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# Response of Three Different Viruses to Interferon Priming and Dithiothreitol Treatment of Avian Cells

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**ABSTRACT**

**We have previously shown that the replication of avian reovirus (ARV) in chicken cells is much more resistant to interferon (IFN) than the replication of vesicular stomatitis virus (VSV) or vaccinia virus (VV). In this study, we have investigated the role that the double-stranded RNA (dsRNA)-activated protein kinase (PKR) plays in the sensitivity of these three viruses toward the antiviral action of chicken interferon. Our data suggest that while interferon priming of avian cells blocks vaccinia virus replication by promoting PKR activation, the replication of vesicular stomatitis virus appears to be blocked at a pretranslational step. Our data further suggest that the replication of avian reovirus in chicken cells is quite resistant to interferon priming because this virus uses strategies to downregulate PKR activation and also because translation of avian reovirus mRNAs is more resistant to phosphorylation of the alpha subunit of initiation factor eIF2 than translation of their cellular counterparts. Our results further reveal that the avian reovirus protein sigmaA is able to prevent PKR activation and that this function is dependent on its double-stranded RNA-binding activity. Finally, this study demonstrates that vaccinia virus and avian reovirus, but not vesicular stomatitis virus, express/induce factors that counteract the ability of dithiothreitol to promote eIF2 phosphorylation. Our data demonstrate that each of the three different viruses used in this study elicits distinct responses to interferon and to dithiothreitol-induced eIF2 phosphorylation when infecting avian cells.**

**IMPORTANCE**

**Type I interferons constitute the first barrier of defense against viral infections, and one of the best characterized antiviral strategies is mediated by the double-stranded RNA-activated protein kinase R (PKR). The results of this study revealed that IFN priming of avian cells has little effect on avian reovirus (ARV) replication but drastically diminishes the replication of vaccinia virus (VV) and vesicular stomatitis virus (VSV) by PKR-dependent and -independent mechanisms, respectively. Our data also demonstrate that the dsRNA-binding ability of ARV protein sigmaA plays a key role in the resistance of ARV toward IFN by preventing PKR activation. Our findings will contribute to improve the current understanding of the interaction of viruses with the host's innate immune system. Finally, it would be of interest to uncover the mechanisms that allow avian reovirus transcripts to be efficiently translated under conditions (moderate eIF2 phosphorylation) that block the synthesis of cellular proteins.**

Interferons (IFNs) comprise a family of multifunctional cytokines that were originally discovered by their strong antiviral activity and which are now recognized as the first barrier that viruses must overcome to establish a productive infection. Of the three IFN types, type I interferon (IFN-α/β) displays the highest antiviral activity, and its expression is induced in many cell types by viral infection or following contact with double-stranded RNA (dsRNA) (1, 2). Type I IFNs are secreted out of the cell where they interact with the ubiquitously expressed type I IFN receptor (IFNAR) complex. This interaction triggers the activation of a signal transduction pathway that leads to increased expression of IFN-stimulated genes (ISGs), thus creating an antiviral state. Subsequent viral infection of IFN-primed cells induces the activation of some of the ISG-encoded proteins, and the antiviral activity of these proteins prevents further dissemination of the virus (3–6). Two of the many ISG-encoded proteins have been shown to play an important role in inhibiting viral protein synthesis within infected cells; they are the 2′,5′-oligoadenylate synthetase (OAS) and the double-stranded RNA (dsRNA)-activated protein kinase (PKR). Increased expression of these enzymes is induced by IFN, but they remain latent until after activation by dsRNA (7, 8). Activated OAS catalyzes the synthesis of short oligonucleotides of the general structure ppp(A2′p5′)nA. These oligonucleotides bind to and stimulate a latent endoribonuclease, designated RNase L, to degrade both cellular and viral RNAs, thus preventing intracellular protein synthesis (9, 10). On the other hand, the interaction of PKR with dsRNA leads to dimerization and kinase activation, which then catalyzes serine/threonine phosphorylation of different substrates, including the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α) (11, 12). Phosphorylation of eIF2α can also be carried by three other well-characterized serine-threonine kinases, PERK (PKR-like endoplasmic reticulum kinase), GCN2 (general control nonderepressible-2), and HRI (heme-regulator inhibitor) (13, 14).

The initiation factor eIF2 plays a key role in the initiation of translation. GTP-bound eIF2 recruits Met-tRNA$_i$ to the 40S ribo-

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somal subunit, thus facilitating recognition of the initiator codon by the scanning 43S complex. Binding of the 60S ribosomal subunit to the preinitiation complex promotes hydrolysis of GTP, releasing eIF2-GDP from the ribosome. In order to start another round of translation initiation, replacement of GDP by GTP on eIF2 is catalyzed by the guanosine nucleotide exchange factor eIF2B. However, when eIF2α becomes phosphorylated on Ser-51, it binds very tightly to eIF2B, thus blocking the capacity of this factor to exchange guanosine nucleotides. As a consequence, the intracellular levels of active eIF2-GTP drastically fall, and protein synthesis initiation is inhibited. Since the intracellular levels of eIF2B are usually lower than those of eIF2, phosphorylation of a fraction of the eIF2α molecules present in the cell is usually enough to disallow the catalytic function of eIF2B. This leads to inhibition of global translation initiation while facilitating the preferential translation of specific stress-related mRNAs, including the mRNA encoding activating transcription factor 4 (ATF4) (15–17).

In order to sustain a productive infection, viruses have evolved a variety of antagonistic strategies to undermine virtually all parts of the IFN system, including inhibition of IFN induction and/or signaling or blocking the synthesis, activation, and/or activity of ISG-encoded proteins (18, 19). In addition, many viruses express products that bind and sequester dsRNA, thus limiting IFN induction and the activation of dsRNA-dependent antiviral enzymes (20, 21). The anti-IFN strategies displayed by a virus not only are virus specific but also vary depending on the type of virus-infected cell (18, 22).

Previous studies from different laboratories, including ours, have shown that replication of avian reovirus (ARV) in cultured avian cells is highly refractory to the antiviral action of chicken IFN (chIFN); thus, the replication of ARV is much more resistant to chIFN than the replication of vaccinia virus (VV), vesicular stomatitis virus (VSV), or Semliki Forest virus (23–25). Subsequent results from our laboratory further suggested that ARV core protein sigmaA, which binds dsRNA irreversibly, plays a key role in protecting ARV against the antiviral effect of IFN by preventing PKR activation (24, 26, 27) although this suggestion was based on indirect evidence. In this study, we have investigated the molecular mechanisms that control the sensitivity of ARV, VV, and VSV toward the antiviral activity of a recombinant chicken IFN-α and toward dithiothreitol (DTT)-induced eIF2α phosphorylation in avian cells.

## MATERIALS AND METHODS

**Cells, viruses, and plasmids.** Chicken embryo fibroblast (CEF) primary cultures were prepared from 9- to 10-day-old chicken embryos (provided by Laboratorios MSD España, Salamanca, Spain) and grown in monolayers in medium 199 supplemented with 10% tryptose phosphate broth and 5% calf serum. Avian DF1 cells and hamster BHK-21 cells (both purchased from ATCC, Manassas, VA) were maintained in monolayers in Dulbecco's modified Eagle's medium (DMEM; Invitrogen). Vaccinia virus Western Reserve (VV), the recombinant vaccinia viruses VV-Luc (28) and VV-S2 (26), and vesicular stomatitis virus Indiana serotype (VSV) were grown on BHK-21 cells, while avian reovirus S1133 strain (ARV) was grown on CEF cells. The construction of plasmids expressing ARV proteins sigmaA and sigmaA R155A has been previously described (26, 29).

**Antibodies and reagents.** Generation of rabbit polyclonal antibodies against ARV proteins sigmaA and μNS has been previously described (26, 30). Rabbit polyclonal anti-chicken PKR (chPKR) antibody was produced by BioSynthesis, using as hapten a synthetic peptide comprising amino acid residues 527 to 550 of chicken PKR. Rabbit polyclonal antibody against E3 VV proteins was a kind gift from Mariano Esteban (Centro Nacional de Biotecnología, Madrid, Spain). Rabbit polyclonal antibodies against human eIF2α (FL-315) and actin (I-19) were from Santa Cruz Biotechnology. Rabbit monoclonal antibody against a synthetic phosphopeptide corresponding to residues surrounding Ser-51 of human eIF2α (9721) was purchased from Cell Signaling Technology, and mouse anti-dsRNA (J2) monoclonal antibody was from Scicons. The J2 antibody, which recognizes dsRNAs of more than 40 bp but not ribosomal RNAs, has been previously used to detect dsRNA intermediates of several viruses (31, 32). The secondary antibodies used in Western blotting (horseradish peroxidase [HRP]-conjugated goat anti-rabbit IgG) and immunofluorescence (Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG) were purchased from Sigma and Invitrogen, respectively.

