

# ***Neisseria gonorrhoeae* Epithelial Cell Interaction Leads to the Activation of the Transcription Factors Nuclear Factor $\kappa$ B and Activator Protein 1 and the Induction of Inflammatory Cytokines**

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## **Summary**

We have studied the effect of human bacterial pathogen *Neisseria gonorrhoeae* (*Ngo*) on the activation of nuclear factor (NF)- $\kappa$ B and the transcriptional activation of inflammatory cytokine genes upon infection of epithelial cells. During the course of infection, *Ngo*, the etiologic agent of gonorrhea, adheres to and penetrates mucosal epithelial cells. In vivo, localized gonococcal infections are often associated with a massive inflammatory response. We observed upregulation of several inflammatory cytokine messenger RNAs (mRNAs) and the release of the proteins in *Ngo*-infected epithelial cells. Moreover, infection with *Ngo* induced the formation of a NF- $\kappa$ B DNA-protein complex and, with a delay in time, the activation of activator protein 1, whereas basic leucine zipper transcription factors binding to the cAMP-responsive element or CAAT/enhancer-binding protein DNA-binding sites were not activated. In supershift assays using NF- $\kappa$ B-specific antibodies, we identified a NF- $\kappa$ B p50/p65 heterodimer. The NF- $\kappa$ B complex was formed within 10 min after infection and decreased 90 min after infection. Synthesis of tumor necrosis factor  $\alpha$  and interleukin (IL)-1 $\beta$  occurred at later times and therefore did not account for NF- $\kappa$ B activation. An analysis of transiently transfected IL-6 promoter deletion constructs suggests that NF- $\kappa$ B plays a crucial role for the transcriptional activation of the IL-6 promoter upon *Ngo* infection. Inactivation of NF- $\kappa$ B conferred by the protease inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone inhibited mRNA upregulation of most, but not all, studied cytokine genes. Activation of NF- $\kappa$ B and cytokine mRNA upregulation also occur in *Ngo*-infected epithelial cells that were treated with cytochalasin D, indicating an extracellular signaling induced before invasion.

The activation of transcription factors and the production of immunomodulatory cytokines is an essential part of the host response to the infection with pathogenic organisms. In activated T cells, monocytes, and macrophages, genes such as those encoding GM-CSF, the inflammatory cytokines IFN- $\beta$ , TNF- $\alpha$ , IL-1 and -6, the receptor for IL-2 $\alpha$  chain, and the monocyte chemotactic protein (MCP)<sup>1</sup>-1/JE are highly induced as a result of the regulatory function of the immediate early response factor nu-

clear factor (NF)- $\kappa$ B (1). Several other transcription factors have been identified that might play a role in regulating immune response genes. These include activator protein 1 (AP-1), which is involved in the induction of a variety of target genes in response to stimulation of cell surface receptors (2) and NF-IL-6 (3).

Cytokines were initially thought to be produced solely by cells of the immune system; however, it is now evident that many nonimmune cells, including epithelial cells, produce cytokines. *Neisseria gonorrhoeae* (*Ngo*), the etiologic agent of gonorrhea, can adhere to and penetrate mucosal epithelial cells during the course of an infection and provoke a strong inflammatory response. Although information about the genetic basis and mechanism of cellular invasion is accumulating, little is known about the cell signaling and its consequences which occur after bacterium-epithelial cell interactions. Analysis of the activated kinases and transcription factors as well as cytokine gene

<sup>1</sup>Abbreviations used in this paper: AP-1, activator protein 1; C/EBP, CAAT/enhancer-binding protein; CRE, cAMP-responsive element; EMSA, electrophoretic mobility shift assay; hGH, human growth hormone; I-309, intercrine 309; MCP, monocyte chemotactic protein; MOI, multiplicity of infection; mRNA, messenger RNA; NF, nuclear factor; *Ngo*, *Neisseria gonorrhoeae*; RT-PCR, reverse transcriptase PCR; TPCK, *N*-tosyl-L-phenylalanine-chloromethyl ketone.

upregulation in eukaryotic cells during the infection process may provide first insight into these mechanisms.

A variety of extracellular factors (mitogens, growth factors, bacterial surface components, etc.) initiate the execution of a complex cellular signaling by binding specific transmembrane receptors onto the eukaryotic cell membrane. The intracellular signaling pathways are complex networks of biochemical reactions that ultimately culminate in specific patterns of nuclear gene expression mediated by transcription factors. Preexisting transcription factors that are involved in immediate early cellular responses can be posttranslationally activated by a variety of mechanisms. Phosphorylation is the most common modification used to activate or repress transcription factors (4). Accordingly, extracellular signals that trigger transcription factor activity may affect transcription factor localization, DNA binding, and the interaction with the basal transcription machinery. In the case of the NF- $\kappa$ B/*rel* family of transcription factors, serine and tyrosine phosphorylation induce or inhibit a proteolytic degradation of I $\kappa$ B $\alpha$  (5–8). Nuclear factor  $\kappa$ B belongs to a multigene family of transcription factors that constitute homo- or heterodimeric proteins with a conserved DNA binding or dimerization domain (9). In its inactive, cytosolic form, NF- $\kappa$ B consists of a dimer of DNA-binding subunits bound to an inhibitor, I $\kappa$ B. Dissociation from the inhibitor is triggered by phosphorylation and mostly by degradation of I $\kappa$ B $\alpha$  (8, 10, 11), and resultant transcription of target genes can occur in response to a number of agents in a broad spectrum of cell lines (12). AP-1 transcription factors are a ubiquitous class of gene regulatory factors that bind specifically to sequences related to the pseudopalindromic AP-1 consensus site (TGA C/G TCA). AP-1 proteins either form Jun-Jun homodimers comprising the members of the Jun family (c-Jun, JunD, and JunB) or Fos-Jun heterodimers derived from the various Fos family members (13).

To understand cellular signaling alterations after pathogen infection in epithelial cells better, we examined the altered expression of cytokines and the activation of transcription factors. Here we demonstrate that *Ngo* induces inflammatory cytokines and chemokines (TNF- $\alpha$ , TGF- $\beta$ , GM-CSF, IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-12, and MCP-1) in various epithelial cells (HeLa, ME180, HaCaT). Before upregulation of cytokine messenger RNA (mRNA), we observed activation of the immediate early transcription factor NF- $\kappa$ B. AP-1 was also activated in *Ngo*-infected cells, but later in the infection process. Transactivation activity of NF- $\kappa$ B is induced by invasive or by merely adherent bacteria, even at a multiplicity of infection (MOI) of 5. Deletion constructs of the IL-6 promoter transfected in HeLa cells confirmed the importance of the NF- $\kappa$ B enhancer element for the activation of the IL-6 gene in *Ngo* infected epithelial cells. Moreover, inactivation of NF- $\kappa$ B by the serine protease inhibitor *N*-tosyl-L-phenylalanine-chloromethyl ketone (TPCK), which suppresses the induction of several cytokine genes, suggests that NF- $\kappa$ B expression is sufficient for the transcriptional activation of cytokine genes in response to *Ngo* infection. The activation of NF- $\kappa$ B and cytokines also occurred in *Ngo*-infected and cytochalasin

D-treated cells, indicating that the cellular signaling is independent of the penetration step of *Ngo* bacteria.

