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Nucleocapsid protein of SARS-CoV activates interleukin-6 expression through cellular transcription factor NF- κ B

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Abstract

High levels of interleukin-6 (IL-6) in the acute stage associated with lung lesions were found in SARS patients. To evaluate the mechanisms behind this event, we investigated the roles of SARS-CoV proteins in the regulation of IL-6. Results showed that the viral nucleocapsid (N) protein activated IL-6 expression in a concentration-dependent manner. Promoter analyses suggested that NF- κ B binding element was required for IL-6 expression regulated by N protein. Further studies demonstrated that N protein bound directly to NF- κ B element on the promoter. We also showed that N protein activated IL-6 expression through NF- κ B by facilitating the translocation of NF- κ B from cytosol to nucleus. Mutational analyses revealed that two regions of N protein were essential for its function in the activation of IL-6. These results provided new insights into understanding the mechanism involved in the function of SARS-CoV N protein and pathogenesis of the virus.

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Keywords: SARS-CoV; N protein; Gene regulation; Virus infection; Pathogenesis; IL-6; Inflammation; NF- κ B

Introduction

Severe acute respiratory syndrome (SARS) is a life-threatening form of atypical pneumonia that emerged in 2003, resulting in 8098 probable cases and 774 deaths around the world (<http://www.who.int/csr/sars>). Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) is the etiological agent for the outbreak of SARS (Peiris et al., 2003). Similar to other coronaviruses in structure, SARS-CoV is an enveloped virus containing a single-stranded, positive-sense RNA genome with 29,700 nucleotides in length that encodes for viral proteins including four structural proteins, spike (S) glycoprotein, matrix (M) protein, small envelope (E) protein, and nucleocapsid (N) protein (Marra et al., 2003).

For coronaviruses, the N protein plays important roles during host cell entry and virus particle assembly and release (Hogue, 1995; Narayanan et al., 2000, 2003). In particular, N protein binds to a defined packaging signal on viral RNA, leading to the

formation of the helical nucleocapsid (Narayanan et al., 2003). A similar function of N protein has been found in other viruses, in addition to coronaviruses (Myers and Moyer, 1997). However, the mechanism by which N protein facilitates virus replication is not established. The N protein of SARS-CoV is 422 amino acids long, sharing only 20–30% homology with the N proteins of other coronaviruses (Marra et al., 2003; Rota et al., 2003). However, it contains a short lysine-rich sequence (362-KTFPPTEPKKDKKKKTDEAQ-381) near the carboxyl terminus, a putative bipartite nuclear localization signal that has not been found in the N proteins of any other known coronavirus (Marra et al., 2003), suggesting the SARS-CoV nucleocapsid protein might have novel functions in pathogenesis. The SARS-CoV N protein has been reported to activate the activator protein 1 (AP1) signal transduction pathway (He et al., 2003) and to induce actin reorganization in COS-1 cells (Sutjit et al., 2004) by forming a dimer by self-association (He et al., 2004).

Interleukin-6 (IL-6) is a pleiotropic cytokine that plays major roles in immunological responses, inflammation, bone metabolism, neoplasia, and aging (Akira et al., 1993; Papanicolaou et al., 1998; Erschler and Keller, 2000). IL-6

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expression is known to be affected by a variety of cytokines and growth factors. It has been reported that activation of IL-6 expression is controlled by the activity of several transcription factors with known consensus sequences in the IL-6 promoter region, including IRF-1 (–267 to –254), AP-1 (–283 to –277), C/EBP (–158 to –145 and –87 to –76), Sp1 (–123 to –119 and –108 to –104), and NF- κ B (–75 to –63) binding sites (Akira and Kishimoto, 1992; Yan et al., 1995). NF- κ B belongs to the Rel family of transcription factors that form homo- or heterodimers through the Rel homology domain (Akira and Kishimoto, 1997; Sen and Baltimore, 1986). In most cells, NF- κ B is present in the cytoplasm bound to a member of the I κ B family of inhibitors (Verma et al., 1995; Carlotti et al., 2000). Upon activation of the cells by cytokines, such as TNF- α and IL-1, viruses, bacteria, or mitogens, I κ B is phosphorylated and then rapidly degraded allowing the translocation of NF- κ B to the nucleus where it binds to specific DNA binding sites.

High serum levels of IL-6 and IL-8 in the acute stage (cytokine storm) associated with lung lesions were found in SARS patients (Hsueh et al., 2004). However, the molecular mechanisms behind this event remain to be determined. In this study, we investigated the roles of SARS-CoV structural proteins in the regulation of IL-6 expression in human lung epithelial cells (A549). In addition, transcription factor involved in the regulation of IL-6 by the viral N protein was also studied. This study partially explains the clinical observation of dramatic cytokine storm and inflammation responses in SARS patients and provides new insights into our understanding the molecular mechanisms involved in the functions of SARS-CoV N protein and pathogenesis caused by the SARS-CoV.

Results

Nucleocapsid protein of SARS-CoV activates the expression of IL-6

It has been shown that high serum levels of IL-6 in the acute stage associated with lung lesions were found in SARS patients (Hsueh et al., 2004). We were, therefore, interested in evaluating the molecular mechanism involved in the expression of IL-6 activated by SARS-CoV. A reporter plasmid (pIL6-luc-651) carrying the luciferase gene under the control of IL-6 promoter was co-transfected with control plasmid (pCMV-tag2B) or plasmid (pCMV-tag2B-N, pCMV-tag2B-S, pCMV-tag2B-M, or pCMV-tag2B-E) containing each of the four viral structural genes of SARS-CoV, respectively, into human airway epithelial cells (A549).

Results from luciferase activity assays of transfected cells revealed that the activity of IL-6 promoter was significantly stimulated in the presence of N protein, slightly enhanced by the S protein, and not changed when M or E protein present, comparing to that of control (Fig. 1A). We also evaluated the status of N, S, M, and E proteins expressed in transfected A549 cells by western blot analysis using anti-flag antibody. Results showed that all four proteins (N, S, M, and E) were expressed in

transfected cells, respectively (Fig. 1B). Further study showed that the level of luciferase activity increased as the concentration of plasmid pCMV-tag2B-N carrying the N gene increased, indicating that the activation of IL-6 promoter regulated by N protein was concentration-dependent (Fig. 1C). However, the level of luciferase activity remained relatively unchanged when M, E, or S protein was presented in transfected cells (data not shown).

To further determine the effect of N protein on the levels of IL-6 protein and mRNA expressed, two additional experiments, enzyme-linked immunosorbent assay (ELISA) and semi-quantitative RT-PCR, were performed. A549 cells were transfected with plasmid pCMV-Tag2B-N or control plasmid pCMV-Tag2B. ELISA results from analyses of transfected cells showed that the level of IL-6 protein was increased in the presence of the viral N protein, comparing to that of controls (Fig. 1D). Results from semi-quantitative RT-PCR also indicated that the N protein could significantly activate the expression of IL-6 mRNA (Fig. 1E). Thus, all results from luciferase activity assays, ELISA, and semi-quantitative RT-PCR were consistent and demonstrated that the N protein of SARS-CoV activated IL-6 expression in human airway epithelial cells.

NF- κ B binding element is required for N protein to activate IL-6 expression

Activation of the IL-6 gene relies on many consensus *cis*-elements including NF- κ B, AP-1, and C/EBP binding sites on the IL-6 promoter (Keller et al., 1996) (Fig. 2A). Since our results showed that the viral N protein activates the expression of IL-6, we further investigated the roles of these *cis*-regulatory elements in the activation of IL-6 expression regulated by N protein. Site-specific mutations or truncated mutations of the IL-6 promoter were generated and used to construct reporter plasmids in which the luciferase gene was under the control of each individual mutant IL-6 promoter.