Dithiothreitol (DTT), the PKR inhibitor imidazole/oxindole (C16), and the synthetic dsRNA poly(I·C) were purchased from Sigma, while alkaline phosphatase was from Promega. Recombinant chicken IFN-α was obtained from AbD Serotec.

**Viral infections, metabolic radiolabeling, and Western blotting.** Semiconfluent monolayers of avian cells, which were untreated or had been treated with a recombinant chicken IFN-α for 24 h, were incubated with the virus indicated in the figure legends for 1 h at 37°C. Then, unadsorbed virus was removed (this moment was considered time zero of infection), and cells were overlaid with medium 199 containing 2.5% fetal calf serum and incubated at 37°C for the times indicated in the text. For metabolic radiolabeling, the cell culture medium was replaced by methionine/cysteine-free medium containing 100 μCi/ml of [$^{35}$S]methionine-cysteine (Hartmann Analytic). The cells were incubated in this medium for 1 h, then washed twice with phosphate-buffered saline (PBS), lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), and finally boiled in Laemmli sample buffer. The samples were subjected to 10% SDS-PAGE and autoradiography. For densitometric analysis, the films were analyzed with a Gel Doc XR system and the Quantity One 1-D Analysis software (Bio-Rad).

For Western blotting, the cells were washed twice with PBS and harvested in buffer I (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5 mM 2-mercaptoethanol, 20% glycerol) supplemented with phosphatase and protease inhibitors. Samples were then boiled for 5 min in Laemmli sample buffer and resolved by SDS-PAGE, and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). To improve the separation of the chPKR isoforms, the gels were allowed to run until the prestained 55-kDa marker band reached the bottom of the gel. Membranes were blocked for 1 h with PBS containing 0.05% Tween 20 and 4% nonfat dry milk and incubated for 2 h with primary antibodies diluted in blocking solution. After several washes, the membranes were incubated for 45 min with HRP-conjugated goat anti-rabbit IgG and visualized by chemiluminescence (Immobilon Western Chemiluminescent HRP substrate; Millipore).

**Transfection and alkaline phosphatase treatment.** Plasmid transfection was done using Lipofectamine 2000 according to the manufacturer's instructions. For dsRNA transfection, 10 μg of poly(I·C) was incubated with 10 μl of Lipofectamine 2000 for 15 min, and the sample was then added to the cell monolayers at a poly(I·C) concentration of 10 μg/ml. For alkaline phosphatase treatment, cells were lysed in buffer I lacking phosphatase inhibitors, and the resulting extracts were diluted with alkaline phosphatase buffer. The samples were then incubated with 167 U/ml of alkaline phosphatase for 1 h at 37°C and finally boiled in Laemmli sample buffer.

**Indirect immunofluorescence.** Cells were grown on coverslips, washed twice with PBS, and fixed for 10 min with 4% paraformaldehyde in PBS. Fixed cells were washed with PBS, permeabilized with 0.5% Triton X-100 in PBS for 3 min, and then blocked with 2% bovine serum albumin

in PBS for 30 min. The cells were subsequently incubated for 2 h with primary antibodies diluted 1:1,000 in blocking buffer, washed three times with PBS, and incubated for 30 min with secondary antibodies and 4′,6′-diamidino-2-phenylindole (DAPI). After several washes with PBS, coverslips were mounted on glass slides. Images were obtained with an Olympus DP-71 digital camera mounted on an Olympus BX51 fluorescence microscope and processed with Adobe Photoshop.

## RESULTS

**PKR activity in IFN-primed virus-infected avian cells.** Previous studies have shown that the synthesis of viral proteins in VSV- and VV-infected CEFs, but not in ARV-infected CEFs, is very sensitive to IFN priming (23–26). Since PKR is an IFN-induced protein that upon activation phosphorylates eIF2α and causes protein synthesis inhibition (11, 12), we sought to determine the role that this kinase plays in the sensitivity of these three viruses toward a recombinant chIFN-α (33). To monitor the degree of PKR activation in avian cells, we wanted to compare the intracellular levels of total versus phosphorylated PKR by Western blotting. We also wanted to monitor intracellular PKR activity by determining the degree of eIF2α phosphorylation in infected avian cells. Test assays revealed that commercial antibodies generated against human eIF2α (total eIF2α) and against a human eIF2α hapten containing phosphorylated Ser-51 (phosphorylated eIF2α) were both able to recognize the eIF2α expressed by chicken cells, so we used these antibodies to monitor the degree of eIF2α phosphorylation in avian cells. In contrast, commercial antibodies generated against human and mouse PKR failed to recognize chPKR, and therefore these antibodies could not be used to monitor the phosphorylation status of chPKR. To try to overcome this problem, we generated rabbit antiserum against a peptide comprising residues 527 to 550 of chPKR and tested whether this antiserum could serve to distinguish the phosphorylated (active) and nonphosphorylated (inactive) chPKR isoforms by Western blotting, an approach that has been successfully used to monitor the activation of mammalian PKRs (34). To accomplish this, IFN-treated avian cells were either untransfected or transfected with PKR activator poly(I·C) and lysed 4 h later, and the resulting extracts were analyzed by immunoblotting, either before or after incubation with alkaline phosphatase. These experiments were performed in CEF cells, as well as in the CEF-derived avian cell line DF1. To improve the electrophoretic separation of the phosphorylated and nonphosphorylated PKR isoforms, the samples were allowed to run much longer than during a routine electrophoretic analysis.

The immunoblot assay shown in Fig. 1A revealed that IFN priming induced PKR expression in the two cell types without promoting eIF2α phosphorylation (compare lanes 1 and 2 and lanes 5 and 6 in panels a and b). This result suggests that the PKR expressed by IFN-treated uninfected chicken cells is catalytically inactive and therefore that the chPKR bands observed in lanes 2 and 6 in panel a of Fig. 1A should correspond to unphosphorylated/inactive PKR. Strikingly, the PKR expressed in IFN-treated CEF cells, but not in DF1 cells, resolved as two bands of different electrophoretic mobilities (Fig. 1A, lane 2 of panel a), suggesting that the various cell types present in the primary CEF cultures express two PKR isoforms of different lengths, a situation similar to that reported for human PKR, where cells of different human tissues express two PKR isoforms, one being 40 residues longer than the other (GeneID 5610; GenBank accession number P19 525) (35, 36). Transfection of dsRNA into IFN-treated avian cells showed different responses in the two cell types. Thus, its transfection into DF1 cells significantly diminished the intensity of the band corresponding to unphosphorylated PKR and caused a concomitant increase in the intensity of a slower-migrating PKR band (Fig. 1A, compare lanes 6 and 7 in panel a), which likely represents a hyperphosphorylated/active PKR isoform since its appearance was accompanied by a great increase in the degree of eIF2α phosphorylation (Fig. 1A, compare lanes 6 and 7 in panel b). This was confirmed by our finding that the slower-migrating PKR band disappeared upon incubation of the cell extract with alkaline phosphatase (Fig. 1A, compare lanes 7 and 8 in panel a). These results validate the use of our anti-chPKR antibody for monitoring PKR phosphorylation/activation in DF1 cells by immunoblotting. We also observed that eIF2α resolved as two protein bands in the dsRNA-transfected DF1 cells (Fig. 1A, panels b and c, lanes 7 and 8), which is in agreement with the reported observations that dsRNA transfection leads to apoptosis and that effector caspases catalyze partial cleavage of eIF2α (37, 38). Surprisingly, incubation of the cell extracts with alkaline phosphatase caused dephosphorylation of PKR but not of eIF2α (Fig. 1A, compare lanes 7 and 8 in panels a and b). A possible explanation for this situation is that phosphorylated eIF2α binds so tightly to the guanosine nucleotide exchange factor eIF2B that alkaline phosphatase access to the phosphorylated Ser-51 of eIF2α present within the eIF2-eIF2B complex is blocked.

The effect of dsRNA transfection on IFN-treated CEF cells was less apparent; we observed a small decrease in the intensity of the faster-migrating PKR band and a moderate increase both in the intensity of an intermediate migrating PKR band and in the degree of eIF2α phosphorylation (Fig. 1A, compare lanes 2 and 3 in panels a and b) although the increase in eIF2α phosphorylation was lower than that observed in DF1 cells (Fig. 1A, compare lanes 3 and 7 in panel b). This, and the fact that the intensity of the intermediate migrating PKR band is highly reduced after alkaline phosphatase treatment (Fig. 1A, compare lanes 3 and 4 in panel a), suggest that the intermediate migrating PKR band might represent a hypophosphorylated/low-activity PKR isoform, which in turn suggests either that transfected dsRNA is not a powerful activator of CEF PKR or that the PKR expressed by CEF cells is not able to reach full phosphorylation.

