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Cowpox Virus and Other Members of the Orthopoxvirus Genus Interfere with the Regulation of NF-κB Activation

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NF-κB comprises a family of transcription factors that regulate key immune processes. In this study, the effects of orthopoxvirus infection upon the activation of NF-κB were examined. During the early phase of infection, cowpox virus can inhibit the induction of NF-κB-regulated gene expression by interfering with the process of IκBα degradation. Although either okadaic acid or tumor necrosis factor (TNF) treatment of infected cells can induce IκBα phosphorylation, further processing of IκBα is inhibited. These results suggest that cowpox virus is capable of inhibiting the activation of NF-κB at a point where multiple signal transduction pathways converge. Other orthopoxviruses affect NF-κB activity, but in a type-specific manner. Raccoonpox virus and vaccinia virus (Copenhagen strain) negatively affect NF-κB induction by TNF. In contrast, the modified vaccinia virus Ankara strain induces NF-κB activation, even in the absence of other stimuli. These findings suggest that orthopoxviruses may affect a broad range of virus–host interactions through their effects upon NF-κB activation. Moreover, because of the central role for NF-κB in immune processes and disease, these type-specific effects may contribute significantly to the immunogenic and pathogenic properties of poxviruses.

Key Words: NF-κB; IκB; TNF; cowpox; poxvirus; pathogenesis; immunity; modified vaccinia Ankara.

INTRODUCTION

NF-κB comprises a family of transcription factors that regulate the expression of numerous genes encoding proteins that are crucial elements of innate and acquired immune responses. In particular, NF-κB increases the expression of many genes, such as those encoding MHC class I, the TAP1 peptide transporter, adhesion molecules, cellular inhibitor of apoptosis proteins (c-IAPs), interferon, tumor necrosis factor (TNF), and other pro-inflammatory cytokines, which are important in response to viral infection (reviewed by Baldwin, 1996; Blackwell and Christman, 1997; Ghosh *et al.,* 1998; Foo and Nolan, 1999). NF-κB also regulates the expression of genes involved in the control of cell proliferation and apoptosis (reviewed by Chen *et al.,* 1999; Baldwin, 2001a,b).

The potency and the broad range of effects of NF-κB-regulated gene expression dictate that the activation of NF-κB transcription factors must be stringently controlled. Current information on the signaling pathways leading to the activation of NF-κB has recently been reviewed by Karin and Ben-Neriah (2000). The primary mechanisms of control can be summarized as follows. In unstimulated cells, the majority of NF-κB is held in the cytoplasm by members of the IκB family of inhibitor proteins; for example, IκBα can control the activation of p50/p65 heterodimer NF-κB. In stimulated cells, NF-κB translocates to the nucleus as a result of the IκBα being degraded. This degradation can be initiated by a variety of stimuli, including cytokines such as TNF, interleukin-1β (IL-1β), and interleukin-18 (IL-18); lipopolysaccharide; phosphatase inhibitors such as okadaic acid; and the activation of the dsRNA-dependent protein kinase (PKR). Each of these factors can induce the activation of kinases, notably the IκB kinase (IKK) complex, which ultimately phosphorylates two serines (ser 32 and ser 36) near the amino terminus of IκBα. The phosphorylated IκBα is recognized as a substrate for ubiquitination, which targets it for degradation by the 26S proteasome. Once released from IκBα, NF-κB translocates to the nucleus where it can function as a transcriptional activator.

The potency and broad range of effects of NF-κB-regulated gene expression also suggest that either viral inhibition, or alternatively, viral induction, of NF-κB activity will markedly affect the interactions between the virus and the host. The nature of these interactions is likely to have important consequences for viral replication, the pathogenic effects of virus infection, and the immunogenic effects of live virus vaccines.

Viruses of several families actively inhibit immune responses (reviewed by Spriggs, 1996; Tortorella *et al.,* 2000), but among these, the poxviruses are especially adept at interfering with immune processes (reviewed by Smith, 1999; Alcami and Koszinowski, 2000; Barrett *et al.,*

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2001; Moss and Shisler, 2001). There are two major contributory factors for this. First, poxvirus genomes typically encode 150–200 proteins. As a result, poxviruses have greater capacities than many other viruses to encode proteins that interfere with host immune responses. Second, in comparison to other DNA viruses, the poxviruses are unusually independent of the host transcriptional machinery, because they encode most of the enzymes and factors needed to effect viral transcription. This property contributes to the abilities of the poxviruses to interfere with the expression of host genes without adversely affecting the expression of viral genes. For these reasons the poxviruses are especially well-suited to affect the activation of NF-κB.

Poxviruses are likely to benefit the most from specific mechanisms for affecting NF-κB function either in infected cells during the early phase of virus replication or in uninfected cells prior to their infection. In general during these phases, host-cell gene expression appears to be fully functional, suggesting that such cells should retain their responsiveness to NF-κB-activating stimuli, as well as the potential to effect NF-κB-regulated gene expression.

Many poxviruses are already known to encode proteins that could affect the activation of NF-κB. For example, several poxviruses produce soluble, secreted versions of receptors for the cytokines TNF, lymphotoxin-α, IL-1β, IL-18, and CD153, each of which can activate NF-κB (Smith *et al.,* 1990, 1991, 1996; Hu *et al.,* 1994; Loparev *et al.,* 1998; Saraiva and Alcami, 2001; unpublished data). Also, some poxviruses encode caspase inhibitors, such as CrmA, which can inhibit caspase-1-mediated processing of pro-IL-1β and pro-IL-18 to the mature, active forms (Ray *et al.,* 1992). There are poxvirus inhibitors of PKR, which might interfere with PKR-dependent activation of NF-κB (Chang *et al.,* 1992; Davies *et al.,* 1992; Kumar *et al.,* 1994). Additionally, it was recently reported that vaccinia virus encodes two proteins that share amino acid similarity with the Toll/IL-1 receptor (TIR) domain and that these are capable of antagonizing signal transduction through IL-1 and Toll-like receptors (Bowie *et al.,* 2000). Conversely, many poxviruses, such as various strains of vaccinia and variola viruses, lack several of these potential inhibitors of NF-κB activation (Goebel *et al.,* 1990; Massung *et al.,* 1993; Shchelkunov *et al.,* 1993; Antoine *et al.,* 1998). Furthermore, many poxviruses encode proteins such as vaccinia growth factor, a protein similar to epidermal growth factor, which is capable of the activation of NF-κB (Blomquist *et al.,* 1984; Brown *et al.,* 1985; Twardzik *et al.,* 1985; Sun and Carpenter, 1998; Biswas *et al.,* 2000). Therefore, different poxviruses may have the potential capacity to affect the activation of NF-κB in a variety of different ways.

Interference with the activation of NF-κB represents an exceptional strategy that a poxvirus could use to counter multiple immune processes through the targeting of a single regulatory process. However, the currently characterized poxvirus proteins that might interfere with NF-κB activation have the potential to affect only a few of the many stimuli and signal transduction pathways that can induce this transcription factor. Moreover, the primary functions of these viral proteins may be unrelated to their potential effects upon NF-κB activation. Despite the importance of NF-κB in immune processes, little is known about the effects of either particular poxvirus proteins, or poxvirus replication in general, upon NF-κB activation in virus-infected cells. Therefore, in this study we have examined the effects of various poxviruses upon NF-κB activation. Further, we have examined the hypothesis that poxviruses may have the ability to interfere comprehensively with the activation of NF-κB by specifically targeting regulatory events that are common to the multiple pathways leading to the activation of NF-κB.