To determine the activities of these mutant promoters, A549 cells were co-transfected with plasmid pCMV-tag2B-N and reporter plasmid containing the luciferase gene driven by each of the mutant IL-6 promoters, respectively. Results from analysis of site-specific mutants of IL-6 gene promoter showed that the level of luciferase activity was increased by 5.3-fold, 4-fold, 5-fold, 5.1-fold, 1.3-fold, and 4.9-fold under the control of full length IL-6 promoter (pIL6-luc-651), or promoters with mutations in *cis*-regulatory elements AP1a (pIL6-luc-651 Δ AP1a), C/EBPa (pIL6-luc-651 Δ C/EBPa), C/EBPb (pIL6-luc-651 Δ C/EBPb), NF- κ B (pIL6-luc-651 Δ NF- κ B), or AP1b (pIL6-luc-651 Δ AP1b), respectively (Fig. 2A). Results from analysis of truncated mutants of IL-6 gene promoter revealed that the level of luciferase activity was increased by 5.3-fold, 5-fold, 5.2-fold, 7.9-fold, and 1.2-fold under the control of full length IL-6 promoter (pIL6-luc-651), or four truncated promoters (pIL6-luc-225, pIL6-luc-100, pIL6-luc-76, pIL6-luc-64), respectively (Fig. 2B). These results suggested that NF- κ B recognition site was required for the activation of IL-6 promoter regulated by N protein.

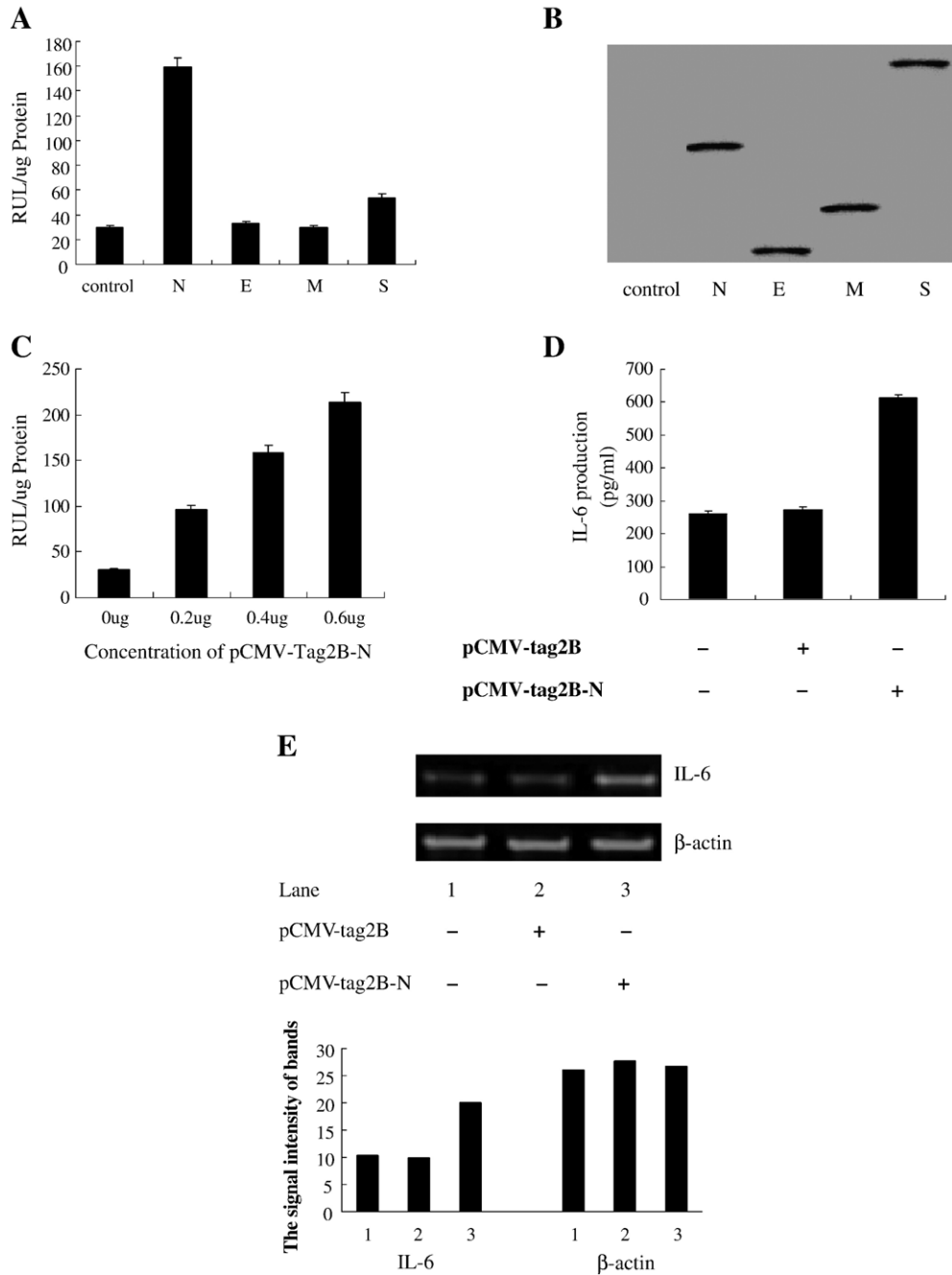


Fig. 1. Determination of roles of SARS-CoV N protein in the activation of IL-6 gene. (A) A549 cells were co-transfected with plasmid pCMV-tag2B-N, pCMV-tag2B-M, pCMV-tag2B-S, or pCMV-tag2B-E expressing N, M, S, or E protein, respectively, along with the reporter plasmid pIL6-luc-651 in which the luciferase gene is under the control of IL-6 promoter. The negative control is parental plasmid pCMV-tag2B. Relative luciferase activity was determined by standard procedures. (B) The expression of N, S, M, or E protein in transfected A549 cells was evaluated by western blot analysis using anti-flag antibody. (C) A549 cells were co-transfected with different amount of plasmid pCMV-Tag2B-N as indicated along with the reporter plasmid pIL6-luc-651. Relative luciferase activities were determined from transfected cells by standard procedures. (D) A549 cells were transfected with plasmid pCMV-Tag2B-N or pCMV-Tag2B. Supernatants of transfected cells were prepared and concentration of IL-6 protein was determined by ELISA using anti-IL-6 antibody. (E) A549 cells (lane 1) were transfected with pCMV-Tag2B (lane 2) or pCMV-Tag2B-N (lane 3). The levels of IL-6 mRNA expressed in transfected cells were determined by RT-PCR using primers specific to IL-6 and the levels of β-actin mRNA produced were measured using primers specific to β-actin. All product bands were visualized and quantified using Gel-Pro Analyzer. The results were expressed as the mean±S.D. of three independent experiments performed in triplicate.

