JOURNAL OF VIROLOGY, May 2005, p. 6043–6051 Vol. 79, No. 10
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.10.6043–6051.2005
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Constitutive Activation of the Transcription Factor NF-κB Results in Impaired Borna Disease Virus Replication

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Received 13 August 2004/Accepted 28 December 2004

The inducible transcription factor NF-κB is commonly activated upon RNA virus infection and is a key player in the induction and regulation of the innate immune response. Borna disease virus (BDV) is a neurotropic negative-strand RNA virus, which replicates in the nucleus of the infected cell and causes a persistent infection that can lead to severe neurological disorders. To investigate the activation and function of NF-κB in BDV-infected cells, we stably transfected the highly susceptible neuronal guinea pig cell line CRL with a constitutively active (IKK EE) or dominant-negative (IKK KD) regulator of the IKK/NF-κB signaling pathway. While BDV titers were not affected in cells with impaired NF-κB signaling, the expression of an activated mutant of IκB kinase (IKK) resulted in a strong reduction in the intracellular viral titer in CRL cells. Electrophoretic mobility shift assays and luciferase reporter gene assays revealed that neither NF-κB nor interferon regulatory factors (IRFs) were activated upon acute BDV infection of wild-type or vector-transfected CRL cells. However, when IKK EE-transfected cells were used as target cells for BDV infection, DNA binding to an IRF3/7-responsive DNA element was detectable. Since IRF3/7 is a key player in the antiviral interferon response, our data indicate that enhanced NF-κB activity in the presence of BDV leads to the induction of antiviral pathways resulting in reduced virus titers. Consistent with this observation, the anti-BDV activity of NF-κB preferentially spread to areas of the brains of infected rats where activated NF-κB was not detectable.

Borna disease virus (BDV), a noncytolytic single-stranded RNA virus, is the only known member of the *Bornaviridae* of the order *Mononegavirales* (51). BDV is highly neurotropic and cell associated and leads to a persistent infection of the central nervous system (54). BDV induces Borna disease (BD), a T-cell-mediated encephalomyelitis, in a wide variety of animals, including cattle, cats, dogs, and birds (reviewed in reference 31). Furthermore, BD virus, nucleic acids, antigens, and antibodies have been detected in the blood of patients with psychiatric diseases (2, 7, 29, 39, 35), although no direct correlation between BDV as a causative agent and a particular mental disorder in humans has yet been demonstrated. The infiltrating immune cells have been characterized as $CD4^+$ or $CD8^+$ T cells and macrophages (6, 13, 43). $CD8^+$ T cells represent the effector cell population (22, 36, 41, 46). No evidence has been presented that antibodies might contribute to neuropathology, although neutralizing antibodies apparently control viral tropism and can prevent the spread of virus from peripheral infection sites to the central nervous system (19, 52). Analyses of BDV-infected brain tissue revealed the presence of mRNAs encoding interleukin-1α (IL-1α), IL-1β, IL-2, IL-6, transforming growth factor beta, tumor necrosis factor alpha, and gamma interferon (IFN-γ) (42, 48). IFN-α/β was also found in BDV-infected cell cultures and was shown to inhibit BDV replication (20, 50, 56). In addition, a variety of chemokine-encoding mRNAs are upregulated in BDV-infected brain tissue (9, 25, 45).

The 8.9-kb negative-strand BDV genome is replicated in the nucleus of the infected cell and codes for at least six different known viral proteins (reviewed in reference 26). The nucleoprotein, which is involved in nuclear transport processes, is present both in the cytoplasm and in the nuclei of infected cells and forms complexes with the phosphoprotein and p10 (5, 59). At least one protein, p24, is phosphorylated at serine residues, suggesting that the function of this protein is controlled by cellular kinases (47, 55). Our knowledge of the interactions of BDV with host cell functions has increased during the last few years. It has been shown that BDV infection interferes with the activation of the Raf/MEK/ERK signaling cascade and that the blockade of this pathway results in reduced viral spread (21, 37). Furthermore, it has been demonstrated that the interaction of the viral nucleoprotein with the Cdc2/cyclin B1 complex results in prolongation of the G_2 phase (38).

The transcription factor NF-κB is involved in the regulation of many cellular processes, including apoptosis and host defense. A variety of stimuli initiate different signaling pathways leading to NF-κB activation. Most of these pathways converge on the IκB kinase (IKK) signalosome complex, which plays a major role in NF-κB activation (24). In addition, NF-κB is activated by multiple families of viruses, including human immunodeficiency virus type 1, human T-cell leukemia virus type 1, the hepatitis B and C viruses, Epstein-Barr virus, vesicular stomatitis virus (VSV), and influenza viruses (reviewed in references 23 and 32). While for some of these viruses, e.g., retroviruses or oncogenic viruses, the activation of this tran-

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scription factor may support viral replication, it is a general belief that NF-κB acts antivirally upon infection with RNA viruses such as VSV or influenza virus (11, 57). RNA virus infections commonly result in the activation of an innate antiviral response mediated by IFN-α/β. This antiviral program is initiated by viral induction of the IFN-β gene through constitutively expressed transcription factors, namely AP-1, interferon regulatory factor 3 (IRF-3), and NF-κB (58). A common viral inducer of NF-κB-dependent responses appears to be double-stranded RNA (dsRNA). Most RNA viruses, including BDV, produce dsRNA-like intermediates, representing a shared molecular pattern that may be sensed by the cell as an alert signal (33).

The interferon regulatory factors (IRFs) belong to a family of transcription factors that commonly possess a novel helix-turn-helix DNA binding motif. Their functional role is regulation of the host defenses, such as innate and adaptive immune responses and oncogenesis, which are mediated through interactions with themselves or other members of this family of transcription factors (54). IRF-3 and IRF-7 are especially important for the induction of an IFN-α/β response. While IRF-7 is an IFN-induced gene, the IRF-3 mRNA is expressed constitutively in all tissues (3). Both IRF-3 and IRF-7 reside in the cytoplasm in a latent form and translocate to the nucleus upon activation (30, 44, 58). Recent reports indicated that Toll-like receptors (TLRs) activate IRF-3 and NF-κB via a newly defined pathway involving inhibitor of κB kinase ε (IKKε) and TANK (TRAF family member-associated NF-κB activator)-binding kinase 1 (TBK-1) for IFN-α/β expression (reviewed in reference 4).