RESULTS

Cowpox virus can specifically inhibit NF-κB-regulated gene expression

To determine whether cowpox virus interferes with NF-κB function, we examined the expression of authentic and artificial NF-κB-regulated genes in virus-infected cells.

First, an RNase protection assay was used to compare the effects of cowpox virus infection and TNF treatment on the quantities of the mRNAs of 11 genes, three of which, *traf1*, *c-iap-1*, and *c-iap-2*, are known to be regulated by NF-κB (Chu *et al.,* 1997; Wang *et al.,* 1998; Schwenzer *et al.,* 1999). TNF stimulation of uninfected HeLa cells, over a 90-min period, resulted in the increased accumulation of TRAF1, c-IAP-1, and c-IAP-2 mRNAs (Fig. 1, lanes 4–7). However, in the virus-infected cells, there was no observable increase in the quantities of these three mRNAs either as a result of the virus infection (Fig. 1, lane 8, in comparison to lane 4) or in response to TNF treatment (Fig. 1, lanes 9–11 in comparison to lanes 5–7). Neither cowpox virus infection nor TNF treatment affected the accumulation of the RNAs of the other genes, suggesting that the virus may specifically affect the expression of NF-κB-regulated genes.

Second, the effects of cowpox virus infection upon the expression of the firefly luciferase gene under the control of an NF-κB-regulated promoter were examined. For control purposes, the effects of virus infection upon the expression of a cotransfected sea pansy luciferase gene, under the control of a constitutively active promoter, were also examined. Transfected HEK 293T cells infected with cowpox virus showed almost no change in firefly luciferase activity in response to TNF treatment,

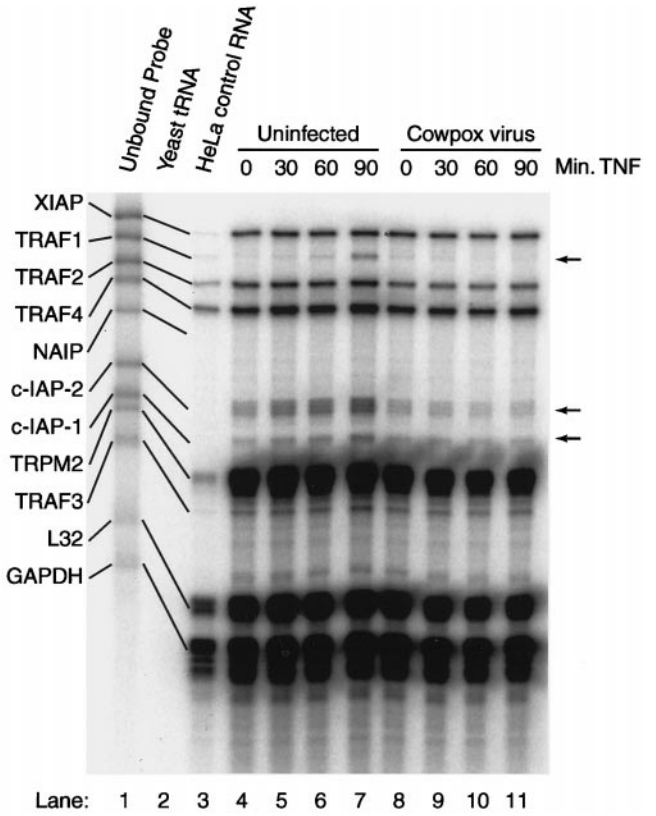
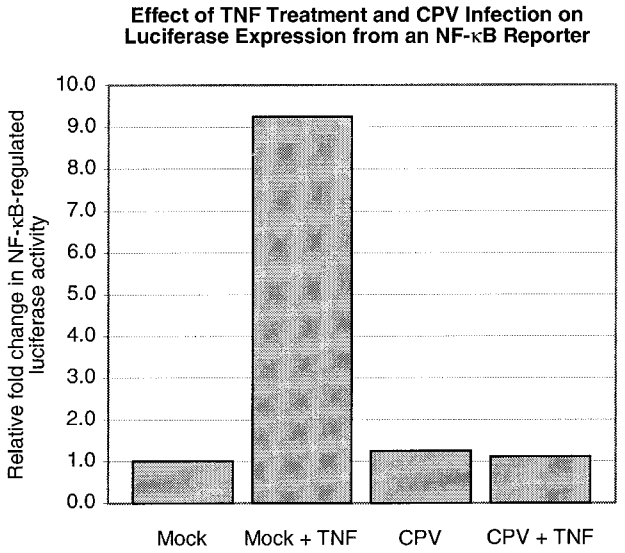


FIG. 1. In HeLa cells infected with cowpox virus, quantities of the mRNAs of NF-κB-regulated genes *traf1, c-iap-1,* and *c-iap-2* are not increased either by virus infection or by TNF treatment. Four hours after infection, uninfected HeLa cells (lanes 4–7) or cowpox virus-infected HeLa cells (10 PFU/cell; lanes 8–11) were treated with TNF (20 ng/ml) for the indicated periods of time. Total cellular RNAs were harvested and analyzed by RNase protection assay using anti-sense RNA probes generated from cDNA templates derived from the indicated genes. Arrows indicate the positions of the protected fragments of TRAF1, c-IAP-1, and c-IAP-2 RNAs. Lane 1 contains unbound probes; lanes 2 and 3 contain assay products resulting from probe hybridization to negative-control yeast tRNAs and positive-control HeLa cell RNAs, respectively. Abbreviations are as follows: XIAP, X chromosome-linked inhibitor of apoptosis protein; TRAF, tumor necrosis factor receptor-associated factor; NAIP, neuronal apoptosis inhibitory protein; c-IAP, cellular inhibitor of apoptosis protein; TRPM2, testosterone-repressed prostate message-2; L32, 50 S ribosomal protein L32; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

whereas TNF treatment of uninfected cells resulted in a 9.2-fold relative increase in firefly luciferase activity over that in untreated cells (Fig. 2).

Infection of the cotransfected cells with cowpox virus did not induce expression of either luciferase gene. Instead, expression of the firefly and sea pansy luciferase genes was about 40% lower in virus-infected cells than uninfected cells (Fig. 2). This reduction almost certainly reflects the progressive general inhibition of host macromolecular synthesis that begins during the late phase of virus replication. This effect is a factor in reporter assays of this type, because after TNF treatment, the cells are normally incubated for 4 h to allow adequate time for the synthesis of the reporter luciferase enzyme. Consequently, even though the virus-infected cells may be treated with TNF during the early phase of virus replication, some of the subsequent expression of the luciferase gene will occur after the late phase of viral replication has begun.

These luciferase data are consistent with the results of the RNase protection experiments, suggesting that cowpox virus infection per se does not induce NF-κB-regulated gene expression, and further, that cowpox virus can specifically inhibit inducible NF-κB-regulated gene expression.



	Average Luciferase Value[1]			
	Sea pansy	Firefly	Ratio[2]	Fold Change[3]
Mock	23.6 +/– 2.7	38.6 +/– 3.2	1.6	1.0
Mock + TNF	18.1 +/– 1.7	273 +/– 14	15.1	9.2
CPV	12.5 +/– 2.0	25.2 +/– 5.6	2.0	1.2
CPV + TNF	14.4 +/– 1.4	25.8 +/– 2.4	1.8	1.1

[1]Average (+/- standard deviation) of values from three transfections
[2]Ratio of average firefly value to average sea pansy value
[3]Relative fold change over the "Mock" ratio number

FIG. 2. Cowpox virus (CPV) inhibits TNF-induced expression of a luciferase gene under the control of an NF-κB-regulated promoter. HEK 293T cells were transiently cotransfected with two vectors: a control vector designed to give constitutive expression of a sea pansy luciferase gene, and an NF-κB reporter vector for the inducible expression of a firefly luciferase gene. At 19 h post-transfection, the cells were either mock-infected with human 143B cell lysate or infected with cowpox virus (10 PFU/cell). Cells were either left untreated or treated with TNF (20 ng/ml) from 4 to 8 h postinfection and then assayed for both sea pansy and firefly luciferase activity. (Table) The average of three independent transfections is represented by each luciferase value. (Bar graph) The graph shows the relative change in firefly luciferase activity compared with untreated, mock-infected cells. The firefly luciferase values are standardized using the sea pansy luciferase values from the constitutively active promoter.