N protein and NF-κB have a synergetic effect on the activation of IL-6

Previous studies have reported that N protein activates NF-κB and such activation is dose-dependent (Liao et al., 2005). To

determine the roles of N protein and NF-κB in the activation of IL-6, we measured the effect of NF-κB inhibitor (MG-132) on the effect of N protein on the activation of IL-6 promoter. Cells were co-transfected with plasmid (pCMV-tag2B-N) and reporter (pIL6-luc-651) and then treated with MG-132 at different

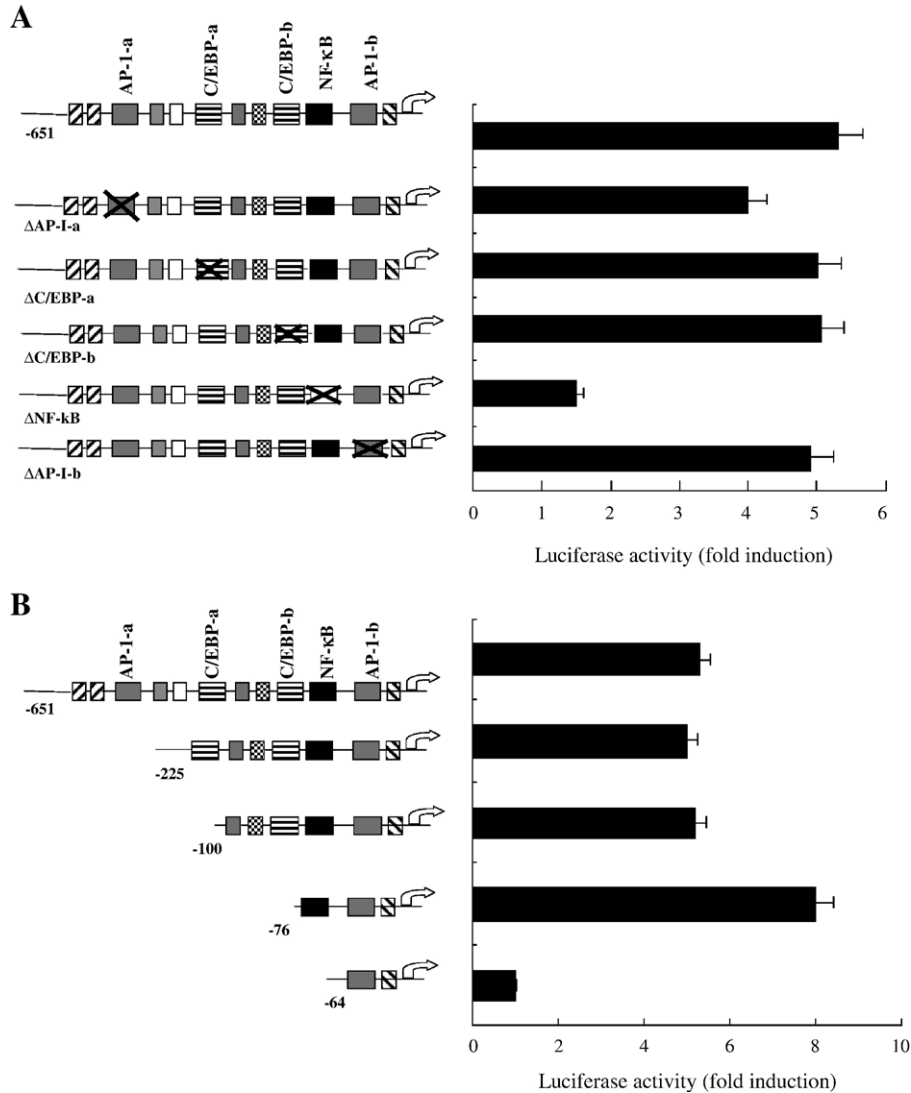


Fig. 2. Functional analysis of *cis*-regulatory elements involved in the activation of IL-6 promoter regulated by N protein. (A) Diagram of site-specific mutants of IL-6 promoter (left panel) and effects of N protein on the activities of these promoters (right panel). (B) Diagram of truncated mutants of IL-6 promoter (left panel) and effects of N protein on the activities of these promoters (right panel). A549 cells were co-transfected with plasmid pCMV-Tag2B-N and reporter plasmid carrying the luciferase reporter gene driven by each of the IL-6 mutant promoters. Promoter activities were determined by measuring the relative luciferase activity in transfected cells. pCMV-Tag2B was used as a negative control (1-fold). Data are expressed as mean+S.D. of three independent experiments.

concentration as indicated. Results from luciferase activity assays revealed that the activity of IL-6 promoter induced by N protein was gradually inhibited as the concentration of MG-132 increased (Fig. 3A) indicating that NF-κB protein was required for the activation of IL-6 promoter regulated by the viral N protein.

The effect of p65, a subunit of NF-κB, on the function of N protein in terms of activation of IL-6 promoter was also determined. A549 cells were co-transfected with plasmid expressing p65 or N protein and the reporter plasmid (pIL6-luc-651) or the mutant plasmid (pIL6-luc-651ΔNF-κB). Result from luciferase activity assays showed that the expression of IL-6 promoter was stimulated in the presence of p65 or N protein (Fig. 3B, lanes 2 and 4) comparing to that of control (Fig. 3B, lane 1). The activity of IL-6 promoter reached a highest level

when both p65 and N proteins present (Fig. 3B, lane 3) indicating that p65 and N proteins had a synergetic effect on the activation of IL-6. However, the NF-κB binding site mutation of IL-6 promoter (pIL6-luc-651ΔNF-κB) abolished the effect of p65 and/or N protein on the activation of IL-6 promoter (Fig. 3B, lanes 5, 6, 7, and 8).

In order to determine the effects of p65 and/or N protein on the expression of IL-6 protein, A549 cells were co-transfected with plasmid expressing p65 or N protein. Results from ELISA of transfected cells demonstrated that N protein (Fig. 3C, lane 3) or p65 protein (Fig. 3C, lane 5) could stimulate the expression of IL-6 protein. ELISA result also showed that N protein could enhance the expression of IL-6 protein activated by the p65 (Fig. 3C, lane 4). This result also suggested that NF-κB and N protein had a synergetic effect on the activation of IL-6 expression.

