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Requirement of a Novel Upstream Response Element in Respiratory Syncytial Virus-Induced IL-8 Gene Expression

Antonella Casola,* Roberto P. Garofalo,* Mohammad Jamaluddin,† Spiros Vlahopoulos,‡ and Allan R. Brasier*‡§

Respiratory syncytial virus (RSV), a enveloped single-stranded negative sense RNA-containing virus, is the major cause of serious lower respiratory tract disease in children (reviewed in Ref. 1). It is estimated that 40–50% of children hospitalized with bronchiolitis and 25% of children with pneumonia are infected with RSV, resulting in 100,000 hospital admissions annually in the US alone (2). RSV infection is also associated with increased morbidity in children with chronic diseases such as bronchopulmonary dysplasia, cystic fibrosis, and congenital heart malformation (3). In addition to the acute morbidity, RSV infection in infancy can lead to long-term consequences, since it has been shown to predispose to the development of hyperreactive airway disease (1), and recurrent episodes of wheezing are often precipitated by subsequent RSV infection (4).

Although the pathophysiology of RSV-induced airway injury is not well understood, autopsy and in vivo studies suggest that the inflammatory response, triggered by the infection of respiratory epithelial cells, is an essential component (5). IL-8 is known to be a potent chemoattractant and activator for neutrophils, T cells, basophils, and primed eosinophils (reviewed in Ref. 6). High concentrations of IL-8 have been detected in nasal and bronchoalveolar lavage fluids of children with RSV respiratory infections (7, 8) and in middle ear effusions of children with viral otitis media (9). It is therefore likely that this cytokine plays a major role in the recruitment of inflammatory cells to the lung following infection by RSV.

Using the model of infecting well-differentiated lung type II alveolar epithelial cells (A549), we have previously shown that RSV replication in airway epithelial cells results in increased IL-8 gene expression and protein release (10). Nuclear run-on assays have shown that the enhanced IL-8 synthesis was primarily due to increased gene transcription. Mutational analysis of the IL-8 promoter demonstrated that NF-κB binding was absolutely required for RSV- and TNF-induced IL-8 gene transcription (10, 11). However, the participation of upstream regulatory elements was not investigated.

Previous studies have shown that regulation of IL-8 gene expression is cell type and stimulus specific (12). The current view on how a cell can respond dynamically to a variety of different stimuli is that combinations of ubiquitous, signal- and tissue-specific activators can be assembled into a nucleoprotein complex called enhanceosome (13). In the enhanceosome model, the arrangement of different activator recognition sites and bound activators generates a network of protein-protein and protein-DNA interactions that is unique to a given enhancer for each stimulus. Each enhanceosome provides a specific activation surface that is chemically and spatially complementary to target surfaces of co-activators and to the basal transcriptional machinery; in this way, they can be recruited to DNA to generate synergistic transcription.

In the few studies performed in alveolar epithelial cells, in which the regulation of IL-8 gene transcription after RSV infection was examined, there has been controversy regarding the need for replicating virus, as well as the promoter region required for IL-8 activation (14–17). Therefore, the purpose of this study was to clarify which promoter elements are involved in regulation of IL-8 gene transcription following RSV infection and to define whether there are different requirements from cytokine stimulation. Our
results indicate that NF-κB binding site is not only necessary, but sufficient for activation of IL-8 transcription after TNF stimulation. In contrast, IL-8 induction by RSV infection requires the participation of several additional upstream response elements. Among them we have identified a previously unrecognized regulatory element, with similarity to the consensus IFN-stimulated response element (ISRE), that plays an important role in RSV-activated gene transcription.

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 (18). The virus titer of the purified RSV pools was 7.5–8.5 log PFU/ml using a methylcellulose plaque assay. No contaminating cytopathics, including IL-1, TNF, IL-6, IL-8, GM-CSF, and IFN, were found in these sucrose-purified viral preparations (19). LPS, assayed using the Limulus hemocyanin agglutination assay, was not detected. Virus pools were aliquotted, quick frozen on dry ice/alcohol, and stored at −70°C until used.