## Materials and Methods

**Human Cell Culture and Infection.** Epithelial cells (HeLa, ME180, HaCaT) were grown in RPMI 1640 (Life Technologies, Eggenheim, Germany) supplemented with 4 mM glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% FCS in a humidified 5% CO<sub>2</sub> atmosphere. Epithelial cells were seeded in tissue culture plates for 48 h before infection. 24 h before infection, the medium was replaced by RPMI 1640 medium supplemented with 10% FCS and 4 mM glutamine. 2 h before infection, the medium was again replaced by RPMI 1640 medium supplemented with 0.1% FCS and 4 mM glutamine. The epithelial cells were infected with *Ngo* given in MOI in a range of 5–100 for different periods of time. For infection experiments, bacteria were centrifuged for 5 min at 500 *g* onto the epithelial cell monolayer. In the experiments using 2 mM cytochalasin D (Sigma Chemical Co., St. Louis, MO) or 30 mM TPCK (Sigma Chemical Co.), the cells were preincubated for 30 min before the bacteria were added. Stimulation of the cells with 10 ng/ml TNF- $\alpha$  (Promega, Heidelberg, Germany) or 40 nM PMA (phorbol 12-myristate 13-acetate; Sigma Chemical Co.) was performed at the indicated time points.

**Bacteria.** Three different *Ngo* strains were used for infection of human epithelial cell lines. The nonpilated, but invasive Opa<sup>+</sup> strain N242 (VP1; P.IA; P<sup>-</sup>; Opa<sub>27</sub>, Opa<sub>27.5</sub>, Opa<sub>28</sub>, Opa<sub>29</sub>, Opa<sub>30</sub>; lipopolysaccharide type L1) has been described (14). The adherent, but noninvasive P<sup>+</sup> strain N138 (P<sup>+</sup> PilE<sub>F3</sub>) and the P<sup>-</sup> Opa<sup>-</sup> control strain N300 (PilE<sub>B1</sub>; opaC<sub>30</sub>::cat, pTH7) are derivatives of strain MS11 (15, 16). Gonococci were routinely grown on phosphate agar as described previously (17). Strain VP1 was additionally grown in 10 ml of RPMI 1640 containing 25 mM Hepes (pH 7.2) for 2 h at 37°C before infection.

**RNA Isolation and Reverse Transcriptase PCR.** Total RNA was isolated using Trizol reagent (Life Tech) as recommended by the manufacturer's instructions. Total RNA (1  $\mu$ g) was reverse transcribed into single-stranded cDNA with Superscript II RT (Life Tech) and oligo(dt) primers. Amplification of cytokine DNA was carried out by PCR with the primers indicated in Table 1. We performed duplex reverse transcriptase (RT)-PCR using  $\beta$ -actin primers as an internal control in each reaction and a subsaturating number of cycles allowed a semiquantitative analysis within the infection kinetics. RT-PCR protocols can be obtained upon request. PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

**ELISA.** Cytokines were assayed in the supernatants of *Ngo*-infected cells by ELISAs. IL-8 and TNF- $\alpha$  ELISA were performed as described by the manufacturer's instructions (R&D Sys., Minneapolis, MN; and Genzyme Corp., Cambridge, MA). GM-CSF ELISA used mouse anti-human GM-CSF as capturing antibody (Genzyme Corp.), rabbit anti-human GM-CSF (Genzyme Corp.) as a secondary antibody, and horseradish peroxidase-labeled goat anti-rabbit IgG (Sigma Chemical Co.) was used as a tracer antibody.

**Preparation of Nuclear Protein Extracts.** At the indicated time points after infection, cytoplasmic and nuclear extracts were prepared by using a nonionic detergent method. In brief, the cells were washed and resuspended in buffer A (18) and 0.15% NP-40 was added. The cells were left on ice for 10 min and subsequently centrifuged at 1,000 *g* for 10 min. The supernatant was used as cytoplasmic extract and the pellet was treated with buffer C (18)

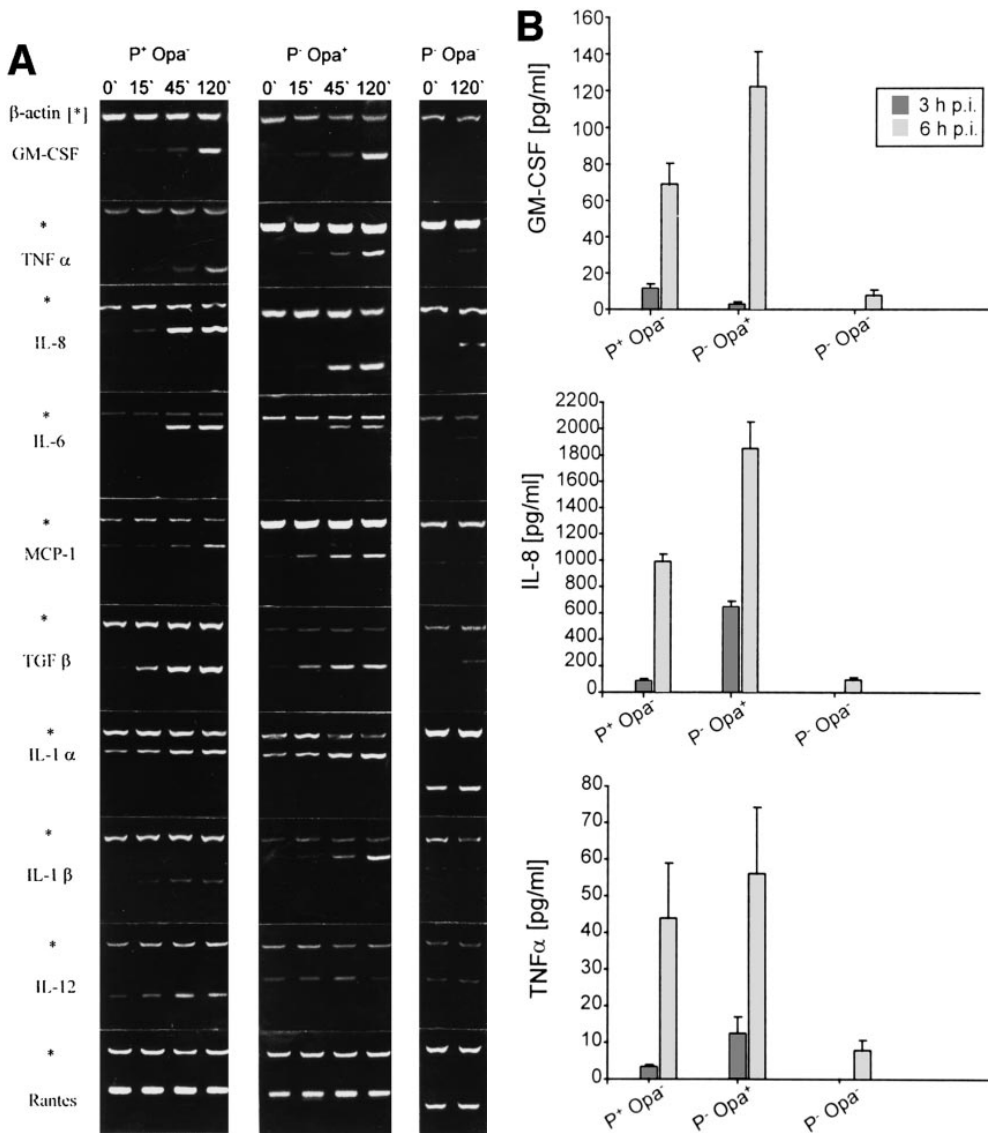
**Table 1.** Oligonucleotide Primers and PCR Product Sizes for 10 Cytokine cDNAs

