VIROLOGY 218, 71–80 (1996)
ARTICLE NO. 0167

NF-κB Activation Is Delayed in Mouse L929 Cells Infected with Interferon Suppressing,

A. HAMID BOULARES, MAUREEN C. FERRAN, and J. LUCAS-LENARD[1]

Molecular and Cell Biology Department, The University of Connecticut, Storrs, Connecticut 06269-3125

Received May 26, 1995; accepted January 22, 1996

Vesicular stomatitis virus (VSV) mutant T1026R1 of the Indiana (IN) serotype is a good inducer of interferon (IFN). This mutant was used to study the activation of NF-κB, a transcription factor necessary for IFN induction, in mouse L929 cells that were stably transfected with a chimeric gene containing the human IFN-β gene promoter attached to the chloramphenicol acetyltransferase (CAT) coding sequence. NF-κB DNA binding activity was detected as early as 30 min after virus adsorption in nuclear extracts, increased up to 4 hr, and then remained constant for at least 6 additional hr. The kinetics of CAT expression correlated with the kinetics of NF-κB nuclear DNA binding activity. Virus entry and delivery of viral components into the cytoplasm were required for NF-κB activation. Exposure of T1026R1 to one hit of UV irradiation nearly completely reduced NF-κB activation. In cells infected with wild-type (wt) VSV (IN), a noninducer of IFN, NF-κB DNA binding activity in the nucleus was delayed for several hours after virus adsorption. Coinfection of wt VSV and T1026R1 resulted in the reduction of T1026R1-promoted NF-κB activation. This inhibitory activity of wt VSV was abolished by one hit of UV irradiation. Under similar conditions expression of the CAT gene was more UV resistant, suggesting that IFN gene expression is regulated at multiple levels.

INTRODUCTION

Viral infections commonly trigger induction of type I interferon (IFN) (i.e., IFN-α and IFN-β) genes in a variety of mammalian cells. The newly produced IFNs, in turn, activate a set of genes whose products are responsible for the antiviral state (for reviews see Samuel, 1991; Sen and Lengyel, 1992).

Vesicular stomatitis virus (VSV) of the Indiana (IN) serotype has been used to study and characterize IFN induction in mammalian cells. Wild-type (wt) VSV (IN) is a poor or noninducer of IFN (Wagner and Huang, 1966). It also severely inhibits both host protein and RNA synthesis in susceptible cells (Wertz and Younger, 1972; Marvaldi *et al.,* 1977, 1978; Weck and Wagner, 1978; Wu and Lucas-Lenard, 1980). In contrast VSV (IN) mutant T1026R1 (Stanners *et al.,* 1977) is an excellent inducer of IFN (Marcus and Sekellick, 1980) and does not inhibit host protein and RNA synthesis until several hours after virus adsorption (Stanners *et al.,* 1977; Dunigan and Lucas-Lenard, 1983). The absence of IFN induction in wt virus-infected cells is thought to result from the presence of one or more virus-coded, IFN suppressors that presumably are defective in the VSV mutants that induce IFN in infected cells (Marcus and Sekellick, 1987). When cells are coinfected with the wt virus and T1026R1, there is no IFN induction, suggesting that the IFN suppression phenotype dominates (Marcus and Sekellick, 1985).

The IFN-β gene promoter is composed of four domains named positive regulatory domains (PRD) I to IV, and a single negative regulatory domain (Goodbourn and Maniatis, 1988; Harada *et al.,* 1989; LeBlanc *et al.,* 1990; Maniatis *et al.,* 1992; Thanos and Maniatis, 1992; Du *et al.,* 1993). IFN-β gene activation by viruses requires several steps, including the binding of IFN regulatory factor 1 (IRF1) to PRD I (Fujita *et al.,* 1988; Harada *et al.,* 1989; Miyamoto *et al.,* 1988; Reis *et al.,* 1992) and the removal of IRF2 from that domain (Harada *et al.,* 1989; Keller and Maniatis, 1991; Palombella and Maniatis, 1992); binding of the activating transcription factor 2 (ATF2), c-jun, and the high-mobility group protein, HMG I(Y), to PRD IV (Du and Maniatis, 1992; Du *et al.,* 1993; Thanos and Maniatis, 1995); and binding of the transcription factor NF-κB to PRD II (Lenardo *et al.,* 1989; Maniatis *et al.,* 1992; Urban *et al.,* 1991). HMG I(Y) also promotes a more efficient binding of ATF2 and NF-κB to the PRD II site (Thanos and Maniatis, 1992). (For a diagram of the human IFN-β promoter and the *cis*-acting elements and *trans*-acting factors necessary for its activation, see Fig. 1 in Du *et al.,* 1993.)

An early step in IFN gene activation is thought to be the translocation of NF-κB into the nucleus. A relatively ubiquitous transcription factor, NF-κB consists of two subunits, p50 and p65, and is complexed to a protein IκB, which keeps the factor in the cytoplasm by masking

[1] To whom correspondence and reprint requests should be addressed.

0042-6822/96 $18.00

its nuclear localization sequence (for reviews see Grimm and Baeuerle, 1993; Thanos and Maniatis, 1995). In order for NF-κB to be translocated to the nucleus, IκB has to be released from the NF-κB complex (Beg *et al.,* 1993; Chiao *et al.,* 1994). Phosphorylation of IκB and its degradation are necessary for its release (Miyamoto *et al.,* 1994).

In this report we characterize the activation of NF-κB in T1026R1-infected mouse L929 cells that have been stably transfected with a chimeric gene, pTWU54, containing the human IFN-β gene promoter attached to the chloramphenicol acetyltransferase (CAT) coding sequence (Twu and Schloemer, 1987). We compare the appearance of NF-κB DNA binding activity in nuclear extracts of cells that have been infected with IFN suppressing (wt) and IFN inducing (T1026R1) VSV. We show that NF-κB activation is delayed in wt virus-infected cells, but not in T1026R1-infected cells. The possible effect of this delay on IFN induction is discussed.

MATERIALS AND METHODS

Cell cultures, viruses, and infection

Monolayers of mouse L929 cells were grown and maintained in Earle's minimum essential medium (MEM) supplemented with asparagine (0.15 g/liter), serine (0.115 g/liter), proline (0.101 g/liter), 5% fetal calf serum, and 5% newborn calf serum on plastic petri dishes at 37°. The heat-resistant strain of the IN serotype of VSV and its mutant T1026R1 isolated by Stanners *et al.* (1977) were grown on either Vero or baby hamster kidney cells. The titers of these virus stocks were between 1 and 3×10^9 PFU/ml. Viruses were exposed to UV light as described by Dunigan and Lucas-Lenard (1983).

Cells were infected with either virus or both at a multiplicity of infection (multiplicity) of 5 PFU/cell unless stated otherwise. Virus was adsorbed in MEM for 1 hr at 37° in the absence of serum, after which complete medium was added.