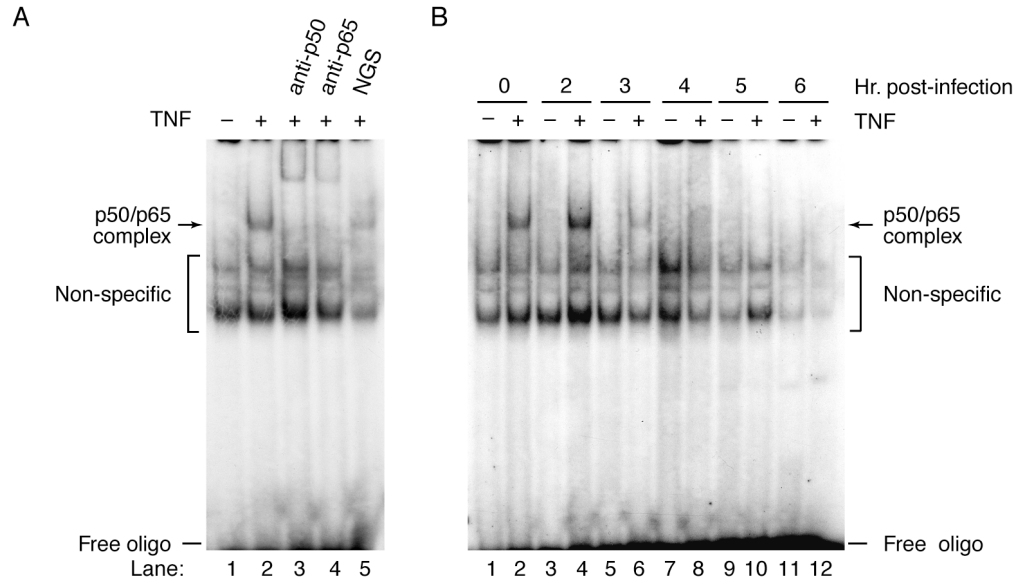


FIG. 3. Cowpox virus inhibits TNF-induced nuclear accumulation of NF-κB capable of binding to κB-site oligonucleotides. Nuclear extracts were prepared both from uninfected HEK 293 cells and from cowpox virus-infected HEK 293 cells (5 PFU/cell) at various times after infection, either with or without TNF treatment (5 ng/ml for 15 min). The nuclear extracts from the cells were incubated with radiolabeled oligonucleotides containing a consensus NF-κB binding-site sequence. Resultant complex formation was analyzed by electrophoretic mobility shift assay (EMSA). (A) TNF treatment induces p50/p65 NF-κB complex formation in uninfected cells. For supershift analysis, prior to being incubated with the oligonucleotides, the extracts were preincubated with goat antibodies against the p50 (lane 3) or p65 (lane 4) subunits of human NF-κB or with normal goat serum (NGS). (B) Uninfected HEK 293 cells (lanes 1–2) or HEK 293 cells infected with cowpox virus (lanes 3–12) were harvested at the indicated hours postinfection; cells were either untreated or treated with TNF for the 15 min prior to harvest.

Cowpox virus interferes with signaling pathways leading to NF-κB activation

Specific inhibition of NF-κB-regulated gene expression by cowpox virus might be predicted to involve early-class gene products that target components of signal transduction pathways leading to the activation of these transcription factors. Therefore, we examined the effects of cowpox virus infection upon NF-κB signaling pathways.

A key regulatory step in the activation of NF-κB-regulated gene expression is the translocation of NF-κB from the cytoplasm to the nucleus. Once in the nucleus, NF-κB can bind to κB elements to contribute to the transcription of specific genes. Electrophoretic mobility shift assays showed that cowpox virus infection inhibits the accumulation of nuclear NF-κB capable of binding to κB sites (Fig. 3). Extracts from uninfected, TNF-treated cells produced a specific complex that was shown by supershift analysis to contain the p50 and p65 subunits of NF-κB (Fig. 3A). Formation of this specific complex was unaffected by the addition of a 100-fold molar excess of oligonucleotides lacking the consensus NF-κB binding site sequence; yet when the extracts were incubated with a 100-fold molar excess of the unlabeled probe, complex formation was no longer detected, demonstrating the binding specificity of the p50/p65 complex for κB elements (data not shown).

Cowpox virus infection resulted in the progressive reduction of the amount of TNF-induced nuclear NF-κB (p50/p65) capable of binding to the κB-site containing oligonucleotides (Fig. 3B). This effect became evident at 3 h postinfection, with little or no complex formation detectable after 4 h of infection. The timing of the viral inhibition of complex formation suggested that viral early gene functions are involved, because in this system, the late phase is not initiated until viral DNA replication begins, at about 4 h after infection.

Evidence that viral late gene functions are not necessary for this inhibition was provided by analysis of complex formation in virus-infected cells cultured in the presence of cytosine-arabinoside (AraC), which prevents viral DNA replication, thereby inhibiting normal intermediate and late viral gene expression (Keck *et al.,* 1990). Under these conditions, nuclear extracts of virus-infected cells still failed to accumulate p50/p65 capable of binding to κB-site oligonucleotides (Fig. 4, lanes 5–8). In uninfected cells, AraC treatment had no effect upon the accumulation of p50/p65 capable of binding to κB-site oligonucleotides after TNF treatment (Fig. 4, lanes 1–4). In these experiments, the efficacy of the AraC treatment in blocking cowpox virus DNA replication was confirmed by hybridization analysis of DNA isolated from the cells, using the *Pst*I F fragment of the cowpox virus genome as a probe (data not shown).

The viral inhibition of the accumulation of nuclear NF-κB capable of binding to κB sites suggested that the virus might interfere with the translocation of NF-κB from the cytoplasm to the nucleus. Such translocation of p50/

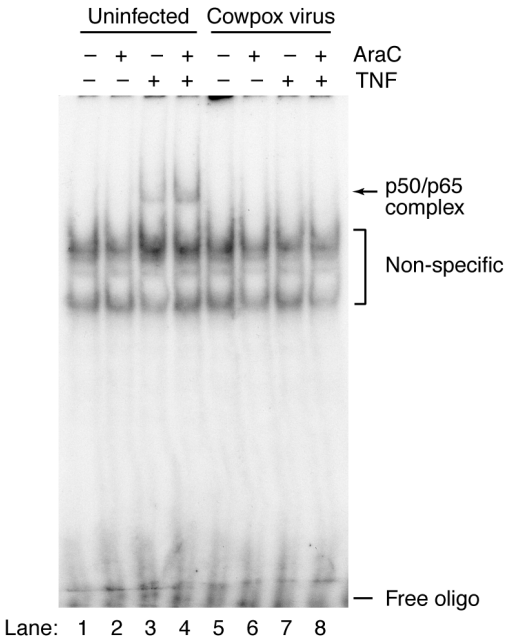


FIG. 4. Late viral gene expression is not required for cowpox virus to inhibit nuclear accumulation of NF-κB capable of complex formation with κB-site oligonucleotides. Nuclear extracts were prepared both from uninfected HEK 293 cells and from cowpox virus-infected HEK 293 cells (5 PFU/cell) at 6 h postinfection. The cells were left untreated (lanes 1 and 5) or were treated with AraC (lanes 2 and 6), TNF (lanes 3 and 7), or both AraC and TNF (lanes 4 and 8). The nuclear extracts from the cells were incubated with radiolabeled oligonucleotides containing a consensus NF-κB binding-site sequence. Resultant complex formation was analyzed by EMSA. AraC treatment was at 40 μg/ml for the entire 6 h; TNF treatment was at 5 ng/ml for 20 min before the cells were harvested.