Factor	Sequence	Product size
		<i>bp</i>
$\beta$ -actin		
Sense	5'-GGCACCACACCTTCTACAATGAG-3'	838
Antisense	5'-CGTCATACTCCTGCTTGCTGATC-3'	
IL-1 $\alpha$		
Sense	5'-GTCTCTGAATCAGAAATCCTTCTATC-3'	421
Antisense	5'-CATGTCAAATTTCACTGCTTCATCC-3'	
IL-1 $\beta$		
Sense	5'-AAACAGATGAAGTGCTCCTTCCAGG-3'	391
Antisense	5'-TGGAGAACACCACTTGTGCTCCA-3'	
IL-6		
Sense	5'-ATGAACTCCTCCTCCACAAGCGC-3'	627
Antisense	5'-GAAGAGCCCTCAGGCTGGACTG-3'	
IL-8		
Sense	5'-AACATGACTTCCAAGCTGGCC-3'	303
Antisense	5'-TTATGAATTCTCAGCCCTCTTC-3'	
IL-12		
Sense	5'-AGGTCAAGGCTATGGTGAGC-3'	189
Antisense	5'-TGCCTTCCAGACACTTACGG-3'	
GM-CSF		
Sense	5'-TGGCTGCAGAGCCTGCTGCTC-3'	432
Antisense	5'-TCACTCCTGGACTGGCTCCC-3'	
MCP-1		
Sense	5'-ATGAAAGTCTCTGCCGCC-3'	303
Antisense	5'-TCAAGTCTTCCGGAGTTTGG-3'	
RANTES		
Sense	5'-ATGAAGGTCTCCGCGGCA-3'	280
Antisense	5'-CTAGCTCATCTCAAAGAG-3'	
TGF- $\beta$		
Sense	5'-GCCCTGGACACCAACTATTGC-3'	335
Antisense	5'-GCTGCACTTGCAGGAGCGCAC-3'	
TNF- $\alpha$		
Sense	5'-CAGAGGGAAGAGTTCCCCAG-3'	325
Antisense	5'-CCTTGGTCTGGTAGGAGACG-3'	

for 10 min to yield the nuclear extract. The disruption of the cytosolic membrane appeared to be more efficient than in the Dignam et al. (18) protocol, without affecting the nuclear envelope (10).

**Electrophoretic Mobility Shift Assays.** Gel retardation assays for the detection of NF- $\kappa$ B were performed either with an H-2K or an Ig $\kappa$  oligo probe as described previously (19). The oligonucleotides containing the NF- $\kappa$ B recognition site were labeled using the large fragment DNA polymerase (Klenow) in the presence of  $\alpha$ [<sup>32</sup>P]deoxy-ATP. The DNA-binding reactions were performed with 20  $\mu$ l binding buffer (2  $\mu$ g poly (dI-dC), 1  $\mu$ g BSA, 5 mM dithiothreitol, 20 mM Hepes, pH 8.4, 60 mM KCl, and 10% glycerol) for 20 min at 30°C. For competition experiments, cold oligonucleotides were used. Supershifts were performed with antibodies as described previously (10). AP-1 DNA-binding activity

was analyzed using oligonucleotides containing the AP-1-binding site: 5'-GATCTTCTAGACCGGATGAGTCATAGCTTG-3'; 5'-CAAGCTATGACTCATCCGGTCTAGAAAGATC-3'. The AP-1 DNA-binding oligonucleotide was labeled using T4 kinase (Boehringer Mannheim GmbH, Mannheim, Germany) in the presence of  $\gamma$ [<sup>32</sup>P]deoxy-ATP. DNA-binding reactions were performed using a binding buffer containing 2  $\mu$ g poly (dI-dC), 1  $\mu$ g BSA, 10 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. Other used oligonucleotides containing DNA-binding sites: cAMP-responsive element (CRE): 5'-CGAGCCCCATTGACGTCAATGGG-GTCGAC-3', 5'-GTCGCCCCATTGACGTCAATGGGGC-TCG-3'; CAAT/enhancer binding protein (C/EBP): 5'-TGC-AGATTGCGCAATCTGCA-3', 5'-TGCAGATTGCGCAAT-



**Figure 1.** *Ngo* infection induces proinflammatory cytokines in epithelial cells. (A) Shown is an analysis of cytokine mRNA levels in ME180 cells in response to *Ngo* infection (the invasive Opa<sup>+</sup> strain, the piliated, noninvasive P<sup>+</sup> strain, and the P<sup>-</sup> Opa<sup>-</sup> control strain) by duplex RT-PCR. Total RNA was isolated at the indicated time points after infection and reverse transcribed into cDNA, and cytokine mRNAs as well as  $\beta$ -actin mRNA were semiquantitated by several cycles of PCR using cytokine-specific primers so that products were below the saturation stage of amplification. Equal RNA was amplified for each sample within an infection kinetic as indicated by the internal  $\beta$ -actin amount. DNA products were separated by electrophoresis on an agarose gel and visualized with ethidium bromide. Shown is an experiment representative of at least three. \*,  $\beta$ -actin. Not shown: IL-2, IL-3, IL-4, IL-5, IL-10, IL-13, I-309, and IFN- $\gamma$  RT-PCR reactions that recognized no transcripts. (B) The secretion of the cytokines TNF- $\alpha$ , GM-CSF, and IL-8 was assayed in ELISA systems 3 h and 6 h after infection (p.i.). Values are means, the standard errors of the means are representative for three experiments.

CTGCA-3'; octamer H2B: 5'-GATCCCAACTCTTACCT-TATTTGCATAAGCGATTCTATAG-3', 5'-GATCCTATA-GAATCGCTTATGCAAATAAGGTGAAGAGTTGG-3'. For the CRE and C/EBP oligonucleotides, the same buffer conditions as indicated for AP-1 were used, whereas for the octamer-binding site NF- $\kappa$ B, shift conditions were used. The reaction products were analyzed by electrophoresis in a 5% polyacrylamide gel using 12.5 mM Tris, 12.5 mM Boric acid, and 0.25 mM EDTA, pH 8.3. The gels were dried and exposed to Amersham TM film (Amersham Buchler, Braunschweig, Germany) at -70°C using an intensifying screen.

**Luciferase and Human Growth Hormone Reporter Assays.** HeLa, HaCat, or ME180 cells at 50–70% confluence were transfected by cationic liposomes (Promega) with 1  $\mu$ g of a luciferase expression plasmid containing four repeats of the NF- $\kappa$ B H-2K-binding site as enhancer element. 24 h after transfection, cells were either infected with *Ngo* strains, stimulated with PMA or TNF- $\alpha$ , or treated with LPS. Luciferase assays were performed at the indicated time points as recommended by the manufacturer's instructions (Promega). The results were recorded on a  $\beta$ -counter (Beckman Wallac 409;

Beckman, Munich, Germany). The data presented are representative of more than three separate experiments.

IL-6 promoter activity was assayed by measuring human growth hormone (hGH) release. The IL-6 promoter reporter gene activity from three different constructs (-524), (-219), and (-49) was assessed by testing for hGH activity in culture supernatants 3 h after infection using a commercially available enzyme immunoassay (Eurogenetics, Tessenderlo, Belgium).