Cytoplasmic and nuclear extract preparations

For the preparation of cytoplasmic and nuclear extracts, cells were washed twice with PBS and lysed by incubation in 500 μl of buffer A (10 m*M* HEPES–KOH, pH 7.9, 10 m*M* KCl, 0.1 m*M* EDTA, 2 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT), 0.5 m*M* phenylmethylsulfonylfloride (PMSF), and 0.3 *M* sucrose) containing 0.1% NP-40 for 8 min at 4°. Cells were then scraped and the lysates were centrifuged at 2500 rpm in a microfuge at 4° for 5 min. The pellet was saved and used for preparation of nuclei. The supernatant was supplemented with glycerol to a final concentration of 20% and then centrifuged at 100,000 *g* for 1 hr. The resultant supernatant was termed the "cytoplasmic extract."

Nuclei were washed with buffer A without NP-40 and then suspended in 30 μl buffer C (20 m*M* HEPES–KOH, pH 7.9, 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 5% glycerol, 1 m*M* DTT, 0.5 m*M* PMSF), for 40 min. The nuclear suspension was then centrifuged at 2500 rpm for 10 min and the supernatant (nuclear extract) was diluted with 1.5 vol of buffer D (20 m*M* HEPES, pH 7.9, 50 m*M* KCl, 0.2 m*M* EDTA, 1 m*M* DTT, 20% glycerol, 0.5 m*M* PMSF). All buffers contained 2 μg/ml pepstatin, aprotinin, and leupeptin. Protein concentrations were determined using the Bio-Rad protein assay.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described by Schutze *et al.* (1992) with some modifications. Extracts (10 μg protein) were incubated in the presence of binding buffer containing 150 m*M* KCl and 1 μg poly(dI–dC) in a total volume of 20 μl for 15 min at 4°. The mixture was then incubated for 15 min at room temperature in the presence of $3–5 \times 10^4$ cpm ^{32}P-5′-end-labeled double-stranded oligodeoxynucleotide κB decameric probe (5′-TCGACAGAGGGGACTTTCCGAGAGGCTCGA-3′) synthesized by the Biotechnology Center of the University of Connecticut. DNA–protein complexes were resolved by 4% polyacrylamide gel electrophoresis (PAGE) in 0.5× Tris–borate–EDTA (TBE) (1× TBE, 90 m*M* Tris, 64.6 m*M* borate, 2.5 m*M* EDTA) and visualized by autoradiography.

Western blot analysis

Cytoplasmic extracts (30 μg) from mock-infected or virus-infected cells were fractionated by 10% SDS–PAGE. The fractionated proteins were then transferred to Immobilon-P membranes (Millipore). The membranes were treated with anti-IκB-α polyclonal antibody (Santa Cruz Biotechnology) and IκB was visualized by enhanced chemiluminescence (Amersham).

Transfection, cell extract preparation, and CAT activity assays

Mouse L929 cells were stably transfected with plasmid pTWU54 (the kind gift of R. H. Schloemer, Indiana University) using calcium phosphate (Strategene Mammalian Transfection kit) according to the manufacturer's directions. Plasmid pTWU54 encodes the CAT gene under the regulation of the 353-bp *Hin*dIII–*Hin*cII fragment of the human IFN-β promoter corresponding to positions −286 to +67. The pTWU54 construct is shown in Fig. 2 of Twu and Schloemer (1987).

The neomycin resistance gene was subcloned into the *Bam*HI site of plasmid pTWU54 and clones containing the plasmids were selected on the basis of their ability to grow in G418. It has been shown by deletion mapping that the 5′ flanking region encompassing positions −125 to −38 relative to the cap site confers full virus inducibil-

ity when this region is attached to a reporter gene (Fujita *et al.,* 1985). When the IFN-CAT gene was induced by poly(I):poly(C), the following modified method of Twu and Schloemer (1987) was used. Poly(I):poly(C) (50 μg/ml) and DEAE–Dextran (10 μg/ml) were added to cells in serum-free medium. After 2 hr at 37° cycloheximide (50 μg/ml) was added for an additional 3.5 hr. After this time period cells were washed twice with serum-free medium and incubated for an additional 3.5 hr in complete medium.

The transfected cells were either mock-infected or infected for 1 hr at 37°. After adsorption the medium was removed and 5 ml of complete medium was added. After incubation at 37° for the time periods indicated in the figure legends, the medium was removed, the monolayers were washed twice with PBS, and 100 μl of 0.25 *M* Tris–HCl (pH 7.8) was added to each plate. Cells were disrupted by three cycles of freeze–thaw and centrifuged for 15 min at 4° in a microfuge. The supernatant was then assayed for CAT activity by thin-layer chromatography as described by Gorman *et al.* (1982). Approximately 100 μg of protein (extract) was added per well. Where indicated the CAT assays were quantitated by the Packard Instant Imager, which allowed determination of the percentage chloramphenicol acetylated as a function of the total cpm in a particular lane in the thin-layer chromatogram. This method eliminated problems of nonlinearity of cpm per band in the autoradiograms and unequal cpm loaded per lane.

RESULTS

Activation of NF-κB and IFN-CAT induction in T1026R1-infected cells

Since NF-κB translocation to the nucleus is one of the first steps in IFN-β gene induction, we wished to characterize the kinetics of this process in cells infected with the IFN-inducing VSV mutant T1026R1. Mouse L929 cells, stably transfected with a chimeric gene containing the human IFN-β gene promoter attached to the CAT coding sequence (see Materials and Methods), were infected with T1026R1. At the time intervals indicated in Fig. 1A, cells were harvested and lysed and the nuclei and cytoplasm were separated by centrifugation. Using a ^{32}P-labeled dsDNA decameric NF-κB binding sequence as probe (see Materials and Methods), we determined the NF-κB DNA binding activity in nuclear extracts by EMSA.

Some NF-κB DNA binding activity was detected as early as 30 min postinfection (Fig. 1A, compare lane 5 with lane 1). This activity continued to increase at a low level until around 2 hr after infection. At 4 hr after infection DNA binding activity increased greatly, probably as a result of viral RNA amplification and increased production of NF-κB activating components. The binding activity remained constant until approximately 16 hr after infection (shown only to 10 hr in Fig. 1A), after which time it began to decrease and ceased at 24 hr. This decrease in activity was most likely a result of cell death, since many cells were lysed by that time postinfection.

The kinetics of IFN-CAT gene induction corresponded relatively closely to the kinetics of NF-κB activation, as measured by CAT activity assays. CAT activity was detected at 2 hr postinfection (Fig. 1B, lane 2), the first time point measured, and increased for up to at least 10 hr. The early appearance of CAT activity is consistent with the detection of IFN mRNA as early as 90 min after induction (Palombella and Maniatis, 1992).