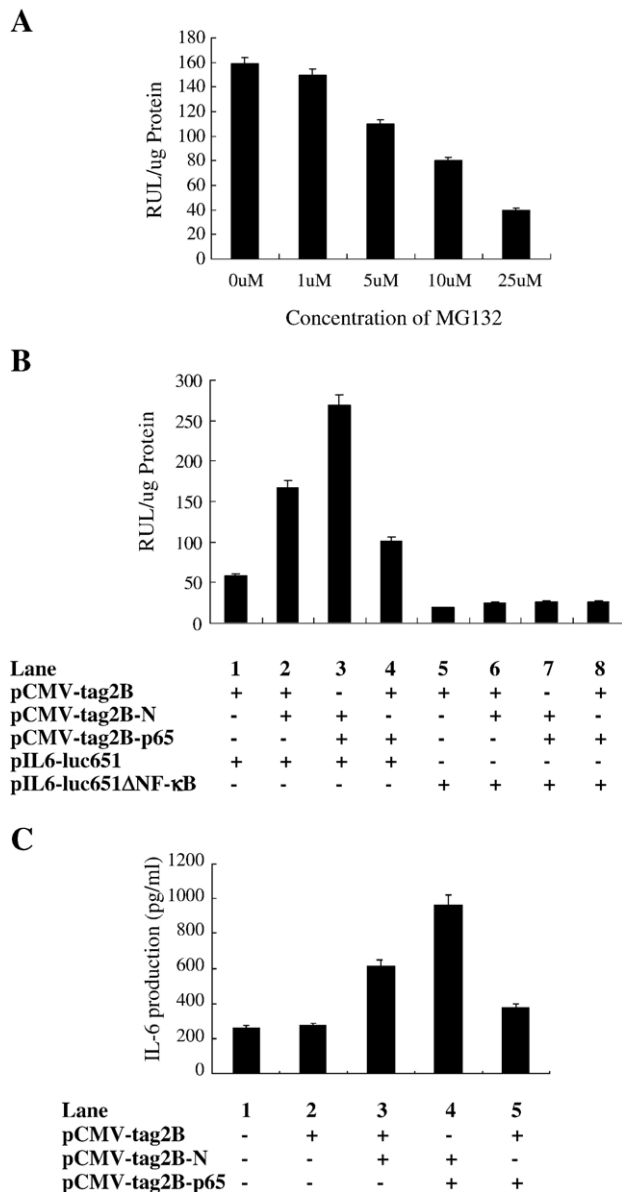


Fig. 3. Determination of the role of NF- κ B in the expression of IL-6 regulated by N protein. (A) A549 cells were co-transfected with pCMV-Tag2B-N and pIL6-luc-651 followed by the treatment with MG-132 at different concentrations as indicated for 24h. Treated cells were then lysed and luciferase activity was measured. (B) A549 cells were co-transfected with pIL6-luc-651 (lanes 1–4) or pIL6-luc-651 Δ NF- κ B (lanes 5, 6, 7, and 8), pCMV-Tag2B-N (lanes 2, 3, 6, and 7) or pCMV-Tag2B (lanes 1, 2, 4–6, and 8), and pCMV-Tag2B-p65 (lanes 3, 4, 7, and 8), respectively. Transfected cells were then lysed and luciferase activity was measured. (C) A549 cells were transfected with pCMV-Tag2B (lanes 2 and 3), or pCMV-Tag2B-N (lanes 3 and 4), or pCMV-Tag2B-p65 (lanes 4 and 5). Transfected cells were lysed and the levels of IL-6 protein produced were then measured by ELISA using anti-IL-6 antibody. The results were expressed as mean \pm S.D. of three independent experiments.

N protein facilitates the translocation of p65 from cytosol to nucleus

Localization to the nucleus is a common feature of coronavirus nucleoproteins (Wurm et al., 2001). Analysis of amino acid sequences revealed that the SARS-CoV N protein contains a putative nuclear export signal (NES) located at po-

sition 220–231 and three putative classical nuclear localization signals (NLS1, NLS2, and NLS3) located at positions 34–44, 257–265, and 369–390, respectively (Timani et al., 2004).

We initially examined the effect of N protein on the translocation of p65 from cytosol to nucleus by transfection of A549 cells with plasmid (pCMV-Tag2B-N) expressing the N protein or with plasmid (pCMV-Tag2B-M) expressing the M protein as a control. At 0, 24, and 48 h post-transfection, cytosol and nucleus fractions were prepared from transfected cells. Levels of p65 presented were determined by western blot using anti-p65 antibody. Results clearly revealed that the level of p65 protein was decreased in cytosol and increased in nucleus as the time of transfection increased in cells transfected pCMV-Tag2B-N (Fig. 4A). However, the level of p65 protein was relatively unchanged in cytosol and in nucleus as the time of transfection increased in cells pCMV-Tag2B-M (Fig. 4B). These results suggested that N protein facilitates the translocation of p65 from cytosol to nucleus.

It has been shown that the full-length sequence of NES of N protein is required for the export of the viral protein out from the nucleus (Timani et al., 2005). To determine the effect of NES of N protein on the activation of IL-6, an N protein mutant (N Δ 220-231) was generated in which the NES sequence was internally deleted. A549 cells were co-transfected with plasmid (pCMV-Tag2B-N Δ 220-231) containing the mutant N gene and the reporter plasmid (pIL6-luc-651). Results from luciferase analysis of transfected cells revealed that the level of IL-6 promoter activity was 11-fold and 5.1-fold in the presence of the mutant N protein (N Δ 220-231) and the wild-type protein, respectively (Fig. 4C). This result suggested that deletion of nuclear export signal of N protein resulted in the additional enhancement of IL-6 activation by at least 2-fold.

N protein binds directly to the NF- κ B recognition elements on the IL-6 promoter

Since our results showed that NF- κ B regulatory element is required for the activation of IL-6 regulated by N protein, it is reasonable for us to assume that SARS-CoV N protein may function through the binding of N protein to NF- κ B regulatory elements on the IL-6 promoter.

To confirm this speculation, we performed electrophoresis mobility shift assay (EMSA) to determine protein–DNA binding between N protein and IL-6 promoter. A549 cells were transfected with control plasmid pCMV-Tag2B (Fig. 5A, lanes 1, 2, and 3) or plasmid pCMV-Tag2B-N (Fig. 5A, lanes 4, 5, 7, and 8). Nuclear extracts were prepared from transfected cells and EMSA was performed with 4 μ g of nuclear extract in binding buffer. To assure the specific binding of transcription factors to the probe, unlabeled double-stranded oligonucleotide competitors were added prior to the addition of labeled probe (Fig. 5A, lanes 2 and 5). To determine whether N protein was specific bound to the promoter, rabbit anti-N polyclonal antibody (Fig. 5A, lanes 1 and 4) or normal rabbit polyclonal antibody (rabbit IgG) (Fig. 5A, lane 8) was incubated with nuclear extracts before adding the binding buffer. DNA probes used in this study were specific for the NF- κ B recognition