Cell culture and infection of epithelial cells with RSV

A549, human alveolar type II-like epithelial cells (American Type Culture Collection, Manassas, VA), were maintained in F12K medium containing 10% (v/v) FBS, 10 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cell monolayers were infected with RSV at multiplicity of 1, in the presence of TNF, 20 ng/ml. At 12 h postinfection or 6 h after TNF stimulation, cells were either infected with RSV at MOI of 1 or stimulated with TNF, 20 ng/ml. At 12 h postinfection or 6 h after TNF stimulation, cells were lysed to independently measure luciferase and β-galactosidase reporter activity, as previously described (20). Luciferase was then normalized to the internal control β-galactosidase activity. All experiments were performed in duplicate or triplicate, using at least two different plasmid preparations.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts of uninfected and infected A549 cells were prepared using hypotonic/nonionic detergent lysis, as previously described (10). To prevent contamination of nuclear extracts with cytoplasmic proteins, isolated nuclei were purified by centrifugation through 1.7 M sucrose buffer A for 30 min, at 12,000 rpm, before nuclear protein extraction. Proteins were normalized by protein assay (Protein Reagent; Bio-Rad, Hercules, CA) and used to bind to duplex oligonucleotides corresponding to the IL-8 RSVRE wild-type (WT) and mutated (MUT; mutations underlined) and to the IL-8 AP-1 binding site: RSVRE (WT), GATCCACCTTTTATTTGATAAGG GAAATAGGAGTGTTA GTGGCATAAACTATTCCTTC TTTA TCC TTCAAAATCTAG; RSVRE (MUT), GATCCACCTTTTATTTGATAAGG GAAATAGGAGTGTTA GTGGCATAAACTATTCCTTC TTTA TCC TTCAAAATCTAG; AP-1, GATCCCAATTGTGATGACCTGTTA GAAATAGGAGTGTTA GTGGCATAAACTATTCCTTC TTTA TCC TTCAAAATCTAG.

Nuclear extracts, used for binding to the AP-1 site, were prepared from control and infected A549 cells that have been serum starved, before and throughout the period of infection, for a total of 24 h.

DNA-binding reactions using the RSVRE probe contained 10–15 μg total protein, 5% glycerol, 12 mM HEPES, 80 mM NaCl, 5 mM DTT, 1 μg of poly(dA-dT), and 40,000 cpm of 32P-labeled double-stranded oligonucleotide in a total volume of 20 μl. Binding reactions for the AP-1 probe contained 10–15 μg total protein, 5% glycerol, 12% HEPES, 80 mM NaCl, 5 mM DTT, 5 mM MgCl2, 0.5 mM EDTA, 1 μg of poly(dI-dC), and 40,000 cpm of 32P-labeled double-stranded oligonucleotide in a total volume of 20 μl. The nuclear proteins were incubated with the probe for 15 min at room temperature and then fractionated by 6% non-denaturing PAGE in TBE buffer (22 mM Tris-HCl, 22 mM boric acid, 0.25 mM EDTA, pH 8). In competition assays, 10 pmol of unlabeled competitors were added at the same time of probe addition. After electrophoretic separation, gels were dried and exposed for autoradiography using Kodak XAR film at ~70°C using intensifying screens.