NF-κB activation is dependent on genome replication and amplified viral RNA synthesis

We wished to determine whether the ability of VSV mutant T1026R1 to activate NF-κB was a UV-sensitive or resistant viral function. Cells were therefore infected with T1026R1 that was exposed to different doses of UV radiation and the ability of these UV-irradiated virus particles to activate NF-κB was examined. As shown in Fig. 2A, lanes 2 and 3, one hit (approximately 54 ergs/mm^2) reduced activation by more than 50%. Two or more hits (Fig. 2A, lanes 4 and 5) reduced activation to levels seen during the early stages of virus infection (Fig. 1, lanes 4 and 5). One hit of UV irradiation to VSV is sufficient to stop virus replication and, hence, secondary transcription of the VSV genome and transcription of the L gene, the gene farthest from the transcription start site (Ball and White, 1976).

Induction of the IFN-CAT gene responded to UV irradiation (Fig. 2B) in a manner similar to that of NF-κB activation. One hit of UV irradiation reduced CAT synthesis by 72%, 3 hits by 81%, and 19 hits by 88%. These results suggested that extensive NF-κB activation and IFN-CAT expression require viral replication and amplified viral RNA synthesis.

Virus entry into cells and delivery of viral components into the cytoplasm are required for NF-κB activation

VSV is thought to enter cells by endocytosis in coated pits and vesicles which fuse with lysosomes (Matlin *et al.,* 1982; Superti *et al.,* 1987). This reaction ultimately leads to virus uncoating and transfer of the viral genome to the cytoplasm. Because of the rapidity with which NF-κB binding activity was detected in nuclear extracts of T1026R1-infected cells (Fig. 1A), we wished to determine whether activation occurs during endocytosis or after the VSV genome enters the cytoplasm.

Ammonium chloride, a lysosomotropic weak base that inhibits low-pH-dependent membrane fusion, has been used to trap VSV in endosomes (Superti *et al.,* 1987). Therefore, T1026R1-infected cells were incubated for 4

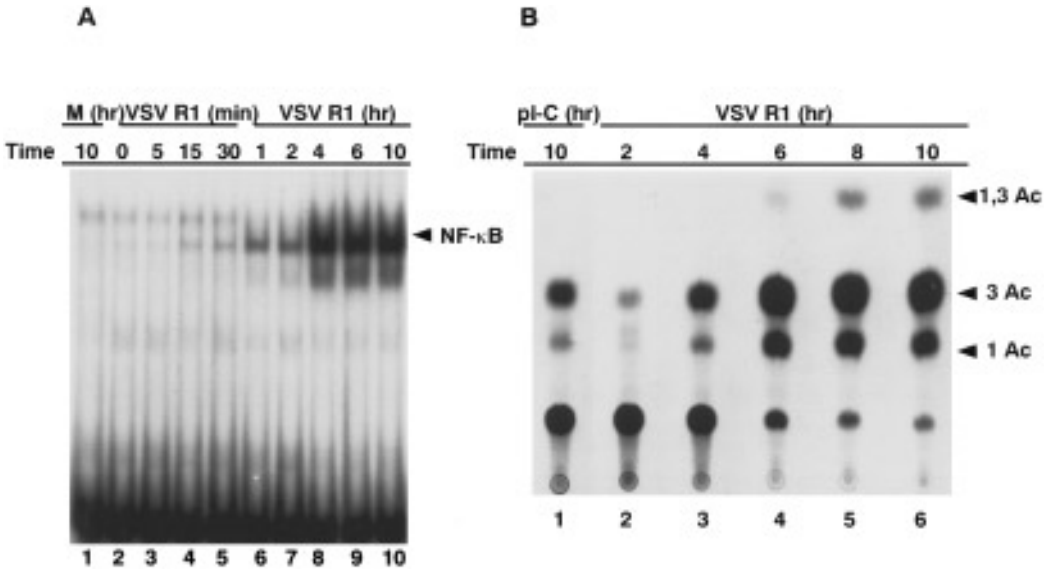

FIG. 1. NF-κB activation and IFN-CAT induction in T1026R1-infected cells. (A) NF-κB binding activity in nuclear extracts from: lane 1, 10-hr mock-infected cells; lanes 2–5, cells infected with T1026R1 for 0, 5, 15, and 30 min, respectively; lanes 6–10, cells infected with T1026R1 for 1, 2, 4, 6, and 10 hr respectively. (B) CAT activity in extracts from: lane 1, cells mock-infected for 10 hr and treated with poly(I):poly(C) as described under Materials and Methods; lanes 2–6, cells infected with T1026R1 for 2, 4, 6, 8, and 10 hr, respectively. The multiplicity in each case was 5 PFU/cell.

hr in the presence or absence of 30 m*M* NH_4Cl, after which time period nuclear extracts were prepared. No NF-κB DNA binding occurred in nuclear extracts from infected cells that were treated with NH_4Cl, but extensive binding took place in cells that were not treated with the salt (Fig. 3, compare lanes 2 and 3). This inhibitory effect was partially relieved when NH_4Cl was removed from cells after 2 hr of treatment and fresh medium was added to cells for an additional 2 hr (Fig. 3A, lane 4) before harvesting the nuclei.