For the present study, the involvement of NF-κB during BDV infection was investigated. BDV infection did not lead to NF-κB activation in CRL cell cultures. An enhanced activation of NF-κB by the expression of an active mutant of IKK resulted in dramatically reduced BDV virus titers concomitant with a specific DNA binding activity of IRFs to an IRF-3/7-responsive element. This indicates that active NF-κB has an antiviral effect on BDV but that BDV avoids activating NF-κB in the host cell. This was also observed in vivo during acute BDV infections of rats, as BDV infectivity was not found in cells in which NF-κB was activated.

MATERIALS AND METHODS

Cell lines, virus, and animals. The guinea pig cell line CRL 1405 was subcloned, and cells that were highly susceptible to BDV infection were used as a standard laboratory cell line for BDV infection (19). Furthermore, Vero cells were used throughout this study. Cells were cultured in Iscove's modified Eagle's medium (IMDM) supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, and 100 U/ml gentamicin.

The fourth rat passage of the Giessen BDV strain He/80 was used for infections (40). In general, adherent cells were infected with a multiplicity of infection (MOI) of 0.01 to 1 in either 96-well or 6-well plates for 1 h in a volume of 25 μl (for 96-well plates) or 200 μl (for 6-well plates) of IMDM–2% FCS. For mock infections, 10% normal rat brain homogenate in IMDM–2% FCS was used. Thereafter, culture medium was added and the cells were cultivated for 5 to 7 days.

Female Lewis rats were obtained from the animal breeding facilities at the Friedrich Loeffler Institut, Bundesforschungsinstitut für Tiergesundheit, Tübingen, Germany. At the age of 6 weeks, the rats were infected intracerebrally in the left brain hemisphere with 0.05 ml of BDV, corresponding to 5×10^3 focus-forming units.

Retroviral infection of CRL 1405 cells. The retroviral expression vectors and stable producer cell lines used for this study were described previously (12). Two days before infection, CRL cells were seeded in six-well plates at a density of 5×10^4 cells/well. For infections, cell culture supernatants containing the retroviral vectors were added to the CRL cells and the culture plates were centrifuged at $100 \times g$ for 3 h. Thereafter, the supernatants were replaced with culture medium. The transfection efficiency was controlled for 2 days after infection, and if the transfection efficiency was below 20%, the infection procedure was repeated to increase the efficiency of infection/transfection, which ranged between 20 and 50%. The cells were cultured in the presence of 1 mg/ml zeocin (Invitrogen) for 2 weeks or until all cells were positive for green fluorescent protein (GFP), which was monitored by fluorometric analysis using a FACSCalibur flow cytometer (Becton Dickinson). Prior to each experiment, the cells were again screened for GFP fluorescence.

Electrophoretic mobility shift assay (EMSA). For preparations of nuclear extracts, 2×10^5 CRL cells were seeded in 60-mm cell culture dishes and either infected with BDV (MOI = 1) or left uninfected on the next day. After 6 days, uninfected cells were stimulated with tetradecanoyl phorbol acetate (TPA; 0.3 μg/ml) for 40 min. Cells were washed twice with cold phosphate-buffered saline (PBS) and then harvested in 400 μl of buffer A (10 mM KCl, 10 mM HEPES [pH 7.9], 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride). After 15 min of shaking at 4°C, 25 μl 10% Nonidet P-40 was added for 2 min, and the nuclei were pellet and resuspended in 50 μl of buffer B (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT). After 20 min of shaking and subsequent centrifugation, the protein concentrations of the nuclear extracts were determined (Bio-Rad, Germany). Eight micrograms of nuclear extract was incubated with 4 μg poly(dI-dC)^2 in binding buffer (0.1 M KCl, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1 mM DTT, and 10% glycerol). After 20 min, 2×10^4 to 5×10^4 cpm of a ^{32}P-labeled double-stranded oligonucleotide (5′-GATCCAGAGGGGACTTTCCG AGTAC-3′) was added to the mixture and further incubated for 10 min at room temperature. Thereafter, the samples were separated in nondenaturing 5% polyacrylamide gels. After drying, the gels were subjected to autoradiography.

Luciferase reporter gene assay. Cells (2.5×10^4) in 24-well plates were transfected with 0.05 μg 3× κB reporter plasmid and 0.1 μl Lipofectamine 2000 (Invitrogen). After 5 h, the cells were stimulated with 0.3 μg/ml TPA. Twenty-four hours after transfection, the cells were harvested in 100 μl of lysis buffer (50 mM sodium morpholineethanesulfonic acid [Na-MES], 50 mM Tris-HCl [pH 7.5], 0.2% Triton X-100, and 1 mM DTT). The lysates were centrifuged for 5 min at $15,000 \times g$. For determination of the luciferase activity, 20 μl of cleared lysate was added to 50 μl of assay buffer (125 mM Na-MES, 125 mM Tris-HCl [pH 7.5], 25 mM magnesium acetate, and 2.5 mg/ml ATP) and quantified in a luminometer (Lumat LB 9501) using 50 μl of substrate solution (0.5 mM Luciferin, 1 mM NaOH). For normalization, protein concentrations were determined by the Bradford method (Bio-Rad).

Infectivity assay. Virus infectivity was determined by the use of CRL 1405 cells. The cells were cultured for 7 days in the presence of different dilutions of BDV-infected cell lysates in flat-bottomed 96-well microtiter plates. Thereafter, cells were fixed with 4% paraformaldehyde-PBS and permeabilized with 1% Triton X-100–PBS. The presence of viral antigens was demonstrated by an immunohistochemical reaction using mouse anti-BDV monoclonal antibodies. The nonspecific binding of immunological reagents was blocked by incubation of the plates with 10% fetal calf serum-PBS. The reaction of monoclonal antibodies with cells was detected by use of a secondary anti-species biotin-labeled antibody (Dianova) and a streptavidin-peroxidase conjugate (Dianova). The reaction was visualized with *ortho*-phenylenediamine and H_2O_2 (Sigma).