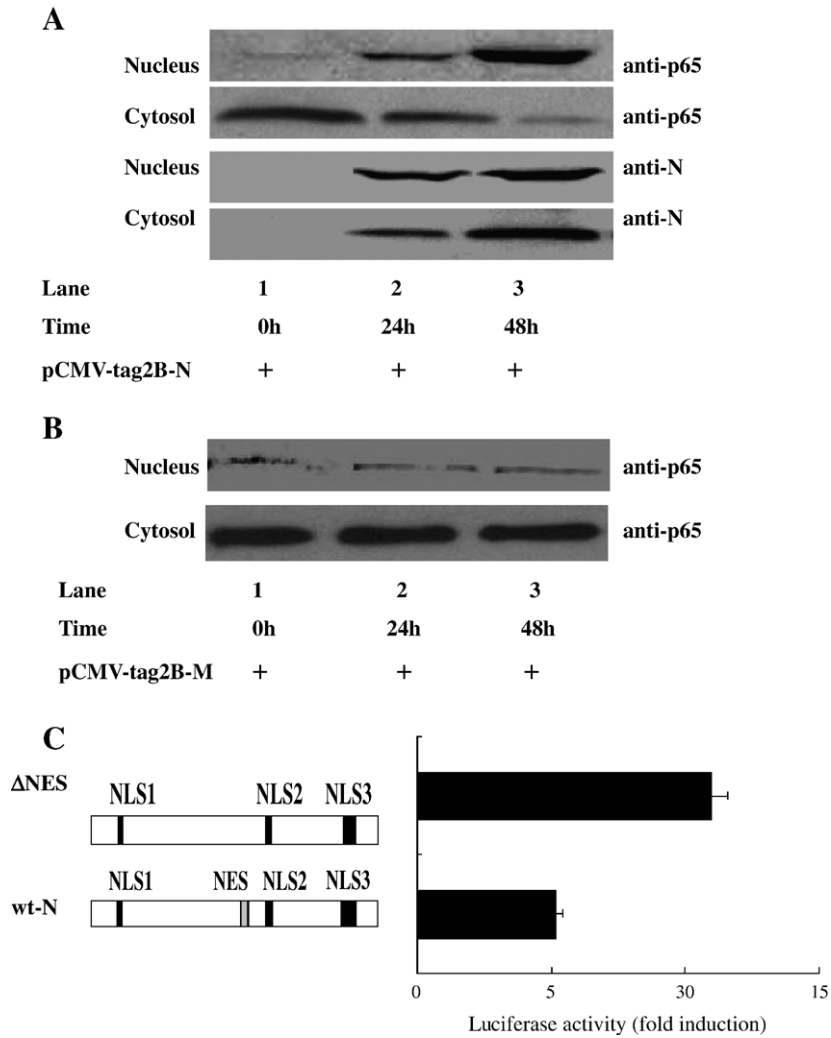


Fig. 4. Determination of the role of N protein in the translocation of NF-κB and activation of IL-6. (A) A549 cells were transfected with plasmid pCMV-Tag2B-N. Cytosol and nucleus fractions of transfected cells were prepared at different time as indicated. Levels of cytosolic and nuclear p65 protein and N protein were determined by western blot using anti-p65 antibody and anti-N antibody, respectively. (B) A549 cells were transfected with plasmid pCMV-Tag2B-M. Cytosol and nucleus fractions of transfected cells were prepared at different time as indicated. Levels of cytosolic and nuclear p65 protein were determined by western blot using anti-p65 antibody. (C) A549 cells were co-transfected with reporter plasmid pIL6-luc-651 along with plasmid pCMV-Tag2B-N or plasmid pCMV-Tag2B-MutNΔ220-231 expressing a mutant N protein (ΔNES) in which a putative nuclear export signal NES located at position 220–231 was deleted. IL-6 promoter activities were determined by measuring the relative luciferase activity in transfected cells. Data are expressed as mean + S.D. of three independent experiments.

element of IL-6 promoter. Samples were then electrophoresed on nondenaturing polyacrylamide gels and subjected to autoradiography. Results from EMSA using NF-κB probes showed that a specific protein–DNA complex was supershifted in cells transfected in the presence of N protein and anti-N polyclonal antibody (Fig. 5A, lane 4).

To further confirm the specific binding of N protein to the NF-κB recognition element on the IL-6 promoter, chromatin immunoprecipitation assays (ChIP) were performed. Chromatin fragments were prepared from A549 cells transfected with plasmid pCMV-Tag2B-N and immunoprecipitated with specific rabbit anti-N polyclonal antibody. Two regions (region a: –225 to +13 and region b: –76 to +13) of IL-6 promoter containing the NF-κB binding sequences were amplified by PCR using specific primers (Table 1), respectively. Results showed that PCR products were only produced from DNA isolated from

cells transfected with pCMV-Tag2B-N in the presence of anti-N antibody (Fig. 5B, lane 4) or directly from input DNA (Fig. 5B, lane 1), but not detected in the presence of control plasmid (Fig. 5B, lane 2) and normal rabbit polyclonal antibody (rabbit IgG) (Fig. 5B, lane 4) or without the treatment of anti-N antibody (Fig. 5B, lane 3). These results demonstrated that the N protein could specifically bind to the NF-κB recognition site on the IL-6 promoter.

Two regions of N protein are essential for the viral protein to activate IL-6 gene

For coronaviruses, the nucleocapsid protein plays an important role during viral packaging, viral core formation, and viral RNA synthesis (Narayanan et al., 2003). In this study, we showed that SARS-CoV N protein plays a role in the activation

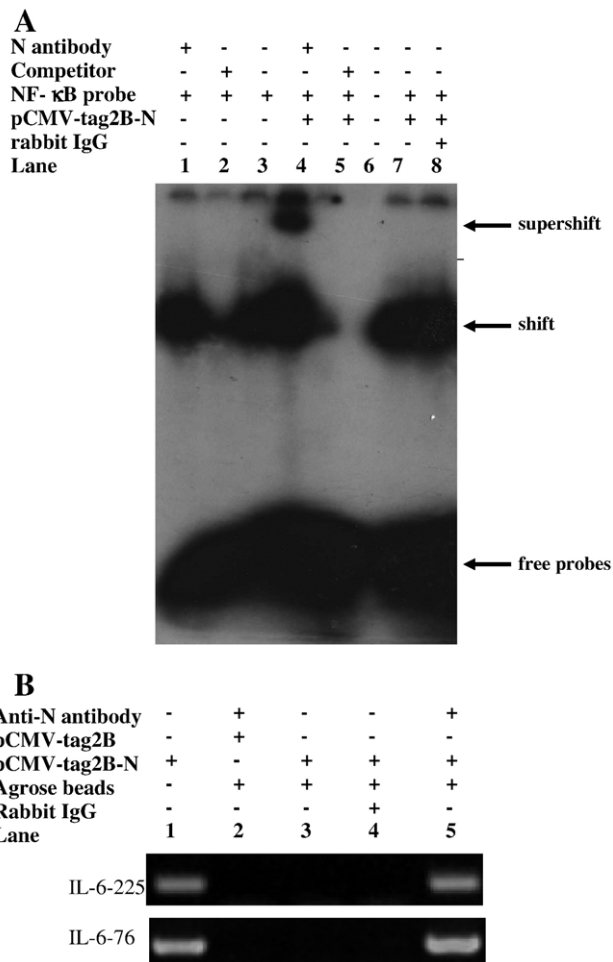


Fig. 5. Determination of interaction between SARS-CoV N protein and IL-6 promoter. (A) Electrophoretic mobility shift assay (EMSA) was performed with nuclear extracts of A549 cells transfected with (lanes 4, 5, 7, and 8) or without (lanes 1–3, and 6) the N gene. NF- κ B probes from nucleotides –76 to –63 were labeled and added to the reactions (lanes 1, 2–5, 7, and 8). Unlabeled double-stranded oligonucleotide competitors of NF- κ B probe were added during preincubation prior to the addition of probes (lanes 2 and 5). For supershift experiments, rabbit anti-N polyclonal antibody (lanes 1 and 4) or normal rabbit polyclonal antibody (rabbit IgG) (lane 8) were incubated with nuclear extracts before adding to the reactions. Samples were electrophoresed on 5% nondenaturing polyacrylamide gel and visualized by autoradiography. Arrows indicate the super-shifted protein–DNA complex, shift band, and free probe. Lane 6: black space. (B) Chromatin immunoprecipitation assay (ChIP) was performed with A549 cells transfected with control pCMV-Tag2B (lane 2) or pCMV-Tag2B-N (lanes 1, 3, 4, and 5). Immunoprecipitated complexes were collected, subjected to PCR amplification, and separated by agarose gel electrophoresis. The IL-6 promoter regions (–225 to +13) and (–76 to +13) were amplified by PCR using specific primers (IL-6-225U and IL-6-651D or IL-6-76U and IL-6-651D), respectively. Lane 1: input DNA as positive control. Lane 4: normal rabbit polyclonal antibody (rabbit IgG) as a negative control.

of IL-6. To further determine the roles of different regions of N protein in the activation of IL-6 expression, we constructed several deletion mutants of the N protein and analyzed the functions of these mutants in the activation of IL-6.

To evaluate the function of each mutant, A549 cells were co-transfected with plasmid carrying the N gene mutant and the reporter plasmid. Ability of truncated N proteins to activate IL-6 expression was measured by luciferase activity assays in trans-

fecting cells (Fig. 6A). Results revealed that deletion of 61 amino acids (N61–422) or 86 amino acids (N86–422) from the N terminus of N protein resulted in a slight increase of IL-6 promoter activity. However, further deletion of 96 amino acids (N96–422) from the N terminus of the viral protein abolished its ability to activate IL-6 expression. These results suggested that the region from amino acids 86 to 96 of N protein was essential for the viral protein to activate the expression of IL-6 gene.

