JOURNAL OF VIROLOGY, Mar. 1996, p. 1554–1563 Vol. 70, No. 3
0022-538X/96/$04.00+0

Inducible Translational Regulation of the NF-IL6 Transcription Factor by Respiratory Syncytial Virus Infection in Pulmonary Epithelial Cells

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Received 25 October 1995/Accepted 5 December 1995

Respiratory syncytial virus (RSV), the most common etiologic agent of epidemic pediatric respiratory disease, infects and replicates in the human airway epithelium, resulting in the induction of cellular gene products essential for immune and inflammatory responses. We describe the effect of RSV infection on nuclear factor-IL6 (NF-IL6) expression, a human basic domain-leucine zipper-containing transcription factor that alone and in combination with other inducible transcription factors regulates the expression of cytokine and adhesion molecule genes. RSV-infected human type II pulmonary alveolar epithelial cells (A549) synthesize a single 45.7-kDa isoform of NF-IL6 rapidly and in a time-dependent manner. NF-IL6 is first detectable after 3 h of infection and continues to accumulate until 48 h (until the cells lose viability). NF-IL6 production could not be induced by UV-inactivated virus, demonstrating the requirement of viral replication for NF-IL6 synthesis. Immunoprecipitation after [^{35}S]methionine metabolic labeling was done to investigate the mechanism for NF-IL6 production. There was robust NF-IL6 protein synthesis within RSV-infected (24 h) cells. Protein synthesis occurred without detectable changes in the abundance or size of the single 1.8-kb NF-IL6 mRNA. RNase protection assay of transfected chloramphenicol acetyltransferase reporter genes driven by either wild-type or mutated NF-IL6 binding sites show a virus-induced increase in NF-IL6-dependent transcription. These studies have demonstrated a novel inducible mechanism for translational control of NF-IL6 synthesis and identify this transcription factor as a potential effector of the host response to RSV infection.

The epithelium represents the principal barrier between the external environment and the internal milieu. Epithelial cell contact with either infectious or noxious agents (UV light, free radicals, or elevated temperature) stimulates a change in gene expression for proteins involved in repair and inflammation (reviewed in reference 44). This is particularly true for the airway epithelium, which is the primary target for many bacterial and viral pathogens. Among them, respiratory syncytial virus (RSV), an enveloped, single-stranded, negative-sense RNA virus, represents the major cause of severe lower respiratory tract disease in infants (41, 53) and in children of any age with underlying heart and lung disease (16, 27). Replication of RSV and other viral pathogens such as parainfluenza virus, rhinovirus, or influenza virus in epithelial cells is known to be a potent stimulus for secretion of cytokines and interferon (2, 13, 14, 34) and expression of adhesion molecules (e.g., intracellular adhesion molecule 1) (32, 38), major histocompatibility complex class I (13), and receptors involved in initiating the apoptotic pathway (50). Identifying the factors that control switches in epithelial cell gene expression, then, is central to the understanding of virus-host interactions.

Nuclear factor-IL6 (NF-IL6), a human basic domain-leucine zipper-containing transcription factor (1, 5), is an important transcription factor participating in inducible gene expression in acute infectious and inflammatory responses (1, 11, 29, 45). Originally identified as a transcription factor that bound to the inducible enhancer of the interleukin-6 (IL-6) gene, NF-IL6 has been shown to regulate the promoter activity of other cytokine genes (IL-8 and granulocyte colony-stimulating factor [11, 20, 45]) and acute-phase reactants (angiotensinogen and serum amyloid A [1, 4, 6]). Gene-targeting (knockout) experiments have shown that NF-IL6 expression is essential for host response to facultative intracellular pathogens, as mice with homozygous disruption of *nf-il6* are susceptible to overwhelming *Listeria monocytogenes* (46) and *Candida albicans* (42) infections. These studies implicate the essential role of NF-IL6 as an activator of gene networks necessary for hepatocyte and macrophage function.

Encoded by a single intronless gene, NF-IL6 mRNA is translated into three different isoforms of 38, 36, and 16 kDa by a leaky ribosomal scanning mechanism (10). The first two products of 38 and 36 kDa contain an alanine-, proline-, and serine-rich NH_2-terminal region that contains kinase-inducible (33, 47) and cell-type-dependent (52) activation domains; in the appropriate cell type, these proteins are transcriptional activators. The smallest, 16-kDa product contains the basic domain-leucine zipper DNA-binding domain (1, 5) and in assays of transient expression functions as a competitive transcriptional attenuator (10).

The expression of NF-IL6 is controlled in a tissue-type-dependent fashion. In the many tissues that express NF-IL6 mRNA, such as the liver and brain, the protein is also produced. In the lung, however, a discrepancy has been noted (9): although normal lung tissue is, in fact, one of the most productive sources of NF-IL6 mRNA, the NF-IL6 protein is almost undetectable (9). This observation suggests that tissue-specific factors are involved in the control of posttranscriptional expression of NF-IL6.

In naturally acquired RSV infection, the primary target for viral replication is the respiratory epithelium. cDNA cloning of

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RSV-infected epithelial cells has identified 10 different viral genes, each coding for a single protein (7). The disulfide-bonded glycoprotein F and the large glycoprotein G, which mediates attachment of the virus to the host cells, are the surface proteins which carry the major antigenic determinants of the virus and appear to play a role in the induction of neutralizing antibodies (reviewed in reference 30). Inhalation or self-inoculation of the virus into the nasal mucosa is followed by spreading to the lower respiratory epithelium by cell-to-cell transfer of the virus along intracytoplasmic bridges (15).

We have therefore investigated the effect of RSV infection of the pulmonary epithelium on the expression and regulation of NF-IL6. We demonstrate that upon RSV infection, the NF-IL6 protein is rapidly synthesized in pulmonary A549 cells in a manner requiring the replicating form of RSV. The NF-IL6 protein synthesized is nuclear and competent to bind to its target DNA and functions as a transactivator. To our surprise, NF-IL6 production is independent of changes in mRNA abundance. We present evidence for a novel virus-inducible translational control of NF-IL6 synthesis in pulmonary epithelial cells.

(Results of this investigation were presented in abstract form at the 77th annual meeting of the Endocrine Society, June 1995, Washington, D.C.)

MATERIALS AND METHODS

Cell culture. Human A549 pulmonary type II epithelial cells and HEp2 cells (American Type Culture Collection, Rockville, Md.) were cultured in Dulbecco's modified Eagle medium containing 10% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml at 37°C in a 5% CO_2 incubator.

RSV preparation. The A2 strain of RSV was propagated on HEp-2 cells and purified by polyethylene glycol precipitation followed by centrifugation in a 35 to 65% discontinuous sucrose gradient as described elsewhere (49). The infectivity of the purified RSV (pRSV) pools, as determined by a plaque forming assay (48), was 7.5 to 8.5 log PFU/ml. RSV-conditioned medium was prepared by infecting monolayers of A549 cells with pRSV at a multiplicity of infection (MOI) of 1 in modified Eagle medium with 2% fetal bovine serum and then incubated at 37°C in 5% CO_2. At 48 h when the cells exhibited cytopathic effect, the supernatant was collected, centrifuged at 3,000 × *g*, and finally exposed to a UV light (254 nm) source at a 10-cm distance for 3 min on ice to inactivate the virus (38). Virus pools and conditioned medium were aliquoted, quick-frozen in liquid nitrogen, and stored at −70°C until used. Purified RSV pools and conditioned media were also screened for the presence of contaminating cytokines. While IL-1, tumor necrosis factor alpha, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor were not found in the purified RSV pools, significant amounts of all these cytokines were present in the RSV-conditioned medium.

