

# Gamma Interferon-Induced, Nitric Oxide-Mediated Inhibition of Vaccinia Virus Replication

NICHOLAS HARRIS, R. MARK L. BULLER,<sup>†</sup> AND GUNASEGARAN KARUPIAH\*

*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases,  
Bethesda, Maryland 20892*

Received 29 September 1994/Accepted 9 November 1994

**Gamma interferon (IFN- $\gamma$ )-induced nitric oxide synthase (iNOS) and nitric oxide (NO) production in the murine macrophage-like RAW 264.7 cells were previously shown to inhibit the replication of the poxviruses vaccinia virus (VV) and ectromelia virus and herpes simplex virus type 1. In the current study, we performed biochemical analyses to determine the stage in the viral life cycle blocked by IFN- $\gamma$ -induced NO. Antibodies specific for temporally expressed viral proteins, a VV-specific DNA probe, and transmission electron microscopy were used to show that the cytokine-induced NO inhibited late protein synthesis, DNA replication, and virus particle formation but not expression of the early proteins analyzed. Essentially similar results were obtained with hydroxyurea and cytosine arabinoside, inhibitors of DNA replication. Enzymatically active iNOS was detected in the lysates of IFN- $\gamma$ -treated but not in untreated RAW 264.7 cells. The IFN- $\gamma$ -treated RAW 264.7 cells which express iNOS not only were resistant to productive infection but also efficiently blocked the replication of VV in infected bystander cells of epithelial origin. This inhibition was arginine dependent, correlated with nitrite production in cultures, and was reversible by the NOS inhibitor *N*<sup>ω</sup>-monomethyl-L-arginine.**

The induction of an antiviral state by interferons (IFNs) is one of the earliest known responses to viral infection and is an essential element in nonspecific host defense mechanisms. IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\omega$  are produced by many different cells in response to virus infection, whereas IFN- $\gamma$  is produced mainly by activated natural killer (NK) and T cells. Each species of IFN can confer protection through the induction of proteins and the activation of enzymatic pathways which interfere with viral replication and block progeny virus production (7, 17, 26, 32, 34, 40).

Some viruses, like vaccinia virus (VV), have evolved mechanisms to counteract the IFN-induced antiviral host response in cells of fibroblastic and epithelial lineages (1, 3, 29, 30). The resistance of VV to IFN- $\alpha$ , - $\beta$ , and - $\omega$  is due, in part, to the presence of two open reading frames, namely, E3L and K3L (1, 3, 6). The products of these two genes and at least one other (29, 30) interfere with the IFN-induced P<sub>1</sub>/eukaryotic initiation factor 2 kinase and 2',5'-oligoadenylate A synthetase antiviral pathways. In contrast, the replication of VV in macrophages is completely restricted by IFN- $\gamma$ , suggesting that either the E3L and K3L gene products cannot overcome the IFN- $\gamma$ -induced inhibition of virus replication in macrophages or the antiviral activity of IFN- $\gamma$  may be mediated by a mechanism(s) other than the aforementioned antiviral pathways. The latter demonstration proved correct with the findings that IFN- $\gamma$ -mediated inhibition of VV, ectromelia virus, and herpes simplex virus type 1 in murine macrophages was through induction of nitric oxide synthase (iNOS) (4, 16). This is the inducible isoform of the enzyme which catalyzes the synthesis of large amounts of nitric oxide (NO) from the guanidino nitrogen of

L-arginine (L-A) (8, 12, 22, 27, 28, 37). IFN- $\gamma$ -induced, high-output NO is known to have potent antimicrobial activity against several classes of pathogens (27, 28). Although IFN- $\gamma$  alone is sufficient to induce iNOS (5, 8, 13), it can also synergize with IFN- $\alpha$  and - $\beta$  and tumor necrosis factor alpha, which, by themselves, are unable to induce the enzyme (8).

The replication cycle of poxviruses proceeds through discrete developmental stages which are regulated genetically by temporally expressed, virally encoded transcription factors (23, 24, 39). The infectious virion contains all of the factors and enzymes necessary for early gene expression. Viral DNA replication begins after virion uncoating and is accompanied by the expression of viral intermediate genes. Late gene expression begins prior to assembly of new virions, and the viral life cycle is completed with the dissemination of progeny virus. Genes of each temporal class are transactivated by proteins encoded by preceding classes, thereby constituting a regulatory cascade. Since poxvirus DNA replication commences after uncoating of the virion, it was thought that the observed NO-mediated inhibition of virus replication may be effective only after exposure of viral DNA. This theory was supported by the observation that purified virus particles are resistant to NO (16). Exposure of virions to high concentrations of NO-producing compounds for several hours had no effect on the infectivity or replication of VV, ectromelia virus, or herpes simplex virus type 1.

We have performed biochemical analyses to determine the developmental stage in the viral life cycle which is blocked by IFN- $\gamma$ -induced NO in RAW 264.7 cells. In addition, we assayed IFN- $\gamma$ -treated RAW 264.7 cells for synthesis of the inducible form of iNOS and investigated their ability to inhibit viral replication in infected, untreated bystander cells.

## MATERIALS AND METHODS

**Viruses.** A crude stock of the WR strain of VV (VV-WR; ATCC VR-1354) propagated in BS-C-1 cells was used for infection of cell cultures in vitro. The inoculum, diluted in phosphated-buffered saline (PBS) to contain 10<sup>6</sup> PFU/ml,

\* Corresponding author. Present address: Division of Cell Biology, The John Curtin School of Medical Research, The Australian National University, P.O. Box 334, Canberra, ACT 2601, Australia. Phone: 61 6 249 26 27. Fax: 61 6 247 8921.

<sup>†</sup> Present address: Dept. of Molecular Biology and Immunology, School of Medicine, St. Louis University, St. Louis, MO 63104.

had no detectable endotoxin (<10 pg/ml) by a chromogenic *Limulus* amebocyte lysate assay (BioWhittaker Inc., Walkersville, Md.). The KOS strain of herpes simplex virus type 1, a gift from M. Challberg, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), was propagated in BS-C-1 cells. The New Jersey strain of vesicular stomatitis virus (VSV-NJ) was kindly provided by A. Hugin (Laboratory of Immunopathology, NIAID, NIH). Supernatants from VSV-infected Vero cell cultures were used as a source of virus to determine the biological activities of IFN- $\alpha$ , - $\beta$ , and - $\gamma$ .