p65 NF-κB is governed by the degradation of IκBα. Accordingly, we next examined the effects of cowpox virus infection upon IκBα degradation. Normally, IκBα is degraded within 15 min of TNF stimulation in TNF-responsive cells. However, in either HEK 293 cells (Fig. 5) or HeLa cells (data not shown), cowpox virus infection resulted in the inhibition of TNF-induced degradation of IκBα. In uninfected cells, IκBα in the cytoplasm was replenished after 90 min of TNF treatment (Fig. 5A). Presumably, this occurred because the synthesis of IκBα is under the control of NF-κB and because mechanisms exist for the desensitization of the pathway to maintain the transience of the IκBα-mediated signaling pathway (reviewed by Ghosh *et al.,* 1998). In cowpox virus-infected cells, there was no apparent change in the quantity of IκBα over the corresponding period after stimulation. Also, TNF treatment of infected cells resulted in the accumulation of a small portion of IκBα with decreased electrophoretic mobility. This modified IκBα, which is detectable on longer exposures of the IκBα immunoblots (Fig. 5B, middle), was specifically detected when the immunoblot was repeated with antibody specific for serine 32-phosphorylated IκBα (Fig. 5B, bottom). This indicates that IκBα in cowpox virus-infected cells can be phosphorylated at serine 32 in response to TNF treatment, and that unlike in uninfected cells, this phosphorylated form of IκBα is not rapidly degraded.

Protein synthesis during infection is required for the inhibition of IκBα degradation by cowpox virus. Infected HEK 293 cells were treated with cycloheximide for the duration of the infection, or for an equivalent amount of time in uninfected cells, and then the effect on TNF-mediated IκBα degradation was assessed. Cycloheximide treatment alone did not alter the quantity of IκBα in the cells (Fig. 6, compare lanes 1 and 5 with 2 and 6) or prevent the uninfected cells from degrading IκBα after TNF stimulation (Fig. 6, lane 4). However, in the presence of cycloheximide, cowpox virus did not inhibit IκBα degradation after TNF stimulation as it did in the absence of cycloheximide (Fig. 6, compare lanes 7 and 8). Taken together with the results from the AraC experiments (Fig. 4), these data suggest that early viral protein synthesis, or virus-induced host protein synthesis, is necessary for

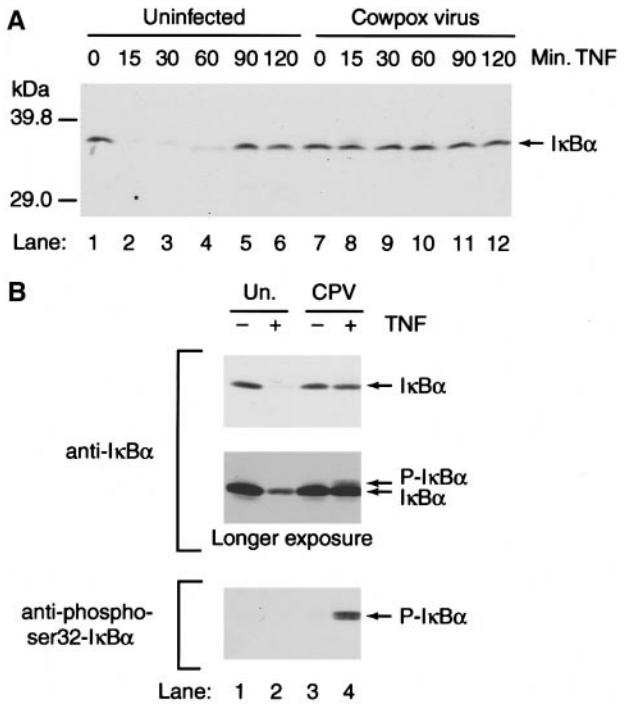


FIG. 5. Cowpox virus inhibits TNF-induced degradation of IκBα. Cytoplasmic extracts were prepared from uninfected cells (Un.) or cells that had been infected with cowpox virus (CPV); where indicated, the cells were treated with 10 ng/ml TNF. Proteins were resolved by polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filters for immunoblot analysis. (A) HEK 293 cells (infected with 5 PFU/cell) were treated with TNF for the indicated number of minutes prior to harvesting the cells at 6 h postinfection. IκBα was detected by immunoblotting with an antibody against human IκBα. (B) HEK 293T cells (infected with 10 PFU/cell) were treated with TNF at 4 h postinfection for 20 min. Top and middle: two exposures of an immunoblot with an antibody against human IκBα. Bottom: the membrane shown in the top and middle was stripped and phosphorylated IκBα was detected with an antibody against a synthetic phospho-Ser32 peptide corresponding to residues 26–39 of IκBα.

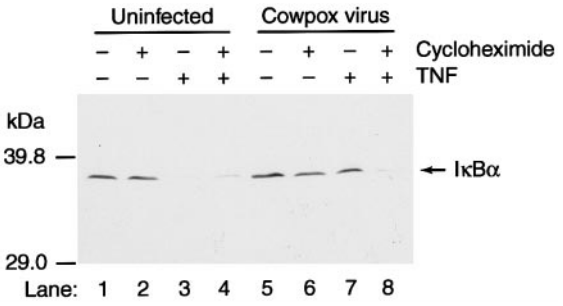


FIG. 6. Protein synthesis is required for cowpox virus to inhibit TNF-mediated IκBα degradation. Cytoplasmic extracts were prepared from HEK 293 cells either left uninfected or infected with cowpox virus (5 PFU/cell). Proteins were resolved by PAGE and transferred to nitrocellulose filters. IκBα was detected by immunoblotting with antibody against human IκBα and visualized by enhanced chemiluminescence. As indicated, the cells were left untreated (lanes 1 and 5) or were treated with cycloheximide (lanes 2 and 6), TNF (lanes 3 and 7), or both cycloheximide and TNF (lanes 4 and 8). At 4 h postinfection the cells were harvested and the extracts were analyzed. Cycloheximide treatment was at 100 μg/ml for the entire 4 h; TNF treatment was at 10 ng/ml for 20 min before the cells were harvested.

cowpox virus to inhibit TNF-mediated activation of NF-κB.

Cowpox virus interferes with the process of IκBα degradation

The inhibition of IκBα degradation by cowpox virus could be attributed to interference either with the pathway leading to the phosphorylation of IκBα or with subsequent processing of appropriately phosphorylated IκBα.

Cowpox viruses may encode multiple inhibitors of components of NF-κB activation pathways. For example, the Brighton Red strain of cowpox virus encodes three soluble, secreted TNF receptors (CrmB, CrmC, and CrmD), one of which, the CrmB protein, is an early gene product (Hu *et al.,* 1994; Smith *et al.,* 1996; Loparev *et al.,* 1998). Thus, one or more of these receptors might contribute to the inhibition of IκBα degradation through interference with the initiation of signaling. However, our data indicate that in this system, cowpox virus does not completely block signaling through the cellular TNF receptors, because TNF treatment of infected cells results in phosphorylation of IκBα on serine 32 (Fig. 5B). Moreover, a cowpox virus variant lacking both copies of the *crmB* gene is still capable of inhibiting TNF-mediated degradation of IκBα (data not shown).