In addition, results showed that deletion of amino acids from 341 to 422 of N protein resulted in the loss of function in the activation of IL-6 and that both N- and C-terminus deletions (N86–341) of N protein abolished the ability of the viral protein to activate IL-6 expression. These results demonstrated that the C-terminus from amino acids 341 to 422 of N protein was also essential for the viral protein to activate the expression of IL-6 gene.

The ability of each plasmid carrying truncated N gene to produce corresponding mutant N protein was confirmed by western blot analysis using anti-N protein antibody (Fig. 6B). Results showed that all truncated N proteins were produced with their sizes as expected.

Discussion

SARS is an emerged infectious disease characterized by persistent fever, respiratory symptoms with lung consolidation, lymphopenia, and respiratory failure in life-threatening cases. IL-6 is a pleiotropic cytokine that plays a major role in the host's response to injury or infection (Snick, 1990). IL-6 expression is tightly regulated and can be induced in macrophages, synovial fibroblasts, endothelial cells, and other cell types. IL-6 plays a crucial role in autoimmune diseases because abnormally high quantities of IL-6 are found in several autoimmune diseases (Hirano, 1992). The elevations of IL-6 and cytokines have been found in the plasma of SARS patients (Hsueh et al., 2004), and can induce the hyperinnate inflammatory response due to the SARS-CoV invasion of the respiratory tract.

It is important to investigate the roles of SARS-CoV in the induction of cytokine, such as IL-6 in the lung cells, which may be the cause or consequence of pulmonary inflammation and immune hyperreactivity in SARS. In this study, we showed that among all SARS-CoV structural proteins (N, S, E, and M) tested (Fig. 1B), only the N protein significantly induced the activation of IL-6 promoter in human airway epithelial cell cultures (Fig. 1A) and such activation was in a dosage-

Table 1

Primers used for generating mutations of IL-6 promoter in this study

AP-1-b-U	5'-AATATTGAGCTGCATGGGAA-3'
AP-1-b-D	5'-TTCCCATGCAGCTCAATATT-3'
C/EBP-b-U	5'-CATTTGTAGTTTCTTTGTTG-3'
C/EBP-b-D	5'-CAACAAAGAAACTACAAATG-3'
IL-6-225-U	5'-CGTGGTACCTAGCCTCAATGA-3'
IL-6-100-U	5'-ATTGGTACCCCTCACCTCC-3'
IL-6-76-U	5'-CCGGGTACCGTGGGATTTC-3'
IL-6-64-U	5'-CAAGGTACCCCATGAGTCT-3'
IL-6-651-D	5'-AGTACCGGAATGCCAAGCTT-3'

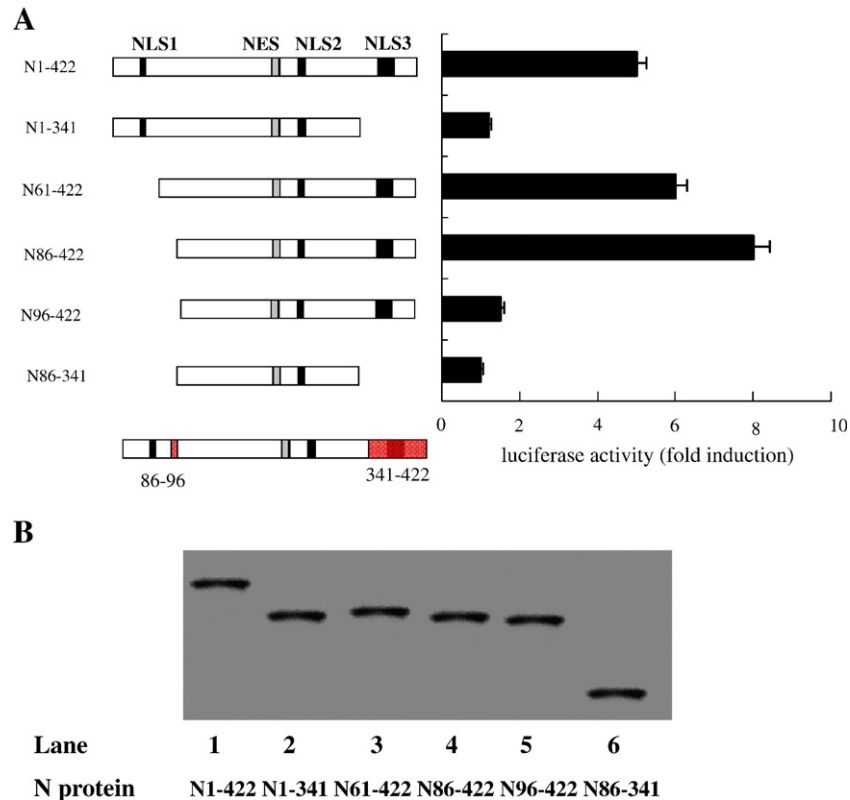


Fig. 6. Functional analysis of deletion mutants of SARS-CoV N protein. (A) Diagram of N protein mutants (left panel) and functional analyses of mutant proteins (right panel). A549 cells were co-transfected with the reporter plasmid pIL6-luc-651 along with plasmid pCMV-Tag2B-N or plasmid containing individual mutant N gene. The effects of each N protein on the activation of IL-6 promoter were determined by measuring luciferase activities of transfected cells. Values correspond to an average of at least three independent experiments. (B) The status of N protein and its mutants expressed in the transfected A549 cells were determined by western blot using anti-N protein antibodies.

dependent manner (Fig. 1C). We also demonstrated that N protein activates the IL-6 promoter (Fig. 1D) and induces IL-6 protein production (Fig. 1E) in A549 cells. These results were consistent with previous clinical findings (Hsueh et al., 2004) and further demonstrated that N protein is, at least partially, responsible for the induction of IL-6 gene expression.

Studies of other coronavirus have reported that the nucleocapsid proteins have a variety of functional activities (He et al., 2004). It has been suggested recently that N protein is a two-domain protein, with the N-terminal from amino acids 50 to 150 as the RNA-binding domain. Our recent results indicated that N protein activates COX-2 gene expression by binding either directly or indirectly to NF- κ B and C/EBP regulatory elements on COX-2 promoter (Yan et al., 2006). Previous studies of the IL-6 promoter indicated optimal IL-6 activation correlated with the NF- κ B sites both *in vivo* and *in vitro*. IL-6 gene activation involves the rapid transport of preexisting p65 to the nucleus after release from a complex with I κ B in the cytosol (Lieberman and Baltimore, 1990).

It is reasonable for us to speculate that N protein activates IL-6 gene expression by binding directly or indirectly to NF- κ B regulatory element on IL-6 promoter. In this study, we demonstrated that NF- κ B regulatory element was essential for N protein function in the induction of IL-6 expression since mutation of such binding site eliminated the activation of IL-6

induced by N protein (Fig. 2). Such mutation of IL-6 promoter also abolished the effect of p65 (a subunit of NF- κ B) and/or N protein on the activation of IL-6 promoter (Figs. 3B and C). In addition, results from electrophoresis mobility shift assay (EMSA) (Fig. 5A) and chromatin immunoprecipitation assays (ChIP) (Fig. 5B) demonstrated that SARS-CoV N protein could bind directly to NF- κ B regulatory elements on IL-6 gene promoter.