Microarray isolation assay

A549 cells were labeled overnight with [3H]methionine and [35S]cysteine and harvested to prepare nuclear extracts. Microarray purification of proteins binding to RSVRE was performed using a two-step biotinylated (B) DNA-streptavidin capture assay (11). In this assay, duplex oligonucleotides were chemically synthesized containing 5′ Bt on a flexible linker (Genosys, The Woodlands, TX). Five hundred micromgrams of nuclear extracts were incubated with 50 pmol of duplex oligonucleotide in the presence of 8 μg poly(dA-dT) (as nonspecific competitor) in 1000 μl (final volume) of binding buffer (8% (v/v) glycerol, 5 mM DTT, 80 mM NaCl, 12 mM HEPES (pH 7.8), at 4°C for 30 min. One hundred microliters of a 0.05 μl of prewashed streptavidin-agarose beads were then added to the sample, and incubated at 4°C for an additional 20 min with gentle rocking. Pellets were washed twice with 500 μl binding buffer, and proteins were eluted from beads with 100 μl of 1 M NaCl. Proteins were desalted on a G50 column and incubated again with the oligonucleotides. The washed pellets were resuspended in 100 μl 1× SDS-PAGE buffer, boiled, and fractionated on a 10% SDS-polyacrylamide gel. After electrophoretic separation, gels were dried and exposed for autoradiography using Kodak XAR film at ~70°C using intensifying screens.

In the microarray isolation/Western blot assay, RSVRE-binding proteins were isolated from control and 12-h RSV-infected nuclear extracts, as previously described, in the absence or presence of 10-fold molar excess of nonbiotinylated RSVRE WT oligonucleotide. After electrophoresis separation, proteins were transferred to polyvinylidene difluoride membrane for Western blot analysis.

Western immunoblot

Cytoplasmic and nuclear proteins were prepared as previously described, fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (11). Membranes were blocked with 5% albumin in TBS-Tween and incubated overnight with a rabbit polyclonal Ab to IRF-1 (Santa Cruz Biotechnology, Santa Cruz, CA). For secondary detection, we used a horseradish-coupled donkey anti-rabbit Ab in the enhanced chemiluminescence assay (Amersham).
deletions of the 5' cells were transiently transfected with plasmids containing serial and cytokine-inducible activity deletions in the IL-8 promoter sequence on RSV

Results

Effects of 5' deletions in the IL-8 promoter sequence on RSV and cytokine-inducible activity

To define the regions of the IL-8 promoter involved in regulating gene expression after RSV infection or TNF stimulation, A549 cells were transiently transfected with plasmids containing serial deletions of the 5' flanking region of the IL-8 gene, linked to the luciferase reporter gene. A schematic diagram of the promoter is shown in Fig. 1. We have previously shown that a -162/+44 nt fragment retains the same RSV and TNF inducibility as longer fragments of the IL-8 promoter (up to -1.4 kb) (10). Luciferase activity was measured after 12 h of RSV infection or 6 h of TNF stimulation, time points that we have previously shown to correspond to peak reporter gene induction after each stimulus (10, 11). As shown in the Fig. 2A, RSV infection induced luciferase activity of the -162/+44 hIL-8/LUC by 6.7-fold compared with uninfected cells. Deletion from -162 to -132 nt reduced to half the inducibility of the luciferase activity (3.7-fold), without affecting basal activity, suggesting the presence of a positive regulatory element in this region of the promoter. Further deletion to -99 nt reduced the basal activity of the promoter by 2-3-fold and completely abolished the RSV-induced luciferase activity, indicating that the sequence between -132 and -99 nt is also critically involved in IL-8 gene activation by RSV. TNF was also a potent inducer of IL-8 transcription, producing a 35-fold increase of the -162/+44 hIL-8/LUC (Fig. 2B). The 5' deletion to -132 nt did not affect the response of the IL-8 promoter to TNF stimulation. Deletion to -99 nt did not change the TNF stimulation of the promoter in terms of fold induction (43-fold), although the overall activity of the promoter was reduced in proportion to the reduction of the basal activity. A further deletion to -54 nt instead greatly diminished, although did not completely abolish, the TNF-induced luciferase activity, demonstrating that the region from -99 to -54 nt is required for IL-8 gene activation by TNF. These data indicate a stimulus-selective role for two regions in the IL-8 promoter up-stream of -99: 1, a region between -132 and -99 nt, that contains a functional AP-1 binding site; and 2, a region from -162 to -132 nt, which has not been investigated. Because this latter region functions as a positive regulatory element only in RSV infection, we term it RSVRE. Therefore, while the presence of RSVRE and AP-1 binding sites is necessary for RSV inducibility of the IL-8 promoter, TNF inducibility of the promoter stimulation mainly requires an intact NF-κB/NF-IL6 binding site.