## Results

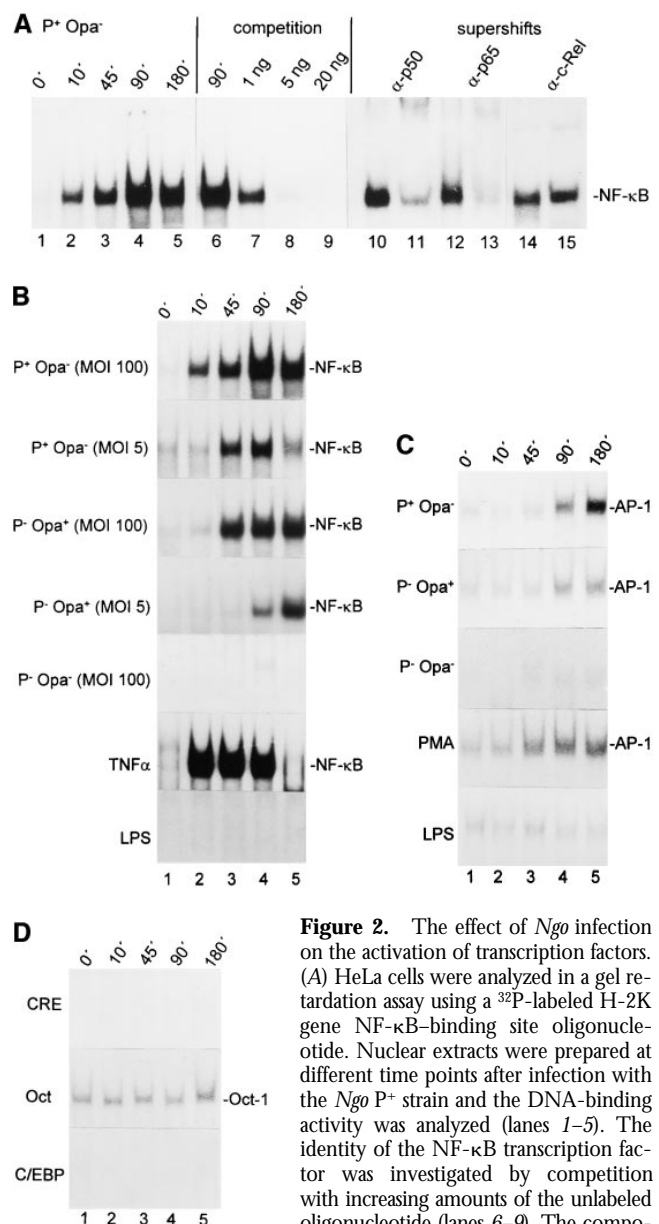
**Induction of Inflammatory Cytokines after Infection of Epithelial Cells with *Ngo*.** To assess changes in cytokine gene expression of human epithelial cells in response to bacterial infection, ME180, HeLa, and HaCa T cells were challenged to infection with *Ngo* at a MOI of 5, as described in Materials and Methods. The assays were performed with different *Ngo* strains and cytokine expression was determined at different time points after infection, i.e., 0, 15, 45,

and 120 min, by reverse transcription (RT-PCR) of total RNA prepared from infected cells. The cytokine mRNA levels were compared with the constitutive  $\beta$ -actin mRNA in the same RT-PCR reaction. The *Ngo* strains used differed with regard to the infection of epithelial cells. Strain N242 ( $Opa^+$ ) enters epithelial cells by binding to heparan sulfate containing surface proteoglycan receptor proteins (14, 20, and Dehio, C., E. Freissler, C. Lanz, O. Gomez-Duarte, G. David, and T.F. Meyer, manuscript submitted). Strain N138 ( $P^+ Opa^-$ ) expresses pili that also confer adherence to epithelial cells (21), but does not express any *Opa* proteins and therefore is not invasive (14). Strain N300 ( $P^- Opa^-$ ) is neither piliated, nor does it express *Opa* proteins (16); consequently this strain adheres only weakly to human cells. Gonococcal infection was routinely monitored by light microscopy of the bacteria using the crystal violet staining technique (22).

Each of the three studied epithelial cell lines expressed inflammatory cytokines in response to *Ngo* infection. As shown in Fig. 1 A, infection of ME180 cells with *Ngo* led to an increased synthesis of several cytokines, i.e., GM-CSF, TNF- $\alpha$ , IL-8, MCP-1, TGF- $\beta$ , and IL-1 $\beta$  as soon as 15 min after infection. Maximum expression was achieved 2 h after infection. Furthermore, the cytokine IL-6 was induced within 45 min after infection and the cytokines IL-1 $\alpha$  and IL-12 showed constitutive mRNA expression with a slight increase upon infection. The chemokine RANTES (regulated on activation, normal T cell expressed and secreted) was constitutively expressed without change in infected cells. Both the adherent ( $P^+$ ) and the invasive ( $Opa^+$ ) gonococci were efficient in cytokine upregulation, whereas the  $P^- Opa^-$  gonococci did not significantly increase cytokine mRNA levels. LPS, a major constituent of the gram-negative bacterial outer membrane and a potent inducer of cytokine mRNA upregulation in CD14 positive cells, did not effect cytokine expression (data not shown). None of the tested cell lines induced detectable levels of mRNA coding for IL-2, IL-3, IL-4, IL-5, IL-10, IL-13, intercrine 309 (I-309), or IFN- $\gamma$  (data not shown).

These studies indicate a coordinate upregulation of inflammatory cytokine mRNA levels in epithelial cells upon infection with *Ngo*. To demonstrate for some of these cytokines that their increased mRNA levels actually lead to an increased protein secretion, we analyzed the cytokine release by ME180 cells in response to bacterial infection. As shown in Fig. 1 B, infection with the  $P^+$  and  $Opa^+$  *Ngo* strains, respectively, resulted in an increase of GM-CSF, IL-8, and TNF- $\alpha$  secretion. The release was weak until 3 h and increased steadily during the following time. The  $P^- Opa^-$  control strain induced GM-CSF, IL-8, and TNF- $\alpha$  release to some extent at 6 h after infection.

***Ngo* Infection Induces the Transcription Factors NF- $\kappa$ B and AP-1.** *Ngo* infection in epithelial cells may also induce alterations in signal transduction pathways that modulate cellular transcription factors. Moreover, the activation of transcription factors and inflammatory cytokine gene expression may be coordinately inducible in *Ngo*-infected cells. Therefore, we investigated whether *Ngo* infection induces transcrip-



**Figure 2.** The effect of *Ngo* infection on the activation of transcription factors. (A) HeLa cells were analyzed in a gel retardation assay using a  $^{32}$ P-labeled H-2K gene NF- $\kappa$ B-binding site oligonucleotide. Nuclear extracts were prepared at different time points after infection with the *Ngo*  $P^+$  strain and the DNA-binding activity was analyzed (lanes 1–5). The identity of the NF- $\kappa$ B transcription factor was investigated by competition with increasing amounts of the unlabeled oligonucleotide (lanes 6–9). The composition of the *Ngo*-induced NF- $\kappa$ B complex was investigated by antibody supershifting and inhibition using different amounts (0.5  $\mu$ l and 2  $\mu$ l) of anti-p50 (lanes 10 and 11), anti-p65 (lanes 12 and 13), or anti-c-Rel antisera (lanes 14 and 15). (B) The NF- $\kappa$ B DNA-binding activity was assayed in response to *Ngo* strains, different MOIs, and different time points (lanes 1–5). As controls, HeLa cells were stimulated with TNF- $\alpha$  (10 ng/ml) or treated with LPS (10  $\mu$ g/ml). (C) AP-1 DNA-binding activity was investigated in response to *Ngo* infection at different time points (lanes 1–5) using a  $^{32}$ P-labeled AP-1 DNA-binding site oligonucleotide as a probe. As controls, cells were stimulated with PMA (40 nM) or treated with LPS (10  $\mu$ g/ml). (D) DNA-binding activity at the CRE, C/EBP, and octamer binding sites were studied in extracts from *Ngo*  $P^+$  strain-infected HeLa cells at different time points (lanes 1–5). Only sections of the autoradiograms containing the protein-DNA complexes are shown. The position of protein-DNA complexes are indicated with arrows.

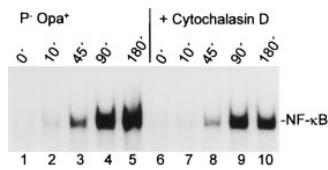
tion factors that could be responsible for inflammatory cytokine gene expression.