Viral infection. A549 cells grown at 70 to 80% confluency were infected with RSV at an MOI of 1. To do this, frozen RSV stock was rapidly thawed and diluted with Dulbecco's modified Eagle medium containing 2% fetal bovine serum. The virus was added immediately to the flasks (0.04 ml of diluted virus per cm^2) after removal of the culture medium. An equivalent amount of sucrose solution was added to the culture which received no RSV. After addition of virus, the flasks were rocked mechanically for 1 h at 37°C and then 0.2 ml of Dulbecco's modified Eagle medium containing 2% fetal bovine serum per cm^2 was added to the culture flasks. The infection was continued for the times indicated in the figures in a 37°C incubator. Vaccinia virus recombinant expressing the individual genes for F and G glycoproteins of RSV and a recombinant control (vSC-8) were kindly provided by Peter L. Collins (Laboratory of Infectious Diseases, National Institutes of Health [36]).

Nuclear extracts. Uninfected and infected A549 cells were scraped from flasks with a rubber policeman, and the cytoplasmic and soluble nuclear extracts were prepared from these cells by nonionic detergent lysis and high-salt extraction as previously described (40). The protein content of the nuclear extract was measured relative to bovine serum albumin with a protein reagent (Bio-Rad, Hercules, Calif.). Approximately 250 μl of nuclear extract was prepared from 10^7 cells containing 5 to 8 μg of protein per μl.

Western blots (immunoblots). Nuclear protein (75 μg) from uninfected or infected cells was loaded into a sodium dodecyl sulfate–12% polyacrylamide gel (SDS–12% PAG) for electrophoretic separation. The protein was transferred to polyvinylidene difluoride membranes by electroblotting at 50 V in 1× CAPS [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11, 10% (vol/vol) methanol] at room temperature for 1 h. The membrane was washed, blocked, and probed with a 1:6,000 dilution of primary anti-NF-IL6 antibody according to the manufacturer's recommendations (Amersham Life Sciences, Arlington Heights, Ill.). Anti-NF-IL6 antibody was generated against a synthetic peptide corresponding to amino acids 66 to 82 of the NF-IL6 protein coupled to keyhole limpet hemocyanin as described elsewhere (40). The secondary antibody was horseradish peroxidase-coupled goat anti-rabbit immunoglobulin G; NF-IL6 was detected by enhanced chemiluminescence (Amersham Life Sciences).

Gel shift assay. DNA-binding assays of NF-IL6 used preparative gel-eluted NF-IL6. Nuclear extract was prepared from A549 cells grown in T-150 flasks as described above. Crude nuclear extracts prepared from uninfected and infected A549 cells were fractionated in a preparative SDS–10% PAG. A 1-cm-wide gel slice containing NF-IL6 was excised, eluted, and renatured by dialysis at 4°C overnight in a buffer containing 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 5 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol as previously described (6). The duplex oligonucleotides used in this assay are as follows (5, 6):

APREM6, GATCCACAGTTGTGATTTCACAACCTGACCAGA
GTGTCAACACTAAAGTGTTGGACTGGTCTCTAG

APREM2, GATCCACATGTTGGATTTCCGATACTGACCAGA
GTGTACAACCTAAAGGCTATGACTGGTCTCTAG

The binding reaction mixture contained 25 μl of gel-eluted NF-IL6, 1 μg of denatured herring sperm DNA, and 20,000 cpm of ^{32}P-APREM6 in a total reaction volume of 30 μl. In the competition experiments labeled probe and nonspecific competitor were added simultaneously. For the gel mobility supershift assay, 1 μl of preimmune sera or NF-IL6 antibody (anti-NF-IL6) was added and incubated for 20 min prior to addition of the probe. The bound and free complexes were separated in a 7% nondenaturing polyacrylamide gel and visualized by autoradiographic exposure as described elsewhere (5, 6).

Northern (RNA) blot. Total RNA was extracted from A549 cells with RNAzol B (Tel-Test, Inc., Friendswood, Tex.). Poly(A)$^+$ mRNA was isolated from total RNA by the PolyATract mRNA isolation system (Promega Corp., Madison, Wis.). Poly(A)$^+$ mRNA (1 μg) was fractionated on a 1.2% agarose-formaldehyde gel and transferred to a nitrocellulose membrane (MSI, Westboro, Mass.). After baking at 80°C for 2 h, the membrane was wet in 10 ml of preheated (37°C) hybridization solution (5× Denhardt's solution, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS, 200 μg of herring sperm DNA per ml, and 50% formamide) and prehybridized at 37°C for 2 h. After prehybridization, hybridization was at 37°C overnight in fresh solution containing ^{32}P-NF-IL6 cDNA probe (2 × 10^6 cpm/ml). The membrane was first washed with 1× SSC–0.1% SDS at room temperature for 15 min and then with 0.25× SSC–0.1% SDS for 10 min. The membrane was dried and exposed to X-ray film (Kodak) for 72 h at −70°C. The same membrane was used for the detection of cyclophilin mRNA. Quantitation was performed on a Molecular Dynamics 425E PhosphorImager after a separate exposure to a PhosphorImager cassette.

Plasmids. The M6-TKCAT plasmid was prepared by cloning the chloramphenicol acetyltransferase (CAT) reporter gene downstream of the 41-bp thymidine kinase promoter driven by three copies of the NF-IL6-specific binding sequences (APREM6) in the pGEM 3Z plasmid (6). The M2-TKCAT plasmid was prepared by inserting three copies of mutated NF-IL6 binding sequences upstream of the thymidine kinase promoter.

Cell transfection. Logarithmically growing A549 cells (10^6) were seeded in eight 6-cm-diameter plates. After 24 h, four plates were transfected with 5 μg of M6-TKCAT plasmid and the other four plates were transfected with 5 μg of M2-TKCAT plasmid by using the transfection reagent DOTAP {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; Boehringer Mannheim}. After 16 h, duplicate plates from each transfection were infected with RSV (MOI, 1) for 24 h. After 48 h, RNA was extracted with RNAzol B and dissolved in 45 μl of denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate, and 0.5% sarcosyl).

RNase protection assay (RPA). To measure CAT reporter activity by RPA, a 250-bp *Eco*RI-*Hin*dIII fragment of CAT was used to synthesize radiolabeled antisense CAT RNA (cRNA) with T7 RNA polymerase and [α-^{32}P]UTP by standard techniques. CAT riboprobe (5 × 10^5 cpm/5 μl) alone or mixed with either sense CAT RNA (control) or an equal amount of RNA from transfected cells in a total volume of 50 μl was hybridized at 37°C for 16 h. Five hundred microliters of RNase working solution (40 μg of RNase A per ml and 1 U of RNase T_1 per ml) was added directly to the hybridization reactions, mixed vigorously for 5 s, and incubated at 37°C for 30 min. Twenty microliters of 10% SDS and 20 μl of proteinase K solution (20 μg/ml) were added to each tube, mixed, and incubated at 37°C for 30 min. The samples were precipitated with isopropyl alcohol and air dried, and the pellet was allowed to air dry with tubes inverted for 20 min. The pellet was suspended in 10 μl of gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heated to 95°C for 5 min, cooled on ice, and loaded onto a 5% acrylamide–8 M urea gel. After drying, the gel was exposed to X-ray film (Kodak) for 48 h at −70°C and quantitated by a separate exposure to a PhosphorImager cassette.