Treatment with interferon. CRL cells were infected with BDV (MOI = 0.1) and at 3 days postinfection (p.i.) were treated with either 100, 10, or 1 U of universal IFN-α/β (PBL Biomedical Laboratories) for an additional 3 days in 3 ml of culture medium. Thereafter, the viral infectivity was determined by the standard assay described above.

Immunohistochemistry. Brain samples were obtained at different time points after infection and were fixed in 4% paraformaldehyde. All tissue sections were stained with hematoxylin-eosin. Immunohistochemistry was carried out for the presence of BDV-specific antigens, using an anti-BDV nucleoprotein-specific mouse monoclonal antibody (38/17C1) (55) as described previously (18), and for the detection of activated NF-κB, using a pNF-κB (p65) phospho-specific rabbit antibody (NEB) at a 1:50 dilution. The staining reaction was enhanced by use of a biotinylated secondary antibody (1:200). For detection, an ABC kit (Vector) was used.

RESULTS

Generation of stably transfected cell lines with mutated IKK/NF-κB signaling pathway. To investigate the role of the IKK/NF-κB signaling module in BDV infection, we used retroviral gene transfer to generate Vero cells and highly BDV-

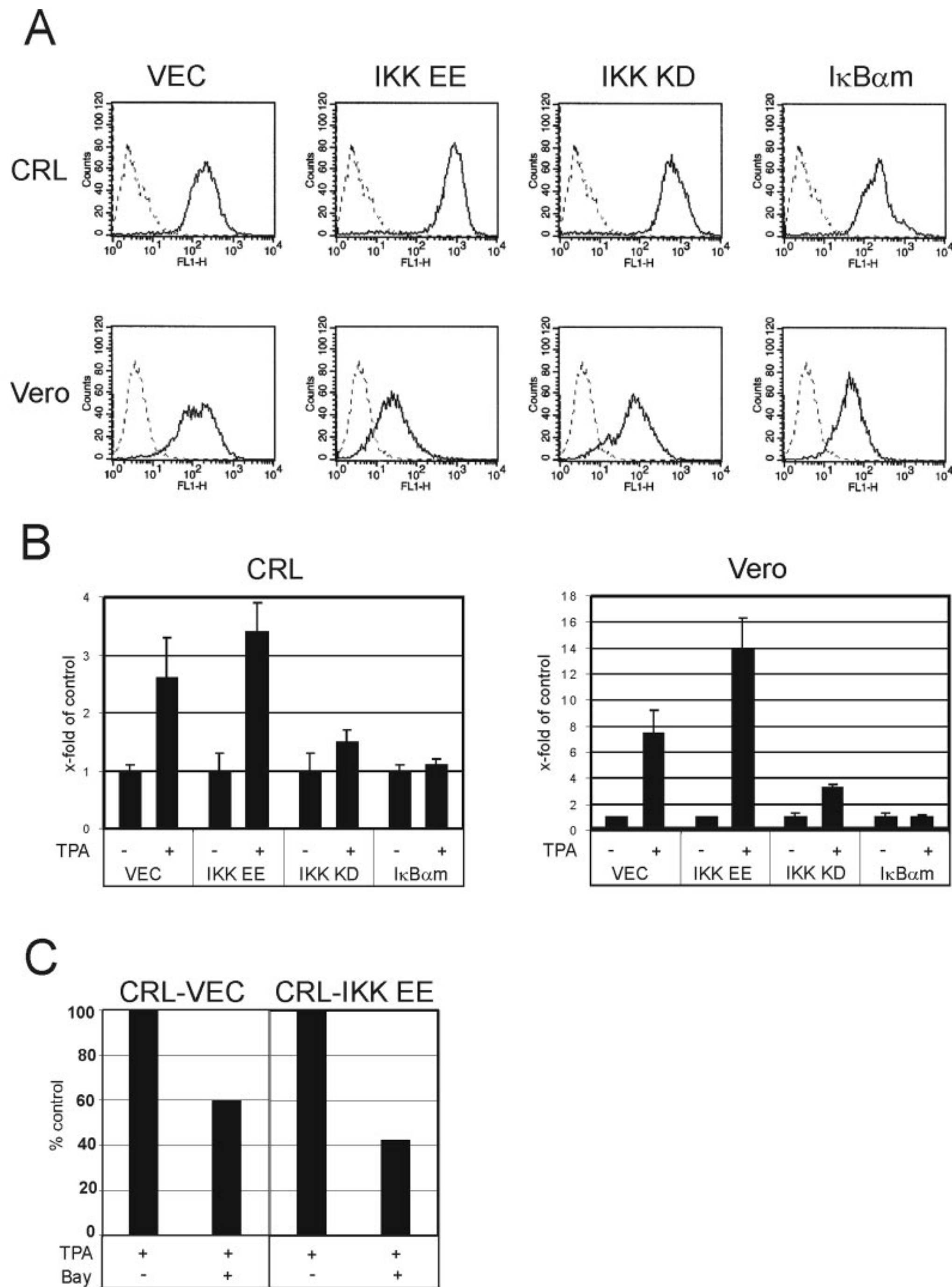

FIG. 1. Characterization of CRL and Vero IKK/IκB mutant cell lines. (A) Fluorescence-activated cell sorting analyses of stably transfected CRL and Vero cells harboring either the empty control vector (VEC), a constitutive form of IKK (IKK EE), a dominant-negative form of IKK (IKK KD) or an inactive mutant of IκB (IκBm). (B) NF-κB-luciferase reporter gene assay. Stably transfected Vero or CRL cells were transfected with a luciferase reporter gene plasmid driven by a 3× κB binding site containing an artificial promoter. Five hours after transfection, cells were treated with 0.3 μg/ml TPA and were harvested 16 h later for luciferase assays to indicate NF-κB activation. The bars represent averages and standard deviations of three independent transfections. Results are shown as fold increases over the control. The relative light units/μg of protein were 3,748.96 ± 122.56 for CRL-VEC cells and 10,193.07 ± 434.62 for CRL-IKK EE cells. (C) Inhibition of NF-κB with Bay 117082 (1 μM).