Statistical analysis

Data from experiments involving multiple samples subject to each treatment were analyzed by the Student Newman Keuls test for multiple pairwise comparisons.

Binding of nuclear factors to the RSVRE and AP-1 sites of the IL-8 promoter

We have previously shown that RSV infection as well as TNF stimulation of A549 cells resulted in increased binding of the sub-units RelA, NF-κB1, and c-Rel to the IL-8 NF-κB site (10, 11). Because the 5' deletion analysis of the promoter showed that the RSVRE and the region containing an AP-1 binding site are two important regulatory elements in RSV-induced IL-8 transcription, we performed EMSA to determine whether RSV infection produced changes in the abundance of DNA-binding proteins that recognize these two regions of the IL-8 promoter. As shown in Fig. 3A, a single nucleoprotein complex (C3) was formed by sucrose cushion-purified control nuclear extracts, using the oligonucleotide
corresponding to the RSVRE, while two other complexes, termed C1 and C2, were faintly detected. RSV infection increased the binding of C1 and C2 starting at 6 h postinfection, with a peak in binding intensity at 12 h postinfection. The sequence specificity of the RSVRE complexes was examined by competition with unlabeled oligonucleotides in EMSA (Fig. 3B). C1 and C2 were competed by the WT oligonucleotide, but not by the MUT one, indicating binding specificity. Specificity was also shown by the absence of C1 and C2 binding to the radiolabeled RSVRE MUT probe. Analysis of the RSVRE sequence identified two potential sites for transcription factor binding: a GATA site between −151 and −147 nt, and a site between −144 and −132 nt, containing an ISRE-like motif in the sense strand and a HNF-3β-like motif in the minus strand (21–23). Mutation of the GATA site did not affect RSVRE-binding and competition assays, using oligonucleotides corresponding to consensus sequences of GATA and HNF binding sites, could not identify the RSVRE as one of them, as neither site was captured by the addition of streptavidin-agarose beads and washed, and the presence of bound IRF-1 was detected by Western blot. As shown in Fig. 7, little IRF-1 was detectable in control cells, but its abundance was greatly increased after RSV infection. IRF-1 detection was reduced when 10-fold excess of nonbiotinylated RSVRE WT oligonucleotide was included as competitor in the initial binding reaction, indicating sequence specificity.

To determine whether IRF-1 was binding to the IL-8 RSVRE, we used a two-step microaffinity isolation/Western blot assay. In this assay, Bt RSVRE was used to bind nuclear extracts of control and 12-h infected A549 cells. RSVRE-binding proteins were captured by the addition of streptavidin agarose beads and washed, and the presence of bound IRF-1 was detected by Western blot. As shown in Fig. 6, little IRF-1 was detectable in control cells, but its abundance was greatly increased after RSV infection. IRF-1 detection was reduced when 10-fold excess of nonbiotinylated RSVRE WT oligonucleotide was included as competitor in the initial binding reaction, indicating sequence specificity.

IRF-1 is a component of the RSVRE-binding complex

As analysis of the RSVRE sequence revealed the presence of an ISRE-like site, we sought to investigate whether RSV infection of A549 cells could activate the viral-inducible transcription factor IRF-1, which modulates transcription of ISRE-containing genes, such as human IFN-β (24). Western blot was performed on cytoplasmic and sucrose cushion-purified nuclear extracts of A549 cells control and infected for various length of time. IRF-1 protein was expressed at low levels in the cytoplasm and nucleus of control cells and was highly inducible following RSV infection, starting between 3 and 6 h postinfection, as shown in Fig. 6.
Effects of site mutations in the promoter on IL-8 gene activation by RSV infection and cytokine stimulation