**Cell lines.** RAW 264.7, a mouse macrophage-like cell line, was maintained in RPMI 1640 (BioWhittaker) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 500  $\mu$ M 2-mercaptoethanol, antibiotics, and 10% fetal calf serum (<5 pg of endotoxin per ml; HyClone Laboratories, Logan, Utah), hereafter referred to as complete RPMI. The human renal epithelial cell line 293 was maintained in Eagle's minimal essential medium (EMEM; BioWhittaker) supplemented with L-glutamine, antibiotics, and 10% fetal calf serum (complete EMEM).

**IFNs, IFN assays, and IFN units.** Purified murine IFN- $\alpha$  (lot 85042) and - $\beta$  (lot 83053) were purchased from Lee Biomolecular Research Laboratories, Inc. (San Diego, Calif.). The IFN activity had been established by the manufacturer by comparative assays with IFN reference reagents provided by the Antiviral Substances Program, NIAID, NIH. Recombinant murine IFN- $\gamma$  (<10 pg of endotoxin) was a gift from Genentech Inc. (South San Francisco, Calif.). The biological activity of each IFN species, on the basis of inhibition of VSV replication, was determined as described elsewhere (15). One unit of IFN is expressed as the reciprocal of the highest dilution of each IFN species which yields 50% inhibition of VSV replication in murine L929 cells.

In order to demonstrate the existence of the IFN- $\gamma$ -induced, L-A-dependent, NO-mediated antiviral pathway in RAW 264.7 cells (16), the effective concentration of IFN- $\gamma$  used for complete inhibition of VV replication, determined by measurement of progeny virus titers, was between 20 and 30 U and was always pretreated in the presence or absence of *N*<sup>ω</sup>-monomethyl-L-arginine (L-NMA).

**Reagents.** L-A, D-arginine (D-A), L-NMA, *N*<sup>ω</sup>-monomethyl-D-arginine (D-NMA), L-cysteine, cytosine arabinoside (Ara C), and hydroxyurea (HU) were obtained from Sigma Immunochemicals (St. Louis, Mo.).

**TEM.** Medium was removed from cultures, and cells were fixed for 30 min with 2.5% glutaraldehyde in Millonig's sodium phosphate buffer (pH 7.9) at 4°C, after which cells were scraped and centrifuged at 500  $\times$  g. Fixation of the cell pellet was continued for a further 90 min at 4°C, after which the fixative was aspirated and replaced with sodium phosphate buffer lacking glutaraldehyde. Samples were stored at 4°C until processed for transmission electron microscopy (TEM).

**Determination of progeny virus titers.** Virus titers in infected cell cultures were determined as described elsewhere (15, 16).

**Coculture of IFN- $\gamma$ -activated RAW 264.7 cells with VV-infected 293 cells.** RAW 264.7 cells ( $2 \times 10^7$  to  $3 \times 10^7$ ) were incubated with 100 U of IFN- $\gamma$  per ml for 20 h in 162-cm<sup>2</sup> flasks (Costar, Cambridge, Mass.) containing 20 ml of complete RPMI 1640. The cells were washed twice in PBS, and  $10^6$  cells were cocultured with  $2 \times 10^5$  VV-infected 293 cells. The 293 cells, infected with VV that had been adsorbed at 1 PFU per cell for 45 min, were washed three times to remove unadsorbed virus. Immediately after mixture of the RAW 264.7 cells with virus-infected cells at a ratio of 5:1, various reagents were added to the cultures in wells of 24-well plates and incubated in a humidified atmosphere containing 5% CO<sub>2</sub> in air at 37°C for 12 to 24 h, depending on the experiment.

**Nitrite and NOS assays.** NO synthesis in cultures was determined by measurement of nitrite (NO<sub>2</sub><sup>-</sup>), a stable product of NO, as described elsewhere (8). For the determination of NOS activity, crude cytosolic fractions (30  $\mu$ g of protein, Bradford method; Bio-Rad) prepared from IFN- $\gamma$ -treated (100 U/ml for 48 h) and untreated RAW 264.7 cells were used in an assay described elsewhere (36).

**Metabolic labeling of viral proteins.** Cells were grown to subconfluency ( $1 \times 10^6$  to  $2 \times 10^6$  cells per well) in 6- or 12-well cluster plates (Costar). The medium was aspirated, and the cells were washed with PBS, following which 1 ml of methionine-minus EMEM supplemented with 5% dialyzed fetal calf serum and 30  $\mu$ Ci of [<sup>35</sup>S]methionine (>1,000 Ci/mmol; Amersham, Arlington Heights, Ill.) was added to each well. After the cells were labeled, the medium was aspirated, and the cells were washed with PBS, harvested in 1 ml of PBS, and centrifuged in a bench-top centrifuge. After PBS was aspirated, cells were lysed in 200  $\mu$ l of lysis buffer (2% Triton X-100, 50 mM Tris-HCl at pH 7.4, 100 mM NaCl, 1 mM EDTA at pH 7.4). Incorporation of [<sup>35</sup>S]methionine was determined by trichloroacetic acid precipitation of labeled proteins onto nitrocellulose membrane and measurement with a  $\beta$ -scintillation counter. Metabolically labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% acrylamide) and fluorography (Amplify; Amersham).