Once we demonstrated that our chPKR antiserum can be used to monitor the activation of the avian kinase by Western blotting, we next analyzed the effect that IFN priming exerts on both protein synthesis and PKR activation in infected avian cells. As a positive control for phosphorylated eIF2α, avian cells were treated with dithiothreitol (DTT), a reducing agent that activates the endoplasmic reticulum kinase PERK to induce eIF2α phosphorylation and protein synthesis inhibition in mammalian cells (39, 40). The results shown in lanes 9 of Fig. 1B and C revealed that DTT was able to induce similar effects in avian cells. IFN priming of uninfected avian cells induced PKR expression but not its activation since it did not promote the appearance of the lower-mobility PKR isoform, did not inhibit cellular protein synthesis, and did not promote eIF2α phosphorylation (Fig. 1B and C, compare lanes 1 and 2), thus confirming that IFN induces the expression of unphosphorylated/inactive PKR in uninfected cells. PKR activation and eIF2α phosphorylation were also not observed in IFN-primed ARV-infected avian cells (Fig. 1B and C, compare lanes 3 and 4 in panels c and d). Curiously, while IFN priming slightly reduced the synthesis of viral proteins in ARV-infected CEF cells,

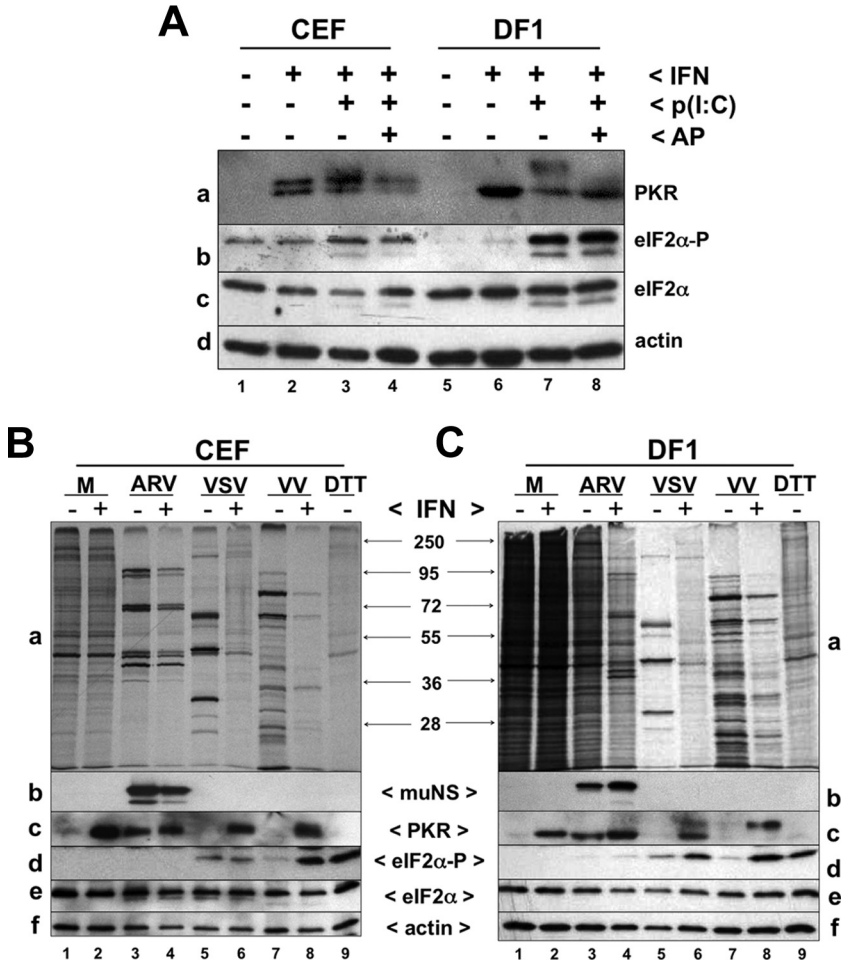


**FIG 1** PKR activation in virus-infected cells. (A) Monolayers of CEF (lanes 1 to 4) or DF1 (lanes 5 to 8) cells were left untreated (lanes 1 and 5) or incubated (lanes 2 to 4 and 6 to 8) with 1,000 U/ml of chIFN for 24 h. One set of the cells was then mock transfected (lanes 1, 2, 5, and 6), and the other set was transfected with 10 μg/ml of poly(I·C) (lanes 3, 4, 7, and 8). The cells were lysed 4 h later, and the resulting cell extracts were processed for Western blotting either before (lanes 1 to 3 and 5 to 7) or after (lanes 4 and 8) 1 h of incubation at 37°C with 167 U/ml of alkaline phosphatase (AP). Immunoblotting was performed with primary antibodies against the proteins indicated at the right of the panels. eIF2α-P, phosphorylated eIF2α. (B and C) Monolayers of CEF or DF1 cells were left untreated (−) or incubated (+) with 1,000 U/ml of chIFN for 24 h and then mock infected (M) or infected with the indicated viruses (ARV, 10 PFU/cell for 10 h; VV, 5 PFU/cell for 9 h; VSV, 5 PFU/cell for 6 h). The mock-infected cells of lanes 9 were incubated with 1 mM DTT for 75 min just before lysis. The cells of panels a were incubated for the last 60 min with 100 μCi of [$^{35}$S]methionine-cysteine before being lysed with RIPA buffer. The radioactive proteins of the extracts were resolved by 10% SDS-PAGE and visualized by autoradiography. The positions of protein markers are indicated between the panels. The cells used for the experiments in panels b to f were lysed at the same time as those of panel a, and the resulting extracts were subjected to Western blot analysis with antibodies against the proteins indicated between the panels.

it slightly increased the synthesis of ARV proteins and diminished the synthesis of cellular proteins in ARV-infected DF1 cells (Fig. 1B and C, compare lanes 3 and 4 in panels a and b), suggesting that IFN priming slightly enhances ARV replication in DF1 cells.

IFN priming of VSV-infected cells induced PKR expression, but most of the protein (~90%) migrated in the position corresponding to unphosphorylated PKR (Fig. 1B and C, compare lanes 5 and 6 in panel c), suggesting that most PKR expressed in these cells is catalytically inactive. This suggestion was further supported by the fact that IFN priming of VSV-infected CEF cells was not accompanied by a significant increase of eIF2α phosphorylation (Fig. 1B, panel d, lane 6), and although a slight phosphorylation rise was observed in VSV-infected DF1 cells (Fig. 1C, panel d, lane 6), this rise was not consistent since it was not detected in other experiments (Fig. 2C, compare lanes 9 and 11 in panel c). On the other hand, IFN priming of VSV-infected cells induced a drastic inhibition of viral protein synthesis and a partial rescue of cellular protein synthesis (Fig. 1B and C, lanes 5 and 6 in panels a, and 2B, lanes 9 and 11 in panel a). Finally, IFN priming of VV-infected cells promoted PKR expression and full kinase activation, as revealed by the electrophoretic mobility shift of PKR toward the lower-mobility isoform and by enhanced levels of phosphorylated eIF2α. These effects were accompanied by a drastic inhibition of viral and cellular protein synthesis (Fig. 1B and C, compare lanes 7 and 8 in panels a, and 2B, compare lanes 5 and 7 in panel a). These results suggest that PKR plays a key role in the sensitivity of VV toward the antiviral effect of IFN in avian cells.