To establish precisely the role of the individual \textit{cis} elements of the IL-8 promoter in conferring responsiveness to either RSV infection or TNF stimulation, we introduced site-directed mutations of these sites in the context of the −162 IL-8 promoter. These mutations correspond to sequences known to disrupt binding of the relevant transcription factor (Fig. 3) (11). A549 cells were transiently transfected with the WT and MUT plasmids, and luciferase activity was measured after 12 h of RSV infection or 6 h of TNF stimulation. As shown in Fig. 8A, the RSV-induced promoter activity was reduced to ∼55% of wild type by site mutations of the RSVRE site. Mutation of the AP-1 site affected both the basal activity and the RSV inducibility of the promoter, the latter being reduced to ∼50%. The NF-IL6 site mutant also showed lower inducibility (60% reduction) compared with the WT promoter, with no effect on the basal activity. When we introduced simultaneous mutations of RSVRE and either the AP-1 or the NF-IL6 binding site, the IL-8 promoter was no longer inducible following RSV infection. These results suggest that all three binding sites contribute to IL-8 promoter induction after RSV infection, and that cooperation among these different sites is required for full activation of the promoter.

When cells were stimulated with TNF (Fig. 8B), the AP-1 site mutant showed a similar fold induction compared with the wild type, and the NF-IL6 site mutant only a modest reduction of the luciferase activity, suggesting that the NF-kB site is sufficient to confer TNF inducibility to the IL-8 promoter.

RSV infection requires the combination of multiple promoter elements to activate transcription

To determine whether these sites could be independently induced by RSV infection or TNF stimulation, A549 cells were transfected with reporter genes containing multimers of the IL-8 AP-1, NF-κB, or NF-IL6 elements ligated upstream of the IL-8 TATA box. As shown in Fig. 9, activity of the NF-κB multimer was highly inducible (18-fold) by TNF, whereas the other sites were not significantly affected by TNF stimulation. Although the AP-1 site was not TNF inducible, it was strongly induced (∼8-fold) by the diacylglycerol agonist, PMA. In contrast, although RSV infection was able to activate the −162/+44 hIL-8/LUC expression, it was unable to stimulate the reporter activity of any isolated multimer. These data suggest that the NF-kB site is the TNF-inducible element of the IL-8 promoter, whose presence is both necessary and
sufficient for TNF-activated IL-8 gene transcription. They also indicate distinct, combinatorial requirements for RSV-induced IL-8 transcriptional activation.

To further investigate the interaction among the different regions of the IL-8 promoter needed in RSV infection to activate gene transcription, we transfected A549 cells with plasmids containing either a copy of the RSVRE together with a copy of the AP-1 site upstream the −299/144 hIL-8 promoter (which itself is not RSV inducible), or two copies of the RSVRE (Fig. 10A). The results showed that the RSVRE could partially restore the RSV inducibility of the −299/144 hIL-8 promoter, as shown in Fig. 8B. These data confirm that RSV induction of IL-8 transcription requires the participation of additional cis elements, implying the requirement of a distinct network of protein-protein and protein-DNA interactions than those required for cytokine stimulation.

Discussion

Under normal conditions, airway epithelial cells represent an important interface between the external environment and the host. Upon infection or injury, they play an important role in initiating the mucosal immune response by producing soluble factors, such as cytokines, that regulate communication among cells of the immune system. The immunomodulatory activity of the airway epithelium is of particular relevance to RSV infection, because the inflammatory response triggered by RSV infection seems to be an essential pathogenic component of RSV-induced lung damage (5). Chemokines are a novel class of small cytokines that are able to recruit and activate leukocytes, and therefore have a significant role as potent mediators of immune/inflammatory responses (25). The C-X-C chemokine IL-8, in particular, is likely to play a major role in the pathogenesis of RSV infection because it is a strong chemoattractant for the majority of inflammatory cells present in the cellular infiltrate of RSV-infected lungs (6, 26), and it has been detected in nasal and bronchial lavage fluids of children infected with RSV (7, 8).