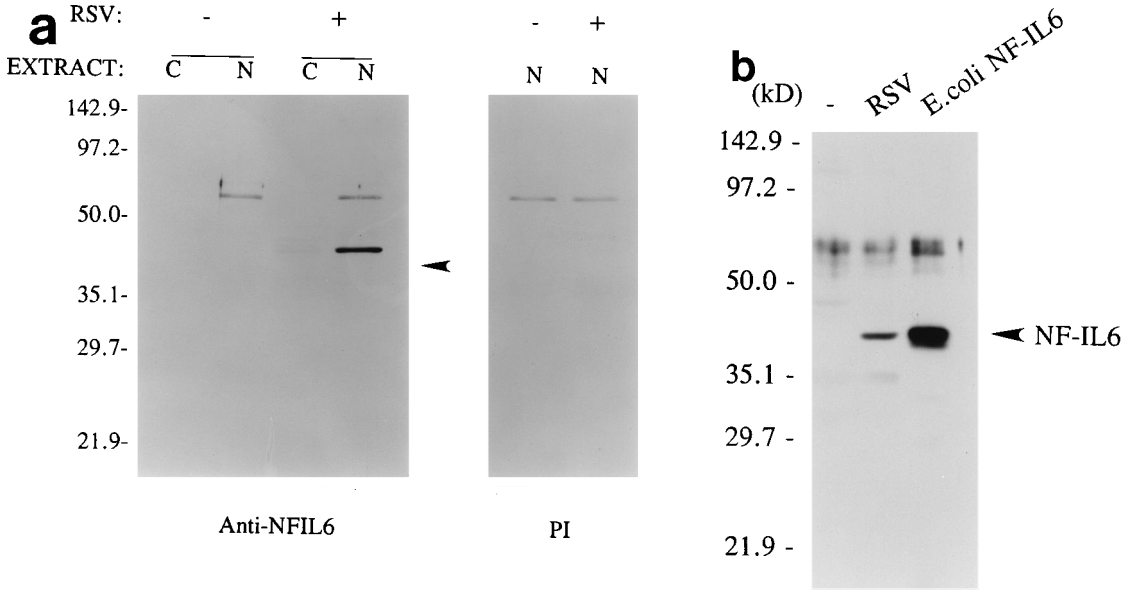

FIG. 1. (a) Induction of nuclear NF-IL6 protein in type II alveolar (A549) cells by RSV infection. A549 were treated with sucrose or infected with purified RSV at an MOI of 1 for 24 h. Cytoplasmic and nuclear extracts were prepared (see Materials and Methods). Equal amounts (50 μg) of nuclear protein (N) from uninfected (−) and infected (+) cells and the cytoplasmic fraction from an equivalent number of cells constituting 150 μg of protein (C) were assayed for NF-IL6 protein by Western blot with anti-NF-IL6 antibody (left panel). A control membrane was incubated with preimmune (PI) rabbit serum (right panel). The NF-IL6-specific band is indicated by the arrowhead. Molecular masses of protein markers (in kilodaltons) are shown on the left. (b) RSV-induced NF-IL6 from A549 cells comigrates with bacterially expressed recombinant NF-IL6 (amino acids 24 to 345). Nuclear protein (50 μg) from uninfected (−) and RSV-infected (24-h infection at an MOI of 1) A549 cells and 5 μg of crude extract of *Escherichia coli* overexpressing NF-IL6 corresponding to the second initiator codon (amino acids 24 to 345) were analyzed for NF-IL6 (arrowhead) by Western blot. Molecular masses of marker proteins are shown on the left. The 45.7-kDa NF-IL6 from RSV-infected cells comigrates with recombinant NF-IL6 (amino acids 24 to 345). Both forms migrate aberrantly in our SDS-PAG system because of alanine-proline insertions in their NH_2 termini (5).

RESULTS

RSV infection enhances steady-state abundance of nuclear NF-IL6. We analyzed the expression of NF-IL6 in cultured human alveolar epithelial cells by Western blot after RSV infection using specific polyclonal antibody to NF-IL6 (40). No NF-IL6 could be detected in cytoplasmic or nuclear extracts of control A549 cultures, and no staining was observed with preimmune antisera (Fig. 1a). In contrast, after 24 h of RSV infection, a preferential nuclear accumulation of NF-IL6 was observed, with a lesser amount detectable in the cytoplasm (Fig. 1a). In estimates of its mobility relative to that of molecular size markers, the RSV-inducible NF-IL6 protein migrates at an apparent size of 45.7 kDa. As an independent confirmation of the nuclear localization of the NF-IL6 antigen, indirect immunofluorescence of RSV-infected (24 h) cells showed a strong nuclear immunofluorescent signal of NF-IL6 in RSV-infected A549 syncytia (data not shown). In Fig. 1b, we compared the size of RSV-induced NF-IL6 antigen with recombinant NF-IL6 (amino acids 24 to 345) as a standard. NF-IL6 is encoded by a single intronless mRNA that contains two in-frame initiator codons that produce translation products (amino acids 1 to 345 and 24 to 345), both containing the epitope recognized by the anti-NF-IL6 antibody (raised to amino acids 66 to 82). The RSV-induced 45.7-kDa NF-IL6 protein exactly comigrates with recombinant NF-IL6 (amino acids 24 to 345), as shown in Fig. 1b.

Time-dependent NF-IL6 induction requires replicative virus. The kinetics of 45.7-kDa NF-IL6 accumulation after RSV infection was explored in Fig. 2. In this particular exposure, NF-IL6 could be detected at 12 h, peaking at 36 h; in overexposed blots, NF-IL6 could be faintly detected by 3 h of RSV infection (the degree of induction was unquantifiable because NF-IL6 is not detectable by Western blots in uninfected cells). To examine whether induction of NF-IL6 is dependent on soluble factors released either as a consequence of RSV infection or as a consequence of direct viral replication, conditioned medium from RSV-infected (48 h) cells was exposed to 254-nm UV light (to inactivate replicative virus) and then used to stimulate logarithmically growing A549 cells (Fig. 2, right panel). Addition of conditioned medium alone, abundant in the cytokines IL-1, tumor necrosis factor alpha, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor, was unable to induce NF-IL6 protein.

The effect of the MOI on NF-IL6 production was examined in Fig. 3. By 24 h of infection, 45.7-kDa NF-IL6 abundance is RSV MOI dependent; NF-IL6 can be detected at a ratio of 0.01 PFU per cell, and the abundance of NF-IL6 saturates at MOIs above 1. In a separate experiment shown in Fig. 4, the effect of 254-nm-UV-inactivated sucrose gradient-purified RSV (inact RSV) was compared with that of the replicative form of RSV, wild-type poxvirus (vaccinia virus), and recombinant vaccinia virus expressing RSV F and G glycoproteins. Only the wild-type RSV was capable of inducing the 45.7-kDa NF-IL6 protein.