**Quantification of viral DNA.** At the end of culture period, medium was aspirated from wells of 293 cell monolayers, and cells were scraped and resuspended in 200  $\mu$ l of PBS. The cells were lysed by three cycles of freeze (-70°C) and thaw (37°C) and sonicated (two 60-s pulses, W-385 sonicator; Heat-Systems-Ultrasonics, Inc., Farmingdale, N.Y.). The DNA was denatured with 0.25 M sodium hydroxide (NaOH) for 10 min at room temperature, and the lysates were then kept on ice, diluted to 0.2 M NaOH-0.1  $\times$  SSC (1  $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and sonicated as described above. A 100- $\mu$ l volume of each sample was blotted onto nylon membrane (GeneScreen Plus; New England

Nuclear) with a Mini fold II slot blotter (Schleicher and Schuell, Keene, N.H.). The membrane was incubated in prehybridization buffer (1% SDS, 1 M NaCl, 200  $\mu$ g of herring sperm DNA per ml) for 15 min at 65°C. Denatured, radiolabeled VV DNA probe (Random Primer DNA labeling kit; Boehringer Mannheim, Indianapolis, Ind.) was added to the prehybridization medium, and the blot was incubated overnight at 65°C. The hybridized blot was washed twice with 2  $\times$  SSC (5 min at room temperature) and then with 2  $\times$  SSC and 1% SDS (30 min at 65°C). The radiolabeled DNA probe hybridized to the blot was then quantitated with a Betagen Betascope blot analyzer.

**Immunoprecipitation.** The VV early gene products, E3L and K1L proteins, were precipitated with the monoclonal antibody (MAb) TW2.3 (43) (a gift of J. Cox) and a rabbit polyclonal antibody (Ab) (a gift of R. Drillien), respectively. Rabbit polyclonal Abs were used to precipitate the viral late gene products of 65 kDa (44) and 70 kDa (also known as 4b) (35). Antibodies (1 to 5  $\mu$ l) were incubated with 50  $\mu$ l of protein A-Sepharose (PAS; 20% [vol/vol]; Sigma) for 1 h at 4°C with agitation. The PAS suspension was centrifuged briefly, the supernatant was aspirated, and the PAS pellet was washed with PBS before being resuspended with precleared cell lysate. Cell lysates were precleared by incubation with 2  $\mu$ l of preimmune serum, 4% PAS, and PBS in a final volume of 250  $\mu$ l for 1 h at 4°C, with agitation. This precleared lysate was used to resuspend the pellet of antibody-bound PAS. The lysate plus antibody-bound PAS suspension was incubated for 4 h at 4°C with agitation. Following centrifugation, the lysate was aspirated and the PAS was washed twice with PBS. Protein was eluted from the PAS with 20  $\mu$ l of PAGE sample buffer (62.5 mM Tris-HCl at pH 6.8 containing 2% SDS, 10% glycerol, 0.0025% bromophenol blue, and 5% 2-mercaptoethanol). The samples were heated at 100°C for 5 min, centrifuged briefly, and then analyzed by PAGE and fluorography.

**Detection of iNOS by immunoblotting.** Cell lysates (30  $\mu$ g of protein, Bradford method; Bio-Rad; for lysate preparation see "Metabolic labeling of viral proteins" above) were resolved by SDS-PAGE on a 4 to 20% gradient gel (Daichi Pure Chemicals, Tokyo, Japan) and probed with a rabbit polyclonal Ab (anti-NO16; a gift from C. Nathan) to the C-terminal peptide (Cys-Nle-Glu-Glu-Pro-Lys-Ala-Thr-Arg-Leu-COOH; synthesized by J. Weidner and R. Mumford) derived from the long form of mouse macrophage iNOS (42). The protein was detected by chemiluminescence (Amersham) and autoradiography.

## RESULTS

**IFN- $\gamma$  inhibits VV protein synthesis.** Treatment of RAW 264.7 cells with IFN- $\gamma$  but not IFN- $\alpha$  or - $\beta$  inhibited VV progeny production in cultures (14). To determine if this effect was due to the inhibition of viral protein synthesis, infected and uninfected RAW 264.7 cells were metabolically labeled with [<sup>35</sup>S]methionine and analyzed by PAGE. The protein profile of VV-infected cells at 20 h postinfection (p.i.) was markedly different from that of uninfected cells shown in Fig. 1. The cellular proteins detectable in uninfected cells were absent from infected cells, as can be expected, since VV infection shuts down host protein synthesis. Late proteins of 97, 69, and 25 to 45 kDa were prominent in the lysates of infected cells. VV late gene expression requires viral DNA synthesis; therefore, virus-infected cells treated with HU, an inhibitor of DNA synthesis, had a protein profile almost identical to that of uninfected cells. A difference between the HU-treated, uninfected, and infected samples was evident in the low molecular mass range of 21.5 to 30 kDa. The protein present in the infected samples but absent from the uninfected lysates is characteristic of an early gene product which would be made before DNA replication.

Treatment of uninfected cells with each of the IFNs had no apparent effect on cellular metabolism, and protein profiles of the IFN- $\alpha$ - and of the IFN- $\beta$ -treated infected cells were indistinguishable from that of untreated infected cells. The protein profile of IFN- $\gamma$ -treated infected cells, however, was almost identical to that of the IFN- $\gamma$ -treated uninfected cells. The protein doublet, between 21.5 and 30 kDa, present in the IFN- $\gamma$ -treated, infected cells but absent from uninfected cells is similar to the product obtained with HU-treated cells. IFN- $\gamma$ , therefore, inhibited the expression of viral proteins, with the apparent exception of a minor number of proteins which comigrated with viral early gene products.

**IFN- $\gamma$ -mediated inhibition of VV late gene expression.** The preceding analysis of metabolically labeled cell lysates indi-