We have previously shown that RSV infection and TNF stimulation of alveolar epithelial cells strongly induce IL-8 gene expression and protein release (10, 11). The enhanced protein synthesis was primarily due to increased gene transcription, and both stimuli required NF-κB activation for gene expression. The mechanisms of IL-8 gene induction have been investigated in a variety of cell types, and deletion and mutational analysis of the promoter indicates that IL-8 gene is activated in a cell-type and stimulus-specific manner (12), suggesting that different sets of nuclear factors might be necessary for IL-8 gene transcription. Because identification of the pleiotropic mechanisms required for IL-8 promoter activation is important for rational design of therapeutic agents that can block its expression in the lung, in this study we performed a detailed analysis of IL-8 promoter activation in airway epithelial cells comparing two important proinflammatory stimuli, RSV infection and TNF stimulation.

Analysis of promoter deletions indicates that the region from nt −99 to +44 is necessary and sufficient for TNF-induced IL-8 transcription. On the other hand, RSV-induced IL-8 activation requires the participation of a previously unrecognized response element.
spanning from −162 to −132 nt, which we term the RSV response element (RSVRE), and a previously characterized element spanning −132 to −100 nt, containing a functional AP-1 binding site. Several studies have investigated the minimal enhancer region of the IL-8 promoter necessary to confer the responsiveness to cytokines such as TNF and IL-1. In human cells derived from fibrosarcoma, astrocytoma, and glioblastoma tumors, as well as in Hela cells, TNF activates IL-8 through a cis element encompassing −97 to −69 nt, a region that contains the NF-IL6 and NF-κB sites (27, 28), similarly to what we have observed in the alveolar epithelial cells. However, the presence of these two binding sites is not always sufficient for IL-8 gene activation. An intact AP-1 binding site, located from −126 to −120 nt, is in fact required for TNF induction of the IL-8 promoter in gastric cancer cells, hepatoma cells, and Jurkat T cells, as well as in a lung epithelial cell line (29, 30). These results suggest that the combination of the minimal enhancer region essential for TNF-induced IL-8 gene activation differs among cell types, possibly due to a different distribution of transcription factors or coactivators of transcription.

Our observation that RSV infection of alveolar epithelial cells requires the presence of two additional regulatory elements for full promoter inducibility, compared with TNF stimulation, indicates the presence of stimulus-specific requirements for IL-8 gene induction within the same cell type. Previous work in alveolar epithelial cells has shown similar inducibility of the −132 and −99/−44 IL-8 promoters after both RSV infection and TNF stimulation (14–16). These studies have also shown IL-8 gene induction with inactivated RSV. In our hands, the UV-inactivated virus is unable to induce IL-8 gene expression (10), and indeed the deletion of the −162 to −132 nt region strongly reduces the RSV inducibility of the IL-8 promoter, while a further deletion to −99 nt completely abrogates it. The differences of our study compared with the previous ones might be explained by their use of nonpurified viral preparations, known to be contaminated by IL-1α and possibly TNF (19), extremely potent IL-8 and NF-κB stimulators, and by the use in transient transfection assays of a nonquantitative reporter gene, such as the chloramphenicol acetyl transferase, in which subtle differences could be missed (31).