To further assess the role that chPKR plays in the sensitivity of VSV and VV toward IFN, we next examined the effect of a specific PKR inhibitor, the oxindole/imidazole derivative C16. This low-

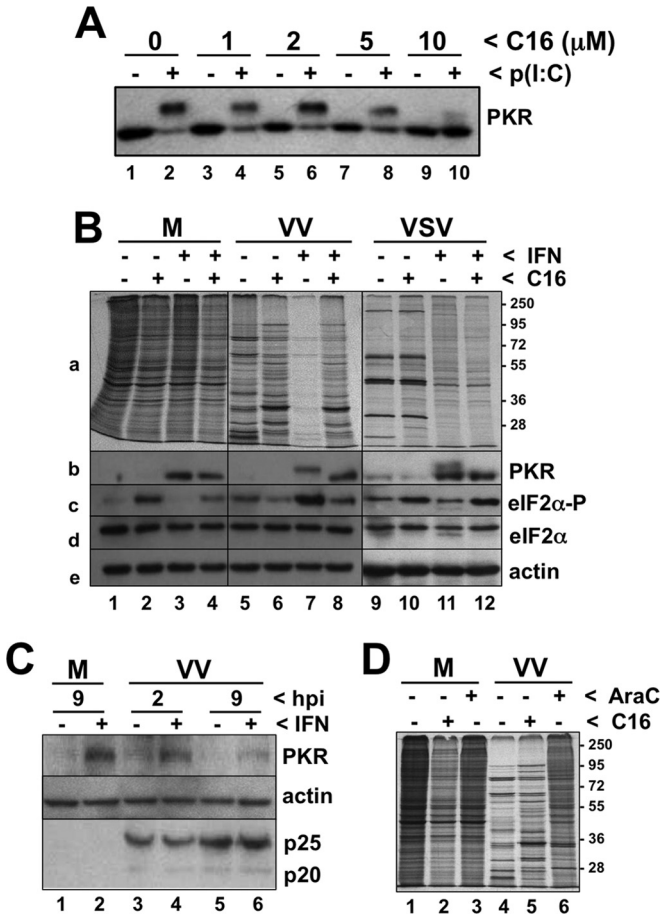


**FIG 2** Effect of PKR inhibitor C16 on PKR activation in DF1 cells. (A) DF1 cell monolayers were primed with IFN for 24 h and then incubated for 5 h with the indicated concentrations of C16. The cells were mock transfected (−) or transfected (+) with 10 μg/ml of poly(I·C) and incubated for another 4 h. The cells were then lysed, and the extracts were subjected to Western blot analysis using anti-chPKR serum as the primary antibody. (B) Monolayers of DF1 cells were left untreated (lanes 1, 2, 5, 6, 9, and 10) or incubated (lanes 3, 4, 7, 8, 11, and 12) with IFN for 24 h, and then the cells used for the experiments shown in the even-numbered lanes were incubated with 10 μM C16 for 30 min. The cells of lanes 1 to 4 were mock infected (M), those of lanes 5 to 8 were infected with 5 PFU/cell of VV, and those of lanes 9 to 12 were infected with 5 PFU/cell of VSV. At 8 hpi for VV-infected cells and at 5 hpi for VSV-infected cells, the cells used for the experiment shown in panel a were incubated for 1 h with 100 μCi of [$^{35}$S]methionine-cysteine and lysed with RIPA buffer, and the radioactive proteins of the extracts were resolved by 10% SDS-PAGE and visualized by autoradiography. The positions of protein markers are indicated on the right. At 9 hpi for VV-infected cells and at 6 hpi for VSV-infected cells, the cells of the experiments shown in panels b to e were lysed, and the resulting extracts were subjected to Western blot analysis with antibodies against the proteins indicated on the right. (C) Monolayers of DF1 cells were left untreated (odd lanes) or incubated (even lanes) with IFN for 24 h, and then the cells of lanes 1 and 2 were mock infected (M), whereas those of lanes 3 to 6 were infected with 5 PFU/cell of VV. At the indicated postinfection times the cells were lysed, and the resulting extracts were subjected to Western blot analysis with antibodies against PKR, actin, and E3 (p25 and p20), as indicated. (D) DF1 cells were untreated (lanes 1, 3, 4, and 6) or treated with 10 μM C16 (lanes 2 and 5) for 30 min. The cells were then mock infected (M; lanes 1 to 3) or infected with 5 PFU/cell of VV (lanes 4 to 6), and 1 h later the cells represented in lanes 3 and 6 were incubated with 40 μg/ml of araC. From 8 to 9 hpi all cells were incubated for 1 h with 100 μCi of [$^{35}$S]methionine-cysteine; the cells were then lysed with RIPA buffer, and the radioactive proteins were resolved by 10% SDS-PAGE and visualized by autoradiography. The positions of protein markers are indicated on the left.

molecular-weight compound is able to prevent PKR autophosphorylation by blocking the ATP-binding site of the kinase and has been shown to be an efficient PKR inhibitor both in cultured cells and in animal models (41, 42). First of all, we tested the ability of this compound to prevent the activation of the PKR expressed by IFN-primed DF1 cells. The results shown in Fig. 2A revealed that the capacity of dsRNA to activate chPKR was inhibited by C16 in a dose-dependent manner and that maximal inhibition was reached at a C16 concentration of 10 μM. This, and our finding that cell viability was not affected by the presence of 10 μM C16 for at least 12 h (data not shown), prompted us to use this inhibitor concentration in subsequent studies with infected cells. Strikingly, C16 induced a certain degree of eIF2α phosphorylation in IFN-primed and -nonprimed uninfected cells (Fig. 2B, panel c, lanes 1 to 4) although this was not accompanied by significant protein synthesis inhibition (Fig. 2B, panel a, lanes 1 to 4). Although we do not understand why C16 promotes eIF2α phosphorylation in uninfected cells and why this was not accompanied by protein synthesis inhibition, our finding that C16 induces eIF2α phosphorylation in cells that do not express detectable PKR levels, like IFN-nonprimed uninfected DF1 cells (Fig. 2B, compare lanes 1 and 2 in panels b and c), suggests that C16 promotes eIF2α phosphorylation through the activation of an eIF2α kinase other than PKR.

When added to IFN-nonprimed VV-infected DF1 cells, C16 did not significantly alter the level of eIF2α phosphorylation or the rate of viral protein synthesis (Fig. 2B, compare lanes 5 and 6 in panels a and c). However, C16 was able to prevent PKR activation, to reduce the degree of eIF2α phosphorylation, and to rescue the synthesis of viral proteins when it was added to IFN-treated VV-infected cells (Fig. 2B, compare lanes 7 and 8 in panels a to c), thus confirming our previous suggestion that PKR plays a key role in the sensitivity of VV toward the antiviral action of IFN in avian cells. In contrast, C16 did not promote the rescue of viral protein synthesis in IFN-treated VSV-infected DF1 cells (Fig. 2B, compare lanes 11 and 12 in panel a) although the inhibitor still promoted the disappearance of the faint band corresponding to the slower-migrating PKR isoform (Fig. 2B, compare lanes 11 and 12 in panel b). These results suggest that IFN blocks the replication of VSV in avian cells mainly through a PKR-independent mechanism, probably acting at a stage of the virus life cycle prior to viral protein synthesis.

That VV is so sensitive to IFN in avian cells and that PKR plays a key role in that resistance are striking results since the replication of VV in most mammalian cells is quite resistant to IFN priming (43), which has been attributed to the action of a plethora of anti-IFN proteins expressed by the VV genome (44). Two of these proteins, E3 and K3, which are encoded by the early viral genes E3L and K3L, have been reported to counteract the antiviral action of PKR (43, 45, 46). Removal of the genes encoding these two anti-IFN proteins renders VV sensitive to IFN priming in many cell lines (47, 48) although it has been suggested that E3 accounts for most anti-PKR activity and that K3 has only a marginal effect (34, 45). The E3 proteins p20 and p25 bind and sequester dsRNA activator (49, 50) although direct interaction of E3 with PKR also appears to be required for preventing PKR activation (51). Three possibilities could account for the inability of E3 to prevent PKR activation in IFN-primed VV-infected avian cells: (i) PKR might block the synthesis of E3 if the kinase becomes active before early viral genes are expressed; (ii) the intracellular levels of E3 might