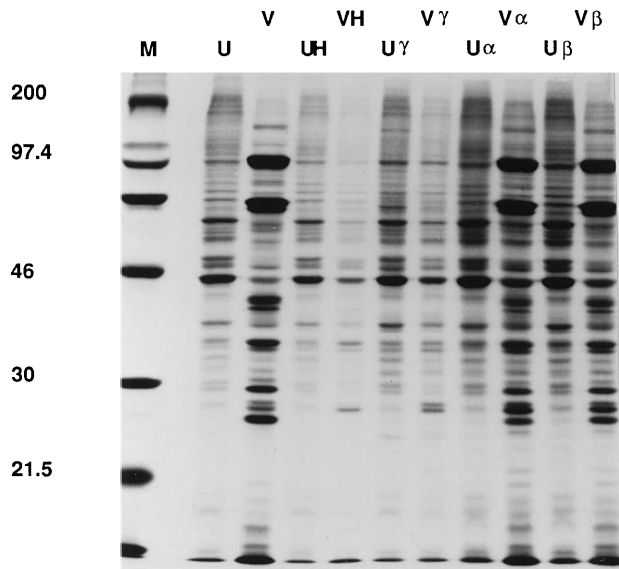


FIG. 1. Inhibition of VV protein synthesis by IFN- $\gamma$ . Cell lysates were made from metabolically labeled ( $^{35}\text{S}$ )methionine uninfected (U) and VV-infected (V) RAW 264.7 cells (10 PFU per cell) 20 h p.i. and analyzed by PAGE and fluorography. Cells were treated with 5 mM HU (H) or with 25 U of IFN- $\gamma$  ( $\gamma$ ), IFN- $\alpha$  ( $\alpha$ ), or IFN- $\beta$  ( $\beta$ ) per ml. Protein molecular mass markers (M) are indicated in kilodaltons.

cated that viral late gene proteins were not expressed in IFN- $\gamma$ -treated cells but suggested that viral early gene proteins were expressed in IFN- $\gamma$ -treated cells. Using antisera specific for early proteins, we confirmed that early genes were expressed in IFN- $\gamma$ -treated cells. Antisera against VV proteins encoded by the open reading frames E3L (25 and 19 kDa) and K1L (32 kDa) precipitated these early gene products from metabolically-labeled lysates of infected but not of uninfected RAW 264.7 cells (Fig. 2A). The diminution of the E3L signal obtained from the VV-infected, IFN- $\gamma$ -treated extract was not observed in other experiments and was attributed to experimental variation. Ara C, an inhibitor of DNA synthesis, had no effect on early gene proteins. Neither IFN- $\gamma$  nor IFN- $\alpha$  had any apparent effect on expression of the early gene proteins, suggesting that the inhibition of VV protein synthesis by IFN- $\gamma$  shown in Fig. 1 occurred subsequently to early gene expression.

The VV 65- and 70-kDa (also known as 4b) late gene proteins were immunoprecipitated from VV-infected but not from uninfected cell lysates (Fig. 2B). Late protein synthesis is dependent on viral DNA replication; therefore, in the presence of the DNA synthesis inhibitor Ara C, neither the 65- nor the 70-kDa late protein was detected. A similar effect was obtained with IFN- $\gamma$ , which had no apparent effect on early gene protein expression but inhibited postreplicative protein synthesis. Neither of the late gene proteins (65 and 70 kDa) was detected in the IFN- $\gamma$ -treated samples (Fig. 2B). Since each temporal class of VV proteins is regulated by proteins of the preceding classes, IFN- $\gamma$  could have inhibited either viral intermediate or viral late gene protein synthesis.

**Absence of VV DNA and virus particles in IFN- $\gamma$ -treated RAW 264.7 cells.** Since VV late protein synthesis is strictly dependent on viral DNA replication, we investigated whether the absence of viral late proteins in IFN- $\gamma$ -treated RAW 264.7 cell lysates could be correlated with inhibition of DNA synthesis. Using radiolabeled viral DNA as a hybridization probe, we could not detect VV DNA in infected, IFN- $\gamma$ -treated cell lysates at 16 h p.i. (Fig. 3A). As expected, a similar observation

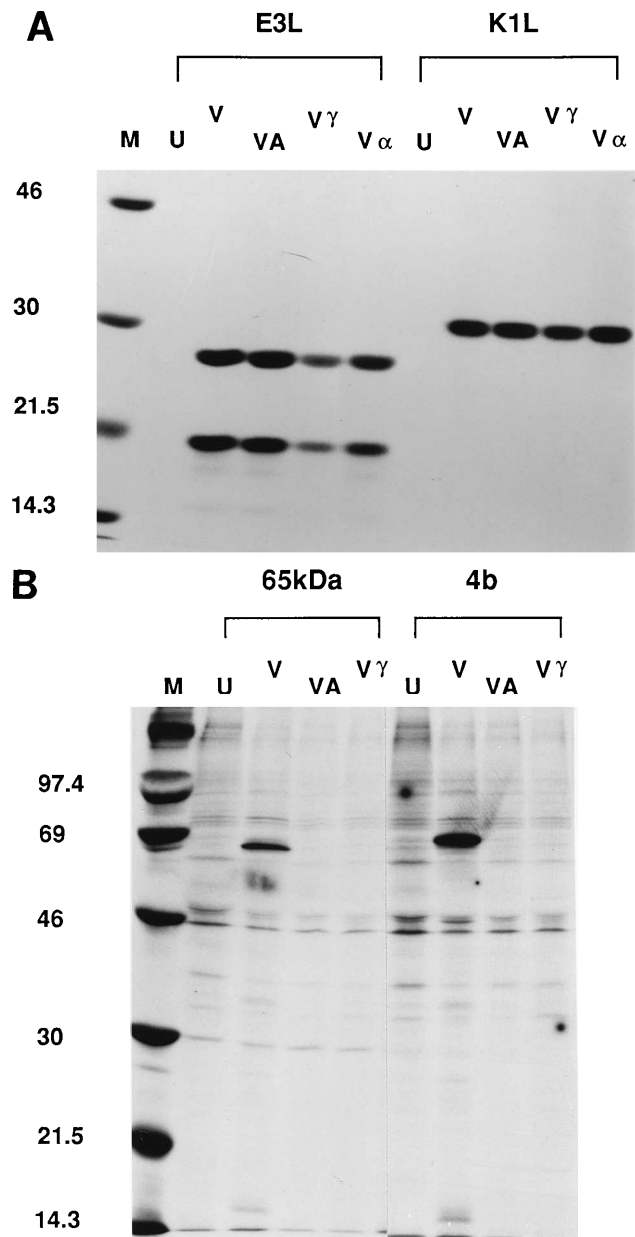


FIG. 2. Inhibition of VV late but not early gene protein synthesis by IFN- $\gamma$ . (A) Lysates of metabolically labeled ( $^{35}\text{S}$ )methionine uninfected (U) and VV-infected (V) RAW 264.7 cells (1 PFU per cell) were analyzed with Ab specific for the viral early gene proteins E3L (25 and 19 kDa) and K1L (32 kDa) 4 h p.i. Cells were treated with 40  $\mu\text{g}$  of Ara C (A) per ml or with 25 U of IFN- $\gamma$  ( $\gamma$ ) or IFN- $\alpha$  ( $\alpha$ ) per ml. (B) Lysates of metabolically labeled RAW 264.7 cells were analyzed with Ab specific for the viral late gene proteins 65 kDa and 4b (70 kDa). Protein molecular mass markers are indicated in kilodaltons.