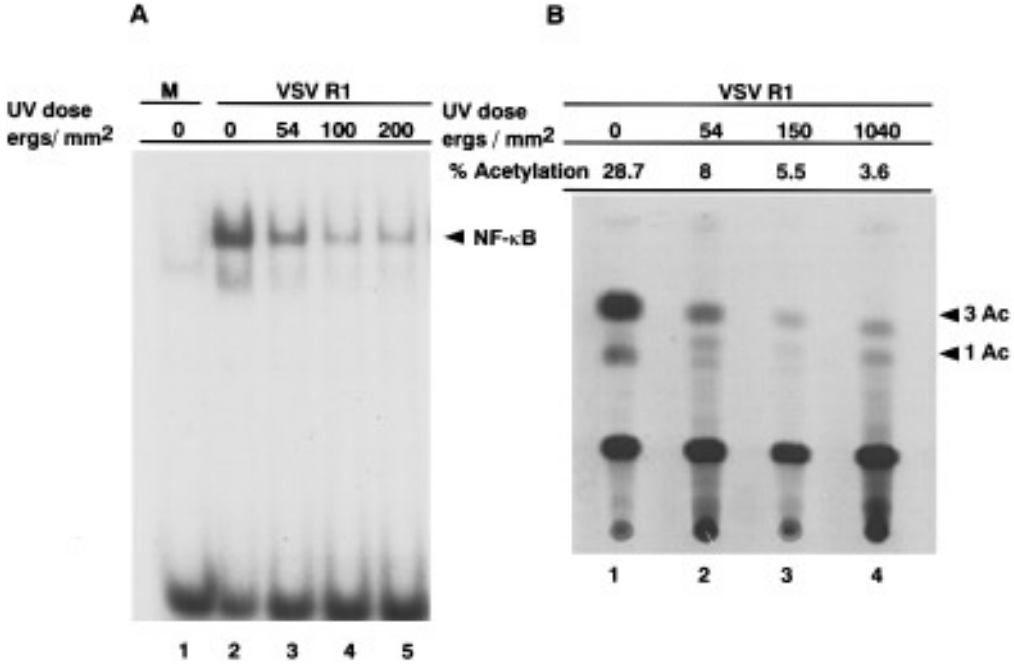

FIG. 2. NF-κB activation and IFN-CAT induction in cells infected with UV-irradiated T1026R1. T1026R1 was irradiated with increasing doses of UV light as described by Dunigan and Lucas-Lenard (1983) and then used to infect monolayers of L cells. (A) NF-κB DNA binding activity in nuclear extracts from: lane 1, mock-infected cells; lane 2, cells infected with T1026R1 for 4 hr; lanes 3–5, cells infected for 4 hr with T1026R1 irradiated at doses 54, 100, and 200 ergs/mm^2, respectively. (B) CAT activity in extracts from: lane 1, T1026R1-infected cells; lanes 2–4, cells infected for 6 hr with T1026R1 that was UV-irradiated at 54, 150, and 1040 ergs/mm^2, respectively.

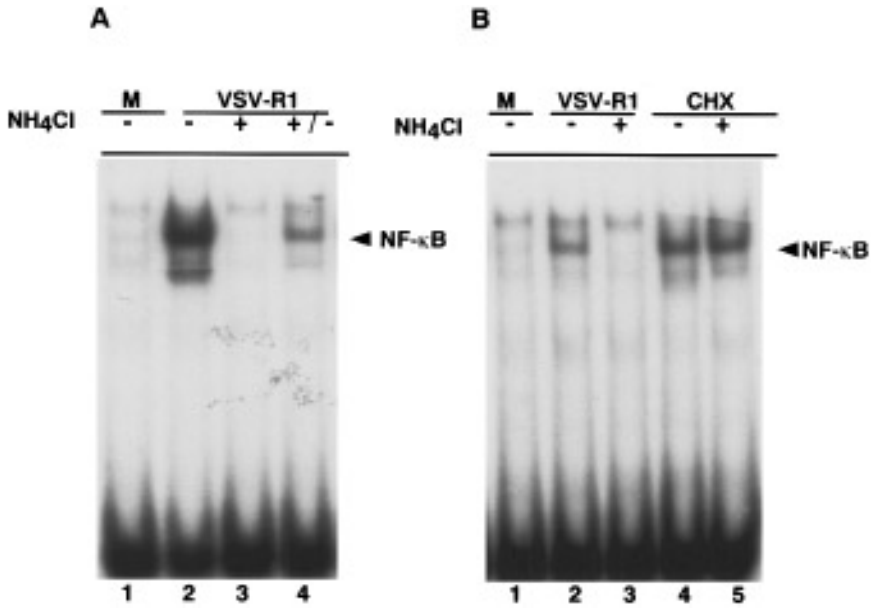

FIG. 3. Effect of NH_4Cl on NF-κB activation in T1026R1-infected cells. (A) NF-κB binding activity in nuclear extracts from: lane 1, mock-infected cells; lane 2, cells infected with T1026R1 for 4 hr; lane 3, cells infected with T1026R1 for 4 hr in the presence of 30 m*M* NH_4Cl; lane 4, cells infected with T1026R1 in the presence of 30 m*M* NH_4Cl for 2 hr and then washed and incubated in fresh medium without the salt for an additional 2 hr. (B) NF-κB binding activity in nuclear extracts from: lane 1, cells mock-infected for 2 hr; lane 2, cells infected with T1026R1 for 2 hr in the absence of NH_4Cl; lane 3, cells infected with T1026R1 for 2 hr in the presence of 30 m*M* NH_4Cl; lane 4, cells treated with 50 μg/ml cycloheximide (CHX) for 2 hr in the absence of NH_4Cl; lane 5, cells treated with cycloheximide (50 μg/ml) for 2 hr in the presence of NH_4Cl.

To determine whether the NH_4Cl had any direct effect on NF-κB activation, we treated cells with a known NF-κB activator, cycloheximide (Wall *et al.,* 1986), in the presence or absence of NH_4Cl. No difference was detected in NF-κB DNA binding activity as shown in Fig. 3B, lanes 4 and 5. These observations suggest that NH_4Cl was functioning to prevent virus entry and delivery of its components into the cytoplasm of cells and was not interfering with NF-κB activation per se. NH_4Cl treatment of T1026R1-infected chick embryo cells has also been reported to reduce IFN induction significantly (Svitlik and Marcus, 1984).

NF-κB activation is delayed in wt virus-infected cells

The suppression of IFN induction in wt virus-infected cells (Marcus and Sekellick, 1987) could result from the inhibition of NF-κB activation. In an attempt to gain some insight into the process of IFN suppression, we infected cells with the wt virus and analyzed nuclear extracts for their NF-κB binding activity. In contrast to the fast activation of NF-κB in T1026R1-infected cells shown in Fig. 1, this transcription factor was not activated until between 4 and 6 hr after infection (Fig. 4A). If IFN induction requires the early activation of NF-κB, then the delay in NF-κB activation could contribute to the suppression of IFN induction.

Interestingly, by 10 hr after infection by wt VSV, the extent of NF-κB activation was approximately the same as in cells infected with T1026R1 for 4 hr (Fig. 4A, lanes 7 and 8). In spite of the late activation of NF-κB, however, there was no corresponding increase in CAT synthesis, as shown in Fig. 4B. These results indicated that NF-κB activation was insufficient to induce the IFN gene and that additional negative regulatory controls were operating, at least at late times during infection.

The delay in activation of NF-κB in wt virus-infected cells was not a result of a change in the cytoplasmic NF-κB pool. Cytoplasmic extracts from wt virus-infected cells retained their capacity to bind the NF-κB probe throughout 10 hr of infection (data not shown), as determined by treating the extracts with sodium deoxycholate to release IκB from the NF-κB:IκB complex (Baeuerle and Baltimore, 1988).

An increase in the multiplicity of wt VSV results in the rapid and extensive activation of NF-κB

If the delay in NF-κB activation was a major cause of the suppression of IFN-CAT gene induction, then by reducing the length of the delay, we should see an induction of CAT synthesis. We attempted to decrease the length of the delay by changing the multiplicity of the infecting wt virus. (The multiplicity of infection in the previous experiment was 5 PFU/cell.)