To confirm that cowpox virus affects the process of signal transduction downstream of the receptor signaling complex, we employed okadaic acid, a protein phosphatase inhibitor that elicits IκBα degradation by a mechanism distinct from that of TNF. Okadaic acid (OA) is thought to induce the phosphorylation and degradation of IκBα by way of preventing any basally phosphorylated, and therefore activated, IκB kinase from being dephosphorylated by cellular phosphatases (Sun *et al.,* 1995; DiDonato *et al.,* 1997). In this way, the okadaic acid maintains or causes an increased accumulation of active IKK inside the cell even in the absence of activating stimuli such as TNF. When uninfected HEK 293 cells were treated with okadaic acid for 1 h, there was a marked decrease in the quantity of IκBα (Fig. 7, top, lane 2). Further, the residual IκBα was distributed between two forms, a form with the same electrophoretic mobility as that in unstimulated cells, and a form with a reduced electrophoretic mobility. After 3 h of okadaic acid treatment, IκBα was not detectable in the uninfected cells. In cowpox virus-infected cells, okadaic acid treatment did not induce an equivalent loss of IκBα; after 3 h, much of the IκBα remained undegraded (Fig. 7, top, lane 8). However, although the IκBα was not degraded, all of the protein was converted to species with decreased electrophoretic mobility, consistent with its conversion to phosphorylated forms. These slower migrating species were confirmed to be serine 32-phosphorylated IκBα by stripping the membrane and repeating the immunoblot with an antibody specific for IκBα phosphorylated at this residue, which is one of the two regulatory serines phosphorylated by IKK (Fig. 7, bottom). This suggests that the IκBα was converted to a potential substrate for ubiquiti-

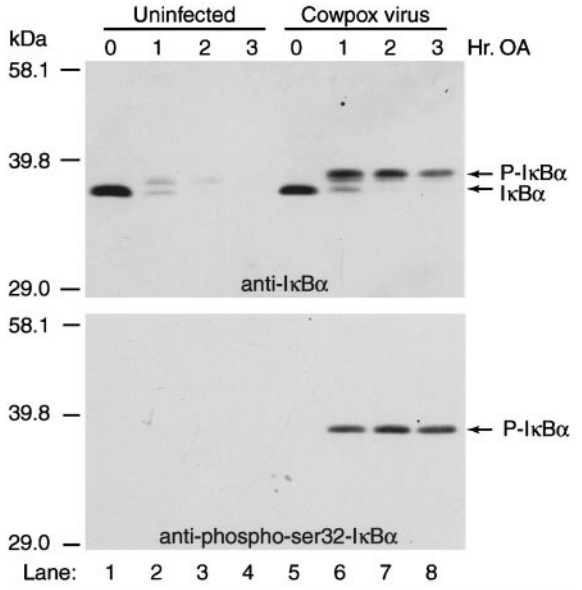


FIG. 7. Cowpox virus inhibits the degradation of phosphorylated IκBα. Cytoplasmic extracts were prepared from either uninfected HEK 293 cells or cells infected with cowpox virus (5 PFU/ml). At 4 h postinfection, the cells were treated with okadaic acid (1 μg/ml) and were then harvested after the indicated periods of time (lanes 2–4 and 6–8); cells that were not treated with okadaic acid (lanes 1 and 5) were harvested at the same time as those treated for 3 h. Proteins were resolved by PAGE and transferred to nitrocellulose filters. (Top) IκBα was detected by immunoblotting with antibody against human IκBα. (Bottom) Phosphorylated IκBα was detected with antibody against a synthetic phospho-Ser32 peptide corresponding to residues 26–39 of IκBα. The detected proteins were visualized by enhanced chemiluminescence.

nation, because IKK is thought to phosphorylate regulatory serines 32 and 36 simultaneously (reviewed by Karin and Ben-Neriah, 2000). Beyond this, the precise nature and extent of IκBα phosphorylation in response to okadaic acid treatment in cells infected with cowpox virus is unknown. Nonetheless, this result indicates that cowpox virus does not inhibit okadaic acid-induced phosphorylation of IκBα; rather, it interferes with the subsequent processing of this phosphorylated IκBα.

The accumulation of phosphorylated IκBα in the okadaic acid-treated, virus-infected cells vs the accumulation of predominantly unphosphorylated IκBα in TNF-treated, virus-infected cells is intriguing (Figs. 5 and 7). This difference could be attributed either to a partial viral inhibition of TNF-mediated IκBα phosphorylation or to an okadaic acid-mediated inhibition of the dephosphorylation of IκBα. Cowpox virus does possess a homolog of the VH1 tyrosine/serine phosphatase encoded by vaccinia virus (Guan *et al.,* 1991; Hakes *et al.,* 1993; unpublished data). However, the phosphatase does not appear to be required for the viral inhibition of NF-κB signaling that we have observed because this inhibition is dependent upon protein synthesis during the early phase of virus replication, whereas the VH1 gene is expressed during the late phase (Rosel *et al.,* 1986). Furthermore, the VH1 phosphatase is relatively insensitive to okadaic acid (Guan *et al.,* 1991). Nevertheless, we have not ruled out the possibility that the VH1 phosphatase might contribute to additional viral effects upon components of NF-κB signaling pathways, because VH1 phosphatase packaged in virus particles (Liu *et al.,* 1995) is capable of dephosphorylating the cellular Stat1 protein, a component of the gamma interferon signaling pathway (Najarro *et al.,* 2001).

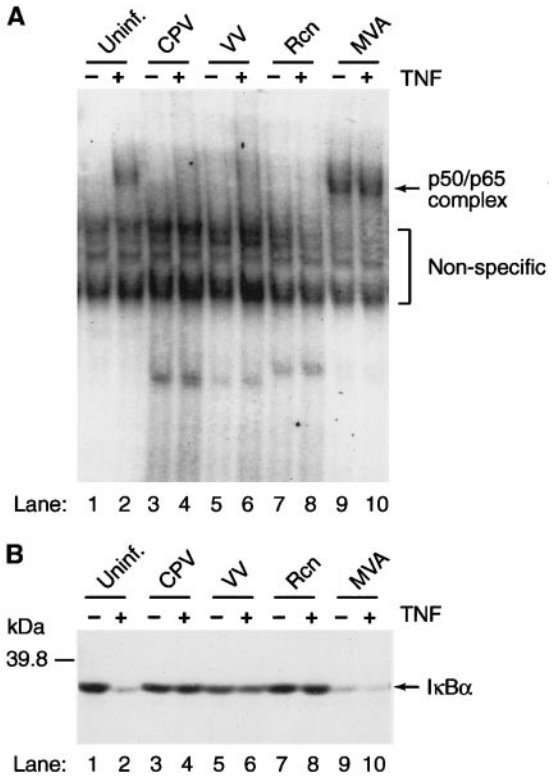


FIG. 8. Orthopoxviruses affect the activation of the NF-κB pathway in a type-specific manner. Nuclear and cytoplasmic extracts were prepared from uninfected and poxvirus-infected cells at 4 h postinfection. Where indicated, the cells were treated with 10 ng/ml of TNF for 20 min before the cells were harvested. (A) HEK 293 cells were infected with cowpox virus (CPV), vaccinia virus Copenhagen strain (VV), raccoonpox virus (Rcn), or modified vaccinia virus Ankara strain (MVA) at 5 PFU/cell. Nuclear extracts from the cells were incubated with radiolabeled oligonucleotides containing a consensus NF-κB binding-site sequence. Resultant complex formation was analyzed by EMSA. (B) HEK 293T cells were infected with CPV, VV, Rcn, or MVA at 10 PFU/cell. Proteins in the cytoplasmic extracts were resolved by PAGE and transferred to nitrocellulose filters. IκBα was detected by immunoblotting with an antibody against human IκBα and visualized by enhanced chemiluminescence.