Induction of NF-IL6 DNA-binding activity. To determine whether NF-IL6 induction results in increased NF-IL6 DNA binding, we measured changes in NF-IL6 DNA-binding activity using a previously described gel renaturation protocol (6). In this assay, a constant 500 μg of nuclear protein from RSV-infected A549 cells was fractionated on a preparative SDS-PAG, the 45.7-kDa NF-IL6 band was excised, and NF-IL6 protein was eluted, renatured by dialysis, and used to bind a high-affinity NF-IL6 DNA binding site (APREM6). This strategy was necessary because of the presence of a nonspecific DNA-binding activity that partially obscured the NF-IL6 band in gel shift assays using crude nuclear protein extracts. DNA-

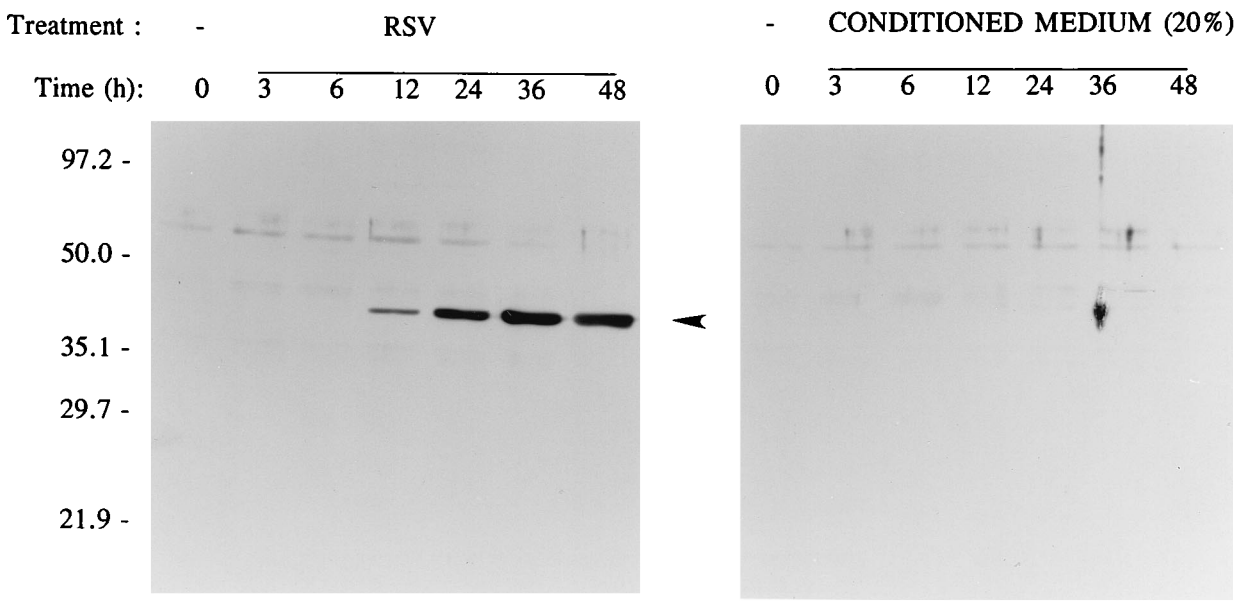

FIG. 2. Time course of NF-IL6 protein induction in A549 cells by RSV infection. (Left) A549 cells infected at an MOI of 1 for 0, 3, 6, 12, 24, 36, and 48 h were harvested and analyzed for NF-IL6 protein (arrowhead) by Western blot. Values on the left are sizes (in kilodaltons) of protein markers. NF-IL6 was detectable in RSV-infected cells in a time-dependent manner. A similar magnitude and kinetics of induction were seen in the larnygeal carcinoma cell line HEp-2 (not shown), indicating that this is a generalized property of lung epithelial cell lines. (Right) Effect of RSV-conditioned medium on NF-IL6 production in A549 cells. A549 cells were cultured without or with 20% (vol/vol) UV-inactivated conditioned medium from RSV-infected cells for the indicated times (top) and assayed for NF-IL6 by Western blot. The UV-inactivated conditioned medium did not induce detectable changes in NF-IL6, indicating that RSV replication, not merely viral attachment or soluble factors secreted from RSV-infected epithelial cells, is necessary for NF-IL6 induction.

binding activity was detected in unstimulated A549 nuclear extracts and increased in a time-dependent fashion to a maximum of a fourfold increase over 36 h (Fig. 5a, left panel). NF-IL6 was detectable in uninfected cells in the gel mobility shift assay (Fig. 5a) but not in Western blots (Fig. 1), because eightfold more protein was used for the gel mobility shift experiments. The presence of NF-IL6 in the DNA complexes was documented in the gel mobility supershift assay (Fig. 5a).

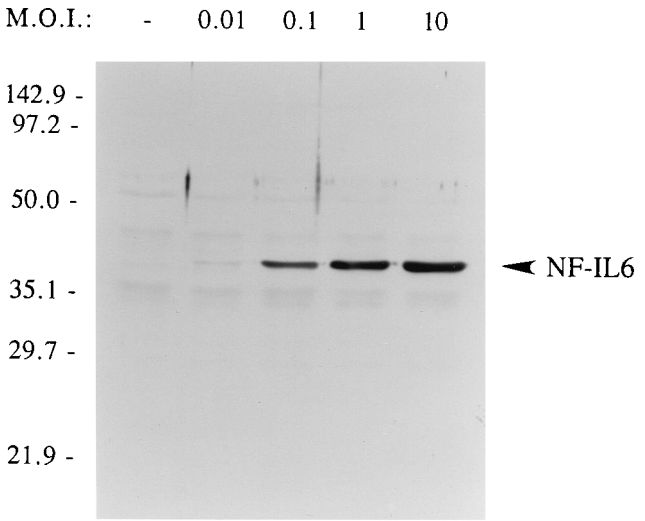

FIG. 3. Effect of MOI on the induction of NF-IL6 protein in A549 cells. A549 cells were not infected (−) or infected with purified virus at the indicated MOI for 24 h. After infection, equal amounts of nuclear protein (75 μg) were assayed for NF-IL6 (arrowhead) by Western blot. Values on the left are molecular masses (in kilodaltons) of protein markers.

Moreover, the DNA-binding activity is sequence specific, being inhibited with homologous APREM6 probe but not its mutated binding site APREM2 (Fig. 5b).

NF-IL6 induction is independent of changes in steady-state mRNA. To investigate the mechanism of NF-IL6 induction, Northern blot analysis was used to quantitate relative changes in NF-IL6 mRNA. Polyadenylated RNA was purified from control and RSV-infected cells, and the 1.8-kb NF-IL6 mRNA was detected by hybridization and autoradiographic exposure (Fig. 6). Surprisingly, NF-IL6 mRNA was as abundant in uninfected cells as in RSV-infected cells (where the increase in NF-IL6 protein abundance was observed). Levels of NF-IL6 mRNA normalized to those of internal control cyclophilin mRNA (as a control for RNA recovery) in each poly(A)$^+$ preparation is shown in Fig. 6b as a mean ± standard error (from four independent experiments). Moreover, induction of NF-IL6 could not be recorded by nuclear run-on analysis (data not shown).