L cells were infected with wt virus or T1026R1 at multiplicities of 1, 10, 25, and 50 PFU/cell for 2 hr. The infected cells were harvested and nuclear extracts were tested

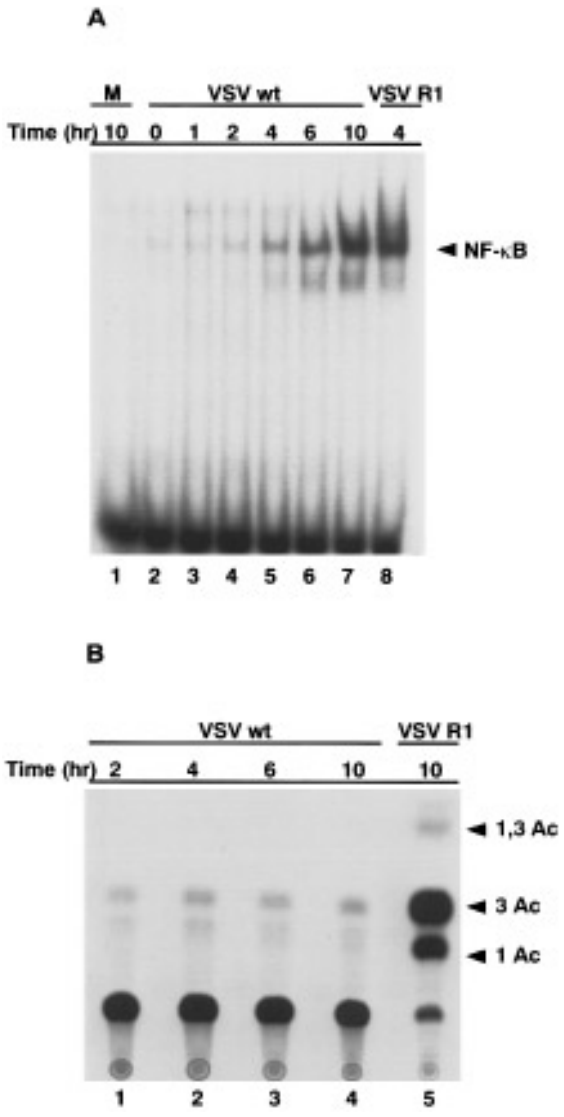

FIG. 4. NF-κB activation and IFN-CAT induction in wt virus-infected cells. Cells were infected with wt virus at a multiplicity of 5 PFU/cell. (A) NF-κB binding activity in nuclear extracts from: lane 1, cells mock-infected for 10 hr; lanes 2–7, cells infected with wt virus at 0, 1, 2, 4, 6, and 10 hr after adsorption, respectively; lane 8, cells infected with T1026R1 for 4 hr. (B) CAT activity in cell extracts from: lanes 1–4, cells infected with wt virus for 2, 4, 6, and 10 hr after adsorption, respectively; lane 5, cells infected with T1026R1 for 10 hr after adsorption.

for their ability to bind to the NF-κB probe. At a multiplicity of 10 PFU/cell, NF-κB binding activity was detected in T1026R1-infected cells, but not in wt virus-infected cells at 2 hr after infection (Fig. 5, lanes 3 and 7). However, at multiplicities of 25 and 50 PFU/cell, NF-κB binding activity was detected at this time point in both wt (Fig. 5, lanes 4 and 5)- and T1026R1 (Fig. 5, lanes 8 and 9)-infected cells, although the extent of activation in wt-infected cells was less than that observed in T1026R1-infected cells. However, no IFN-CAT expression was ever detected in wt virus-infected cells under these same conditions of high multiplicity (data not shown). These results indicated that activation of NF-κB per se was insufficient to induce the IFN-CAT gene.

Presence of inhibitors of both IFN-CAT expression and NF-κB activation in wt VSV-infected cells

The fact that in most cases the extent of NF-κB activation was less in wt virus-infected cells than in T1026R1-infected cells suggested the presence of an inhibitor of NF-κB activation in the former cells. To test this hypothesis, we infected cells with wt virus or T1026R1 (which does not contain IFN suppressor activity) alone and with the two together for 2 or 4 hr and assayed nuclear extracts for NF-κB binding activity. As mentioned earlier, the IFN suppressor phenotype is dominant (Marcus and Sekellick, 1985).

NF-κB binding activity was high in extracts from T1026R1-infected cells and low in wt virus-infected cells at both time points (Fig. 6B, lanes 2, 3, 6, and 7). In cells infected with both viruses, the NF-κB activation capacity of T1026R1 was reduced considerably (Fig. 6B, lanes 4 and 8). The same results were obtained when CAT activity was measured (Fig. 6A, lanes 1–3). Expression of the IFN-CAT gene was high in T1026R1-infected cells and negligible in cells infected with the wt virus alone and with the two viruses together.

To determine whether the IFN-CAT suppressor and the NF-κB activation inhibitor were the same, we compared their sensitivity to UV irradiation. In these experiments wt virus was subjected to UV irradiation prior to

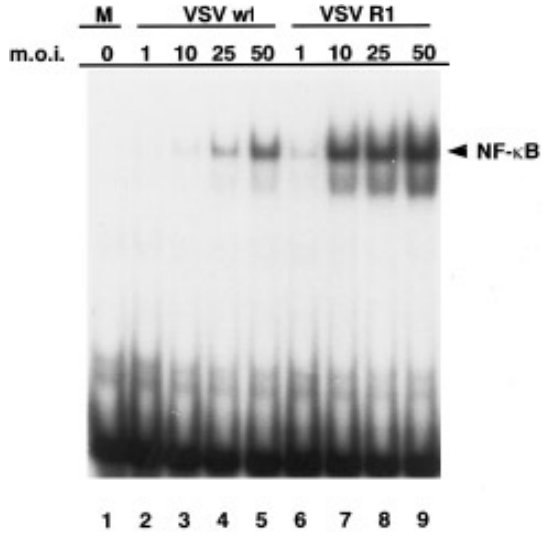

FIG. 5. Effect of multiplicity on NF-κB activation. L cells were infected with an increasing multiplicity of either wt virus or T1026R1. At 2 hr after adsorption NF-κB binding activity was measured in nuclear extracts from: lane 1, mock-infected cells; lanes 2–5, cells infected with wt virus at multiplicities of 1, 10, 25, and 50 PFU/cell, respectively; lanes 6–9, cells infected with T1026R1 at multiplicities of 1, 10, 25, or 50 PFU/cell, respectively.