Subconfluent monolayers of HeLa cells were infected with *Ngo*. At different time points after challenge, nuclear

protein extracts were prepared and analyzed for the levels of cellular transcription factor DNA-binding activity by using a panel of radiolabeled oligonucleotides corresponding to the DNA-binding sites of five transcription factor families (NF- $\kappa$ B, AP-1, CRE, C/EBP, and octamer factors). As Fig. 2 A shows, when the NF- $\kappa$ B-binding site (H-2K) was used for the electrophoretic mobility shift assay (EMSA), an enhanced binding of nuclear proteins was observed in HeLa cells within 10 min after infection with the P<sup>+</sup> strain (Fig. 2 A, lanes 1 and 2). The DNA-binding activity increased within 90 min, and was already reduced after 180 min (Fig. 2 A, lanes 3–5). To examine the specificity of the DNA-binding capability induced by adherence of the P<sup>+</sup> strain, nonlabeled double-stranded oligonucleotide was added for competition. A decrease in the amount of bound complex was observed as the concentrations of unlabeled NF- $\kappa$ B consensus sequence increased (Fig. 2 A, lanes 6–9). The most prominent form of transcription factor NF- $\kappa$ B has been described as a heterodimer consisting of two proteins, p50 and p65 (23). Furthermore, members of the NF- $\kappa$ B/*rel* family of proteins can form homodimers and heterodimers (24). The nature of the proteins that bind to the  $\kappa$ B sequence were characterized using supershift assays. Experiments were performed in which the nuclear extracts were preincubated with either an anti-p50, anti-p65, or anti-c-Rel antiserum before addition of the <sup>32</sup>P-labeled oligonucleotide. The results shown in Fig. 2 A (lanes 10–15) indicate that the anti-p50 antibody (0.5  $\mu$ l and 2  $\mu$ l antiserum, lanes 10 and 11) and the anti-p65 antibody (0.5  $\mu$ l and 2  $\mu$ l antiserum, lanes 12 and 13) led to a significant reduction and to supershifts of the NF- $\kappa$ B complex. The anti-c-Rel antibody affected only slightly the NF- $\kappa$ B–DNA complex (0.5  $\mu$ l and 2  $\mu$ l antiserum, lanes 14 and 15). Therefore, both p50 and p65 represent the predominant protein species in the  $\kappa$ B DNA-binding activity present in P<sup>+</sup> strain–infected HeLa cells.

To evaluate whether cellular NF- $\kappa$ B activation is at variance in epithelial cells infected with adherent versus invasive *Ngo* strains, we compared NF- $\kappa$ B DNA-binding activity in HeLa cells infected with either the P<sup>+</sup> or Opa<sup>+</sup> strain. At a MOI of 100, the noninvasive P<sup>+</sup> strain induced stronger and earlier activation of NF- $\kappa$ B than the Opa<sup>+</sup> strain, whereas no NF- $\kappa$ B activation was observed in cells infected with the P<sup>–</sup> Opa<sup>–</sup> control strain or LPS-treated cells (Fig. 2 B). The activation of NF- $\kappa$ B in response to *Ngo* infection was delayed compared to the NF- $\kappa$ B activation in response to TNF- $\alpha$ , but showed a similar potential to induce DNA-binding activity. The activation of the immediate early transcription factor NF- $\kappa$ B in *Ngo*-infected epithelial cells was also inducible at a MOI of 5. This indicates highly specific *Ngo*-induced signaling leading to downstream activation of NF- $\kappa$ B.

Activation of AP-1 in response to *Ngo* infection was studied using an AP-1 consensus DNA-binding oligonucleotide. Protein–DNA complexes were identified in HeLa cells infected with either the P<sup>+</sup> or the Opa<sup>+</sup> strain in EMSA using an oligonucleotide containing the AP-1-binding site, indicating that members of the c-jun/c-fos family were activated (Fig. 2 C). AP-1 DNA-binding activity was observed 90 min after infection and was as strong as AP-1 ac-



**Figure 3.** Inhibition of invasion of *Ngo* Opa<sup>+</sup> bacteria using cytochalasin D does not affect NF- $\kappa$ B activation. Time-dependent activation of NF- $\kappa$ B in response to *Ngo* Opa<sup>+</sup> strain infection in HeLa cells (lanes 1–5) and in cells treated for 30 min

before the infection with cytochalasin D (lanes 6–10) is shown. Only a section of the autoradiogram containing the protein–DNA complex is shown. The position of the NF- $\kappa$ B–DNA complex is indicated with an arrow.

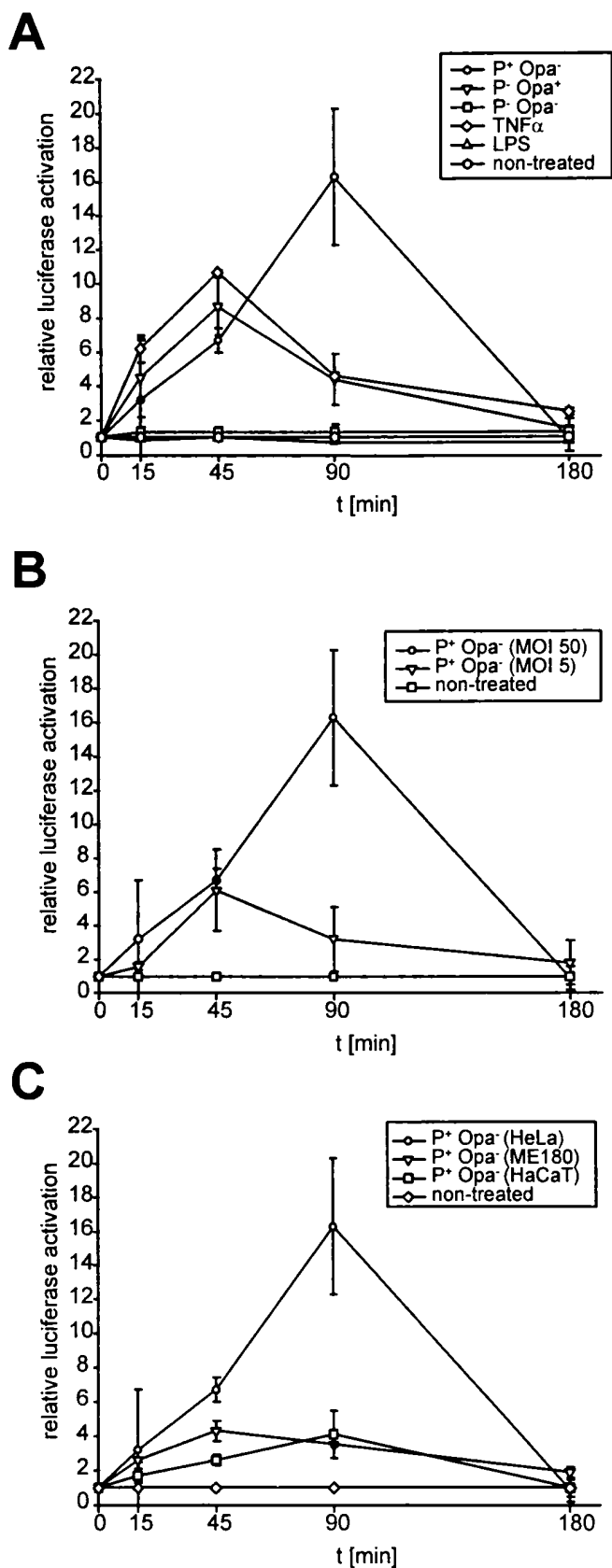
tivation in response to PMA, which induces AP-1 within 10 min. No AP-1 activation was observed in N300 (P<sup>–</sup> Opa<sup>–</sup>)–infected or LPS-treated cells. In contrast to the experiments performed to study NF- $\kappa$ B activation in response to *Ngo* infection, AP-1 activation was only observed reproducibly in epithelial cells, which were infected in the presence of FCS. The removal of serum of epithelial cells induced AP-1 activation leading to a constitutive high AP-1 DNA-binding activity at the time of infection, which became enhanced only marginally during *Ngo* infection.