RSV enhances de novo synthesis of NF-IL6 protein. To determine if NF-IL6 protein was induced as a consequence of increased translation, [^{35}S]methionine pulse-labeling was used to determine relative NF-IL6 synthetic rates in control or RSV-infected (24 h) A549 cultures (Fig. 7). No NF-IL6 labeling could be detected in control cells pulse-labeled for 1 h. In fact, NF-IL6 synthesis could not be detected after labeling for up to 4 h, indicating that the synthetic rate of NF-IL6 in control uninfected cells is extremely low (data not shown). In contrast, labeled 45.7-kDa NF-IL6 was immunoprecipitable after 1 h of incorporation by 24 h after RSV infection, indicating that de novo synthesis of NF-IL6 is partly responsible for the NF-IL6 induction after RSV infection.

NF-IL6 binding sites are RSV-inducible enhancers. Figure 8 shows the results of a transient transfection assay to determine whether NF-IL6 induced by RSV functions as a transcriptional

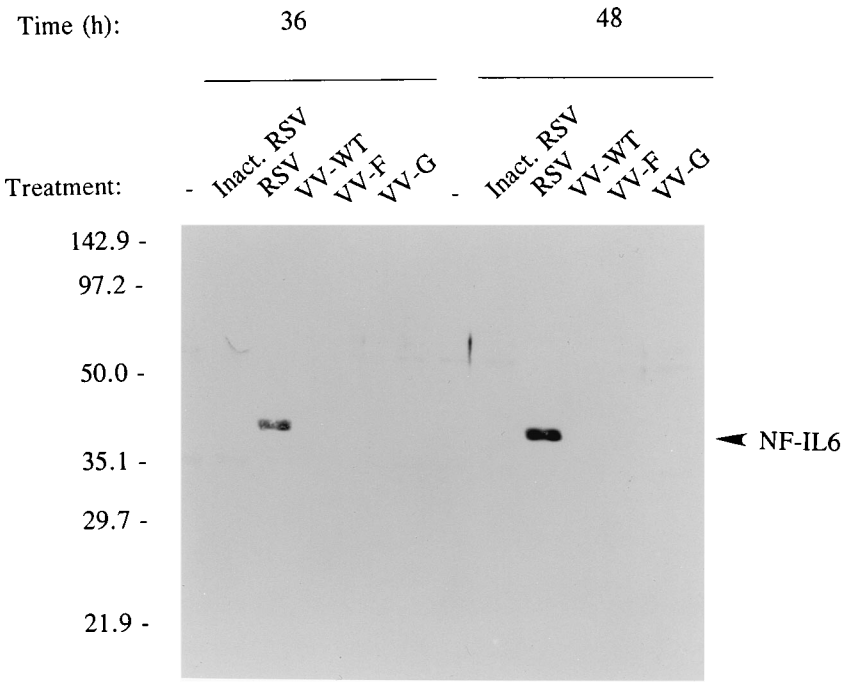

FIG. 4. RSV replication is necessary for the induction of NF-IL6. A549 cells were infected either with sucrose alone (−), sucrose gradient-purified RSV (RSV), UV-inactivated RSV (Inact. RSV), wild-type vaccinia virus (VV-WT), recombinant vaccinia virus expressing RSV F protein (VV-F), or recombinant vaccinia virus expressing RSV G protein (VV-G) at an MOI of 1 for 36 and 48 h. Equal amounts of nuclear protein (70 μg) from each treatment were assayed for NF-IL6 (arrowhead) by Western blot. Only replication-competent RSV induced NF-IL6 abundance; UV-inactivated RSV, VV-WT, VV-F, and VV-G did not. The masses of protein standards (in kilodaltons) are shown on the left.

activator. A549 cells were transiently transfected with CAT reporter genes driven by three copies of wild-type NF-IL6 binding sites (M6-TKCAT) or mutated NF-IL6 binding sites (M2-TKCAT) and infected for 24 h with RSV, and reporter activity was determined by RPA. The reporter genes, cRNA probe, and size of the anticipated protected fragment are schematically diagrammed in Fig. 8. In our hands, the RPA detected changes in CAT mRNA over a 100-fold difference in input RNA (Fig. 8). In A549 cells transfected with M6-TKCAT, corresponding to the wild-type NF-IL6 binding sites, RSV infection produces a fourfold increase in reporter mRNA. In contrast, M2-TKCAT reporter was less active in control cells than the M6-TKCAT reporter, indicating the effect of constitutive NF-IL6 protein detectable by gel shift assay (Fig. 5). Importantly, M2-TKCAT mRNA was not inducible by RSV infection, an observation reproduced in three independent experiments. We therefore conclude that RSV replication results in the transcriptional activation of NF-IL6 binding sites in type II pulmonary epithelium.

DISCUSSION

Our data indicate that human alveolar type II epithelial cells synthesize the NF-IL6 transcription factor in response to RSV replication. RSV infection stimulates the translational synthesis of a single NF-IL6 isoform comigrating with recombinant NF-IL6 (amino acids 24 to 345). That the RSV-inducible immunoreactive species represents NF-IL6 is supported by the following data. (i) The anti-NF-IL6 antibodies used in this study recognize and supershift NF-IL6 in 3T3-L1 adipocytes in parallel with other anti-NF-IL6 antibodies (40). The anti-NF-IL6 antibodies also recognize recombinant NF-IL6 (Fig. 1b). (ii) The antigen in SDS-PAGs has sequence-specific NF-IL6 DNA-binding activity when eluted and renatured (Fig. 5). (iii) RSV induces a transcriptional activity specific for NF-IL6 binding sites but not mutated NF-IL6 binding sites. Taken together, we conclude that RSV replication induces NF-IL6 synthesis. Moreover, the isoform of NF-IL6 produced comigrates with NF-IL6 (amino acids 24 to 345), indicating that the RSV induction is probably specific for the second translational initiation codon. The induction of NF-IL6 is independent of changes in NF-IL6 mRNA, and using immunoprecipitation of [^{35}S]methionine-labeled proteins, we show an increased rate of NF-IL6 synthesis. Thus, the accumulation of NF-IL6 after RSV infection is due to increased translation of the preformed NF-IL6 mRNA.