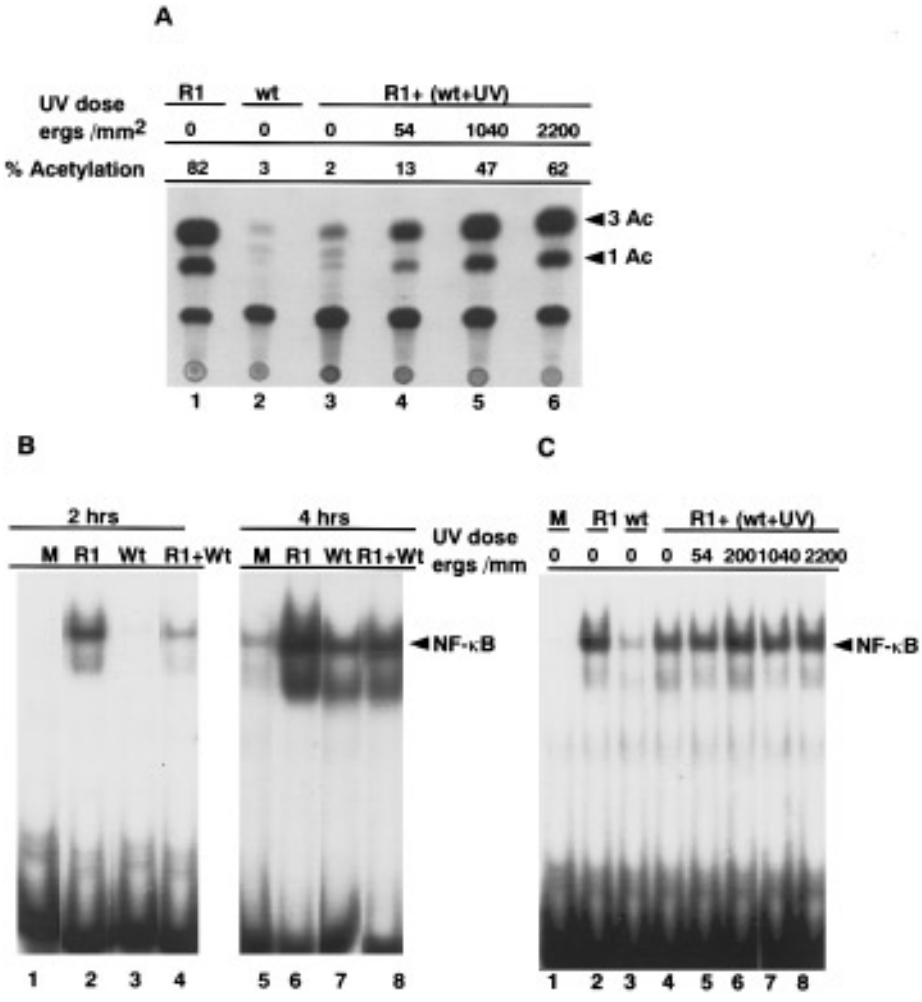

FIG. 6. Effect of wt virus and UV-irradiated wt virus infection on IFN-CAT gene expression and NF-κB activation in T1026R1-infected cells. L cells infected with T1026R1 (multiplicity, 5 PFU/cell) were coinfected with wt virus or UV-irradiated wt virus (multiplicity, 5 PFU/cell) and the effect on NF-κB activation and CAT induction was determined. (A) CAT induction in cell extracts from: lane 1, T1026R1-infected cells; lane 2, wt virus-infected cells; lane 3, cells infected with both T1026R1 and wt virus; lanes 4–6, cells infected with both T1026R1 and wt virus that was UV-irradiated at doses of 54, 1040, or 2200 ergs/mm^2, respectively. (B) NF-κB binding activity in nuclear extracts from: lanes 1 and 5, cells mock-infected for 2 or 4 hr, respectively; lanes 2 and 6, cells infected with T1026R1 for 2 or 4 hr, respectively; lanes 3 and 7, cells infected with wt virus for 2 or 4 hr, respectively; lanes 4 and 8, cells infected with both viruses for 2 or 4 hr, respectively. (C) NF-κB binding activity in nuclear extracts from: lane 1, mock-infected cells; lane 2, cells infected with T1026R1 for 2 hr; lane 3, cells infected for 2 hr with wt virus; lane 4, cells infected with both T1026R1 and wt virus; lanes 5–8, cells infected with T1026R1 and wt virus that was UV-irradiated at doses of 54, 200, 1040, or 2200 ergs/mm^2, respectively. The cpms in the NF-κB band in (C) were: lane 1, 548; lane 2, 2492; lane 3, 1377; lane 4, 1778; lane 5, 2402; lane 6, 3237; lane 7, 2160; and lane 8, 2620.

use in double infections. Since the inducer of NF-κB activation and of IFN induction is very sensitive to UV irradiation (Fig. 2A), it was presumed that the UV-irradiated wt virus no longer contained substantial IFN-inducing or NF-κB activation activity. Inducing activity in coinfection experiments was provided by T1026R1, which was not UV irradiated. These assays measured only the suppressor activity of the wt virus.

As shown (Fig. 6A), it took a rather high dose of UV irradiation, greater than 2200 ergs/mm^2 (equivalent to about 40 hits to the genome), to reduce the IFN-CAT suppressor activity of the wt virus from 98 to 24% (Fig. 6A, lanes 3–6). The high resistance of the IFN-CAT suppressor to UV irradiation suggested that input virions (in addition to other factors) were contributing to the inhibition of CAT expression. In an analogous experiment NF-κB binding activity in nuclear extracts from cells coinfected with T1026R1 and UV-irradiated wt virus was determined (Fig. 6C). In this experiment the extent of radioactivity in each band was quantitated by excising the bands from the gel and counting them in a liquid scintillation counter. One hit (54 ergs/mm^2) of UV irradiation to the wt virus essentially (96% of T1026R1 control activity) inactivated its ability to inhibit NF-κB activation (see leg-

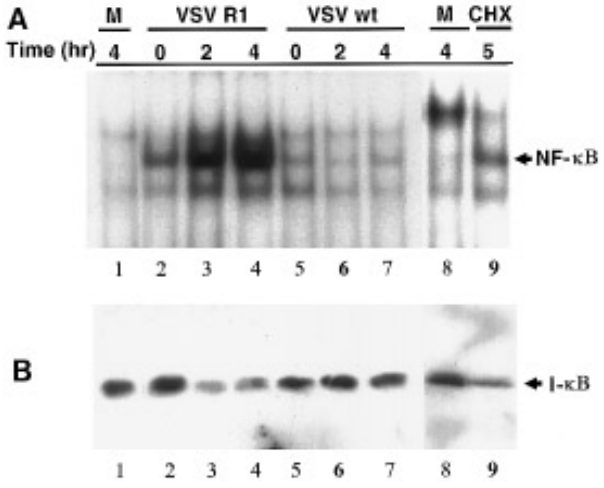

FIG. 7. Correlation between NF-κB activation and IκB degradation in wt- and T1026R1-infected cells. (A) NF-κB binding activity in nuclear extracts from: lanes 1 and 8, cells mock-infected for 4 hr; lanes 2–4, cells infected with T1026R1 for 0, 2, and 4 hr, respectively; lanes 5–7, cells infected with wt virus for 0, 2, and 4 hr, respectively; lane 9, cells treated with 50 μg/ml of cycloheximide (CHX) for 5 hr to activate NF-κB. (B) Western blot of cytoplasmic extracts from cells described in (A) carried out as described under Materials and Methods using anti-IκB antibody.

end to Fig. 6 for data). Therefore, it appears that the NF-κB activation inhibitor and the IFN-CAT suppressor are different components.