The DNA-binding capability of other transcription factors investigated with oligonucleotides containing the consensus recognition sequence for other basic leucine zipper factors, the C/EBP and the palindromic CRE, as well as the octamer-binding site, were not enhanced by *Ngo* infection (Fig. 2 D).

*Ngo*–Epithelial Cell Interaction Induces Activation of NF- $\kappa$ B and Inflammatory Cytokines. The highly invasive Opa<sup>+</sup> *Ngo* strain was used to determine whether cellular invasion by the bacteria was a prerequisite for the activation of inflammatory cytokines and transcription factors. Previously, we demonstrated the apparent role of the actin cytoskeleton in the gonococcal entry process by the inhibition of gonococcal uptake by cytochalasin D (14, 17). Since the recruitment of F-actin by *Ngo* is not affected by cytochalasin D treatment, we assume the integrity of the actin microfilaments is required to complete the bacterial entry process.

To study whether signaling in HeLa cells infected with Opa<sup>+</sup> *Ngo* is dependent on the bacterial penetration step, we infected the eukaryotic cells either in the presence or absence of 2 mM cytochalasin D. As shown in Fig. 3, despite of the treatment with cytochalasin D, the Opa<sup>+</sup> *Ngo* were still able to efficiently activate the transcription factor NF- $\kappa$ B. The observation that inhibition of epithelial cell invasion by cytochalasin D treatment does not cause alterations in the downstream signaling suggests that adherence, rather than cellular invasion, is responsible for the induction of the signal. This assumption was supported by cytokine expression data obtained by measuring the release of TNF- $\alpha$ , GM-CSF, and IL-8 in VP1-infected and cytochalasin-treated HeLa cells. Herein, Opa<sup>+</sup> *Ngo*-infected HeLa cells induced a cytokine release that was at similar levels independent of the presence or absence of cytochalasin D (data not shown).

*Ngo* Infection Induces Transactivation at the NF- $\kappa$ B-binding Sequence. To quantify the NF- $\kappa$ B activation in epithelial cells elicited by *Ngo* infection, HeLa cells were transfected



**Figure 4.** Kinetics of NF- $\kappa$ B transactivation activity in *Ngo*-infected epithelial cells. Epithelial cells at 50–70% confluence were transfected with 1  $\mu$ g of a luciferase expression plasmid containing four repeats of the

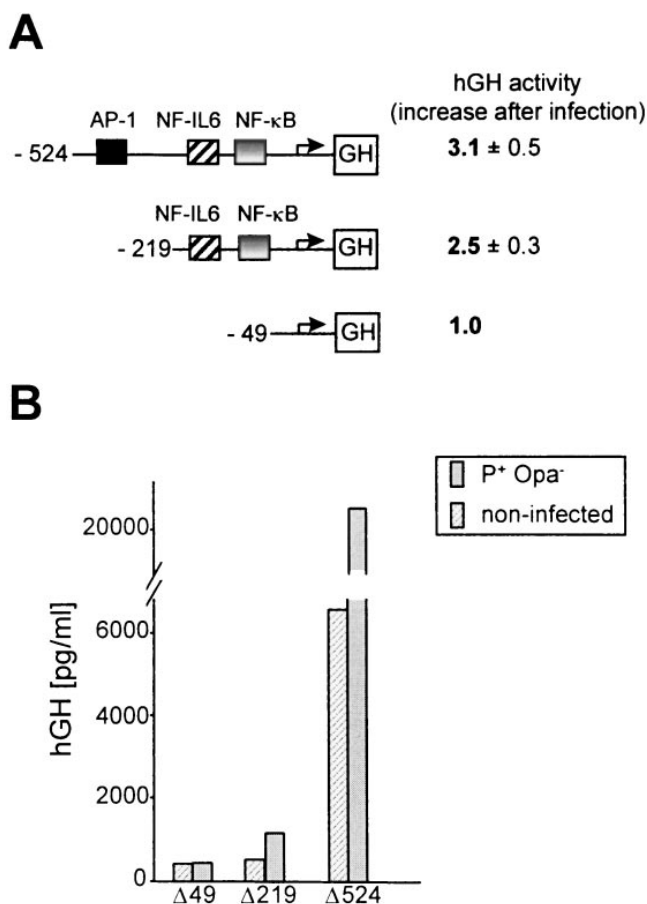
with a plasmid (pGL2-Luc/H-2K) containing four tandem repeats of the H-2K NF- $\kappa$ B DNA-binding sequence linked to a luciferase gene. As shown in Fig. 4 A, epithelial HeLa cells transfected with this promoter construct exhibited a substantial capacity to express luciferase activity in response to different stimuli. Cells infected at a MOI of 50 with either the P<sup>+</sup> or the Opa<sup>+</sup> strain, and TNF- $\alpha$ -treated cells showed a rapid increase in luciferase activity ( $\sim$ three- to sixfold) within 15 min. In cells infected with the P<sup>+</sup> strain, luciferase activity steadily increased during the first 90 min showing a  $\sim$ 16-fold activation as compared to nontreated cells, whereas cells infected with the invasive (Opa<sup>+</sup>) strain, and TNF- $\alpha$ -treated cells have their maximal luciferase activity (9- and 11-fold, respectively) 45 min after infection. In all cases, NF- $\kappa$ B transactivation decreased rapidly from the peak activity to the near basal level within 180 min. Control *Ngo* (P<sup>-</sup> Opa<sup>-</sup>) -infected cells or LPS-treated HeLa cells showed no enhanced NF- $\kappa$ B transactivation compared to the nontreated control cells.

The inducibility and strength of the NF- $\kappa$ B transactivation is influenced by the MOI as shown in Fig. 4 B. Infection of HeLa cells with P<sup>+</sup> *Ngo* at a MOI of 5 or 50 induced an approximately sixfold higher luciferase activity compared to noninfected cells at 45 min after infection. In contrast to the infection of cells at MOI 50, the luciferase activity in cells infected with MOI 5 declines rapidly.

The level of NF- $\kappa$ B transactivation depends on the epithelial cell type (Fig. 4 C). The highest level of transactivation in response to P<sup>+</sup> *Ngo* infection was observed in HeLa cells (16-fold within 90 min after infection), whereas HaCaT and ME180 cells revealed an approximately fourfold NF- $\kappa$ B transactivation.

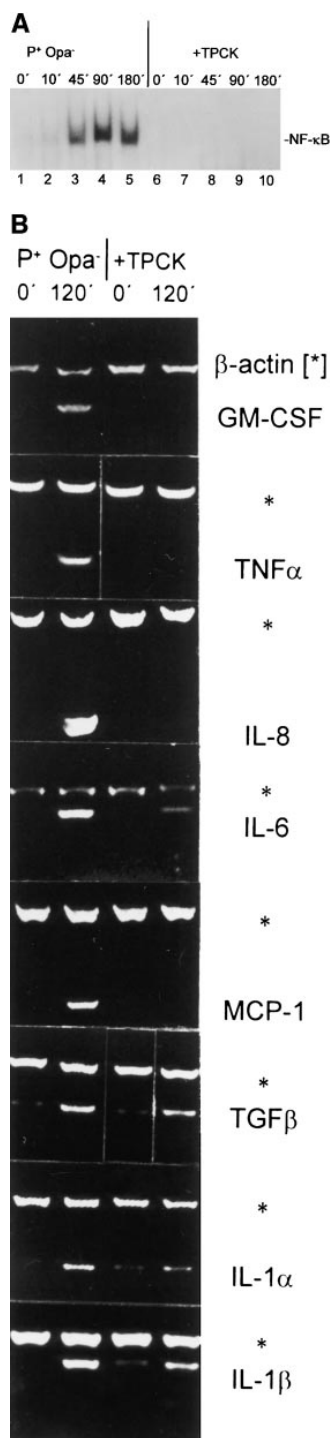
**Activation of IL-6 Promoter Enhancer Elements by *Ngo* Infection.** As shown in Fig. 1 A, IL-6 mRNA is upregulated in response to *Ngo* infection. Therefore, the following experiment was aimed to investigate the molecular mechanism mediating this effect. To this end, deleted forms of IL-6 promoter fragments linked to the hGH gene as a reporter gene were used (Fig. 5 A), and plasmids were transiently transfected into HeLa cells. Promoter activity was assayed after *Ngo* infection by analysing the activity of the hGH in cell-free culture supernatants. Infection experiments were performed for 3 h in the presence of 10% FCS. Transcriptional induction by infection with P<sup>+</sup> *Ngo* was observed in all promoter constructs with the exception of the promoter construct deleted upstream of position -49, containing no enhancer elements. As much as approximately threefold

