorescence microscopy. In uninfected cells, RelA staining is concentrated in a perinuclear halo (arrowheads). Upon RSV infection, the immunoreactivity is entirely nuclear. Note the presence of syncytia in RSV-infected cells, indicating a cytopathic effect.

An inflammatory gene network controlled by NF-κB. Using two different experimental approaches—nuclear run-on transcription rate assays of endogenous IL-8 transcription and transient transfection analysis of IL-8 reporter genes—we observed an approximately fivefold increase in IL-8 transcription 12 to 24 h after RSV infection; this magnitude and kinetics closely parallel the changes in IL-8 mRNA abundance. The temporal appearance of NF-κB in the RSV-infected nucleus is coincident with both the increase in endogenous IL-8 gene transcription and the increase in activity from transfected IL-8-linked reporter genes. Mutations in the NF-κB binding site completely abrogate the RSV induction of transfected reporter genes. Taken together, our studies indicate that NF-κB binding is essential for RSV-inducible IL-8 promoter activation. NF-κB is an inducible transcriptional activator implicated in the inducible expression of viral (43, 48), cytokine (15), and acute-phase reactant genes (6, 8, 35) by binding to regulatory sites within the promoters of these genes (reviewed in references 3 and 55). Of relevance to pulmonary inflammation, NF-κB not only activates the expression of IL-8 (33, 59) but also activates expression of many other inflammatory gene products, such as tumor necrosis factor alpha (15), ICAM-1 (44), IL-6 (60), and granulocyte colony-stimulating factor (16), cytokines whose expression is known to be enhanced by RSV infection (2, 20, 41). Although an important subset of inflammatory gene products is likely to be controlled by NF-κB, we note that RSV can stimulate the expression of different inflammatory gene subsets through the effect of other nuclear factors; we earlier reported that pRSV induces the translation of the nuclear factor–IL-6 (NF-IL-6) transcriptional activator, a protein with DNA-binding activity different from that of NF-κB (30). Our data indicate that NF-IL-6 binding alone is not sufficient for IL-8 induction. The functional interaction, if any, between NF-IL-6 and RelA in IL-8 promoter activation in A549 cells will require additional study. We speculate that NF-κB activation may be responsible for activating a subset of gene networks of inflammatory molecules and cell surface receptors required for immune activation in the airway mucosa.

Activation of the potent RelA transcription factor complex in RSV infection. Our studies are the first to demonstrate RelA activation by a mucosa-restricted virus. RelA is a 65-kDa member of the NF-κB family of transcription factors sharing a homologous 300-amino-acid NH_2-terminal *rel* homology domain. The *rel* family of proteins include the postranslationally processed members NF-κB1 and NF-κB2, produced by proteolytic processing of large 105- and 110-kDa precursor proteins, and the unprocessed Rel family members RelA (NF-κB p65), c-Rel, and RelB. RelA is recognized as a potent transactivating protein by virtue of two transactivating domains unique to its COOH terminus. We demonstrate that RelA is contained within the RSV-inducible C1 and C2 nucleoprotein complexes in A549 cells because subunit-specific antibodies quantitatively supershift these complexes. We were unable to convincingly demonstrate the presence of other NF-κB subunits in these complexes with identical reagents and conditions that produced supershifts of other NF-κB complexes in hormone-inducible NF-κB binding from hepatocytes (6, 35). The distinct electrophoretic mobility of the C1 and C2 complexes,

each containing RelA, may be the result of RelA heterodimerizing with other as-of-yet unidentified NF-κB subunits. For example, we note that a faint reduction in the C2 complex occurred in the presence of the anti-NF-κB1 antibody, perhaps indicating the presence of NF-κB1 in this complex. Additional studies, such as biochemical fractionation of nuclear proteins or UV cross-linking, will be required to definitively establish the composition of C1 and C2 complexes. Importantly, the NF-κB binding site in the IL-8 promoter, containing the nearly palindromic sequence 5′-TGGAATTCCTC-3′, is a preferred sequence for binding RelA homodimers, as shown by the PCR-based binding site selection strategy by which the IL-8 site was preferentially recognized from a pool of random nucleotides (33).