susceptible CRL cells stably overexpressing IKK either as dominant-negative (IKK KD) or constitutively active (IKK EE) mutants. Furthermore, a nondegradable mutant of the inhibitor of NF-κBα (mIκBα) and a control cell line expressing the empty retroviral vector (VEC) were generated. Since the vector used expresses GFP from the same mRNA as the transgene, successful transduction could be controlled by fluorocytometric analysis prior to each experiment of this study (Fig. 1A). TPA stimulation resulted in the transcriptional activation of NF-κB in CRL and Vero cells stably transfected with the

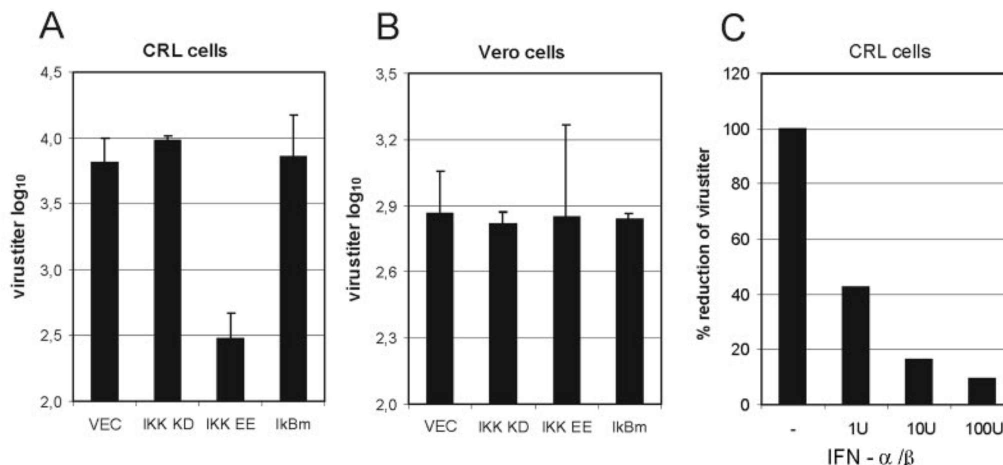

FIG. 2. Reduction of infectious virus in CRL-IKK EE cells is due to IFN-α/β. CRL cells (A) and Vero cells (B) were infected with BDV (MOI = 0.01) and cultured for 6 days. Thereafter, the cells were harvested and the infectivity of 2×10^6 cells/ml was determined by a standard focus-forming assay. (C) Inhibition of BDV by IFN-α/β. CRL cells were infected with BDV (MOI = 0.1) and at 3 days p.i. were treated with either 100, 10, or 1 U of human IFN-α/β. After 6 days of cultivation, the cells were harvested and the BDV infectivity was estimated by a standard focus-forming assay. The bars represent averages and standard deviations of three independent virus titrations.

VEC construct or IKK EE. Reduced transcriptional activation of NF-κB was found in IKK KD cells, while mIκBα cells showed an efficient block of NF-κB activation, as demonstrated with the luciferase reporter gene assay (Fig. 1B). Thus, IKK EE expression resulted in a slightly enhanced NF-κB activity, while in cells expressing the dominant-negative mutant, NF-κB was functionally knocked down. The relative light units/μg of protein presented in Fig. 1B were 3,748.96 ± 122.56 for CRL-VEC cells and 10,193.07 ± 434.62 for CRL-IKK EE cells without TPA stimulation, demonstrating that NF-κB activity was already increased in IKK EE cells compared to controls. Furthermore, NF-κB activity induced by TPA stimulation could be inhibited by using the specific inhibitor Bay 117082 (Fig. 1C).

Reduced BDV titers in CRL-IKK EE cells. IKK/IκB mutant CRL cell lines were infected with BDV at an MOI of 0.01 for 6 days to scrutinize whether manipulation of the IKK/NF-κB signaling pathway has an influence on BDV replication. Cells were lysed and virus titers were determined by use of a standard focus-forming assay. In CRL-VEC, CRL-IKK KD, and CRL-mIκBα cells, similar virus titers of roughly 4.0 $\log_{10}/10^6$ cells were found. In contrast, in CRL-IKK EE cells, the virus titers were strongly reduced (2.5 $\log_{10}/10^6$ cells) compared to the other mutant cell lines (Fig. 2A). The same results were obtained when CRL mutants were infected at an MOI of 0.1 (for CRL-VEC, 4.1 $\log_{10}$; for CRL-IKK KD, 4.1 $\log_{10}$; for CRL-IKK EE, 2.6 $\log_{10}$; for CRL-mIκBα, 4.4 $\log_{10}$). Since Vero cells have a chromosomal deletion of the alpha and beta-1 interferon genes, they are not able to produce IFN-α/β (14). IKK/IκB mutant Vero cell lines were infected with BDV at an MOI of 0.01 for 6 days to further investigate the role of the IKK/NF-κB signaling pathway and IFN-α/β in BDV replication in more detail. Although in contrast to the case for CRL cells, BDV virus titers were generally lower in Vero-VEC, Vero-IKK KD, and Vero-mIκBα cells (roughly 2.8 $\log_{10}/10^6$ cells), no reduction in the BDV infectivity of Vero-IKK EE cells was found (Fig. 2B). Again, after infection at an MOI of 0.1, similar results were found (data not shown). These results indicate that BDV infectivity seems to be controlled by active NF-κB, most likely through the induction of IFN-α/β. Therefore, supernatants of BDV-infected CRL-IKK/IκB mutant cell lines were collected and tested for IFN-α/β activity in a VSV-based bioassay. Interferon was detectable in small amounts only in the supernatants of BDV-infected CRL-IKK EE cells, but not in those of other infected cell lines or uninfected cells (data not shown). To demonstrate that IFN-α/β is able to control BDV in cell culture, we infected CRL cells with BDV, and 3 days after infection, treated them with IFN-α/β. Surprisingly, at this time already a treatment with 1 U of IFN-α/β led to a 50% reduction in the virus titer. Incubation with 100 U of IFN-α/β resulted in a reduction of 90%, indicating the high sensitivity of BDV to IFN-α/β (Fig. 2C).