NF- $\kappa$ B H-2K-binding site as enhancer element as described in Materials and Methods. Luciferase assays from whole cellular extracts were performed at the indicated time points after infection. The results are expressed as fold induction against nontreated cells. (A) HeLa cells were either infected with different *Ngo* strains, stimulated with TNF- $\alpha$  (10 ng/ml), treated with LPS (10  $\mu$ g/ml), or left untreated. (B) HeLa cells were infected with *Ngo* P<sup>+</sup> strain at a MOI of 5 and 50, or left uninfected. (C) Different epithelial cells (HeLa, HaCaT, ME180) were infected with *Ngo* at a MOI of 50 or left untreated. The results are expressed as fold induction against nontreated cells from each cell line. The data presented are representative of more than three separate experiments.



**Figure 5.** *Ngo* infection induces expression of IL-6 promoter-hGH constructs transfected in HeLa cells. (A, left) Schematic representation of the deleted IL-6 promoter constructs. The transcription start site is designated with a black arrow. (A, right, and B) HeLa cells were transfected by cationic liposomes with 3  $\mu$ g of plasmids. Cells were maintained in medium containing 10% FCS for 24 h. 1 h before the infection, the medium was exchanged and the cells either untreated or infected with the *Ngo* P<sup>+</sup> strain at a MOI of 100. hGH activity was assessed in culture supernatants by ELISA, and the results of three independent experiments expressed as fold induction against noninfected cells.

hGH activity was observed from the longest promoter construct deleted upstream of position  $-524$  containing the NF- $\kappa$ B, NF-IL-6, and AP-1 enhancer elements (Fig. 5 A). With this construct, the exchange of the medium before the infection already led to high hGH levels (Fig. 5 B). Transfection of the promoter construct upstream of position  $-219$  lacking the binding site for the transcription factor AP-1 (position  $-283$  to  $-277$ ) caused a drastic reduction of the residual hGH release, but did not affect the inducibility of hGH release by *Ngo* infection. Although the NF-IL-6-binding site at position  $-158$  to  $-145$  has been demonstrated to be involved in transcriptional activation of the IL-6 promoter (25), we observed no activation of NF-IL-6 at the C/EBP DNA-binding site in response to *Ngo* infection by EMSA (Fig. 2 D). Thus, NF-IL-6 does not appear to contribute in IL-6 promoter activation. These data rather suggest that NF- $\kappa$ B plays a crucial role for the transcriptional activation of the IL-6 promoter upon *Ngo* infection.



**Figure 6.** Inhibition of NF- $\kappa$ B activation by TPCK blocks the induction of cytokine genes in *Ngo*-infected epithelial cells. (A) Nuclear extracts from HeLa cells were prepared at different time points after infection (*Ngo* P<sup>+</sup> strain), incubated with a <sup>32</sup>P-labeled oligonucleotide containing the NF- $\kappa$ B H-2K DNA-binding site, and analyzed for NF- $\kappa$ B activation in an EMSA (lanes 1–5). Additionally, cells were treated with the serine protease inhibitor TPCK 30 min before the infection (lanes 6–10). Only a section of the autoradiogram containing the protein-DNA complexes is shown. The position of the NF- $\kappa$ B-DNA complexes is indicated with an arrow. (B) Shown is an analysis of cytokine mRNA levels in HeLa cells in response to *Ngo* P<sup>+</sup> strain infection by duplex RT-PCR either in the absence or presence of the protease inhibitor TPCK. Total RNA was isolated at the indicated time points after infection and reverse transcribed into cDNA, and cytokine mRNAs as well as  $\beta$ -actin mRNA were semiquantitated by several cycles of PCR using cytokine-specific primers so that products were below the saturation stage of amplification. Equal RNA was amplified for each sample within an infection kinetic as indicated by the internal  $\beta$ -actin amount. DNA products were separated by electrophoresis on an agarose gel and visualized with ethidium bromide. Shown is an experiment representative of at least three. \*,  $\beta$ -actin.

*Ngo* Infection Induces Cytokine mRNA Upregulation Is Blocked by NF- $\kappa$ B Inactivation. From the experiments described above, we suggest that the activation of the transcription factor NF- $\kappa$ B represents a critical event in *Ngo* infection of HeLa cells. To study the importance of NF- $\kappa$ B in the downstream signaling after *Ngo* infection, we asked whether the blockage of NF- $\kappa$ B activation by the addition of the serine protease inhibitor TPCK could cause a block of cytokine mRNA upregulation. As shown in Fig. 6 A, TPCK effectively blocked I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B



activation in response to P<sup>+</sup> *Ngo* infection as determined in the gel retardation assay. The TPCK inhibitor does not generally or nonspecifically affect DNA-binding proteins since, for example, Oct-1 DNA-binding activity remains unaffected (data not shown). To determine if cytokine mRNA upregulation is affected by *Ngo* infection of TPCK pretreated cells, we investigated a panel of cytokine genes as shown in Fig. 1 A. TPCK pretreatment of HeLa cells led to a strong inhibition of GM-CSF, TNF- $\alpha$ , IL-8, IL-6, and MCP-1 cytokines mRNAs synthesis upon infection with P<sup>+</sup> *Ngo* (Fig. 6 B). Marginal or no blockage of mRNA synthesis was observed for the cytokines IL-1 $\alpha$ , IL-1 $\beta$ , and TGF- $\beta$ . Thus, in most of the cases studied, the activation of the immediate early factor NF- $\kappa$ B is sufficient for the transcriptional activation of cytokine genes in response to infection with *Ngo*.

## Discussion

Since the mucosal epithelial surface forms the first barrier that impedes the penetration of microorganisms, we used this cell type to study the activation of cytokine expression as a response to an infection with *Ngo*. Cytokines are important modulators of the antigen-specific immune response and thus influence the immune surveillance. If cytokines are induced upon the initial contact of a pathogen with mucosal surfaces, this response could be crucial to the outcome of an infection. Our current investigation supports this notion and reveals that *Ngo* induces the upregulation of a variety of so-called proinflammatory cytokines. Infection with either adherent P<sup>+</sup> *Ngo* or invasive Opa<sup>+</sup> *Ngo* leads to a rapid, direct, and specific activation of a panel of cytokine genes. Our data indicate that physical invasion of epithelial cells by *Ngo* is not a prerequisite of cytokine upregulation. Thus, it appears that the bacterium eukaryotic cell contact is already sufficient to induce an efficient downstream signaling.