The delay in NF-κB activation in wt virus-infected cells occurs at the level of IκB degradation

NF-κB is activated by many components and under various conditions, not all of which lead to IFN induction, as seen above in Fig. 4. Phosphorylation of IκB in the NF-κB:IκB complex and its degradation are essential for NF-κB activation (Miyamoto *et al.,* 1994). We wished to determine whether there was a correlation between NF-κB activation and IκB degradation in T1026R1- and wt virus-infected cells. In this experiment L cells were infected with T1026R1 or wt virus and at 0, 2, and 4 hr, the presence of IκB in the cytoplasm was examined using Western blot analysis. As shown in Fig. 7B, IκB was detected in mock-infected cells (lane 1) and at the 0 time point in T1026R1-infected cells (lane 2). However, IκB was present in much smaller amounts in T1026R1-infected cells at both 2 and 4 hr after infection. The decrease in amount of IκB correlated with an increase in NF-κB activation in nuclear extracts from T1026R1-infected cells (Fig. 7A, lanes 3 and 4). The amount of IκB in wt virus-infected cell lysates was unchanged throughout 4 hr of infection (Fig. 7B, lanes 5–7), consistent with the lack of NF-κB activation at these time points (Fig. 7A, lanes 5–7). In the control samples shown in Fig. 7A and B, lanes 8 and 9, the activation of NF-κB by cycloheximide was accompanied by IkB degradation.

DISCUSSION

NF-κB DNA binding activity was detected early in infection in VSV T1026R1-infected mouse L cells (Fig. 1A). This fast activation, seen also when cells are subjected to other stimuli such as TNFα (Miyamoto *et al.,* 1994), phorbol myristate acetate (Sen and Baltimore, 1986), and H_2O_2 (Schreck *et al.,* 1992), is very important in the early response of cells to external stimuli. The rapid activation of NF-κB coincided with the early appearance of IFN-CAT gene expression seen by 2 hr after infection (Fig. 1B). NF-κB activation leveled off at approximately 4 hr after infection, whereas CAT activity continued to increase up to at least 16 hr. Virus entry and release of its components into the cytoplasm were required for NF-κB activation. Extensive NF-κB activation and IFN-CAT gene expression also depended on viral replication and amplified RNA synthesis, since one hit of UV radiation to the viral genome nearly completely reduced these activities (Fig. 2). These results suggested that the IFN inducer in these L cells must be synthesized in relatively large amounts to be effective.

The observation that NF-κB activation was delayed for between 4 and 6 hr in cells infected with the wt virus was of particular interest because it provided a possible explanation for the suppression of IFN induction in mouse L cells. The activation of NF-κB at late times during infection and at early times at high multiplicities of infection without corresponding expression of the CAT reporter gene was not deemed to diminish the importance of the NF-κB activation delay. Under these conditions both host transcription and translation are severely inhibited (Dunigan *et al.,* 1986). Therefore, even if NF-κB was activated, transcription of the CAT gene could not have taken place.

The presence of two seemingly different inhibitors of the IFN induction process in wt virus infected cells was also of interest. The NF-κB activation inhibitor was blocked by one hit of UV irradiation to the VSV genome, whereas the CAT expression inhibitor was much more UV resistant. The capacity of VSV (wt) to inhibit host transcription is also a highly UV resistant property (Dunigan *et al.,* 1986), leading us to speculate that what we measured in terms of CAT expression was the ability of the virus to shut off host transcription.

As shown in these studies, IFN induction is an early event in T1026R1-infected cells. Within 2 hr after infection, both NF-κB was activated and the CAT reporter gene was expressed. (RNA synthesis is not inhibited during the early hours of infection by this virus.) In contrast, in wt virus infected cells, NF-κB activation was delayed early in infection. By the time NF-κB was activated, host RNA transcription was so severely inhibited (as noted above) that any IFN gene expression was precluded. Therefore, it seems that there is a delicate bal-

ance between NF-κB activation and host DNA transcription. To block IFN induction the wt virus regulates these two activities by viral coded suppressors, which are probably defective in mutant T1026R1.

The correlation of IκB degradation with NF-κB activation in T1026R1-infected cells suggested that IκB must have been phosphorylated, since phosphorylation is a prerequisite for its degradation (Miyamoto *et al.,* 1994). The lack of IκB degradation in wt virus-infected cells under conditions in which NF-κB activation was delayed, in turn, implied that the delay may have resulted from failure of IκB to be phosphorylated. Therefore, the putative inhibitor of NF-κB activation described above may act by regulating the phosphorylation of IκB. Further studies are necessary to clarify the role of the inhibitor in this process.

ACKNOWLEDGMENTS

We thank Carol Menditto for her valuable technical assistance, Dawna Houston for quantitating our CAT assays, and Dr. Robert Schloemer for kindly providing plasmid pTWU54. This research has benefited from use of the Animal Cell Culture Facility and the Macromolecular Characterization Facility of the Biotechnology Center at the University of Connecticut. This research was supported by the University of Connecticut Research Foundation and by USDA Grant 93-37204-9335.

REFERENCES

Baeuerle, P. A., and Baltimore, D. (1988). Activation of DNA-binding activity in an apparently cytoplasmic precursor of NF-κB transcription factor. *Cell* 53, 211–217.

Ball, L. A., and White, C. N. (1976). Order of transcription of genes of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. USA* 73, 442–446.

Beg, A. A., Finco, T. S., Nantermet, P. V., and Baldwin, A. S., Jr. (1993). Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: A mechanism for NF-κB activation. *Mol. Cell. Biol.* 13, 3301–3310.

Chiao, P. J., Miyamoto, S., and Verma, I. M. (1994). Autoregulation of IκBα activity. *Proc. Natl. Acad. Sci. USA* 91, 28–32.

Du, W., and Maniatis, T. (1992). An ATF/CREB binding site is required for virus induction of the human interferon β gene. *Proc. Natl. Acad. Sci. USA* 89, 2150–2154.

Du, W., Thanos, D., and Maniatis, T. (1993). Mechanism of transcriptional synergism between distinct virus-inducible enhancer elements. *Cell* 74, 887–898.

Dunigan, D. D., and Lucas-Lenard, J. M. (1983). Two transcription products of the vesicular stomatitis virus genome may control L cell protein synthesis. *J. Virol.* 45, 618–626.

Dunigan, D. D., Baird, S., and Lucas-Lenard, J. M. (1986). Lack of correlation between the accumulation of plus-strand leader RNA and the inhibition of protein and RNA synthesis in vesicular stomatitis virus infected mouse L cells. *Virology* 150, 231–246.

Fujita, T., Ohno, S., Yasumitsu, H., and Taniguchi, T. (1985). Delimitation and properties of DNA sequences required for the regulated expression of human interferon-β gene. *Cell* 41, 489–496.

Fujita, T., Sakakibara, J., Sudo, Y., Miyamoto, M., Kimura, Y., and Taniguchi, T. (1988). Evidence for a nuclear factor(s), IRF-1, mediating induction and silencing properties to human IFN-beta gene regulatory elements. *EMBO J.* 7, 3397–3405.