Other poxviruses affect the activation of NF-κB in a type-specific manner

Cowpox virus was chosen for the initial studies on the effects of virus infection on NF-κB activation because cowpox virus possesses one of the largest genomes among viruses in the orthopoxvirus genus. Consequently, it was judged to be a good system in which to begin the search for viral mechanisms capable of affecting the activation of NF-κB.

While most of the viral genes essential for *in vitro* growth are conserved between the orthopoxvirus species, those conferring accessory functions that affect virus–host interactions *in vivo* differ markedly among species and strains of virus. Therefore, we were interested in whether the cowpox virus inhibition of the NF-κB activation was a property common among related poxviruses.

To address this question, three different strains of vaccinia virus (Copenhagen, Western Reserve, and modified vaccinia Ankara) and one strain of raccoonpox virus (V71-I-85A) were examined. Parenthetically, none of these strains of vaccinia virus encodes any secreted TNF receptors (Goebel *et al.,* 1990; Howard *et al.,* 1991; Smith *et al.,* 1991; Antoine *et al.,* 1998). Each was investigated for the effects of viral infection on, first, the quantities of nuclear NF-κB capable of binding to κB sites, and second, IκBα degradation in HEK 293 cells either with or without TNF treatment. Interestingly, the viruses acted in a type-specific manner to produce diametrically different outcomes on the NF-κB activation pathway. Infection with vaccinia virus Copenhagen strain, vaccinia virus Western Reserve strain (data not shown), or raccoonpox virus did not induce either the nuclear accumulation of p50/p65 NF-κB capable of binding to κB sites (Fig. 8A) or the degradation of IκBα (Fig. 8B). Similar to cowpox virus, each of these three viruses prevented TNF induction of these events. Additionally, we noted the

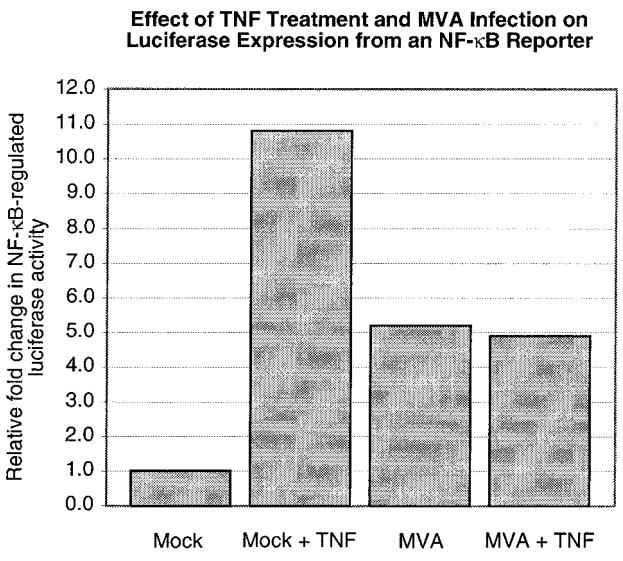
presence of minor amounts of apparently virus-specific, protein–DNA complexes in the nuclear extracts of poxvirus-infected cells (e.g., Fig. 8A, lanes 3–10). The nature of these complexes has yet to be determined. In sharp contrast to these viruses, infection with the modified vaccinia virus Ankara strain (MVA) induced both the nuclear accumulation of NF-κB capable of binding to κB sites (Fig. 8A, lane 9) and the degradation of IκBα (Fig. 8B, lane 9), even in the absence of TNF treatment. Of note, since MVA is severely host-range restricted compared with other poxviruses, MVA can also induce IκBα degradation in BHK cells (data not shown), which are permissive for MVA replication (Carroll and Moss, 1997; Drexler *et al.,* 1998). This suggests that initiation of IκBα degradation by MVA is independent of the ability of the virus to replicate in a cell.

To determine whether MVA infection can result in the activation of NF-κB-regulated gene expression, we examined the effects of virus infection upon the expression of luciferase reporter genes transfected into HEK 293T cells (Fig. 9). Under the conditions of this assay, MVA induced a fivefold reduction in the constitutive expression of sea pansy luciferase in comparison to uninfected cells. This is consistent with the more rapid interference with host gene expression by MVA in comparison to cowpox virus, most likely as a result of the shorter replication cycle of MVA. If quantities of the NF-κB-regulated firefly luciferase were normalized to quantities of sea pansy luciferase, then MVA induced an apparent fivefold increase in NF-κB-regulated luciferase gene expression, which was not increased further by the addition of TNF. However, to interpret this as evidence that MVA does induce NF-κB-regulated luciferase gene expression, it is necessary to assume that the progressive viral inhibition of host gene expression affects the reporter gene and the reference gene equivalently, though it is not certain if this is the case during an MVA infection. Despite this caveat, such an interpretation is consistent with the previous results showing the induction of NF-κB signaling events as a consequence of MVA infection.

In summary, several orthopoxviruses do not activate NF-κB signaling upon infection and possess the ability to interfere with signal transduction pathways leading to NF-κB activation. Other orthopoxviruses, such as MVA, may activate NF-κB signaling pathways upon infection.

DISCUSSION

This study demonstrates that poxviruses can affect NF-κB-regulated gene expression in the infected host cell. Further, it shows that poxviruses exert their effects, either negatively or positively, in a type-specific manner. Importantly, it shows that poxviruses, by inhibiting the degradation of IκBα, even after it has been phosphorylated, target a point of convergence of multiple signal pathways leading to the activation of NF-κB.



	Average Luciferase Value[1]			
	Sea pansy	Firefly	Ratio[2]	Fold Change[3]
Mock	24.0 +/– 2.9	34.6 +/– 3.7	1.4	1.0
Mock + TNF	17.0 +/– 1.2	263 +/– 7.1	15.6	10.8
MVA	4.6 +/– 0.6	34.5 +/– 1.4	7.5	5.2
MVA + TNF	5.4 +/– 0.5	37.8 +/– 2.2	7.0	4.9

[1]Average (+/- standard deviation) of values from three transfections
[2]Ratio of average firefly value to average sea pansy value
[3]Relative fold change over the "Mock" ratio number

FIG. 9. The effect of the modified vaccinia virus Ankara strain on the expression of a luciferase gene under the control of an NF-κB-regulated promoter. HEK 293T cells were transiently cotransfected with two vectors: a control vector designed to give constitutive expression of a sea pansy luciferase gene; and an NF-κB reporter vector for the inducible expression of a firefly luciferase gene. At 19 h posttransfection, the cells were either mock-infected with BHK cell lysate or infected with MVA (10 PFU/cell). Cells were either left untreated or treated with TNF (20 ng/ml) from 4 to 8 h postinfection and then assayed for both sea pansy and firefly luciferase activity. (Table) The average of three independent transfections is represented by each luciferase value. (Bar graph) The graph shows the relative change in firefly luciferase activity compared with untreated, mock-infected cells. The firefly luciferase values are standardized using the sea pansy luciferase values from the constitutively active promoter.

By targeting NF-κB-regulated gene expression, the virus interferes with one of the central regulatory mechanisms of the immune system, enabling the virus to suppress the expression of numerous proteins that contribute to the immune response against infection. Indeed, many of these host proteins, or the processes in which these proteins are engaged, are known to be targeted by poxviruses through other means. These different mechanisms of poxviral interference with NF-κB activation may well be complementary rather than redundant mechanisms. For example, viral soluble, secreted receptors for ligands that can activate NF-κB may contribute to

the interference with NF-κB-regulated gene expression in uninfected cells as well as in virus-infected cells. This apparent multiplicity of mechanisms of inhibition underscores the advantage that might be gained by viral suppression of NF-κB-regulated gene expression, presumably because this could delay or impair host immune responses.