Previous studies have reported that N protein can activate transcriptional factor NF- κ B and such activation is dose-dependent (Liao et al., 2005). In this study, we further demonstrated that the viral N protein is required for the activation of NF- κ B in the regulation of IL-6 expression. Our results revealed that the activity of IL-6 promoter induced by N protein was eliminated by the presence of NF- κ B inhibitor, MG-132 (Fig. 3A). In addition, we also showed that the viral N protein and the host transcription factor p65 protein had a synergistic effect on the activation of IL-6 (Figs. 3B and C), such effect was abolished when NF- κ B recognition site was mutated (Fig. 3B). Finally, we found that N protein facilitates the translocation of p65 from cytosol to nucleus (Fig. 4A). However, the detailed mechanisms involved in the activation of IL-6 expression regulated by the viral N protein and the transcriptional factor NF- κ B need to be further investigated.

Localization to the nucleolus is a common feature of coronavirus N proteins. This feature helps disrupt host cell

division to promote virus assembly and sequester ribosomes for translation of viral proteins (Wurm et al., 2001). SARS-CoV N protein localized to the cytoplasm and nucleus of mammalian cells (Timani et al., 2005). Our results showed that the C-terminus (from amino acids 341 to 422) of N protein was essential for the viral protein to activate the expression of IL-6 gene, since deletion of this region resulted in the loss of function in the activation of IL-6 (Fig. 6). Because this region contains a putative nuclear localization signal (NLS3), the failure of this mutation to activate IL-6 expression might be due to the loss of its ability to target to the nucleus.

Analysis of truncated N proteins also revealed that the region from amino acids 86 to 96 of N protein was essential for the viral protein to activate the expression of IL-6 gene (Fig. 6A). This result was in agreement with the finding that through multi-alignment of total 19 sequences of the coronavirus N proteins including that of SARS-CoV, one conserved structural region at amino acids 81 to 140 was found to perform critical functions (Wang et al., 2003).

The biological functions of coronavirus N protein are thought to participate in the replication and transcription of viral RNA and to interfere with the cell cycle of host cells (Parker and Masters, 1990; Kuo and Masters, 2002). The N proteins of many coronaviruses are highly immunogenic and abundantly expressed during infection (Liu et al., 2001; Narayanan et al., 2003). However, its intracellular effects on host gene regulation remain largely unknown. The cytoplasmic localization of SARS-CoV N protein is reported recently (Chang et al., 2004), suggesting that N protein may interact with unknown cytoplasmic and nuclear proteins to regulate host gene expression.

Our results demonstrated that SARS-CoV N protein interacted with the host transcriptional factor NF- κ B to regulate IL-6 expression, which may partially explain the clinical observation of dramatic cytokine storm and inflammation responses in SARS patients. These results provide new insights into our understanding of the mechanism involved in the functions of SARS-CoV N protein and pathogenesis of SARS-CoV. In addition, these data would also provide new insights into the development of novel vaccine and therapeutics for the prevention and treatment of SARS.

Materials and methods

Cell culture

Human airway epithelial cell line (A549) was cultured in Ham's F12K medium (Sigma), supplemented with 10% fetal bovine serum, 100 μ g/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in a 5% CO₂ incubator.

Plasmid construct

A plasmid (pIL6-luc-651) containing a 651-bp fragment of the human IL-6 gene promoter located directly upstream of the transcriptional start site and its site-specific mutants pIL6-luc-651 Δ C/EBPa, pIL6-luc-651 Δ NF- κ B, and pIL6-luc-651 Δ AP-

1a were kindly provided by Oliver Eickelberg (University of Giessen) (Eickelberg et al., 1999). The AP-1 site b and C/EBP site b were mutated by using QuickChange site-directed mutagenesis kit (Stratagene). The AP-1b site consensus sequence (positions –61 to –55, 5'-TGAGTCT-3') was changed to 5'-TGCAGCT-3' (Baccam et al., 2003). The C/EBPb site consensus sequence (positions –86 to –76, 5'-CTTATCAAAT-3') was changed to 5'-ACTACAAACT-3' (Galien et al., 1996). The truncated mutants of IL-6 promoter mIL6-luc-225, mIL6-luc-100, mIL6-luc-76, and mIL6-luc-64 were created by using specific primers (Table 1). Site-specific mutants and truncated mutants of IL6 promoter were also sub-cloned into the luciferase expression vector to yield plasmids, pIL6-luc-651 Δ AP-1b and pIL6-luc-651 Δ C/EBPb, pIL6-luc-225, pIL6-luc-100, pIL6-luc-76, and pIL6-luc-64, respectively.

Four viral structural proteins (N, M, S, and E) of SARS-CoV strain WHU (GenBank Accession No. AY394850) were amplified by RT-PCR from RNA isolated previously from SARS-CoV infected Vero E6 cells (Zhu et al., 2005) using corresponding primers listed in Table 2. The PCR products were cloned into *Eco*RI and *Bam*HI sites of pCMV-tag2B (Stratagene) to generate plasmid pCMV-tag2B-N, pCMV-tag2B-M, pCMV-tag2B-S, and pCMV-tag2B-E, respectively. The internal deletion of the N gene (MutN Δ 220-231) was performed by using QuickChange site-directed mutagenesis kit (Stratagene), and its truncation mutants N1-422, N1-341, N61-422, N86-422, N96-422, and N86-341 were amplified by PCR using corresponding primers listed in Table 3. All mutant N genes were cloned into *Eco*RI and *Bam*HI sites of pCMV-tag2B (Stratagene) to generate plasmids pCMV-tag2B-N Δ 220-231, pCMV-tag2B-N1-422, pCMV-tag2B-N1-341, pCMV-tag2B-N61-422S, pCMV-tag2B-N86-422, pCMV-tag2B-N96-42, and pCMV-tag2B-N86-341, respectively. The eukaryotic expression plasmid pCMV-tag2B-p65 carrying the coding regions of p65 under control of the CMV promoter was a gift of Hongbin Shu (Wuhan University, China). All constructs were verified by DNA sequencing.

Generation of polyclonal antisera

Subcloning of N gene into prokaryotic expression vector, expression and purification of N protein were described previously (Timani et al., 2004). New Zealand rabbits were kept in conventional conditions and were handled in compliance with Wuhan University (Wuhan, China) guidelines for animal care and use. Rabbit was immunized with 0.1–0.2 mg of

Table 2
Primers used for amplification of genes of SARS-CoV in this study

Nu	5'-AGCTGGATCCATGTCTGATAATGGACCCCAATCAAAC-3'
Nd	5'-AGCTGAATTCATCATGAGTGTTTATGCCTGAGT-3'
Mu	5'-AGCTGGATCCGCTTATCATGGCAGACAACGGTACT-3'
Md	5'-AGCTGAATTCATCTGTTGTCACCTACTGTACTAGC-3'
Eu	5'-AGCTGGATCCATGTACTATTCTGTTTCGGAAGAAAC-3'
Ed	5'-AGCTGAATTCCTAGTTCGTTTAGACCAGAAGATC-3'
Su	5'-AGCTGGATCCACATGTTTATTTTCTTATTATTCTTAC-3'
Sd	5'-AGCTGAATTCAGTGGCTGTGCAGTAATTGATCC-3'

Table 3
Primers used for generating mutations of the N gene in this study

MutNΔ220-231-U	5'-GGCTAGCGGAGGTGGTGAACCTGCCGAGAGCAAAGTTTCTGGTAAAGGCC-3'
MutNΔ220-231-D	5'-GGCCTTACCAGAACTTTGCTCTCGGCAGTTTACCACCTCCGCTAGCC-3'
N1-341-D	5'-CTTAAGGGCATCCAATTTAATGGCT-3'
N61-422-U	5'-CCGGATCCAAGGAGGAACCTTAGATTC-3'
N86-422-U	5'-AAGGATCCTACTACCGAAGACTAC-3'
N96-422-U	5'-AAGGATCCGCAAATGAAAGAGCTC-3'

purified recombinant N protein and was injected subcutaneously at multiple sites on its back. Booster injections were given 2 weeks and 4 weeks later. Blood was drawn from the rabbit at 5 weeks following the immunization, the blood was allowed to clot at 4 °C, and the antiserum was recovered by centrifugation at 5000×g for 10 min at 4 °C. A controlled serum was done by injecting normal saline at the same conditions.