was made with lysates from virus-infected cells treated with the DNA synthesis inhibitor Ara C, while viral DNA was detected in untreated virus-infected cells. Consistent with the absence of viral DNA and late protein synthesis, virus particles were undetectable by TEM in RAW 264.7 cells treated with either IFN- $\gamma$  (Fig. 3B) or Ara C (Fig. 3C). These cells were indistinguishable from uninfected cells (data not shown), whereas nontreated, virus-infected cells contained virus particles at different stages of maturation (Fig. 3D). Our failure to detect progeny virus in Ara C- or IFN- $\gamma$ -treated cultures (data not

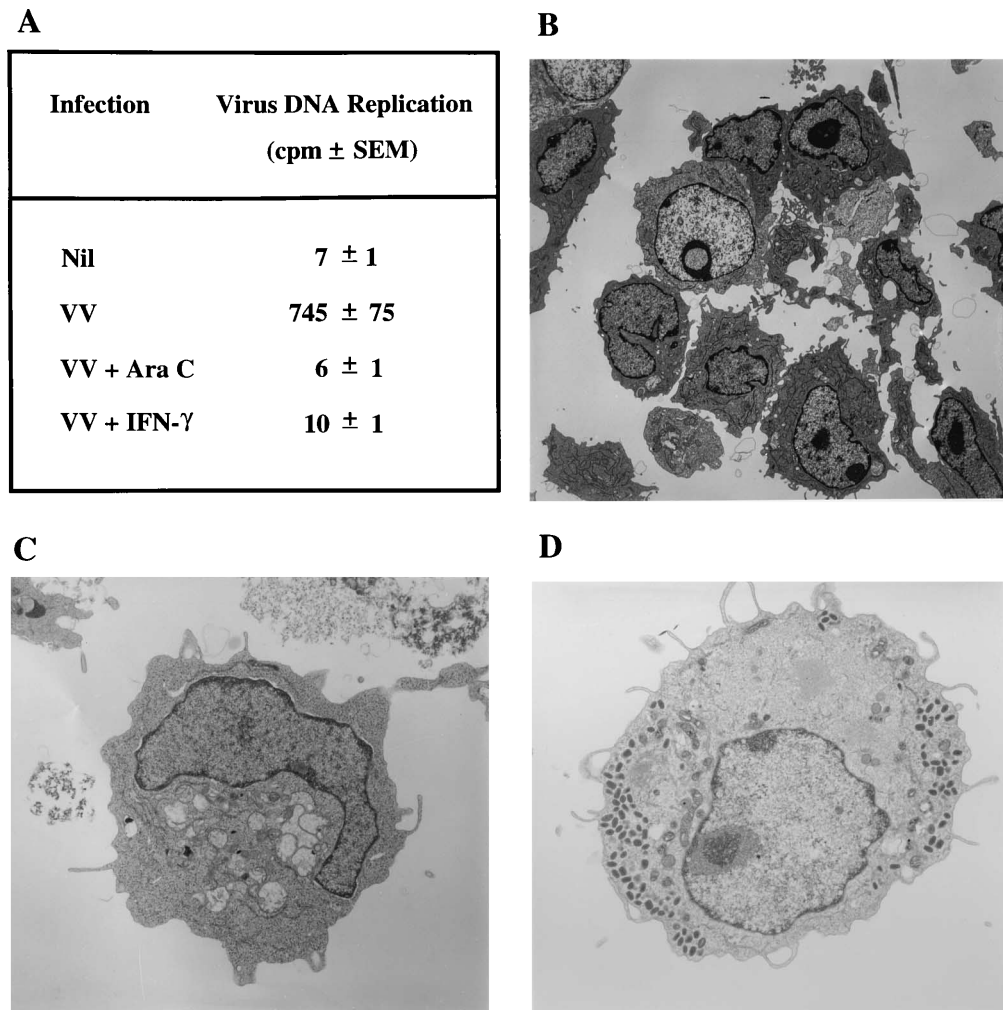


FIG. 3. Inhibition of VV DNA synthesis and virus particle formation by IFN- $\gamma$ . (A) The lysates of VV-infected RAW 264.7 cells (1 PFU per cell) were analyzed at 20 h p.i. by filter hybridization with radiolabeled VV genomic DNA ( $3 \times 10^6$  to  $5 \times 10^6$  cpm per filter). Cells were treated with either the DNA synthesis inhibitor Ara C or with IFN- $\gamma$ . TEM analysis was performed as described in the text. (B) Infected, IFN- $\gamma$ -treated cells (magnification,  $\times 2,000$ ); (C) infected, Ara C-treated cells (magnification,  $\times 6,300$ ); (D) infected, untreated cells (magnification,  $\times 6,300$ ).

shown) is consistent with the above findings. As reported previously (16), the IFN- $\gamma$ -induced inhibition of VV replication could be substantially reversed with L-NMA, and this correlated with nitrite levels in cultures (data not shown).

**Treatment of RAW 264.7 cells with IFN- $\gamma$  induces iNOS.** Abortive VV infection of IFN- $\gamma$ -treated RAW 264.7 cells was attributed to NO. In order to demonstrate that iNOS was indeed induced in the RAW 264.7 cells, we analyzed lysates of cells treated with IFN- $\gamma$ . An antipeptide Ab to murine macrophage iNOS detected the enzyme by Western analysis (immunoblotting) (Fig. 4). The Ab reacted with a protein of approximately 130 kDa, the molecular mass of macrophage-expressed iNOS. Although the Ab cross-reacted with other cellular proteins, no specific reactivity was detected in the lysates of untreated RAW 264.7 cells. The iNOS expressed in the RAW 264.7 cells was biologically active with a specific activity of  $313 \text{ pmol mg}^{-1} \text{ min}^{-1}$ , and no activity was detected in the lysates of untreated RAW 264.7 cells.