Posttranscriptional control mechanisms of NF-IL6 expression. Like that of other members of the C/EBP transcription factor family, the abundance of NF-IL6 is controlled by a variety of developmental, tissue-specific, and inducible cues (1, 9, 10, 40). NF-IL6 encodes a single RNA transcript that produces three different translation products of 38, 36, and 16 kDa, as a consequence of alternative utilization of three in-frame initiator codons (9, 10). A similar phenomenon has been reported for the family member C/EBPα (37), indicating that alternative initiation is a generalized phenomenon of the C/EBP transcription factor family. In tissues expressing NF-IL6, the relative proportions of the three translated products are maintained at a ratio of 1:3:1, with the second initiation codon being preferentially utilized (10). Selection of the three initiation codons has been proposed to be the consequence of a leaky ribosome scanning mechanism (9, 23). In this model, the 43S ribosomal complex scans the 5′ untranslated region (UTR) until an initiator codon is reached. After GTP hydrolysis, the 60S ribosomal subunit enters the complex and peptide synthesis occurs (31, 39, 43). Sequences flanking the AUG initiation codon influence the efficiency of the translation ini-

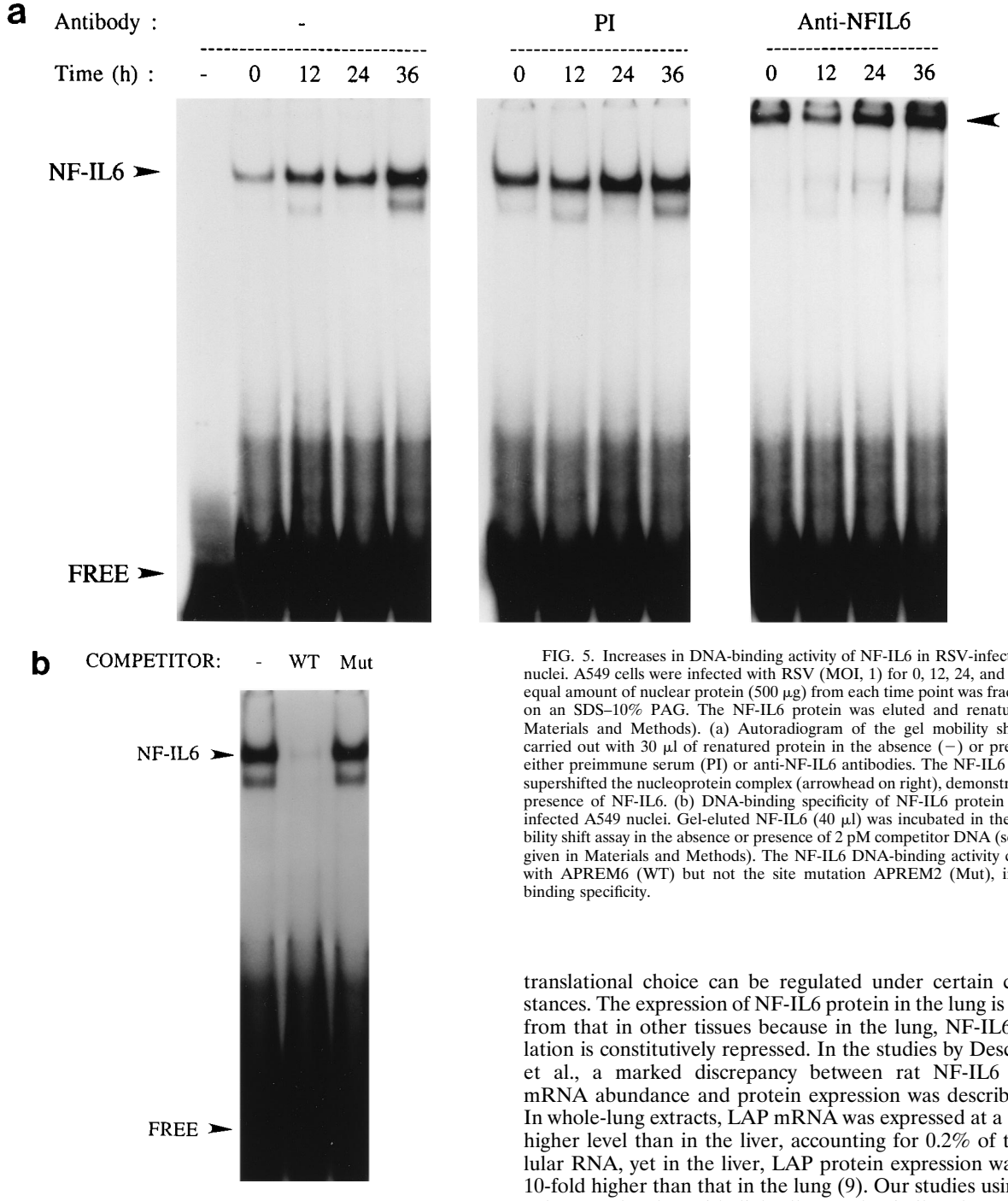

FIG. 5. Increases in DNA-binding activity of NF-IL6 in RSV-infected A549 nuclei. A549 cells were infected with RSV (MOI, 1) for 0, 12, 24, and 36 h. An equal amount of nuclear protein (500 μg) from each time point was fractionated on an SDS–10% PAG. The NF-IL6 protein was eluted and renatured (see Materials and Methods). (a) Autoradiogram of the gel mobility shift assay carried out with 30 μl of renatured protein in the absence (−) or presence of either preimmune serum (PI) or anti-NF-IL6 antibodies. The NF-IL6 antibody supershifted the nucleoprotein complex (arrowhead on right), demonstrating the presence of NF-IL6. (b) DNA-binding specificity of NF-IL6 protein in RSV-infected A549 nuclei. Gel-eluted NF-IL6 (40 μl) was incubated in the gel mobility shift assay in the absence or presence of 2 pM competitor DNA (sequences given in Materials and Methods). The NF-IL6 DNA-binding activity competes with APREM6 (WT) but not the site mutation APREM2 (Mut), indicating binding specificity.

tiation, and this may be one explanation for the preferential translation beginning at the second AUG codon. In NF-IL6 mRNA, the context surrounding the second AUG initiator codon (ATCC**AUG**G) is more closely homologous to the consensus Kozak initiation sequence (CCA/GCC**AUG**G) than is the first AUG sequence (22).

Although the sequence context of the NF-IL6 AUG codon plays an important role in its selection for translational initiation, other factors influence this choice. For example, the ratio of 36-kDa to 16-kDa NF-IL6 translation products is changed during terminal liver differentiation (9, 10), indicating that translational choice can be regulated under certain circumstances. The expression of NF-IL6 protein in the lung is unique from that in other tissues because in the lung, NF-IL6 translation is constitutively repressed. In the studies by Descombes et al., a marked discrepancy between rat NF-IL6 (LAP) mRNA abundance and protein expression was described (9). In whole-lung extracts, LAP mRNA was expressed at a 10-fold higher level than in the liver, accounting for 0.2% of the cellular RNA, yet in the liver, LAP protein expression was 5- to 10-fold higher than that in the lung (9). Our studies using type II human alveolar epithelial cells reveal a similar phenomenon, with detectable mRNA but little NF-IL6 protein synthesis in unstimulated control cells. Importantly, our data extend these observations to show that NF-IL6 translation is inducible by viral infection.