Transfection and luciferase assays

Co-transfection of luciferase reporter plasmid with relevant recombinant plasmids into cells was carried out by mixing 0.2 µg of reporter plasmid and 0.4 µg testing plasmids with 2 µl Sofast™ transfection reagents (Xiamen Sunma Biotechnology Co, Ltd.). The mixture was then added to each well of 24-well plates with A549 cells growing at 70% confluence. After incubation for 24 h, the cells were harvested for luciferase activity assays.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR analysis was performed to determine IL-6 mRNA level. After 24 h post-transfection, total RNA was isolated from transfected cells using a TRIzol reagent (Invitrogen), and cellular RNA was amplified by reverse-transcribed PCR with a random primer and then amplified by PCR using IL-6 specific primers listed in Table 4. β-actin primer set was used as an internal control. The PCR products were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide. All product bands were visualized and quantified using Gel-Pro Analyzer (Beijing Junyi-Dongfang Electrophoresis instrument Co., Ltd., China).

Enzyme-linked immunosorbent assay (ELISA)

The level of IL-6 protein produced was assessed by ELISA. After transfection, secreted IL-6 protein was quantified in 100 µl culture supernatants using an IL-6 ELISA test (Jingmei Biotech, China) as recommended by the supplier.

Table 4
Sequences of primers for RT-PCR and EMSA

β-actin-U	5'-ATGATATCGCC GCGCTCG-3'
β-actin-D	5'-CGCTCGGTGAGGATCTTCA-3'
IL-6mRNA-U	5'-AGCAAAGAGGCACTGGCAGAAAACA-3'
IL-6mRNA-D	5'-AGAAGAAGGAATGCCCATTAACAAC-3'
NF-κB probe-U	5'-CAAATGTGGGATTTTCCCATGA-3'
NF-κB probe-D	5'-TCATGGGAAAATCCCACATTTG-3'

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation assay was done as previously described with slight modifications (Wu et al., 2003; Yan et al., 2006). Monolayer of A549 cells (70% confluent) was incubated for 24 h after transfection, and then formaldehyde was added to the culture medium to a final concentration of 1%. The cells were then washed twice in PBS, scraped, and lysed in lysis buffer (1% SDS, 10 mM Tris-HCl, pH 8.0, 1 mM PMSF, 50 mg/ml of both aprotinin, and leupeptin) for 10 min on ice. The lysates were sonicated on ice and the debris was removed by centrifugation at 12,000 rpm for 15 min at 4 °C. One-fourth of the supernatant was used as DNA input control. The remaining supernatant was diluted 10-fold with dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) and incubated with antibody against N protein overnight at 4 °C. Immunoprecipitated complexes were collected using protein A/G agarose beads. The pellets were washed with dialysis buffer (2 mM EDTA, 50 mM Tris-HCl, pH 8.0). Samples were incubated at 67 °C for 5 h to reverse formaldehyde crosslink. DNA was precipitated with ethanol and extracted three times with phenol/chloroform. Finally, pellets were resuspended in TE buffer and subjected to PCR amplification using IL-6 promoter specific detection primer (Table 1). The PCR products were resolved by agarose gel electrophoresis.

Western blot analysis

Whole cell lysates were prepared by lysing A549 cells with PBS pH 7.4 containing 0.01% Triton-100, 0.01% EDTA, and 10% cocktail protease inhibitor (Roche, Germany). The cytosolic and nuclear protein fractions were separated as described. Cells were washed with ice-cold PBS and collected by centrifugation and the pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.5 mM DTT, 10% protease cocktail inhibitor) for 15 min on ice, and vortexed for 10 s. Nuclei were pelleted by centrifugation at 13,000 rpm for 1 min. Supernatants containing cytosolic proteins were collected. Lysates were centrifuged at 13,000 rpm for 10 min. The supernatants were boiled for 5 min with equal volumes of 2× gel loading buffer (100 mM Tris, 10% β-mercaptoethanol, 20% glycerol, 4% SDS, 2 mg/ml bromophenyl blue). One hundred micrograms of cultured cell lysates was electrophoresed in 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. Nonspecific IgGs were blocked with 5% nonfat dried milk before being incubated with a rabbit polyclonal anti-N protein antibody or mouse monoclonal anti-NF-κB antibody

(Santa Cruz). Protein bands were detected using SuperSignal Chemiluminescent (Pierce, IL).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously with some modification (Liu et al., 2007). Cells were washed with cold PBS twice and scraped into 1 ml of cold PBS. Cells were harvested by centrifugation for 15 s and incubated in 2 packed cell volumes of buffer A (10 mM HEPES, pH 8.0, 0.5% Nonidet P-40, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 200 mM sucrose) for 5 min at 4 °C with flicking the tube. The crude nuclei were collected by centrifugation for 30 s; pellets were rinsed with buffer A, resuspended in 1 packed cell volume of buffer B (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, and 1.0 mM DTT), and incubated on a rocking platform for 30 min at 4 °C. Nuclei were clarified by centrifugation for 5 min, and the supernatants were diluted 1:1 with buffer C (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT). Cocktail protease inhibitor tablets were added to each type of buffer. Nuclear extracts were frozen in liquid N₂ and stored at –70 °C until use. NF-κB oligonucleotides were synthesized by Takara (Takara Biotech Co. Ltd., Japan) based on the cognate human IL-6 promoter sequence (Table 4). The complimentary oligonucleotides were annealed and purified according to the manufacture's protocol. Each probe was end-labeled with [γ^{32} P] ATP using T4 polynucleotide kinase (Takara biotech). EMSA was performed by incubating 2 μg of nuclear extract with a labeled probe (15,000 cpm, 10 fmol) in binding buffer (4 mM Tris–HCl, 12 mM HEPES–KOH, pH 7.9, 60 mM KCl, 12% glycerol, 0.5 mM EDTA, and 1 mM DTT) containing 1 μg of poly(dI–dC) for 25 min at room temperature. To assure the specific binding of transcription factors to the probe, the probe was chased by 100-fold molar excess of cold wild-type. For supershift experiments, 2 μg of purified polyclonal antibody was incubated with nuclear extracts on ice for 30 min before adding to the binding buffer. Samples were electrophoresed on 5% nondenaturing polyacrylamide gel with running buffer (0.25× TBE containing 22.5 mM Tris–borate, 0.5 mM EDTA), and the gels were dried and subjected to autoradiography.

Statistical analysis

All of the experiments were reproducible and were carried out in duplicates or quadruplicates. Each set of experiments was repeated at least three times with similar results and a representative one is shown. The results are presented at the means ± S.D. Student's *t*-test for paired samples was used to determine statistical significance. Differences were considered statistically significant at a value of $P \leq 0.05$.

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