**NO produced by IFN- $\gamma$ -activated RAW 264.7 cells inhibits VV replication in contiguous cells.** The preceding experiments demonstrated that VV infection of IFN- $\gamma$ -induced, iNOS-ex-

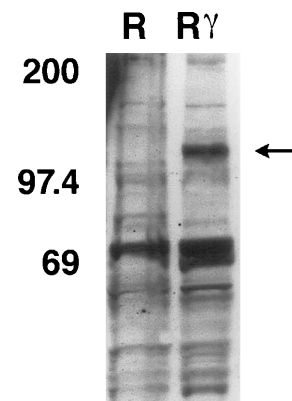


FIG. 4. iNOS expression in IFN- $\gamma$ -treated RAW 264.7 cells. Ab specific for iNOS was used to detect the protein in lysates of RAW 264.7 cells. The cells were untreated (R) or treated with 100 U of IFN- $\gamma$  (R $\gamma$ ) per ml for 48 h. Cell lysates (30  $\mu\text{g}$ ) were fractionated by PAGE, transferred to nitrocellulose, and analyzed with Ab to iNOS. The antigen was detected by enhanced chemiluminescence. The arrow indicates the position of iNOS, which is approximately 130 kDa. Protein molecular mass markers are indicated in kilodaltons.

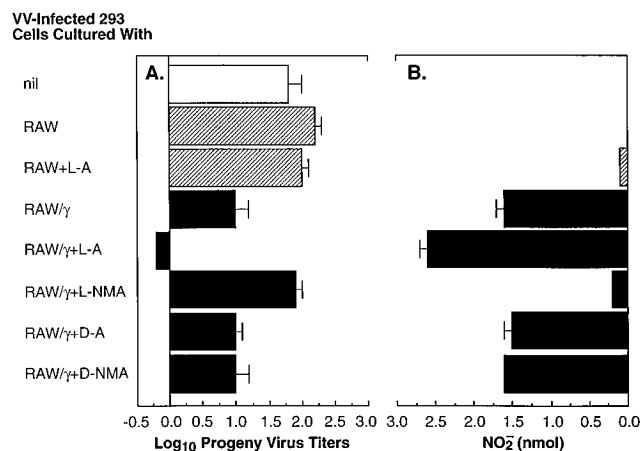


FIG. 5. Inhibition of VV replication by IFN- $\gamma$ -induced NO. RAW 264.7 cells, either untreated (RAW) or treated with 100 U of IFN- $\gamma$  (RAW/ $\gamma$ ) per ml for 20 h, were cocultured with VV-infected (1 PFU per cell) human 293 cells at a ratio of 5:1. Control cultures (nil) contained only VV-infected 293 cells. At 16 h p.i., progeny virus titers (A) and NO<sub>2</sub><sup>-</sup> levels (B) in the cultures were determined. Cocultures were supplemented with 0.5 mM L-A or D-A and 1 mM L-NMA or D-NMA. Error bars indicate the standard errors of the means of triplicate cultures in each group.

pressing RAW 264.7 cells was abortive. We next addressed the question of whether the antiviral effect of IFN- $\gamma$ -induced NO was confined to the treated macrophages or whether it was transmissible to bystander cells. Accordingly, IFN- $\gamma$ -treated RAW 264.7 cells were cocultured with VV-infected 293 cells. L-A, L-NMA, and the stereospecificity controls D-A and D-NMA were used to determine whether inhibition of virus replication, if any, could be attributed to an arginine-dependent, NO-mediated mechanism(s). Coculture of untreated RAW 264.7 cells with VV-infected 293 cells, with or without addition of exogenous L-A, had a negligible effect on VV propagation (Fig. 5A). In contrast, there was more than a 10-fold (1 log<sub>10</sub> PFU) reduction in progeny titers when IFN- $\gamma$ -treated RAW 264.7 cells were cocultured with VV-infected cells. Addition of 0.5 mM L-A to cultures completely abolished progeny virus recovery. When L-NMA, an analog of L-A and a competitive inhibitor of NOS, was added, inhibition of virus replication was nearly completely reversed and progeny yields were comparable to those of controls. D-A and D-NMA, enantiomeric analogs of L-A and L-NMA, respectively, had virtually no effect on viral replication and titers of progeny virus. When IFN- $\gamma$ -treated RAW 264.7 cells were separated from the infected 293 target cells by a semipermeable membrane, no inhibitory effect on progeny virus yields was noted (data not shown), suggesting a requirement for effector-to-target cell contact. These results, which also correlated with nitrite production (Fig. 5B), are consistent with an arginine-dependent, NO-mediated mechanism of inhibition of viral replication. In addition, they demonstrate the importance of the proximity of effector to target cell. NO produced by IFN- $\gamma$ -activated RAW 264.7 cells inhibited virus replication in infected but untreated bystander cells.

## DISCUSSION

Antigen-specific T-cell-mediated immune responses are essential for recovery from most primary viral infections. In the absence of early, nonspecific defense mechanisms, however, the specific immune responses alone are insufficient to combat infection (15, 25). The IFNs are among the earliest known macromolecules produced by the host to counter viral infec-

tions. Their antiviral activity may be mediated in at least two ways. First, IFNs can prevent the multiplication of viruses by inducing an antiviral state in host cells (7, 17, 32, 34, 40). Second, they can activate other effector cells, such as macrophages, to confer antiviral protection. Among the IFNs, IFN- $\gamma$  is the most potent macrophage-activating factor and the only known cytokine with the capacity to induce iNOS in macrophages by itself.