The results of site-directed mutation experiments clearly show that the RSVRE, AP-1, and NF-IL6 binding sites are all necessary for RSV-induced activation of the IL-8 promoter, while TNF stimulation requires mainly an intact NF-κB site. Our data are in agreement with previous studies showing that AP-1 and NF-IL6 binding sites are important in RSV-induced IL-8 promoter activity (14, 15). However, our results also indicate the involvement of a previously unidentified regulatory element of the IL-8 promoter, the RSVRE, that plays an important role in activation of IL-8 gene expression during RSV infection. The RSVRE shows sequence similarity with the consensus ISRE site, and our results suggest that can bind the transcription factor IRF-1, which is activated in A549 cells following RSV infection. IRF-1 belongs to a growing family of transcription factors, the IRFs. To date, 10 members of this family have been identified, and their expression is either constitutive and/or inducible after IFN/cytokine stimulation or in response to viral infection (24). IRF-1 is of particular interest, as this virus-inducible protein activates IFN-β, a gene highly expressed in RSV-infected epithelium (M. Jamaluddin, unpublished data). Interestingly, on the IFN-β gene, IRF-1 activates transcription only when NF-κB is coexpressed (32), indicating a common mechanism of activation with our studies on IL-8. Our observation extends the sites that cooperate with IRF-1 to include NF-IL6 and AP-1. Whether these sites are promoter/context specific or is a more general requirement of IRF-1 will need further investigation. Our data also show that IRF-1 is binding within a larger complex with additional proteins because we observed an unknown 70-kDa protein in the microarray-binding assay (Fig. 4). Future studies will determine whether other members of the IRF family, such as IRF-3 and IRF-7, which have also been shown to play an important role in IFN-γ gene induction (33), can bind to the RSVRE and regulate IL-8 promoter activation following RSV infection. The RSVRE is not only stimulus specific, but is possibly also cell type specific, because we were not able to demonstrate its involvement in IL-8 gene regulation in a similar model of intestinal epithelial cells infected with rotavirus (A. Casola, unpublished data).

Several studies have suggested an important role for NF-IL6 in cytokine-induced IL-8 promoter activation (27, 34, 35). Our mutational experiments show only a modest reduction of TNF inducibility of the NF-IL6-MUT −162/+44 IL-8 LUC, suggesting that the NF-κB site is the major regulatory element in TNF-induced IL-8 activation. Furthermore, we were not able to show NF-IL6 binding after TNF stimulation, either by gel-shift assay or microarray isolation (11). A possible explanation is that the role of NF-IL6 was tested in the context of the −162/+44 IL-8 promoter. In this study, other binding sites, such as AP-1, could have redundant activity and could be used alternatively for NF-IL6 site.

Further evidence that the mechanisms of activation of IL-8 promoter are different in the RSV infection and TNF stimulation is provided by the results of transient transfections of IL-8 multimer binding sites. Although TNF is able to stimulate the reporter activity of plasmids containing multimers of the NF-κB site, and not of the NF-IL6 and AP-1 sites, none of the multimers is activated by RSV infection. The RSV-induced reporter activity is instead partially reconstituted by the addition of the RSVRE to the uninducible −99/+44 IL-8 promoter, suggesting again that RSV infection requires a different network of protein-protein and protein-DNA interactions to activate IL-8 gene transcription, in comparison with cytokine stimulation. Although both TNF and RSV can induce NF-κB nuclear translocation and DNA binding, the ability of NF-κB to transactivate the IL-8 promoter is different between the two stimuli. This difference in NF-κB activation could be due to the induction of distinct intracellular signaling pathways affecting posttranslational modifications of NF-κB and the recruitment of different coactivators to the IL-8 promoter. The activity of transcription factors is regulated at multiple levels: the level of synthesis, subcellular localization, and posttranslational modifications. RelA/p65 has been shown to be phosphorylated either constitutively or in an inducible manner by stimuli such as LPS, IL-1, and TNF (36–38). This event has been associated with an increase in p65 transcriptional activity, without modification of nuclear translocation or DNA-binding affinity, and with increased association with the coactivators p300/CPB (37).

Our findings that multiple binding sites contribute to the IL-8 promoter induction after RSV infection and that cooperation among these different sites is required for full activation of the promoter support the enhanceosome model for IL-8 gene transcription. An enhanceosome is a nuclear protein complex assembled at a given enhancer, in which various combinations of ubiquitous, signal- and tissue-specific activators allow different interactions with coactivators and with the basal transcriptional machinery, recruiting them to DNA to generate synergistic transcription (13, 39). In the case of alveolar epithelial cells, we have demonstrated that NF-κB is necessary and sufficient for activation of IL-8 transcription after TNF stimulation. In contrast, IL-8 induction by RSV infection requires the cooperation of various elements of the promoter. Among them we have identified a previously unrecognized ISRE-like regulatory element that plays an important role in RSV-activated IL-8 gene transcription.
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