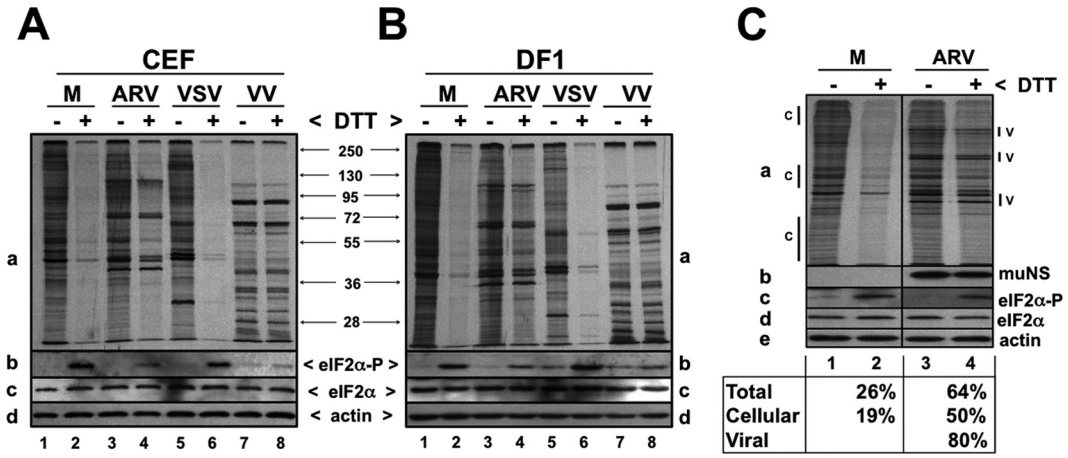


**FIG 3** Effect of DTT on protein synthesis and eIF2 phosphorylation in avian cells. Monolayers of CEF (A) and DF1 (B and C) cells were mock infected (M) or infected with ARV (10 PFU/cell for CEF cells and 50 PFU/cell for DF1 cells) or with 5 PFU/cell of VSV or VV, as indicated. The cultured medium of the cells used for the experiments shown in the even-numbered lanes was supplemented with 1 mM DTT 1 h before lysis. The cells of panels a were incubated for 45 min before lysis with 100 μCi of [$^{35}$S]methionine-cysteine; cells were then lysed with RIPA buffer (at 6 hpi for VSV-infected cells and at 9 hpi for the rest of the cells), and radioactive proteins of the extracts were resolved by 10% SDS-PAGE and visualized by autoradiography. The positions of protein markers are indicated between the panels. (C) The regions of the lanes selected for densitometric analysis of cellular (c) and viral (v) protein bands are indicated at both sides of panel a. The cells of panels b to e were lysed at 9 hpi, and the resulting extracts were subjected to Western blot analysis with antibodies against the proteins indicated between panels A and B or on the right of panel C. The samples shown on panel C were run on the same gel, but internal lanes were removed. For densitometric analysis of the protein bands in panel a, the intensities of the whole lanes (Total) or of the selected lane regions indicated at both sides of the figure (c, cellular proteins; v, viral proteins) were measured, and the percentages of intensities relative to lane 1 are indicated below lane 2, while the percentages of intensities relative to lane 3 are shown below lane 4.

not be sufficient to sequester all intracellular dsRNA generated in these cells; and (iii) E3 might be unable to block PKR activation if it fails to interact with the PKR of avian origin. To try to understand why E3 is not able to prevent PKR activation in avian cells, we performed a Western blot analysis to compare the intracellular levels of E3 proteins in IFN-primed and -nonprimed DF1 cells. The results shown in Fig. 2C revealed that IFN priming did not inhibit the production of the E3 proteins p25 and p20 in VV-infected DF1 cells at early or late times postinfection (p.i.), suggesting that expression of early VV genes takes place normally in IFN-primed avian cells, which in turn suggests that PKR activation takes place after the mRNAs of these genes have been translated. This suggestion is further supported by our finding that IFN priming of avian cells does not inhibit the expression of an avian reovirus gene controlled by an early VV promoter (see Fig. 5).

Curiously, although the incubation of VV-infected DF1 cells with the PKR inhibitor C16 did not alter the rate of viral protein synthesis, it induced a change in the expression pattern of the viral polypeptides. Thus, the inhibitor promoted the expression of specific viral proteins and altered the rate of synthesis of several VV polypeptides (Fig. 2B, compare lane 5 with lanes 6 and 8 in panel a). One possibility is that C16 might block the expression of intermediate and late VV genes, allowing the virus to express exclusively its early genes. To test this hypothesis, we compared the pattern of the viral proteins expressed in VV-infected DF1 cells when these cells were incubated in the presence of either C16 or cytosine arabinoside (araC). Cytosine arabinoside is a nucleoside analog that has been shown to block the replication of the VV genome in mammalian cells, thus preventing the expression of intermediate and late viral genes (52). araC was similarly effective on avian cells since the pattern of VV polypeptides expressed in the presence of the inhibitor was quite different from the one obtained in its absence (Fig. 2D, compare lanes 4 and 6) but similar to the ones reported for the VV polypeptides synthesized in mammalian cells in the presence of araC or at early infection times (52). However, the pattern of the viral proteins synthesized in the presence of araC was different from the one generated in the presence of C16 (Fig. 2D, compare lanes 5 and 6). These results suggest that C16 induces deregulation of the temporal expression pattern of the VV genome, allowing the virus to express early, intermediate, and late genes at the same time, although this suggestion requires experimental confirmation.

**Different responses of the three viruses to DTT-induced eIF2α phosphorylation.** One of the mechanisms used by the host cell to inhibit viral replication and spread is the phosphorylation of the alpha subunit of the translation initiation factor eIF2, and therefore viruses use a variety of different countermeasures to reduce the intracellular levels of phosphorylated eIF2α. Thus, some viruses inhibit the activation and/or the activity of eIF2α kinases, while others downregulate eIF2α phosphorylation or induce dephosphorylation of the initiation factor subunit (53, 54). Finally, some viruses express mRNAs that are able to initiate translation at concentrations of phosphorylated eIF2α that preclude the translation of most cellular mRNAs (55). To assess whether the viruses employed in this study use strategies to deal with eIF2α phosphorylation, we examined the capacity of virus-infected avian cells to counteract the ability of DTT to induce eIF2α phosphorylation. DTT was added to the medium of infected cells at late infection times in order for the cells to have time to express and accumulate viral proteins. The results revealed that incubation with 1 mM DTT for 1 h was very effective in inducing eIF2α phosphorylation and in blocking protein synthesis in uninfected avian cells (Fig. 3A and B, compare lanes 1 and 2 in panels a and b). Similar results were obtained when VSV-infected cells were incubated with DTT (Fig. 3A and B, compare lanes 5 and 6 in panels a and b), suggesting both that VSV does not use counter-

measures to downregulate DTT-induced eIF2α phosphorylation and that the translation of VSV mRNAs is very sensitive to eIF2α phosphorylation. In contrast, DTT did not provoke eIF2α phosphorylation or protein synthesis inhibition when added to VV-infected avian cells (Fig. 3A and B, compare lanes 7 and 8 in panels a and b), suggesting that VV expresses/induces factors that are able to prevent eIF2α phosphorylation or to remove phosphate groups from phosphorylated eIF2α. Finally, treatment of ARV-infected avian cells with DTT induced a slight increase in the level of eIF2α phosphorylation and caused inhibition of cellular protein synthesis but not of viral protein synthesis (Fig. 3A and B, compare lanes 3 and 4). Furthermore, examination of lanes 3 and 4 in panels a of Fig. 3A and B suggested that the inhibition of cellular protein synthesis induced by DTT in ARV-infected cells is less pronounced than that observed in uninfected cells. To confirm this suggestion, we performed a densitometric analysis of the cellular and viral protein bands obtained from uninfected and ARV-infected DF1 cells when untreated or treated with DTT. The results revealed that DTT inhibited the synthesis of cellular proteins in uninfected cells by 80%, but the inhibition reached only 50% in ARV-infected cells (Fig. 3C), probably because of reduced eIF2α phosphorylation. These results suggest both that ARV uses strategies to downregulate eIF2α phosphorylation and that the translation of ARV mRNAs is less sensitive to eIF2α phosphorylation than that of their cellular counterparts.

**The capacity of dsRNA to activate PKR is diminished in ARV-infected cells.** The results shown so far suggest that ARV uses strategies to prevent PKR activation and eIF2α phosphorylation, so we next examined whether the infection of avian cells with ARV reduces the capacity of dsRNA to activate PKR. For this, CEF and DF1 cells were primed with IFN, and 24 h later the cells were either infected with ARV or left uninfected. At 6 h p.i. (hpi) the cells were transfected with the synthetic dsRNA poly(I·C) and lysed 4 h later, and the resulting cell extracts were subjected to Western blot analysis. The results revealed that dsRNA transfection caused PKR activation in uninfected cells, as detected both by the appearance of the lower-mobility PKR band and by increased eIF2α phosphorylation (Fig. 4A and B, compare lanes 1 and 2). PKR activation was more evident in DF1 cells, where the PKR band corresponding to nonphosphorylated PKR was drastically reduced and replaced by a lower-mobility PKR isoform (Fig. 4B, panel b, lane 2). Our results further revealed that the capacity of transfected poly(I·C) to promote PKR activation was downregulated in ARV-infected avian cells, as detected by reduced phosphorylation of both PKR and eIF2α (Fig. 4A and B, compare lanes 3 and 4 in panels b and c). These results suggest that ARV expresses or induces factors that are able to prevent PKR activation.