In the macrophage-like RAW 264.7 cells, IFN- $\gamma$  inhibited VV DNA replication, late gene protein synthesis, and virus particle formation but had no effect on early gene protein expression. This phenomenon was reproduced when VV-infected RAW 264.7 cells were treated with the DNA synthesis inhibitor HU or Ara C. The failure to detect VV DNA, late proteins, or virus particles in IFN- $\gamma$ -, Ara C-, or HU-treated cells correlated with an absence of progeny virus in these cultures (data not shown). Because inclusion of L-NMA in cultures could reverse the IFN- $\gamma$ -induced inhibition of VV replication, the block in progeny virus production was attributed to NO (data not shown and reference 16). It appeared that the IFN- $\gamma$ -induced, NO-mediated inhibition of viral replication affected events directly associated with DNA replication.

Of interest was the finding that the viral early gene product E3L, known to block the IFN-induced P<sub>1</sub>/eukaryotic initiation factor 2 kinase antiviral pathway (3), was made in infected IFN- $\gamma$ -treated RAW 264.7 cells yet virus replication was completely restricted. This observation suggested that the E3L protein was not effective in overcoming IFN- $\gamma$ -mediated inhibition of VV replication in the macrophage-like cells and that the process is carried out by a different mechanism(s). Alternatively, it is possible that the amount of IFN- $\gamma$  used in these experiments was insufficient to induce effective quantities of P<sub>1</sub> kinase and/or 2',5'-oligoadenylate A synthetase, and hence the observed lack of any effect of E3L. It is known that IFN- $\gamma$  is not as efficient as IFN- $\alpha$ , - $\beta$ , and - $\omega$  with respect to induction of either of these antiviral pathways (33, 41). Since IFN- $\gamma$  has pluripotent effects on cellular processes, we endeavored to minimize events that were secondary or fortuitous that resulted from its treatment with only that amount of cytokine which blocked virus replication completely but which could be substantially reversed by L-NMA (16). Indeed, with increasing concentrations of IFN- $\gamma$ , accompanied by an increase in nitrite levels, the reversal of inhibition of VV replication in macrophages with L-NMA proved difficult. At IFN- $\gamma$  concentrations higher than those used in the current study, L-NMA reduced nitrite levels without restoring viral replication. This suggested that other antiviral mechanism(s), in addition to that mediated by NO, were induced at higher concentrations of IFN- $\gamma$ . On the basis of these findings, the reported inverse relationship between progeny virus titers and nitrite levels resulting from the treatment of macrophage-like cells with increasing concentrations of IFN- $\gamma$  (21), therefore, is not evidence for the inhibition of virus replication resulting exclusively from an NO-mediated mechanism.

The induction of iNOS in macrophages may be an important antiviral strategy and could be operative in at least two ways. First, abortive infection of iNOS-expressing macrophages would protect the functional integrity of macrophages and help limit virus spread to secondary tissues. Second, the transmissibility of NO by IFN- $\gamma$ -activated macrophages to contiguous cells complements an antiviral defense mechanism designed to further restrict viral dissemination. NO produced by macrophages at foci of infection could potentially retard virus replication in contiguous cells prior to the actions of antiviral T cells and Ab (2).

NO produces many and varied physiological responses in cells (22, 27). It is difficult, therefore, to discriminate between activity which may be virus specific and that which is a result of inhibition of cellular metabolism. At the molecular level, NO is known to inhibit enzymes that require iron and sulfur prosthetic groups for catalytic activity by forming nitrosyl-iron-sulfur complexes (19, 27, 31). Enzymes which are targets for NO-mediated inactivation include *cis*-aconitase of the citric acid cycle (9), NADH:ubiquinone oxidoreductase, and succinate:ubiquinone oxidoreductase of the mitochondrial electron transport chain (10–12, 38). NO can also inhibit DNA synthesis by inactivating the rate-limiting enzyme ribonucleotide reductase (18, 20). To understand the mechanism of NO-mediated inhibition of virus DNA replication, it will be necessary to identify both viral and cellular targets of nitrosylation. Studies on the putative targets of inactivation by NO in this system are currently under way.

#### ACKNOWLEDGMENTS

We thank Carl Nathan, Bernard Moss, and members of their respective laboratories and Herbert C. Morse III and Jack Bennink for critical reading of the manuscript and constructive suggestions. We are grateful to Robert Drillien, Josephine Cox, Carl Nathan, and Qiaowen Xie for the gift of antibodies and to Cristina Cassetti for assistance in the presentation of the figures.