Pulmonary virus infection activates NF-IL6 by distinct mechanisms. In contrast to many other viruses which shut off translation of cellular mRNAs (reviewed in reference 24), RSV infection does not appear to globally affect protein or RNA synthesis (26). To our knowledge, these observations are the first description of inducible translation of NF-IL6 protein from preformed NF-IL6 mRNA. Inducible translation of a preformed mRNA allows a rapid synthesis of the NF-IL6 pro-

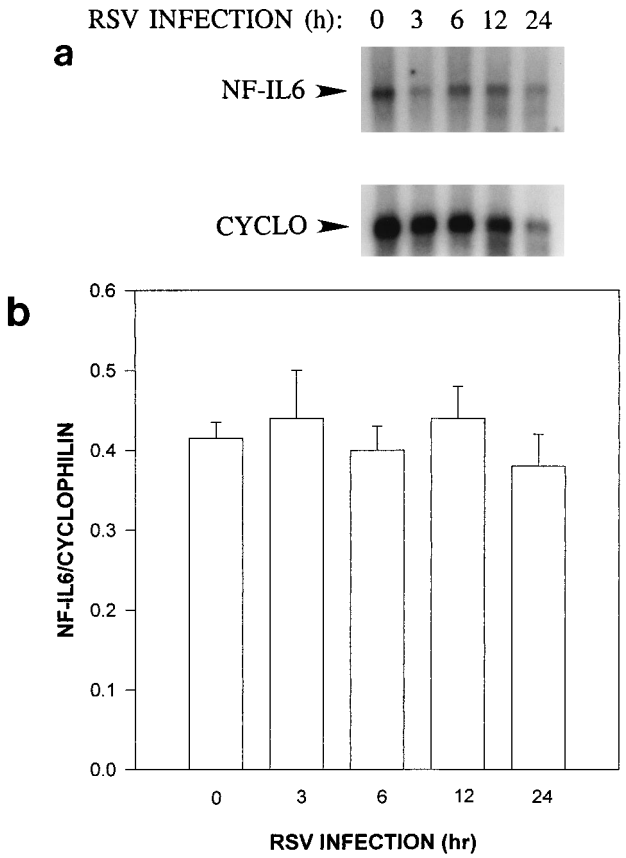

FIG. 6. Northern blot analysis of NF-IL6 mRNA in A549 cells. (a) Poly(A)$^+$ RNA (1 μg) isolated from A549 cells infected with RSV (MOI, 1) for 0, 3, 6, 12, and 24 h was analyzed for NF-IL6 mRNA by Northern blot. Shown is a representative autoradiographic exposure demonstrating the single 1.8-kb NF-IL6 mRNA. The same membrane was used for the detection of cyclophilin mRNA (CYCLO) shown below as a control for RNA recovery. Hybridization signals of the NF-IL6 and cyclophilin bands were quantitated with a 425E PhosphorImager, and the ratio of NF-IL6/cyclophilin band intensity was calculated. (b) The normalized data are presented as a bar graph showing that no significant changes in NF-IL6 mRNA occur in RSV-infected A549 cells.

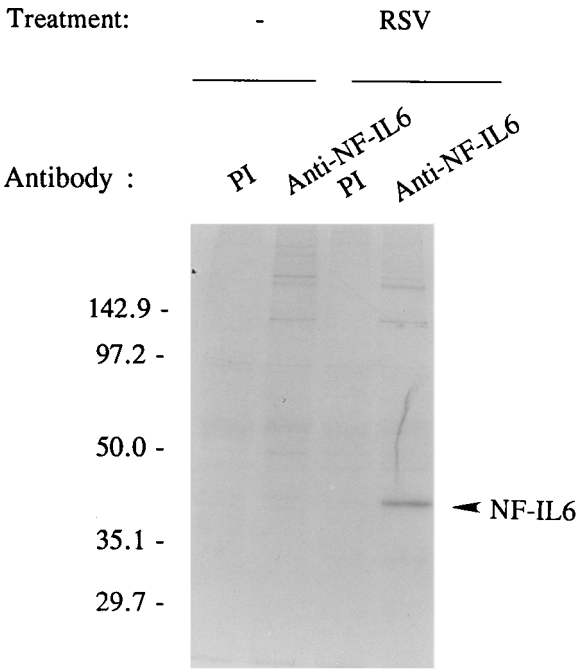

FIG. 7. De novo synthesis of NF-IL6 is increased in RSV-infected A549 cells. Autoradiogram of immunoprecipitation assay of [^{35}S]methionine-labeled A549 cells treated with sucrose alone (−) or infected with RSV (MOI, 1) for 24 h. [^{35}S]methionine-labeled nuclear protein was isolated and immunoprecipitated with NF-IL6-specific antibody or preimmune serum (PI). Masses (in kilodaltons) are shown on the left. No NF-IL6 synthesis could be detected in control cells. In contrast, the 45.7-kDa NF-IL6 is synthesized in RSV-infected cells (arrowhead).

tein with the minimum expenditure of cellular energy. Moreover, RSV induces NF-IL6 translation specifically for one isoform of NF-IL6, we believe amino acids 24 to 345, and not for NF-IL6 (amino acids 1 to 345) because our antibody recognizes recombinant NF-IL6 (amino acids 1 to 345) and NF-IL6 (amino acids 24 to 345) equally well and these proteins can be distinguished in our SDS-PAG system (not shown).

The mechanism for enhanced translation of preformed mRNA used by RSV is contrasted with a recently reported effect of influenza virus infection in HeLa cells (50). Influenza virus infection increased, rapidly and transiently (over 2 to 4 h), the DNA-binding activity of preformed NF-IL6 protein without changing its steady-state levels. That this effect could be reproduced by double-stranded RNA left the suggestion that double-stranded RNA kinase could be an important virus-inducible activator for posttranslational activation of NF-IL6 DNA-binding activity. Thus, NF-IL6 activity appears to be inducible by several distinct posttranslational mechanisms by viral infection within the same epithelial cell type.

Possible mechanisms for enhanced NF-IL6 translation. Although few examples of gene selective virus-inducible translational regulation have been reported, translational control systems are important during embryogenesis, when several general mechanisms emerge (reviewed in references 8 and 24). Translational initiation rates can be controlled by proteins stabilizing structures in the 5′ UTR. One example is the iron regulatory protein (IRP) that binds the ferritin 5′ UTR mRNA (the iron response element [IRE]) in the absence of cellular iron (21). IRP inhibits translation by preventing the binding of the 43S small ribosomal subunit-initiation factor complex. In the presence of cellular iron, IRP releases ferritin mRNA, allowing translation rates to increase 40- to 100-fold. Alternatively, mRNA can be masked after translation by incorporation into a ribonucleoprotein particle by association with RNA-binding phosphoproteins whose association with RNA is dependent on 3′ UTR sequences and/or phosphorylation-dephosphorylation (28). Finally, 3′ UTR sequences can control translation by controlling the length of the poly(A) tail; this in turn influences the formation of the cap binding complex (12). Which mechanism(s) is operative for RSV induction of NF-IL6 will require further study. We note that NF-IL6 contains a stable stem-loop structure at its 5′ UTR (with a free energy for formation of −101 kcal/mol [ca. −423 kJ/mol]). However, the potential stem-loop structure in the NF-IL6 5′ UTR is distinct from the ferritin IRE because there is no sequence similarity in nucleotides recognized by IRP and the free energy for stem-loop formation in the NF-IL6 5′ UTR is much greater. Moreover, there are no obvious changes in the size of the polyadenylated NF-IL6 mRNA after RSV infection to suggest a mechanism involving changes in cytoplasmic polyadenylation.