Conversely, viral activation of NF-κB-regulated gene expression would be expected to stimulate and intensify immune responses. Viruses of many other families are known to activate NF-κB, particularly those that take advantage of NF-κB-regulated mechanisms of transcription of viral genes (reviewed by Hiscott *et al.,* 2001). It is less clear whether poxviruses might benefit from the activation of NF-κB. Nevertheless, the finding that MVA, a virus closely related to other strains of vaccinia virus, is capable of activating NF-κB by virtue of infection alone is significant. Although the precise mechanisms involved in the activation of NF-κB by MVA have yet to be determined, this finding suggests that orthopoxviruses of other types may also possess this capacity. The MVA genome does not contain any genes that do not have counterparts in the genomes of most other orthopoxviruses (Antoine *et al.,* 1998). Thus, the viral activation of NF-κB may reflect the loss of viral function in MVA, rather than the presence of an MVA-specific inducer of NF-κB. Alternatively, variants of genes that are conserved among the orthopoxviruses may confer different capacities to induce NF-κB activation. In this case, it is also possible that the mechanisms of MVA-mediated activation of NF-κB might not involve the same signaling pathways targeted by cowpox and vaccinia viruses to inhibit TNF-mediated activation of NF-κB. Such an activation process might pre-empt, or circumvent, or be dominant over any inhibitory mechanisms that MVA may possess in common with cowpox virus. In either of these models, the degree of activation of NF-κB is likely to be dependent upon both the type of virus and the type of cell infected.

The results of this study may have several implications for the pathogenesis of poxvirus infections.

First, most of the relatively avirulent poxviruses that we have examined inhibit NF-κB activation. This suggests that in host species natural to the virus, the ability to inhibit NF-κB may be a contributory factor in the impairment of the immune response to infection. This may facilitate a productive infection without preventing the eventual clearance of the virus and recovery of the host.

Second, limited viral activation of NF-κB, which might occur in the context of a nonproductive infection, such as MVA infection of humans, may stimulate the immune response against that infection. However, since MVA infection appears to be self-limiting, viral activation of NF-κB might also be somewhat limited. This would be beneficial from the standpoint of vaccine efficacy. It may be a major contributory factor to the immunogenicity of MVA-derived vaccines, which appear to be as effective as other live vaccinia virus vaccines despite the inability of MVA to replicate in humans (Sutter *et al.,* 1994; Moss *et al.,* 1996; Carroll *et al.,* 1997; Belyakov *et al.,* 1998; Ramírez *et al.,* 2000). In effect, the activation of NF-κB may act as an adjuvant during these vaccinations.

Third, viral activation of NF-κB in the context of a productive infection might stimulate the immune response, which could lead to an accelerated clearance of the virus, or alternatively, result in prolonged overexpression of NF-κB-regulated genes. In the former case, such a response may be protective for the host and limit viral replication, perhaps contributing to a limitation on the *in vivo* host range of the virus without necessarily restricting the *in vitro* host range. An orthopoxvirus that exhibits host range properties of this type is variola virus. Although variola virus is capable of replicating in cells derived from a wide variety of nonhuman species, it lacks the ability to sustain replication in any animal except humans (reviewed by Fenner *et al.,* 1988a). One hypothesis to explain this is that variola virus might induce the limited activation of NF-κB in these nonhuman species, resulting in an enhanced immune response that curtails viral replication *in vivo*. However, if the host were to fail to limit the viral replication, prolonged NF-κB activation could lead to the pathology associated with this host response. The precedence for this is the endotoxemia that is associated with many bacterial infections and for which the activation of NF-κB is a contributory factor (Barnes and Karin, 1997; Böhrer *et al.,* 1997; Christman *et al.,* 1998; Armstead *et al.,* 1999; Neish *et al.,* 2000). In this regard it is noteworthy that fatal effects of variola virus infections of humans have similarities to the effects of bacterial toxemia (Koplan and Foster, 1979; Fenner *et al.,* 1988b). The molecular mechanisms contributing to mortality and morbidity associated with infections by highly pathogenic orthopoxviruses such as variola and monkeypox viruses are poorly understood. For these reasons it may be highly instructive to determine the properties of some of these orthopoxviruses with respect to their effects on NF-κB-regulated gene expression.

Finally, the results of this study may have several implications for the processes contributing to health and disease in a context broader than that of poxvirus infections. Several studies have suggested that NF-κB-regulated gene expression may be a major contributory factor in the pathogenic processes associated with a variety of infectious and noninfectious diseases, including rheumatoid arthritis, asthma, sepsis, neurodegenerative disorders, and various cancers (reviewed by Chen *et al.,* 1999; Karin and Ben-Neriah, 2000; Baldwin, 2001a,b; Mattson and Camandola, 2001). Therefore, a greater understanding of the mechanisms by which poxviruses affect the activation of NF-κB may assist the develop-

ment of new methods to control the pathogenic effects of NF-κB activation in a number of these diseases.

MATERIALS AND METHODS

Cell culture

Human embryonic kidney (HEK) 293 and HEK 293T cells (kindly provided by Dr. K. Burns, University of Lausanne) were maintained in minimal essential medium (MEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco BRL). Human osteosarcoma 143B cells were maintained in MEM supplemented with 5% FBS. Syrian hamster kidney BHK-21 (BHK) cells were maintained in MEM alpha (Gibco BRL) supplemented with 5% FBS. HeLa cells were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 5% FBS.

Reagents

Lyophilized recombinant human tumor necrosis factor-alpha (TNF; R&D Systems Inc., Minneapolis, MN) was reconstituted at a concentration of 10 μg/ml in a sterile-filtered solution of phosphate-buffered saline containing 0.1% bovine serum albumin (Sigma, St. Louis, MO); aliquots were stored at −80°C. Cycloheximide and AraC were obtained from Sigma. Okadaic acid (product no. O 9381, Sigma) was resuspended at 25 μg/ml in MEM plus 5% ethanol, according to the manufacturer's instructions, and stored at −20°C. Rabbit polyclonal anti-IκBα IgG (C-21), goat polyclonal anti-NF-κB p50 (NLS)-G IgG, and goat polyclonal anti-NF-κB p65 (C-20)-G IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Normal goat serum was obtained from Vector Laboratories, Inc. (Burlingame, CA). Phospho-IκBα (Ser32) antibody was obtained from New England Biolabs Inc. (Beverly, MA).

Virus stocks

Cowpox virus (Brighton Red strain), vaccinia virus (Copenhagen and Western Reserve strains), and raccoonpox virus (V71-I-85A strain) stocks were grown in human 143B cells. The modified Ankara strain of vaccinia virus (kindly provided by Dr. B. Moss, NIH) was grown in BHK cells as described by Carroll and Moss (1997).

Infection and TNF treatment of cells

Cells were plated the day before infection at approximately 10^5 cells/cm^2. To achieve an accurate multiplicity of infection for each experiment, the total number of cells per dish were determined by hemocytometer counts of cells in duplicate dishes. Cells were infected at 5 or 10 PFU/cell in PBS using a total volume of 300–500 μl for 6-cm dishes or 100 μl for 12-well plates. Cells were incubated with the inoculum for 45 min at 37°C. The inoculum was removed and 1.5-ml fresh medium was added per 6-cm dish, or 1 ml medium was added per well of the 12-well plates. For TNF treatment, an appropriate volume of the concentrated stock of TNF was added directly to the culture medium.