#### REFERENCES

- Beattie, E., J. Tartaglia, and E. Paoletti. 1991. Vaccinia virus-encoded eIF-2 $\alpha$  homolog abrogates the antiviral effect of interferon. *Virology* **183**: 419–422.
- Blandon, R. V., A. J. Hapel, P. C. Doherty, and R. M. Zinkernagel. 1976. Lymphocyte-macrophage interactions and macrophage activation in the expression of antimicrobial immunity in vivo, p. 367–401. *In* D. S. Nelson (ed.), *Immunology of the macrophage*. Academic Press, New York.
- Chang, H.-W., J. C. Watson, and B. L. Jacobs. 1992. The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **89**: 4825–4829.
- Croen, K. D. 1993. Evidence for an antiviral effect of nitric oxide. *J. Clin. Invest.* **91**:2446–2452.
- Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon- $\gamma$  genes. *Science* **259**:1739–1742.
- Davies, M. V., O. Elroy-Stein, R. Jagus, B. Moss, and R. J. Kaufman. 1992. The vaccinia virus K3L gene product potentiates translation by inhibiting double-stranded-RNA-activated protein kinase and phosphorylation of the alpha subunit of eukaryotic initiation factor 2. *J. Virol.* **66**:1943–1950.
- DeMaeyer, E., and J. DeMaeyer-Guignard. 1988. *Interferons and other regulatory cytokines*. Wiley, New York.
- Ding, A. H., C. F. Nathan, and D. J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. *J. Immunol.* **141**:2407–2412.
- Drapier, J.-C., and J. B. Hibbs, Jr. 1986. Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. *J. Clin. Invest.* **78**:790–797.
- Granger, D. L., and A. L. Lehninger. 1982. Sites of inhibition of mitochondrial electron transport in macrophage-injured neoplastic cells. *J. Cell Biol.* **95**:527–535.
- Granger, D. L., R. R. Taintor, J. L. Cook, and J. B. Hibbs, Jr. 1980. Injury of neoplastic cells by murine macrophages leads to inhibition of mitochondrial respiration. *J. Exp. Med.* **65**:357–370.
- Hibbs, J. B., Jr., R. R. Taintor, Z. Vavrin, D. L. Granger, J.-C. Drapier, I. J. Amber, and J. R. Lancaster. 1990. Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron, p. 189–223. *In* S. Moncada and E. A. Higgs (ed.), *Nitric oxide from L-arginine: a bioregulatory system*. Elsevier Science Publishers, B.V., Amsterdam.
- Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel, and M. Aguet. 1993. Immune response in mice that lack the interferon- $\gamma$  receptor. *Science* **259**:1742–1745.
- Karupiah, G. Unpublished data.
- Karupiah, G., T. N. Fredrickson, K. L. Holmes, L. H. Khairallah, and R. M. L. Buller. 1993. Importance of interferons in recovery from mousepox. *J. Virol.* **67**:4214–4226.
- Karupiah, G., Q.-W. Xie, R. M. L. Buller, C. Nathan, C. Duarte, and J. D. MacMicking. 1993. Inhibition of viral replication by interferon- $\gamma$ -induced nitric oxide synthase. *Science* **261**:1445–1448.
- Kerr, I. M., and G. R. Stark. 1992. The antiviral effects of the interferons and their inhibition. *J. Interferon Res.* **12**:237–240.
- Kwon, N. S., D. J. Stuehr, and C. F. Nathan. 1991. Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. *J. Exp. Med.* **174**:761–767.
- Lancaster, J. R., and J. B. Hibbs, Jr. 1990. EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc. Natl. Acad. Sci. USA* **87**:1223–1227.
- Lepoivre, M., F. Fieschi, J. Coves, L. Thelander, and M. Fontecave. 1991. Inactivation of ribonucleotide reductase by nitric oxide. *Biochem. Biophys. Res. Commun.* **179**:442–448.
- Melkova, Z., and M. Esteban. 1994. Interferon- $\gamma$  severely inhibits DNA synthesis of vaccinia virus in a macrophage cell line. *Virology* **198**:731–735.
- Moncada, S., R. M. J. Palmer, and E. A. Higgs. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**:109–142.
- Moss, B. 1990. Regulation of vaccinia virus transcription. *Annu. Rev. Biochem.* **59**:661–688.
- Moss, B. 1994. Vaccinia virus transcription, p. 185–205. *In* R. C. Conaway and J. W. Conaway (ed.), *Transcription: mechanisms and regulation*. Raven Press, Ltd., New York.
- Muller, U., U. Steihoff, L. F. L. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* **264**:1918–1921.
- Nathan, C. 1992. Interferon and inflammation, p. 265–290. *In* J. I. Gallin, I. M. Goldstein, and R. Snyder (ed.), *Inflammation: basic principles and clinical correlates*. Raven Press, Ltd., New York.
- Nathan, C. 1992. Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6**:3051–3064.
- Nathan, C. F., and J. B. Hibbs, Jr. 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.* **3**:65–69.
- Pacha, R. F., R. J. Meis, and R. C. Condit. 1990. Structure and expression of the vaccinia virus gene which prevents virus-induced breakdown of RNA. *J. Virol.* **64**:3853–3863.
- Paez, E., and M. Esteban. 1984. Resistance of vaccinia virus to interferon is related to an interference phenomenon between the virus and the interferon system. *Virology* **134**:12–28.
- Pellat, C., Y. Henry, and J.-C. Drapier. 1990. IFN- $\gamma$ -activated macrophages: detection by electron paramagnetic resonance of complexes between L-arginine-derived nitric oxide and non-heme iron proteins. *Biochem. Biophys. Res. Commun.* **166**:119–125.
- Samuel, C. E. 1991. Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. *Virology* **183**:1–11.
- Samuel, C. E., and G. S. Knutson. 1983. Mechanism of interferon action: human leukocyte and immune interferons regulate the expression of different genes and induce different antiviral states in human amnion U cells. *Virology* **130**:474–484.
- Sen, G. C., and P. Lengyel. 1992. The interferon system. A bird's eye view of its biochemistry. *J. Biol. Chem.* **267**:5017–5020.
- Sodeik, B., G. Griffiths, M. Ericsson, B. Moss, and R. W. Doms. 1994. Assembly of vaccinia virus: effects of rifampin on the intracellular distribution of viral protein p65. *J. Virol.* **68**:1103–1114.
- Stuehr, D. J., H. J. Cho, N. S. Kwon, M. F. Weise, and C. F. Nathan. 1991. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci. USA* **88**:7773–7777.
- Stuehr, D. J., and M. A. Marletta. 1987. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon- $\gamma$ . *J. Immunol.* **139**:518–525.
- Stuehr, D. J., and C. F. Nathan. 1989. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med.* **169**:1543–1555.
- Traktman, P. 1990. The enzymology of poxvirus DNA replication. *Curr. Top. Microbiol. Immunol.* **163**:93–123.
- Vilcek, J. 1990. Interferons, p. 3–38. *In* M. B. Sporn and A. B. Roberts (ed.), *Peptide growth factors and their receptors*, vol. 2. Springer-Verlag, New York.
- Wallach, D., M. Fellous, and M. Revel. 1982. Preferential effect of  $\gamma$  interferon on the synthesis of HLA antigens and their mRNAs in human cells. *Nature (London)* **299**:833–836.
- Xie, Q.-W., H. J. Cho, J. Calaycay, R. A. Mumford, K. M. Swiderek, T. D. Lee, A. Ding, T. Troso, and C. Nathan. 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256**:225–228.
- Yuen, H., J. H. Cox, J. W. Yewdell, J. R. Bennink, and B. Moss. 1993. Nuclear localization of a double-stranded RNA-binding protein encoded by the vaccinia virus E3L gene. *Virology* **195**:732–744.
- Zhang, Y., B.-Y. Ahn, and B. Moss. 1994. Targeting of a multicomponent transcription apparatus into assembling vaccinia virus particles requires RAP94, an RNA polymerase-associated protein. *J. Virol.* **68**:1360–1370.