RSV is a negative-sense RNA virus of the paramyxovirus family whose primary target for replication is the airway epithelium (17). RSV replication appears to be important for NF-IL6 synthesis, because we are unable to reproduce the inductive phenomenon by addition of conditioned medium or

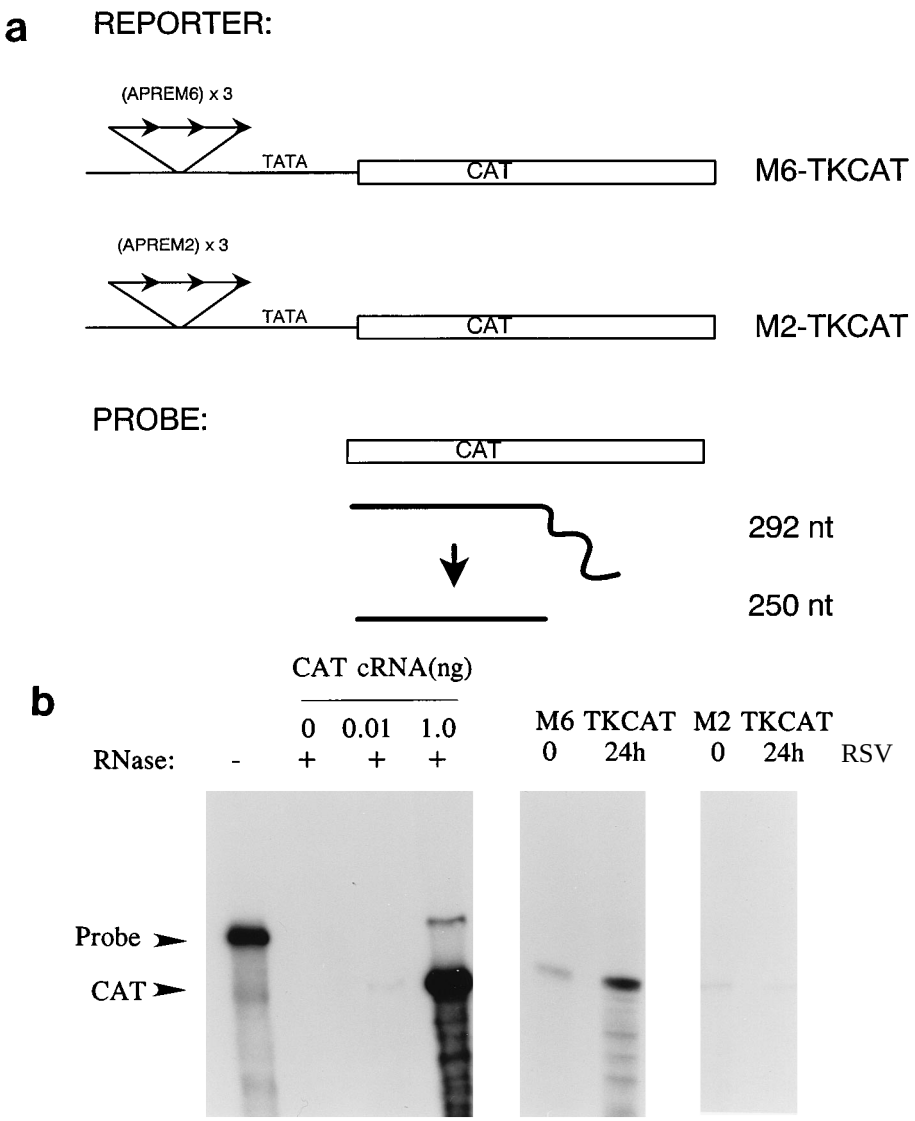

FIG. 8. RSV-induced NF-IL6 in A549 cells is transcriptionally active. Transcriptional activity of NF-IL6 was determined by RPA using the CAT reporter gene. (a) Schematic diagram of CAT reporter genes and riboprobe. A 250-bp *Eco*RI-*Hin*dIII fragment of the CAT gene was cloned into pGEM3Z. Sense and antisense CAT RNAs were generated from this plasmid with SP6 and T7 RNA polymerase, respectively (see Materials and Methods). nt, nucleotides. (b) RPA of NF-IL6-driven reporters in A549 cells. Autoradiograph of RPA of CAT reporter gene activity in RSV-infected A549 cells. A549 cells were transfected with either 5 μg of M6-TKCAT (containing three copies of NF-IL6 binding sites) or M2-TKCAT (containing three copies of mutated NF-IL6 binding sequences). After 16 h of transfection, cells were treated with sucrose or infected with RSV (MOI, 1) for 24 h. Equal amounts of RNA (50 μg) were analyzed for CAT reporter gene mRNA by RPA. Migration of the CAT probe and protected CAT fragment after RNase digestion are indicated by arrowheads. M6-TKCAT-transfected cells showed a fourfold increase in CAT mRNA levels, whereas in M2-TKCAT-transfected cells there was no increase of CAT mRNA with RSV infection.

UV-inactivated virus. Coincident with NF-IL6 synthesis, viral antigens are detectable within 9 h after infection (3, 35) and infectious virus is secreted by 11 to 13 h after infection (25, 51). Thus, a temporal relationship is present between viral protein expression and NF-IL6 synthesis. Presently, it remains speculative whether NF-IL6 induction is the consequence of intracellular expression of a specific RSV-encoded gene product or the result of some nonspecific cellular stress produced as a consequence of RSV replication (such as alterations in redox potential).

Potential roles of NF-IL6 in RSV-infected epithelium. After contact with noxious or infectious agents, epithelial cells induce and sustain inflammatory and immunological events by producing a variety of effector substances, including cytokines (reviewed in reference 44) and cell surface receptors involved in either the immune response or programmed cell death (50). RSV infection of human airway epithelial cells results in the enhanced expression of the cytokines IL-8, granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor (among others [2, 14, 34]). The chemotactic and proinflammatory activities of these cytokines initiate and sustain the pronounced inflammatory response seen in RSV infection (reviewed in reference 19). We note that these genes share dependence on the NF-IL6 transcription factor for their expression (1, 11, 29, 45) and that the cytokine mRNAs increase in parallel with the kinetics of NF-IL6 protein abundance (14). This raises the speculation that inducible translation of NF-IL6 during RSV replication in the airway epithelial

cell is an important mechanism for the coordinate expression of the proinflammatory networks of cytokine genes. The expression of Fas, a receptor involved in the initiation of apoptosis, is controlled by NF-IL6 in HeLa epithelial cells (50), as is the expression of intercellular adhesion molecule 1 (18). More experiments will be required to determine the spectrum of genes activated by NF-IL6 and their role in the epithelial cell-initiated response to RSV infection.

In conclusion, we report the novel mechanism of RSV induction of the potent NF-IL6 transcription factor in airway epithelial cells. NF-IL6 protein synthesis increases in response to replicative virus independent of changes in mRNA, constituting a distinct mechanism for NF-IL6 virus induction by pathogenic pulmonary virus infection. Understanding the mechanisms responsible for switches in gene expression by infected airway epithelium identifies potential therapeutic targets for modulating the inflammation seen after RSV infection that is pathogenic for RSV-induced pulmonary disease. It will be of interest, and potential therapeutic benefit, to antagonize the induction of NF-IL6 and determine its effects on viral infection.

ACKNOWLEDGMENTS

We thank Mona Sabry and Fang Mei for reagents and help with experiments.

This work was supported by grants from the Texas Advanced Research Program (ATP/ARP 004952001); Child Health and Human Development, NIH (R30HD 27841); and NIAID (AI/HL 15939-14A1). M.J. is a MacLaughlin Postdoctoral Fellow. A.R.B. is an Established Investigator of the American Heart Association.

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