The upregulation of cytokine mRNA and the release of cytokines were observed in several different human epithelial cell lines. The *Ngo*-induced cytokines TNF- $\alpha$ , IL-6, and IL-1 have been implicated in the defense of bacterial infections and promote bactericidal activity of leukocytes. In addition, the three cytokines can activate T and B lymphocytes. Their primary function may therefore be host protective (26), although adverse effects towards the host cannot be ruled out. The other cytokines induced by *Ngo* infection have a major inflammatory activity. The cytokine GM-CSF induces granulocyte/macrophage populations and TGF- $\beta$  represents a neutrophil chemoattractant. Moreover, TGF- $\beta$  also exerts activities that oppose or downregulate inflammatory processes. The chemokine IL-8 induces leukocyte chemotaxis and the chemokine MCP-1 attracts monocytes/macrophages. As epithelial cells represent the entry sites for bacterial infections, the activation of these chemokines functions as an early warning system at a time when bacterial products are unavailable to the stimulation of circulating leukocytes (26). The array of proinflammatory cytokines produced by epithelial cells overlaps, but also exhibits

several important differences from that produced by cells of the monocyte/macrophage lineage. Thus, no high levels of IL-12 p40 or IL-1 were expressed by epithelial cells (27, 28). Furthermore, the array of cytokines expressed by epithelial cells markedly differs from that characteristic of T cells, mast cells, eosinophils, and NK cells (29–31). Thus, none of the *Ngo*-infected epithelial cells expressed mRNA for IL-2, IL-3, IL-4, IL-5, IL-10, IL-13, I-309, and IFN- $\gamma$ . Taken together, our findings support the notion that epithelial cells are an integral component of the host's nonspecific immune system. This concept is consistent with other reports. For example, *E. coli* induces IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8, but not TNF- $\alpha$  in epithelial cells (32, 33). Invasive *Salmonella*, *Yersinia*, *Shigella*, and *Listeria* species have been shown to activate a wide range of cytokines like MCP-1, GM-CSF, IL-8, and TNF- $\alpha$  (34, 35). Herein, the bacterial attachment seems to be the signal enhancing the epithelial cell cytokine responses (33, 36). LPS effectively induces cytokine production from macrophages, but only poorly induces epithelial cytokine responses. The poor LPS response may be explained in part by lack of CD14 on epithelial cells (37).

The rapid production of proinflammatory cytokines by epithelial cells involves the activation of immediate early transcriptional activators. Cellular transcription factors are participants in the host response to infection. The promoters of genes important in the immune response like TNF- $\alpha$ , IL-6, IL-8, and MCP-1 contain binding domains for a number of transcription factors, including NF- $\kappa$ B. As confirmed by many other studies, the immediate early transcription factor NF- $\kappa$ B plays a critical role in host immune response to bacterial and viral pathogens (9). Gel retardation assays using nuclear extracts from *Ngo*-infected epithelial cells and a  $\kappa$ B consensus motif resulted in activation of NF- $\kappa$ B-*rel* complexes as compared with nuclear extracts from uninfected cells. Supershift experiments with antibodies against p50 and p65 led to upshifts or the disappearance of the NF- $\kappa$ B complex, suggesting that the protein-DNA complex is composed of the p50 and p65 proteins. The NF- $\kappa$ B activation in epithelial cells occurred within 10 min after infection with P<sup>+</sup> *Ngo* at an MOI of 100 and the induction of cytokine mRNAs were detected after 15 min of infection. High specificity for NF- $\kappa$ B activation was evident from *Ngo* infection experiments using a MOI of 5, which also showed strong NF- $\kappa$ B DNA binding in the EMSA, even with a delay in time. Similarly, transient transfection assays revealed that the NF- $\kappa$ B site is strongly activated in response to *Ngo* infection (Fig. 4). In contrast to NF- $\kappa$ B, AP-1 was induced at an MOI of 100 within 90 min after infection. Further, transcription factors like C/EBP, CRE, or octamer DNA-binding factors tested for their activation in *Ngo*-infected epithelial cells were either not activated or maintained their activity.

Experiments with cultured epithelial cells suggest that gonococcal entry into epithelial cells involves both the polymerization of actin microfilaments (14, 17) and microtubuli (38, and Dehio, C., E. Freissler, C. Lanz, O. Gomez-Duarte, G. David, and T.F. Meyer, manuscript submitted).

Since we speculated that NF- $\kappa$ B activation and cytokine activation might already be triggered by adherent *Ngo* through as yet undefined surface proteins, we studied the cytokine activation in the presence of the microfilament disrupting agent cytochalasin D. In our experiments, we observed that the inhibition of the internalization of invasive *Ngo* by cytochalasin D affected neither NF- $\kappa$ B DNA binding (Fig. 3) nor cytokine release (data not shown) supporting the notion that *Ngo* adhesion to the surface of epithelial cells triggers signals leading to NF- $\kappa$ B activation. An invasion independent activation of NF- $\kappa$ B was described for *Listeria monocytogenes* on macrophages (39), whereas in the case of *Shigella flexneri* invasion was discussed as a prerequisite for NF- $\kappa$ B activation (40).

The transient transfection assay using deletion mutants of the IL-6 promoter revealed that the NF- $\kappa$ B site is primarily responsible for *Ngo*-dependent induction of IL-6 (Fig. 5). This is in line with other studies that have shown that the NF- $\kappa$ B-binding site is crucial for the activation of the IL-6 promoter (41). Both the induction of the IL-6 mRNA and the activation of the transcription factor NF- $\kappa$ B occur rapidly, indicating that *Ngo* infection causes posttranslational modifications of preexisting NF- $\kappa$ B. AP-1, as well as the NF-IL-6 transcription factor, have previously been shown to be involved in the transcriptional activation of the IL-6 gene by compounds such as PMA (42). Some evidence, however, indicates that AP-1 and NF-IL-6 do not participate in the immediate *Ngo*-induced upregulation of IL-6 transcripts. Transcriptional activation of the IL-6 promoter requires an intact NF- $\kappa$ B site, whereas deletion of the AP-1 enhancer element does not abolish inducibility by *Ngo* infection. Further, EMSA data clearly show that AP-1 DNA-binding activity occurs later than IL-6 mRNA up-

regulation, and DNA-binding activity of NF-IL-6 at the C/EBP-binding site in response to *Ngo* infection was not observed.

To test if NF- $\kappa$ B could sufficiently contribute to cytokine mRNA upregulation, we inhibited the degradation of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ , which usually undergoes complete proteolytic degradation after NF- $\kappa$ B stimulation (24). Inhibition of I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B activation was achieved using the cell-permeable serine protease inhibitor TPCK. Under these conditions, concomitant infection of HeLa cells with P<sup>+</sup> *Ngo* inhibited the upregulation of cytokine mRNAs of TNF- $\alpha$ , GM-CSF, IL-6, IL-8, and MCP-1 (Fig. 6). Thus, the immediate early transcription factor NF- $\kappa$ B has a major role in the transactivation of these cytokine genes in response to *Ngo* infection. In contrast, the cytokine gene promoters of IL-1 $\alpha$ , IL-1 $\beta$ , and TGF- $\beta$ , which exert either weak or no NF- $\kappa$ B enhancer elements, still showed upregulation of their mRNAs in response to *Ngo* infection. Interestingly, the elimination of the transactivation activity of NF- $\kappa$ B is used as an immune evasion strategy by the African swine fever virus. This virus produces an inhibitor of NF- $\kappa$ B, A238L, which shows homology to I $\kappa$ B $\alpha$ . A238L interacts with NF- $\kappa$ B to prevent transcription and causes an almost complete shutdown of proinflammatory cytokines (43).

Since both the adherent P<sup>+</sup> *Ngo* and the invasive Opa<sup>+</sup> *Ngo* strongly induce NF- $\kappa$ B, AP-1, and proinflammatory cytokines, we assume complex bacterial stimuli are responsible for triggering the multiple signals in human cells. Experiments are now in progress to determine which signaling pathways could account for the induction of the transcription factors NF- $\kappa$ B and AP-1 in *Ngo*-infected epithelial cells.

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