**The ARV protein sigmaA is able to prevent PKR activation.** It has been previously shown that the ARV protein sigmaA binds dsRNA very tightly in an irreversible and sequence-independent manner (27, 56, 57), and two reports have provided evidence that sigmaA antagonizes the interferon-induced cellular response against ARV by preventing PKR activation (24, 26). However, the anti-PKR activity of sigmaA was based on indirect evidence since at that time we were unable to directly monitor chPKR activation and eIF2α phosphorylation in avian cells. Once these conditions were established, we reexamined the anti-PKR effect of sigmaA. We performed the experiments in DF1 cells because the phosphorylated and nonphosphorylated PKR isoforms expressed by these cells are better resolved electrophoretically than the ones

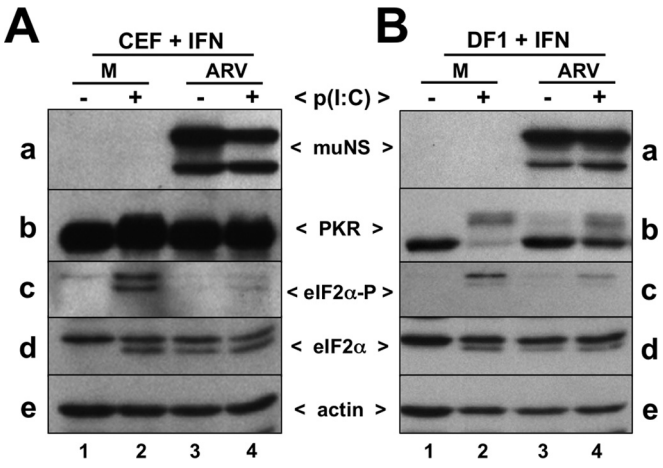


**FIG 4** Effect of ARV infection on the capacity of dsRNA to activate PKR. Monolayers of IFN-primed CEF (A) and DF1 (B) cells were mock infected (M) or infected with ARV (10 PFU/cell for CEF cells and 20 PFU/cell for DF1 cells), as indicated. At 6 hpi the cells were mock transfected (−) or transfected (+) with 10 μg/ml of poly(I·C), as indicated. The cells were lysed 4 h later, and the resulting extracts were subjected to Western blotting with antibodies against the proteins indicated in the middle of the figure.

expressed by CEF cells. In a first attempt to determine the anti-PKR activity of sigmaA, we used two recombinant VVs, one that contains the sigmaA-encoding gene (VV-S2) (26) and one that contains the luciferase gene (VV-Luc) (28), with both genes controlled by early viral promoters. IFN-primed DF1 cells were infected with these viruses, and one set of cells was labeled for 1 h at 8 hpi with [$^{35}$S]methionine-cysteine for protein synthesis analysis, whereas the other set was lysed at 9 hpi for Western blot analysis. As expected, IFN priming of uninfected cells induced PKR expression, but the protein was expressed as an inactive kinase because we could not detect either a shift of PKR isoforms, eIF2α phosphorylation, or protein synthesis inhibition (Fig. 5A, compare lanes 1 and 2 in panels a, c, and d). On the other hand, IFN priming of VV-Luc-infected cells induced not only PKR expression but also a PKR shift toward the phosphorylated slower-mobility isoform (Fig. 5A, compare lanes 3 and 4 in panel c), and this was accompanied by both increased eIF2α phosphorylation and the inhibition of viral protein synthesis (Fig. 5A, compare lanes 3 and 4 in panels a and d). In contrast, IFN priming of the cells infected with the sigmaA-expressing virus VV-S2 did not promote a PKR shift, eIF2α phosphorylation, or the inhibition of viral protein synthesis (Fig. 5A, compare lanes 5 and 6 in panels a, c, and d), indicating that sigmaA expression protects VV against the antiviral action of IFN by blocking PKR activation, which in turn supports our previous suggestion that PKR plays a key role in the sensitivity of VV toward IFN in avian cells. Furthermore, the capacity of IFN to inhibit viral protein synthesis in cells infected with VV-Luc, but not in the ones infected with VV-S2, was dose dependent (Fig. 5B). However, the two recombinant VVs were similarly resistant to DTT treatment (Fig. 5C), which again suggests that VV expresses factors that are able to prevent eIF2α phosphorylation.

The crystal structure of a bacterially expressed recombinant sigmaA has been solved, and two key arginine residues (Arg-155 and Arg-273) involved in dsRNA binding have been identified because mutation of either of these two residues abolishes dsRNA binding (27). To determine whether there is a correlation between

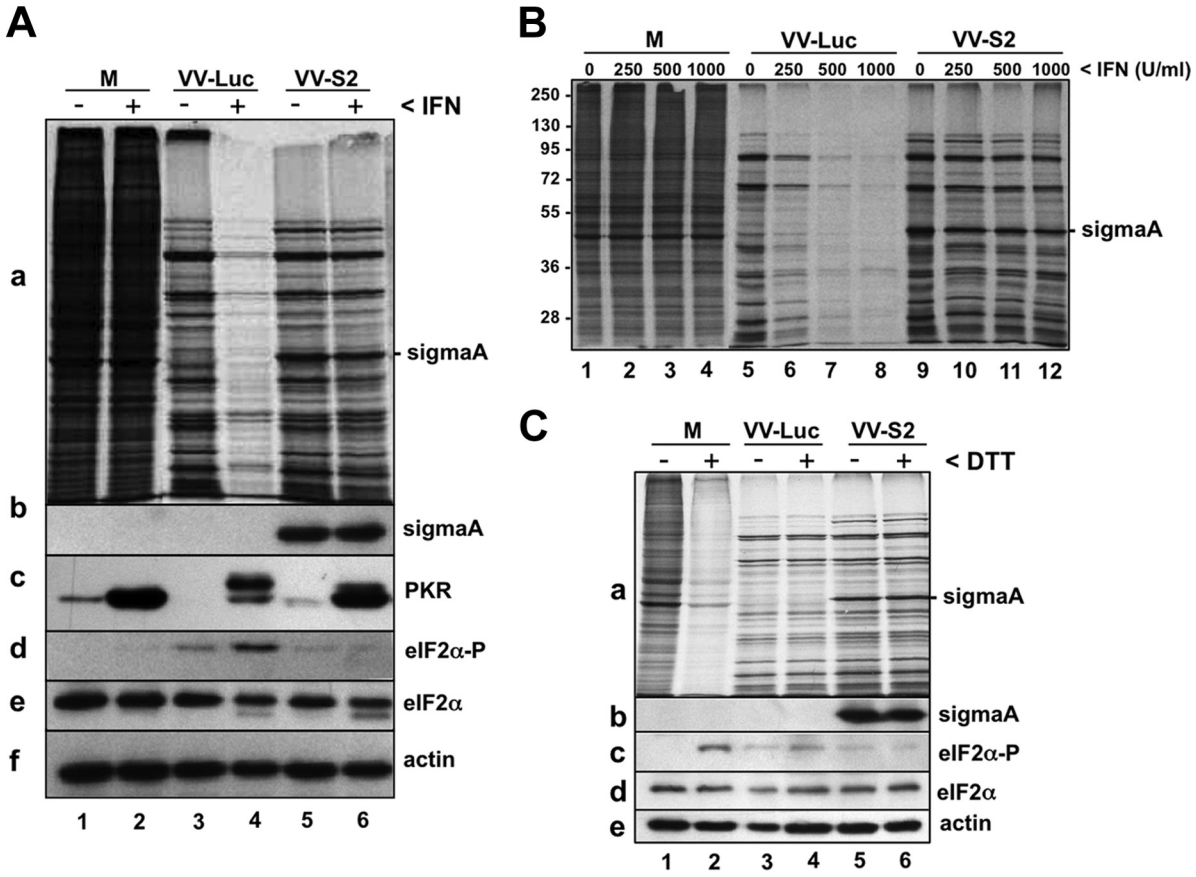


**FIG 5** Sensitivity of recombinant vaccinia viruses VV-Luc and VV-S2 toward IFN and DTT. (A) Monolayers of DF1 cells that had been IFN primed (+) or left unprimed (−) were mock infected (M) or infected with 5 PFU/cell of the indicated recombinant vaccinia viruses. At 8 hpi the cells used in the experiment shown in panel a were incubated for 1 h with 100 μCi of [$^{35}$S]methionine-cysteine and lysed with RIPA buffer, and the radioactive proteins of the extracts were resolved by 10% SDS-PAGE and visualized by autoradiography. At 9 hpi the cells used in the experiments shown in panels b to f were lysed, and the resulting extracts were subjected to Western blot analysis with antibodies against the proteins indicated at the right. The samples used for the experiment shown in panel a were run on the same gel, but an internal lane between lanes 2 and 3 was removed. (B) Monolayers of DF1 cells primed with the IFN concentrations shown at the top of the figure were mock infected (M) or infected with the indicated recombinant vaccinia viruses indicated. At 8 hpi the cells were radiolabeled and processed as described for panel A(a). (C) Monolayers of DF1 cells were mock infected (M) or infected with the indicated recombinant vaccinia viruses. At 8 hpi the cells were incubated for 1 h with 1 mM DTT, and then one set of cells was processed for Western blotting with the antibodies indicated at the right of panels b to e. The other set was incubated for the last 45 min with 100 μCi/ml of [$^{35}$S]methionine-cysteine, and then processed as described for panel A(a). The position of sigmaA is indicated at the right of the panels.