RNase protection assays

Total cellular RNAs were prepared using the Roche (Indianapolis, IN) High Pure RNA Isolation Kit according to the manufacturer's instructions. The hAPO-5 multi-probe cDNA template set (PharMingen, San Diego, CA) was used to generate the anti-sense RNA probes for the RNase protection assays, which were performed using the PharMingen Riboquant Multi-Probe RNase Protection Assay System according to the manufacturer's protocol. Briefly, RNAs isolated from 5×10^6 cells, in a 2 μl vol, were mixed with 8 μl radiolabeled probe (3.5 cpm/μl) in hybridization buffer. The mixture was placed in an oven prewarmed to 90°C, and then the temperature setting was immediately changed to 56°C (to allow the temperature to ramp down slowly) for overnight hybridization. The next day, any remaining unhybridized probe or cellular RNA was digested with RNase A, and then the RNase A was removed by digestion with proteinase K. Intact RNA was purified by phenol:chloroform extraction followed by ethanol precipitation. The RNA was resuspended in 10 μl of 1× loading buffer (PharMingen) and half of the sample was resolved by PAGE on a 5% acrylamide gel. The protected probe was detected by autoradiography of the dried gel.

Transfections and luciferase assays

HEK 293T cells were seeded onto 12-well plates the day before being transfected to give 10^6 cells per well. Cells were transfected according to the manufacturer's protocol using 1.5 μl LipofectAMINE 2000 reagent (Gibco BRL) and 0.5 μg DNA, in serum-free MEM, per each well. The DNA was a 10:1 mix of NF-κB reporter vector to constitutive expression vector. The NF-κB reporter vector used was the Stratagene (La Jolla, CA) pNFκB-Luc plasmid, which gives inducible NF-κB-dependent expression of firefly (*Photinus pyralis*) luciferase driven by a synthetic promoter comprising a TATA box preceded by five direct repeats of the sequence 5′-TGGGGACTTTCCGC-3′ containing the NF-κB binding element first identified in the kappa light chain gene enhancer (Sen and Baltimore, 1986). The constitutive expression vector used was the Promega (Madison, WI) pUC-based pRL-TK vector, which gives low-level constitutive expression of sea pansy (*Renilla reniformis*) luciferase from the promoter of the herpes virus thymidine kinase gene. At 15.5 h post-transfection, FBS was added to the medium for a final concentration of 10%. At 19 h post-transfection, the culture medium was removed, and the cells were infected with 10 PFU/cell, as described above. At 4 h postinfection, the cells were treated with 20 ng/ml TNF and harvested 4 h later at 8 h postinfection (27 h post-transfection). To harvest

the cells and to determine luciferase values, the Promega Dual-Luciferase Reporter Assay System was used according to the manufacturer's instructions, with slight modifications. Briefly, the cells were scraped into the culture medium and collected by a 10-s centrifugation at 14,000 rpm in a Sorvall MC 12B centrifuge with an FA-MICRO/1.5 ml rotor. The supernatant was removed and the cells were centrifuged again for about 3 s to allow for the removal of any residual medium. The cell pellet was lysed in 200 μl of 1× passive lysis buffer (Promega) for 5–10 min at room temperature. The lysates were clarified by 5 min of centrifugation, and the supernatants were was analyzed for firefly and sea pansy luciferase activity. For luciferase value determination, 50 μl of luciferase assay reagent II (Promega) was mixed with 10 μl of cell lysate and analyzed for firefly luciferase activity with a Turner Design TD-20/20 luminometer. Then 50 μl of Stop & Glo reagent (Promega) was added for a determination of sea pansy luciferase activity.

Preparation of cytoplasmic and nuclear extracts

Extracts were prepared according to a modified version of the methods described by Dignam *et al.* (1983) and Lægreid *et al.* (1994). Briefly, the cells were scraped into the culture medium and collected by a 10-s centrifugation at 14,000 rpm in a Sorvall MC 12B centrifuge with an FA-MICRO/1.5 ml rotor. The supernatant was removed by aspiration and the cell pellet was lysed in approximately 3 vol of CE buffer [10 mM HEPES, 10 mM KCl, 0.1 mM EDTA (pH 8.0), 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 0.05% NP-40, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 20 mM β-glycerophosphate, 10 mM NaF, and 1 mM Na_3VO_4]. After a 5-min incubation on ice, the nuclei were pelleted with a 20-s centrifugation, and the supernatant was saved as the cytoplasmic extract. The pelleted nuclei were gently washed with 5–10 vol of CE buffer before being pelleted again with a 20-s centrifugation. Nuclear extracts were then prepared by resuspending the nuclei in approximately 3 vol of NE buffer [20 mM HEPES, 25% glycerol, 0.4 M NaCl, 1 mM EDTA (pH 8.0), 1 mM EGTA, 0.5 mM PMSF, 1 mM DTT, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 20 mM β-glycerophosphate, 10 mM NaF, and 1 mM Na_3VO_4]. The nuclei were incubated in NE buffer for 10–30 min on ice, and then the debris was pelleted at 14,000 rpm in an Eppendorf 5415 centrifuge for 10 min at 4°C. Alternatively, the suspension of nuclei in NE buffer was sonicated for approximately 1 min on ice to disrupt the nuclear membranes prior to the centrifugation step. The supernatant was saved as the nuclear extract and stored at −80°C.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed using a modification of the techniques described by Baldwin *et al.* (1991) and the manufacturer's instructions for the Promega Gel Shift Assay Core System. Briefly, an oligonucleotide containing an NF-κB binding site consensus sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was obtained from Promega and end-labeled with [γ-^{32}P]ATP [Amersham Pharmacia Biotech (Amersham), Piscataway, NJ]. The unincorporated ATP was removed by passage of the oligonucleotide through a Sephadex G-25 (Amersham) column buffered with TEN [0.1 M NaCl, 10 mM Tris–Cl (pH 8.0), and 1mM EDTA (pH 8.0)]. Nuclear extracts, prepared as described above, were incubated with the radiolabeled κB-site-containing oligonucleotide in gel shift buffer [5× buffer is 5 mM $MgCl_2$, 2.5 mM EDTA (pH 8.0), 2.5 mM DTT, 250 mM NaCl, 50 mM Tris, 25 ng/ml poly(dI-dC) · poly(dI-dC), 20% glycerol] for 20 min at room temperature. Samples were separated by gel electrophoresis through a 5% acrylamide/TGE [25 mM Tris, 190 mM glycine, 1 mM EDTA (pH 8.0)] nondenaturing gel. The gel was dried and subjected to autoradiography.

Western blot analysis

Extracts were analyzed using an SDS-PAGE system (Laemmli, 1970). Cytoplasmic extracts, prepared as described above, were mixed with an equal volume of 2× treatment buffer (0.125 M Tris–Cl, 4% SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% bromphenol blue, pH 6.8), boiled for 5 min, and electrophoretically separated on a polyacrylamide gel with a 4% stacking gel and a 12.5% running gel. The proteins were transferred to a nitrocellulose membrane in transfer buffer [25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS, pH 8.2 (Towbin *et al.,* 1979)]. The membrane was blocked with 5% milk in Tris-buffered saline/0.05% Tween 20 (TBST). Specific proteins were detected with primary antibody diluted 1:5000 in 5% milk/TBST followed by a secondary incubation with donkey anti-rabbit antiserum conjugated to horseradish peroxidase (Amersham) diluted 1:5000 in TBST. The membrane was then incubated in enhanced chemiluminescence substrate (Amersham), and autoradiography was used to detect the specific proteins.

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