Electrophoretic mobility shift assay revealed no activation of NF-κB by BDV. To analyze whether BDV as an RNA virus interferes with the IKK/NF-κB signaling cascade, we infected the CRL-IKK/IκB mutant cell lines with BDV (MOI = 1) for 6 days. Thereafter, the cells were harvested and the nuclei were isolated and screened in an EMSA for DNA binding activity of activated NF-κB as described in Materials and Methods. As shown in Fig. 3A, neither CRL-VEC cells that functioned as a negative control nor CRL-IKK EE, CRL-IKK KD, or CRL-mIκBα cells showed NF-κB binding after BDV infection. In contrast, when cells were stimulated with TPA, NF-κB binding was found for CRL-VEC and CRL-IKK EE cells. In addition, NF-κB activation in CRL-IKK KD cells was reduced compared to that in CRL-VEC and CRL-IKK EE cells, while in CRL-mIκBα cells, TPA was unable to stimulate NF-κB (Fig. 3A).

Besides the viral activation of NF-κB, the activation of IRF-3/7 is another hallmark of an efficient antiviral response leading to IFN-α/β production. Therefore, the binding of IRFs to the PRI/III region of the IFN-β promoter, the sequence to which IRF-3 and -7 bind, was investigated during BDV infection. No specific IRF-DNA binding was observed for wild-type CRL cells (Wt) or CRL-VEC cells up to 120 h postinfection (p.i.). In contrast, specific PRI/III binding complexes were

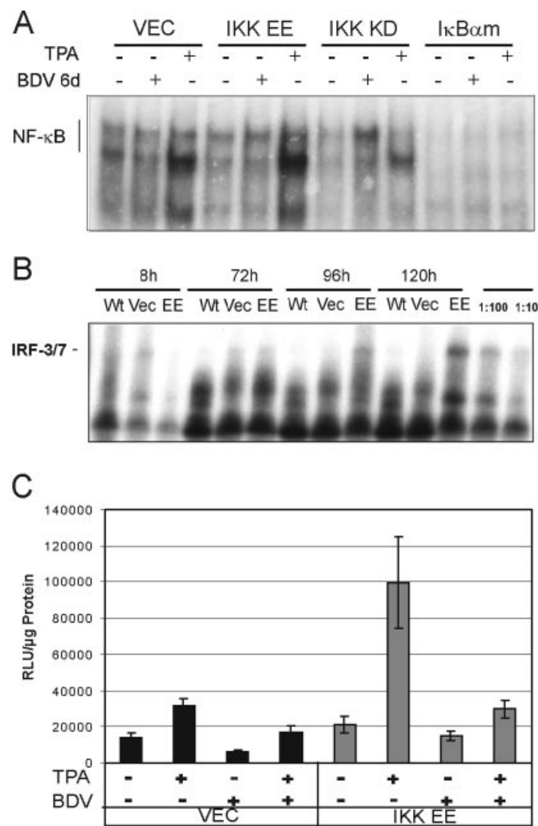

FIG. 3. Lack of NF-κB activation after BDV infection. For EMSAs, IKK/IκB mutant CRL cells were infected with BDV (MOI = 1) on day 0 or left uninfected, and the cells were stimulated with TPA (0.3 μg/ml) for 40 min on day 6 p.i. Thereafter, nuclear extracts were isolated and incubated with ^{32}P-labeled double-stranded oligonucleotides specific for NF-κB (A) or IRF-3/7 (B). The samples were then separated in nondenaturing 5% polyacrylamide gels. After drying, the gels were subjected to autoradiography. (C) NF-κB-luciferase reporter gene assay. Stably transfected CRL-VEC or CRL-IKK EE cells were infected with BDV (MOI = 1), and 6 days later, these BDV-infected cells together with uninfected cells were seeded into 24-well plates and transfected with a luciferase reporter gene plasmid driven by a 3× κB binding site containing an artificial promoter on the following day. Six hours after transfection, uninfected cells and individual samples of BDV-infected cells were treated with 0.3 μg/ml TPA and harvested 16 h later for luciferase assays. The bars represent averages and standard deviations of three independent transfections.

found for BDV-infected CRL-IKK EE cells at 96 h p.i. and were more pronounced 120 h after infection. To verify the specificity of binding, we coincubated samples with unlabeled competitive PRI/III specific probes at two different concentrations (1:100 and 1:10). Consequently, the IRF-specific oligonucleotide complex disappeared due to competition (Fig. 3B, right end).

Furthermore, we investigated whether BDV infection leads to the activation of NF-κB transcriptional activity in an NF-κB promoter-dependent reporter gene assay. In this case, TPA stimulation of CRL-VEC or CRL-IKK EE cells resulted in NF-κB activation (Fig. 3C). In contrast, BDV infection of CRL-VEC or CRL-IKK EE cells did not result in NF-κB-dependent transcription activity. More surprisingly, even the activation of BDV-infected CRL-VEC or CRL-IKK EE cells with TPA did not result in an increase in NF-κB activity compared to control cells (Fig. 3C). This might suggest that BDV inhibits NF-κB activation with a block that cannot be overcome by TPA stimulation.