the dsRNA binding and the anti-PKR activities of sigmaA, we compared the capacities of wild-type sigmaA and its mutant R155A (in which Arg-155 has been replaced by alanine) to prevent dsRNA-induced PKR activation. To accomplish this, DF1 cells were transfected with the empty pcDNA plasmid or with recombinant plasmids expressing sigmaA or its R155A mutant, and 3 h later the cells were treated with IFN for 18 h and finally transfected with poly(I·C) for another 4 h. One set of the cells was then processed for immunofluorescence analysis, and the other set was lysed for Western blot analysis. The immunofluorescence pictures shown in Fig. 6A revealed that the sigmaA protein (lane 2), but not the R155A mutant (lane 3), colocalized with transfected dsRNA in the cell cytoplasm, thus confirming that sigmaA, but not the R155A mutant, binds dsRNA. The Western blot analysis shown in Fig. 6B revealed that transfection of dsRNA into cells containing either the empty plasmid or the R155A-expressing plasmid promoted a PKR shift toward the lower-mobility hyperphosphorylated band (compare lanes 1 and 2 and lanes 5 and 6). In contrast, this shift hardly took place in the cells transfected with the sigmaA-expressing plasmid (compare lanes 3 and 4). These results not only confirm that sigmaA is able to prevent the activation of PKR induced by dsRNA but also indicate that this property relies on the capacity of sigmaA to bind and sequester activator dsRNA.

## DISCUSSION

Previously published studies have revealed that the replication of ARV in cultured chicken cells is much more resistant to the antiviral action of chIFN than the replication of VV and VSV (23, 24, 26). In this study, we have tried to elucidate the molecular mechanisms that control the susceptibility of these three viruses toward chIFN in avian cells by examining protein synthesis, PKR activation, and eIF2 phosphorylation. Our results reveal that each of these viruses elicits different responses to IFN priming.

Previous studies from different laboratories reported conflicting results regarding the stage of the VV life cycle blocked by IFN in avian cells. Thus, the results of Bialy and Colby (58) suggested that chIFN inhibits the synthesis of VV transcripts in CEF cells, whereas the results of Esteban and Metz (59) indicated that the

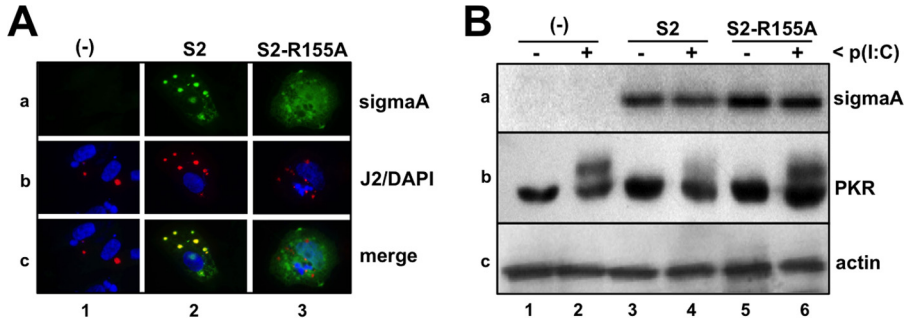


**FIG 6** Effect of sigmaA dsRNA-binding activity on its anti-PKR function. (A) Monolayers of DF1 cells were transfected for 3 h with an empty pcDNA plasmid (−) or with recombinant pcDNA plasmids containing inserts encoding wild-type sigmaA (S2) or the sigmaA R155A mutant (S2-R155A). The cells were then primed with IFN for 18 h and subsequently transfected with 10 μg/ml of poly(I·C) for another 4 h. Finally, the cells were fixed with formaldehyde and immunostained with green for sigmaA and red for dsRNA and with DAPI (blue). Stained cells were visualized by fluorescence microscopy. (B) Monolayers of DF1 cells were transfected for 3 h with the same plasmids used for the experiment shown in panel A and then incubated in the presence of 1,000 U/ml of IFN for 18 h. One set of cells was then mock transfected (−) while the other set was transfected (+) with 10 μg/ml of poly(I·C). The cells were incubated for another 4 h and then lysed, and the resulting extracts were subjected to Western blot analysis with antibodies against the proteins indicated at the right. S2, VV-S2 expressing sigma A; S2-R155A, VV-S2 expressing sigmaA R155A.

cytokine induces viral protein synthesis inhibition while stimulating the synthesis of early VV transcripts. On the other hand, the study by Grün et al. (60) suggested that priming CEF cells with IFN caused inhibition of VV protein synthesis by promoting the degradation of early viral transcripts, while the data of Degen et al. (61) revealed that the degradation of VV mRNAs cannot be solely responsible for the inhibition of protein synthesis observed in IFN-treated VV-infected CEF cells. The results presented in the current study indicate that priming of avian cells with a recombinant chIFN-α induces viral protein synthesis inhibition and that PKR plays a key role in that inhibition. This conclusion is based on our findings that priming of VV-infected avian cells with IFN provokes PKR activation, eIF2α phosphorylation, and the inhibition of viral and cellular protein synthesis. Furthermore, our finding that IFN priming does not reduce the intracellular levels of two proteins expressed by early VV genes, like VV E3 and ARV sigmaA (Fig. 2C and 5A, respectively), suggests that PKR activation does not take place until after the parental virus expresses the genes controlled by early promoters. Thus, it seems that the dsRNA required to activate PKR is generated in IFN-primed avian cells during the expression of the intermediate/late genes and that PKR activation at this stage precludes intracellular viral amplification by blocking ribosomal translation. This situation is reversed when IFN-primed VV-infected cells are cultured in the presence of the specific PKR inhibitor C16 or when VV expresses the ARV anti-PKR protein sigmaA.

The results of this study also reveal that the IFN sensitivity of wild-type VV in avian cells is similar to that reported for VV mutants lacking the E3L gene in mammalian cells (48, 50). Thus, the E3L-lacking VV mutants are very sensitive to IFN priming but become IFN resistant when they express specific dsRNA-binding proteins from other viruses, like the mammalian reovirus protein sigma3 or the influenza virus NS1 protein (62, 63). Similarly, we have found that VV replication in avian cells changes from IFN sensitive to IFN resistant when VV expresses the ARV dsRNA-binding protein sigmaA. Thus, it appears that E3 is either nonfunctional in avian cells or that its concentration within IFN-primed VV-infected CEF cells is not high enough for sequestering all the dsRNA generated in these cells during virus transcription. Our previous finding that RNase L is not active in IFN-treated VV-infected CEF cells (26) suggests that OAS remains inactive because of the dsRNA-binding activity of E3, despite the fact that chicken IFN induces very high intracellular levels of OAS in CEF cells (24, 64). This, in turn, suggests that E3 is functional in avian cells and that the lack of anti-PKR activity that we found in IFN-primed VV-infected avian cells could be due to the inability of E3 to heterodimerize with chPKR although this suggestion requires experimental confirmation.

In contrast to IFN priming, eIF2α is not phosphorylated, and viral protein synthesis is not inhibited when VV-infected avian cells are treated with DTT even though DTT efficiently induces eIF2α phosphorylation and protein synthesis inhibition in both uninfected and VSV-infected avian cells. These results suggest that VV-infected avian cells contain factors that prevent PERK-induced eIF2α phosphorylation. The most likely candidate is the VV-encoded K3 protein. This protein, which is encoded by a viral early gene, has homology to the amino-terminal region of eIF2α and acts as a nonphosphorylatable PKR pseudosubstrate to competitively inhibit eIF2 phosphorylation (65, 66). Although we do not have specific antibodies to analyze the intracellular K3 concentration, we assume that, as with E3 and sigmaA, IFN priming does not inhibit the synthesis of K3. Then, if this protein is able to prevent PERK-induced eIF2α phosphorylation in avian cells, why is K3 able to block eIF2α phosphorylation in DTT-treated cells but not in IFN-treated cells? One possibility is that K3 might act as a pseudosubstrate for chicken PERK but not for chicken PKR since it has been shown that rapid evolution of PKR in different species alters its sensitivity to K3 while maintaining its eIF2α phosphorylation activity (67). Another possibility is that intracellular viral amplification would be required for K3 to reach the threshold concentration necessary for preventing eIF2α phosphorylation. That goal could not be achieved in IFN-primed cells because viral amplification would be blocked by PKR activation, whereas high levels of early viral proteins like K3 would accumulate in DTT-treated cells because the inhibitor was added to the medium at late infection times.