Using subcellular fractionation, Western immunoblot analysis, and indirect immunofluorescence microscopy, we have demonstrated that one mechanism for the RSV-enhanced activation of RelA binding is through changes in its subcellular localization. In unstimulated cells, RelA immunofluorescence is concentrated in a perinuclear halo. Upon RSV infection, the perinuclear RelA becomes diffusely nuclear with the exception of nucleoli. In unstimulated cells, nuclear transport of RelA is blocked by the inhibitor of NF-κB (IκB), a family of proteins that bind and mask the RelA nuclear localization sequences (4). In response to inducers, IκB is serine phosphorylated at its NH_2 terminus (10). Phosphorylation targets IκBα for proteolysis by the 26S ubiquitin-proteasome complex (10, 12). Once freed from its inhibitor, RelA is able to enter the nucleus. More detailed studies will be necessary to determine specific IκB family members expressed in airway epithelial cells, which ones are associated with RelA, and how RSV infection modifies their activity.

Although our studies are the first to describe RelA activation by a mucosa-restricted virus, NF-κB activation has been reported in myeloid cells as a consequence of chronic lentivirus infection (45, 47), acute Sendai virus infection of fibroblastic and renal carcinoma cells (22), and picornavirus infection of alveolar epithelial cells (60). Lentivirus infection of myeloid cells activates NF-κB chronically; given the ability of lentivirus to stimulate cytokine production, many of which can themselves stimulate NF-κB, it is difficult to determine whether RelA activation is a direct effect of viral replication or a secondary paracrine phenomenon. Our data indicate that for the paramyxovirus RSV, replication in airway epithelial cells is a necessary and sufficient event for RelA activation. In contrast to our observations, others have shown a more rapid induction of NF-κB binding after exposure of respiratory epithelial cells to RSV (39). Although the NF-κB subunits were not characterized in that study, the differences in kinetics may be due to the use of a nonpurified viral preparation, shown previously to be contaminated by cytokines, including IL-1α (44), a known NF-κB stimulant (8). Finally, picornavirus (rhinovirus) infection has been shown to activate multiple NF-κB/*rel* family members in pulmonary epithelial cells, a phenomenon that results in IL-6 secretion during this infection (60).

Different kinetics of RelA activation: distinct intracellular signalling pathways? The kinetics of RelA activation in response to RSV infection occur, perhaps, surprisingly slowly, requiring 6 to 12 h before significantly detectable NF-κB protein accumulates in the nucleus. This contrasts with an extremely rapid NF-κB activation in response to hormone receptors (such as IL-1α and tumor necrosis factor alpha), in which NF-κB is activated within minutes (8, 11, 35, 46), and the relatively slower activation observed during rhinovirus infection (60). These observations imply that although NF-κB activation may be a final common pathway for infectious and inflammatory signalling, this event is mediated by distinct intracellular signalling mechanisms. During RSV infection, we note that the kinetics of RelA activation coincide with the production of viral antigens, detectable within 9 h after infection (5). This time course and the observation that both NF-κB production and IL-8 production do not occur in response to exposure to RSV-conditioned medium or to UV-inactivated virus argue that the process of active viral replication is necessary to trigger NF-κB binding and subsequent IL-8 synthesis. In fact, others have reported that IL-8 gene expression (at 24 h) is also dependent on viral replication (18). Our studies demonstrate that the mechanism for this induction is via the RelA transcription factor. Viral products of replication such as free radicals (48) and double-stranded RNA (57) are potent activators of NF-κB in certain cell types. It is interesting to speculate that the expression of one or more viral gene products may be responsible for activating RelA translocation.

In conclusion, we have demonstrated that cytoplasmic-nuclear translocation of the RelA transcription factor is required for inducible IL-8 transcription in alveolar epithelial cells by RSV infection. RelA is selectively induced from other NF-κB family members in a kinetic manner distinct from hormone receptor activation and from other cold virus infection in airway epithelial cells and is absolutely dependent on viral replication. RelA regulates the activities of many other cytokine promoters whose expression is influenced by RSV and whose activities are important for the pathogenesis of pulmonary inflammation seen with bronchiolitis. We speculate that RelA may be an efficacious target for immunomodulatory therapeutics in virus-induced pulmonary inflammation.

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