Goodbourn, S. E. Y., and Maniatis, T. (1988). Overlapping positive and negative regulatory elements of the human β-interferon gene. *Proc. Natl. Acad. Sci. USA* 85, 1447–1451.

Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2, 1044–1051.

Grimm, S., and Baeuerle, P. A. (1993). The inducible transcription factor NF-κB: Structure–function relationship of its protein subunits. *Biochem. J.* 290, 297–308.

Harada, H., Fujita, T., Miyamoto, M., Kimura, Y., Maruyama, M., Furia, A., Miyata, T., and Taniguchi, T. (1989). Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN inducible genes. *Cell* 58, 729–739.

Keller, A. D., and Maniatis, T. (1991). Identification and characterization of a novel repressor of β-interferon gene expression. *Genes Dev.* 5, 868–879.

LeBlanc, J.-F., Cohen, L., Rodrigues, M., and Hiscott, J. (1990). Synergism between distinct enhanson domains in viral induction of the human beta interferon gene. *Mol. Cell. Biol.* 10, 3987–3993.

Lenardo, M. J., Fan, C.-M., Maniatis, T., and Baltimore, D. (1989). The involvement of NF-κB in β-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. *Cell* 57, 287–294.

Maniatis, T., Wittemore, L. A., Du, W., Fan, C.-M., Keller, A. D., Palombella, V. J., and Thanos, D. N. (1992). Positive and negative regulation of the human interferon-β gene transcription. *In* "Transcriptional Regulation" (S. McKnight and K. Yamamoto, Eds.), pp. 1193–1220. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Marcus, P. I., and Sekellick, M. J. (1980). Interferon induction by viruses. III. Vesicular stomatitis virus: Interferon-inducing particle activity requires partial transcription of gene N. *J. Gen. Virol.* 47, 89–96.

Marcus, P. I., and Sekellick, M. J. (1985). Interferon induction by viruses. XIII. Detection and assay of interferon induction suppressing particles. *Virology* 142, 411–415.

Marcus, P. I., and Sekellick, M. J. (1987). Interferon induction by viruses. XV. Biological characteristics of interferon inducing-suppressing particles of vesicular stomatitis virus. *J. Interferon Res.* 7, 269–284.

Marvaldi, J. L., Lucas-Lenard, J., Sekellick, M. J., and Marcus, P. I. (1977). Cell killing by viruses. IV. Cell killing and protein synthesis inhibition by vesicular stomatitis virus require the same gene functions. *Virology* 79, 267–280.

Marvaldi, J. L., Sekellick, M. J., Marcus, P. I., and Lucas-Lenard, J. (1978). Inhibition of mouse L cell protein synthesis by ultraviolet-irradiated vesicular stomatitis virus requires viral transcription. *Virology* 84, 127–133.

Matlin, K. S., Reggio, H., Helenius, A., and Simons, K. (1982). Pathway of vesicular stomatitis virus entry leading to infection. *J. Mol. Biol.* 156, 609–631.

Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T., and Taniguchi, T. (1988). Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory element. *Cell* 54, 903–913.

Miyamoto, S., Maki, M., Schmitt, M. J., Hatanaka, M., and Verma, I. M. (1994). Tumor necrosis factor α-induced phosphorylation of IκBα is a signal for its degradation but not dissociation from NF-κB. *Proc. Natl. Acad. Sci. USA* 91, 12740–12744.

Palombella, V. J., and Maniatis, T. (1992). Inducible processing of interferon regulatory factor-2. *Mol. Cell. Biol.* 12, 3325–3336.

Reis, L. F., Harada, H., Wolchok, J. D., Taniguchi, T., and Vilcek, J. (1992). Critical role of a common transcription factor, IRF-1, in the regulation of IFN-beta and inducible genes. *EMBO J.* 11, 185–193.

Samuel, C. E. (1991). Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective activiral activities. *Virology* 183, 1–11.

Schreck, R., Meier, B., Mannel, D. N., Droge, W., and Baeuerle, P. A. (1992). Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J. Exp. Med.* 175, 1181–1194.

Schutze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Kronke, M. (1992). TNF activates NF-κB by phosphatidylcholine-specific phopholipase C-induced "acidic" sphingomyelin breakdown. *Cell* 71, 765–776.

Sen, R., and Baltimore, D. (1986). Inducibility of K immunoglobulin enhances binding protein NF-κB by a posttranslational mechanism. *Cell* 47, 921–928.

Sen, G. C., and Lengyel, P. (1992). The interferon system. A bird's eye view of its biochemistry. *J. Biol. Chem.* 267, 5017–5020.

Stanners, C. P., Francoeur, A. M., and Lam, T. (1977). Analysis of VSV mutant with attenuated cytopathogenicity: Mutation in viral function, P, for inhibition of protein synthesis. *Cell* 11, 273–281.

Superti, F., Seganti, L., Ruggeri, F. M., Tinari, A., Donelli, G., and Orsi, N. (1987). Entry pathway of vesicular stomatitis virus into different host cells. *J. Gen. Virol.* 68, 387–399.

Svitlik, C., and Marcus, P. I. (1984). Interferon induction by viruses. XI. Early events in the induction process. *J. Interferon Res.* 4, 585–602.

Thanos, D., and Maniatis, T. (1992). The high mobility group protein HMG I(Y) is required for NF-κB-dependent virus induction of the human IFN-beta gene. *Cell* 71, 777–789.

Thanos, D., and Maniatis, T. (1995). NF-κB: A lesson in family values. *Cell* 80, 529–532.

Twu, J. S., and Schloemer, R. H. (1987). Transcriptional trans-activating function of hepatitis B virus. *J. Virol.* 61, 3448–3453.

Urban, M. B., Schreck, R., and Baeuerle, P. A. (1991). NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO J.* 10, 1817–1825.

Wagner, R. R., and Huang, A. S. (1966). Inhibition of RNA and interferon synthesis in Krebs-2 cells infected with vesicular stomatitis virus. *Virology* 28, 1–10.

Wall, R., Briskin, M., Carter, C., Govan, H., Taylor, A., and Kincade, P. (1986). A labile inhibitor blocks immunoglobulin K-light-chain-gene transcription in a pre-B leukemic cell line. *Proc. Natl. Acad. Sci. USA* 83, 295–298.

Weck, P. K., and Wagner, R. R. (1978). Inhibition of RNA synthesis in mouse myeloma cells infected with vesicular stomatitis virus. *J. Virol.* 25, 770–780.

Wertz, G., and Youngner, J. (1972). Inhibition of protein synthesis in L cells infected with vesicular stomatitis virus. *J. Virol.* 9, 85–89.

Wu, F.-S., and Lucas-Lenard, J. (1980). Inhibition of ribonucleic acid accumulation in mouse L cells infected with vesicular stomatitis virus requires viral ribonucleic acid transcription. *Biochemistry* 19, 804–810.