Distribution of active NF-κB and BDV infectivity in the rat brain. Activated NF-κB, defined by immunohistological staining using a phospho-specific antibody directed against the p65 subunit, is regularly found in cells of the normal rat brain (28). Depending on the rat strain, phosphorylated p65 can be detected either in the hippocampus or in the cortex. In the Lewis rat, NF-κB activity is more pronounced in the cortex, but a few neurons with activated NF-κB are also detectable in the hippocampus (28). After BDV infection of Lewis rats with a rat-adapted virus strain, BDV was first detected in the hippocampus. During infection, the virus spread to the cortex and the cerebellum. By day 21 p.i., BDV-infected cells could be found in almost all compartments of the brain. When the distributions of activated NF-κB and a BDV-specific nucleoprotein were compared, it was obvious that in the early phase of the disease, between day 12 and day 15 p.i., BDV was found in areas where activated NF-κB was almost absent. As shown in Fig. 4A and B, phospho-p65-specific staining was predominantly found in an area of the cortex. In the same region considerably less BDV antigen was detectable, as demonstrated by the use of serial brain sections; in contrast, BDV was found in a region closely adjacent to areas with activated NF-κB (Fig. 4C and D). The observation that BDV was almost absent in areas with NF-κB activity was supported by the distribution of BDV in Purkinje cells of the cerebellum. When we investigated serial brain sections for the presence of BDV and activated NF-κB, we predominately found BDV infection only in Purkinje cells in which NF-κB was not activated (Fig. 4E and F).

In contrast, in the entire hippocampal area only a few cells were found to be positively stained for phospho-p65 (Fig. 4G) by day 12 p.i., whereas the BDV antigen could easily be detected in large amounts, mostly in the CA3 region of the hippocampus (Fig. 4H). Most interestingly, not only was the number of cells that were positive for activated NF-κB in the hippocampus different from that in the cortex, but also the distribution of NF-κB within the cell differed. In the hippocampus, phospho-p65-specific signals were predominately found punctually around the nuclei (Fig. 5A), while in the cortex the nuclei of the neuronal cells were fully stained (Fig. 5B). At later time points after BDV infection, activation of NF-κB in the hippocampus was found, as exemplified on day 18 p.i. (Fig. 5C). Thus, during the early phase of the disease, BDV prefers areas where activated NF-κB is less dominant or even absent, whereas later during infection activated NF-κB and BDV are both found in conjunction throughout the brain.

DISCUSSION

A wide variety of viruses activate the transcription factor NF-κB. Although this transcription factor may support the replication of some of these viruses (e.g., retroviruses or oncogenic viruses) (23), in general RNA virus-mediated NF-κB activation appears to act antivirally. Infections with these viruses commonly result in the activation of an innate antiviral response mediated by IFN-α/β. The antiviral activity of the host cell is initiated by immediate viral induction of the IFN-β

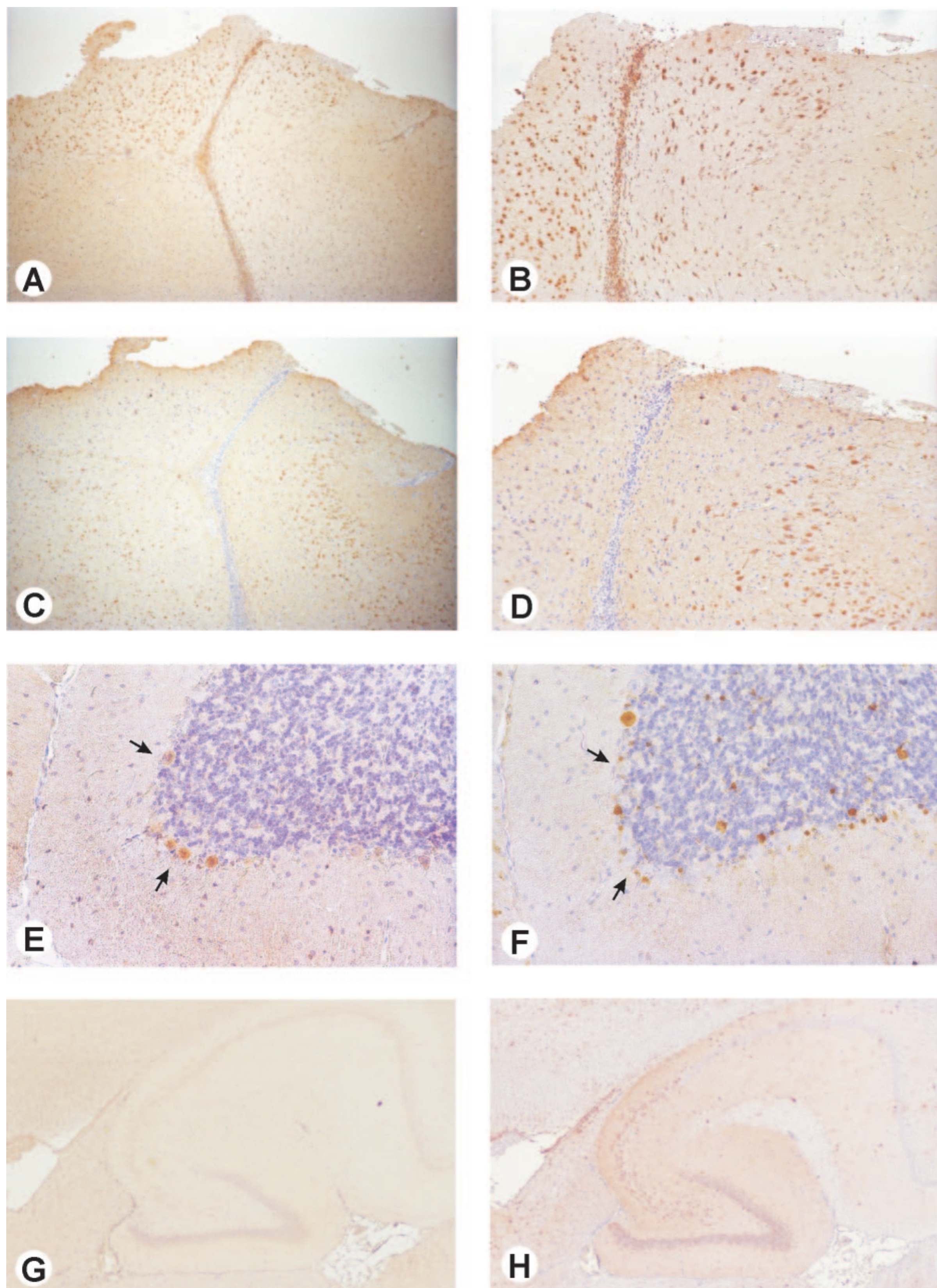

FIG. 4. Immunohistochemistry revealed different distributions of NF-κB and BDV in the rat brain. Brain samples were obtained at different time points after infection and were fixed in 4% paraformaldehyde. (A) Distribution of activated NF-κB in the cortex of a BDV-infected rat brain (18 days p.i.) detected with the pNF-κB p65-specific rabbit antibody. Magnification, ×10. (B) Same section as panel A. Magnification, ×20. (C) Distribution of BDV in the cortex of a rat brain (18 days p.i.) detected with an anti-nucleoprotein mouse monoclonal antibody (38/17C1).

gene. This process requires the cooperative activation of several transcription factors, namely, AP-1, IRF-3, IRF-7, and NF-κB (11). The constitutively expressed IRF-3 protein and the inducible factor IRF-7 are the critical transcription factors required for virus-induced IFN production. IRF-3 resides in the cytoplasm in a latent form and translocates to the nucleus upon viral infection and in response to dsRNA (reviewed in reference 54). Recently, it was demonstrated that IKKε/TBK-1, an IKK-related kinase, is responsible for IRF-3 and IRF-7 phosphorylation (15, 49).

BDV is a nonsegmented RNA virus whose replication occurs in the nuclei of infected cells. BDV leads to a persistent infection in vitro and in vivo. In the present study, we demonstrated that BDV infections of highly susceptible CRL cells did not induce NF-κB or IRF-3/7 activation. Only in CRL cells with constitutively activated IKK did BDV infection lead to the translocation and consequently activation of IRFs as late as 96 h p.i., becoming more pronounced 120 h after infection. This late activation of IRFs by BDV is not surprising since BDV replication is rather slow (8). The fact that IRF activation was not found in CRL cells after BDV infection was more surprising. Recently, it was shown that the NS3/A4 viral protease of hepatitis C virus is able to suppress IRF-3 activation and consequently promote hepatitis C virus replication (16). Whether a similar mechanism is used by BDV cannot be determined since no protein with a similar activity to that of NS3/A4 has been identified for BDV so far. We were also unable to find an activation of NF-κB after BDV infections of CRL cells. Even the costimulation of BDV-infected cells with TPA did not cause the NF-κB activity levels seen in CRL-VEC or CRL-IKK EE cells that were stimulated with TPA alone. Thus, it seems that a viral compound might be responsible for causing inhibition of the activation of NF-κB that cannot be overcome with TPA, a potent NF-κB activator.

Again, the question rises as to whether BDV fails to activate NF-κB or has an active inhibitory effect on NF-κB/IRF activation. Recently, it was shown that IKKε and TBK-1 are responsible for the coordination of IRF-3/NF-κB activation (15). It has been proposed that Toll-like receptor 3 (TLR3) recognizes dsRNA provided through viral infection (1). TLR3 can stimulate a multicomponent protein complex that activates IKKε and TBK-1 (15). Thus, one might argue that BDV actively inhibits the IRF/NF-κB activation that is performed by viral interference within this regulatory protein complex. This hypothesis is supported by the fact that IRF activation and IFN production are indeed found in BDV-infected CRL-IKK EE cells. Since IKKε and TBK-1 synergize with TANK (TRAF family member-associated NF-κB activator) to promote an interaction with IKK-γ of the IKK signalosome complex (10), the constitutive activation of IKK could provide an appropriate amount of the TANK/IKKε/TBK-1 complex required for IRF activation. As a possible result of this activation, which leads to IFN-α/β production, BDV replication was reduced in BDV-

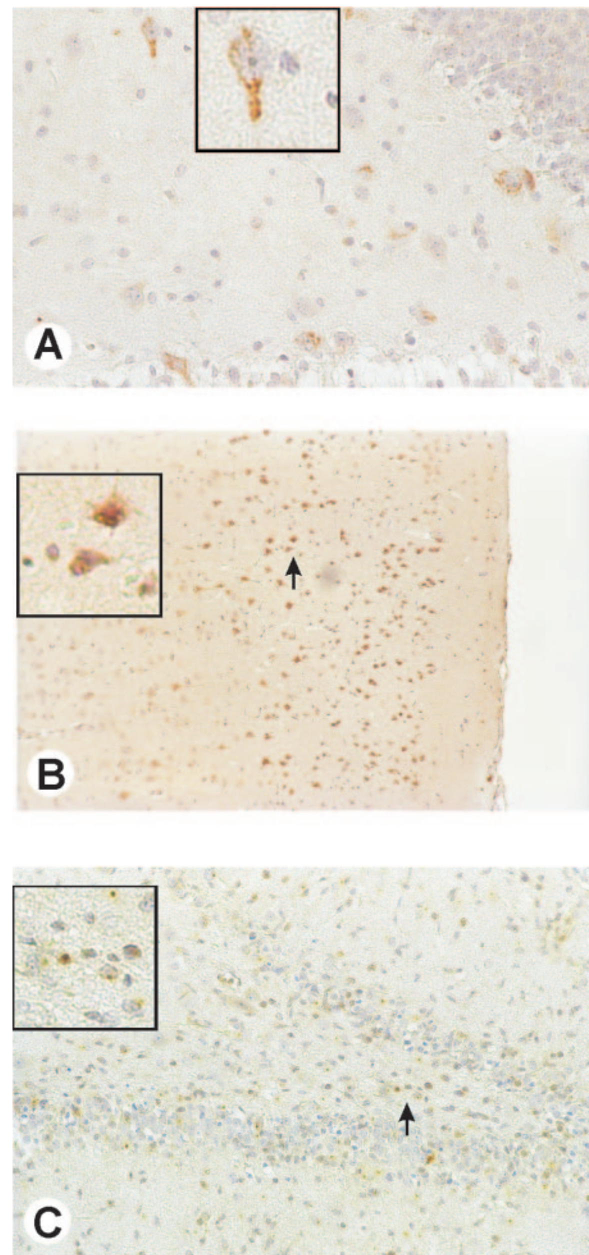

FIG. 5. Activation of NF-κB in the hippocampus late after BDV infection. Brain samples were fixed in 4% paraformaldehyde. (A) Perinuclear p65 staining in the hippocampus, as detected with the pNF-κB p65-specific rabbit antibody. Magnification, ×40. (B) Nuclear staining of p65 in cells of the cortex. Magnification, ×20. (C) Nuclear staining of p65 in cells of the hippocampus 18 days after BDV infection. Magnification, ×20. Arrows indicate areas of enlargement. The staining reaction was enhanced by the use of a biotinylated secondary antibody. For detection, an ABC kit was used.

Magnification, ×10. (D) Same section as panel C. Magnification, ×20. (G) NF-κB staining of the hippocampus (day 12 p.i.). Magnification, ×10. (H) The serial section shown in panel E was used for BDV staining of the hippocampus (day 12 p.i.). Magnification, ×10. (E) NF-κB p65 staining in the cerebellum at 18 days p.i. Magnification, ×40. (F) Detection of BDV nucleoprotein in the cerebellum 18 days after BDV infection. Magnification, ×40. Arrows indicate Purkinje cells. The staining reaction was enhanced by the use of a biotinylated secondary antibody. For detection, an ABC kit was used.

infected CRL-IKK EE cells and IFN was detectable exclusively in CRL-IKK EE cells, not in CRL-VEC, CRL-IKK KD, or CRL-mIκBα cells. Since the amount of IFN was low in the supernatants of CRL-IKK EE cells, one might argue that a second signal other than NF-κB is required for IFN activation. In contrast, when Vero mutant cell lines were used for BDV infection, no inhibition of BDV replication was found, even in Vero-IKK EE mutant cells. Since Vero cells have a genetic defect in interferon production (14), Vero-IKK EE cells are not able to produce IFN even after BDV infection. When IFN-α/β was added to either CRL cells (Fig. 2C) or Vero cells (20), BDV replication was efficiently blocked, indicating that BDV replication in general is susceptible to IFN (56). This is further supported by the fact that BDV can replicate in cultured embryo cells of IFN-α/β knockout mice but not in wild-type control cells (50). Nevertheless, in this respect the in vivo situation might be rather different. For example, IFN-α can be demonstrated by reverse transcription-PCR to be present in the brains of normal rats, and IFN-α-specific mRNA does not significantly increase during persistent BDV infection (48). Since reverse transcription-PCR cannot distinguish between different brain areas, the question of whether during the early phase of BDV infection the virus is only found in areas where IFN-α is not present or if in vivo the effectiveness of IFN-α is limited still remains unanswered.

To address the possible in vivo relevance of our in vitro findings, we infected adult Lewis rats with BDV. The distribution and correlation of BDV with NF-κB activation were examined by immunohistochemistry. Activation of NF-κB was detected with an antibody directed against the phosphorylated p65 (RelA) subunit of the dimeric NF-κB transcription factor. Active NF-κB was found in the cerebral cortex, and to a lesser extent, in the hippocampus of the normal rat brain. Activation of the transcription factor NF-κB plays a critical role in preventing neuronal cell death (53, 60). In contrast, the inhibition of NF-κB promotes apoptosis in neurons (17, 27). Consequently, activated NF-κB seems to participate in normal rat brain functioning, reflecting a distinct state of neuronal activity or differentiation (28). At early time points after BDV infection, BDV spread to regions where NF-κB was not activated. During the peak of BDV infection, between days 18 and 21 p.i., cells expressing viral antigens were found in the hippocampus and the cortex and also to a lesser extent in the cerebellum. At this time point, the activation of NF-κB was increased, most probably due to the presence of cytokines that promote NF-κB activation in BDV-infected rat brains (34, 42, 45, 48). The fact that NF-κB promotes neuronal activity or differentiation seems contradictory to the biology of BDV. Recently, we reported that BDV prefers slow proliferative host cells and that BDV is able to reduce the host cell cycle by interfering with the Cdc2/cyclin B1 complex (38). Therefore, it is tempting to speculate that BDV avoids the infection of neurons with active NF-κB during the early phase of infection to ensure effective virus spread and, most obviously, viral persistence. In contrast, at the peak of BDV infection, at about day 21 p.i., BDV infectivity and NF-κB activity were found in the same regions of the brain, indicating that the inhibitory effect of BDV on NF-κB activity can be overcome in vivo, most probably due to the activation of various cytokines that function as potent NF-κB activators in the brain, such as interleukin-1 (38).

In summary, these findings demonstrate that after BDV infection, the hallmark transcription factors IRF and NF-κB that are necessary for the expression of various genes involved in the innate immune response of the virus-infected cell are not activated in CRL cells. Whether this is due to active inhibition by the virus itself and if this observation can be generalized cannot be answered yet. Our in vivo findings for BDV-infected rats show a negative correlation between the activation of NF-κB and viral replication, and we therefore propose that nonactive IRF-3/NF-κB is a prerequisite for the establishment of a persistent infection both in vitro and in vivo.

ACKNOWLEDGMENTS

We thank Lothar Stitz for a critical reading of the manuscript and for helpful discussions. Furthermore, we thank Winfried Kramer and Mandy Laukner for technical assistance.

This study was supported in part by grants Pla 256/1-2 and WO 554/2-1 of the Deutsche Forschungsgemeinschaft and by grant 729.59-8/1 of the Forschungsprogramm Baden-Württemberg. Furthermore, this work is part of the activities of the VIRGIL European Network of Exellence on Antiviral Drug Resistance supported by a grant (LSHM-CT-2004-503359) from the Priority 1 "Life Sciences, Genomics and Biotechnology for Health" programme in the 6th Framework Programme of the EU.

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