In the case of VSV we observed that IFN priming of avian cells induced the following: (i) the activation of a small PKR fraction, (ii) a slight increase in eIF2α phosphorylation, (iii) the arrest of viral protein synthesis, and (iv) a partial rescue of cellular protein

synthesis. Furthermore, we found that the PKR inhibitor C16 was unable to rescue the synthesis of VSV proteins in IFN-primed cells. Taken together, these data suggest that chIFN induces a blockade of VSV replication at a step prior to viral protein synthesis. Accordingly, IFN has been reported to inhibit the replication of VSV in mammalian cells at stages of the virus life cycle like penetration, decapsidation, and/or viral transcription (68–73). On the other hand, our findings that eIF2α is phosphorylated and that VSV protein synthesis is arrested in DTT-treated cells indicate both that VSV does not express factors for preventing PERK-mediated eIF2 phosphorylation and that translation of VSV mRNAs is very sensitive to phosphorylation of this initiation factor, as has been previously documented (34). Taken together, these results suggest that ISGs other that PKR are involved in the IFN-induced arrest of VSV replication in avian cells, a situation already reported for the replication of this virus in mammalian cells (73–76).

In contrast to replication of VV and VSV, the replication of ARV in avian cells is quite resistant to IFN priming even though efficient expression of both type I interferons and PKR takes place in ARV-infected avian cells (77). Thus, it appears that ARV triggers anti-IFN strategies for efficient replication in IFN-primed avian cells, and the results of this study suggest that one strategy is the prevention of PKR activation since we have found that PKR does not become active upon IFN priming of ARV-infected avian cells. This suggests that ARV expresses/induces factors that prevent PKR activation, and one likely candidate is the ARV core protein sigmaA, a protein that binds tightly to dsRNA in an irreversible manner (27, 56, 57). Previous studies have provided the following indirect evidence that sigmaA inhibits PKR activation: (i) the capacity of extracts of ARV-infected cells to overcome the translational inhibitory activity of dsRNA in reticulocyte lysates is lost upon removal of protein sigmaA; (ii) a recombinant protein sigmaA fused to maltose-binding protein is able to relieve the translation-inhibitory activity of dsRNA in reticulocyte lysates by blocking the activation of endogenous dsRNA-dependent enzymes; (iii) transient expression of sigmaA in HeLa cells rescues gene expression of a vaccinia virus mutant lacking the E3L gene; (iv) insertion of the sigmaA-encoding gene into vaccinia virus confers protection for the virus against interferon in chicken cells; and (v) expression of recombinant sigmaA in mammalian cells interferes with PKR function (24, 26). In the present work we provide direct evidence that ARV infection and sigmaA expression are both able to downregulate PKR activation and eIF2α phosphorylation. We further show that the capacity of sigmaA to prevent PKR activation is linked to its dsRNA-binding activity since a sigmaA mutant lacking this activity displayed a reduced ability to prevent PKR activation (Fig. 6B). Thus, sigmaA should be included in the list of viral proteins that not only display structural functions but also have anti-IFN properties, like the nucleoprotein of arenaviruses (78), the glycoprotein of hantaviruses (79), and the matrix proteins of both VSV and Thogoto virus (80, 81).

It has been reported that the E3L protein of VV prevents PKR activation not only by sequestering dsRNA activator but also by binding to PKR and forming a ternary complex with PKR and dsRNA (51). This prompted us to assess whether the ARV protein sigmaA similarly interacts with chPKR, but such interaction could not be detected when two-hybrid pulldown assays or colocalization experiments were performed (data not shown), suggesting that the anti-PKR activity of sigmaA relies exclusively on its dsRNA-binding activity. This would explain why both PKR and RNase L remain inactive in IFN-primed ARV-infected avian cells (26).

Our results also demonstrate that DTT treatment of ARV-infected avian cells induces moderate phosphorylation of eIF2α and that this causes a drastic inhibition of cellular protein synthesis, whereas viral protein synthesis is hardly affected. Furthermore, DTT-induced eIF2α phosphorylation and cellular protein synthesis inhibition are more pronounced in uninfected cells than in ARV-infected cells, suggesting that the inhibition of cellular protein synthesis is a direct response to eIF2α phosphorylation. These results further indicate that the translation of ARV mRNAs is more resistant to eIF2α phosphorylation than the translation of their cellular counterparts.

Some cellular and viral transcripts have been reported to be quite resistant to eIF2α phosphorylation (15, 55, 82). One class of these mRNAs, such as those encoding activating transcription factor 4 (ATF4), contain upstream open reading frames (uORFs) in their 5′ untranslated regions and require these uORFs and eIF2α phosphorylation for efficient translation of the main ORF (15, 82–84). Another class of transcripts contain internal ribosome entry site (IRES) or downstream loop (DLP) structures, which allow them to recruit Met-tRNA$_i^{met}$ in the absence of eIF2 (40, 82, 85–95). On the other hand, phosphorylation of eIF2α has been reported to occur in cells infected with rotavirus, a member of the Reoviridae family; under these restrictive conditions, the synthesis of most cellular proteins is inhibited while viral transcripts are efficiently translated (96), suggesting that translation of rotavirus transcripts is quite resistant to eIF2α phosphorylation. Finally, some IRES-containing cellular and viral transcripts have been reported to use alternative initiator factors, such as 2A, 5B, 2D, and MCT1/DENR, to replace the function of eIF2 for initiator tRNA delivery (55, 82). Since most ARV transcripts are monocistronic and do not contain uORFs in their short 5′ untranslated regions (97), it will be interesting to assess whether ARV mRNAs utilize an eIF2-independent mode of translation and, if so, whether they use alternative noncanonical factors for initiator tRNA recruitment.

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## REFERENCES

1. **Diebold S.** 2010. Innate recognition of viruses. Immunol Lett **128:**17–20. http://dx.doi.org/10.1016/j.imlet.2009.09.010.
2. **Koyama S, Ishii KJ, Coban C, Akira S.** 2008. Innate immune response to viral infection. Cytokine **43:**336–341. http://dx.doi.org/10.1016/j.cyto.2008.07.009.
3. **Li MM, MacDonald MR, Rice CM.** 2015. To translate, or not to translate:

viral and host mRNA regulation by interferon-stimulated genes. Trends Cell Biol **25:**320–329. http://dx.doi.org/10.1016/j.tcb.2015.02.001.
4. **Sadler AJ, Williams BR.** 2008. Interferon-inducible antiviral effectors. Nat Rev Immunol **8:**559–568. http://dx.doi.org/10.1038/nri2314.
5. **Takaoka A, Yanai H.** 2006. Interferon signalling network in innate defence. Cell Microbiol **8:**907–922. http://dx.doi.org/10.1111/j.1462-5822.2006.00716.x.
6. **de Weerd NA, Samrajiwa SA, Hertzog PJ.** 2007. Type I interferon receptors: biochemistry and biological functions. J Biol Chem **282:**20053–20057. http://dx.doi.org/10.1074/jbc.R700006200.
7. **Hovanessian AG.** 2007. On the discovery of interferon-inducible, double-stranded RNA activated enzymes: the 2′-5′oligoadenylate synthetases and the protein kinase PKR. Cytokine Growth Factor Rev **18:**351–361. http://dx.doi.org/10.1016/j.cytogfr.2007.06.003.
8. **Krug RM.** 2014. Viral proteins that bind double-stranded RNA: countermeasures against host antiviral responses. J Interferon Cytokine Res **34:**464–468. http://dx.doi.org/10.1089/jir.2014.0005.
9. **Chakrabarti A, Jha BK, Silverman RH.** 2011. New insights into the role of RNase L in innate immunity. J Interferon Cytokine Res **31:**49–57. http://dx.doi.org/10.1089/jir.2010.0120.
10. **Silverman RH.** 2007. Viral encounters with 2′,5′-oligoadenylate synthetase and RNase L during the interferon antiviral response. J Virol **81:**12720–12729. http://dx.doi.org/10.1128/JVI.01471-07.
11. **Garcia MA, Meurs EF, Esteban M.** 2007. The dsRNA protein kinase PKR: virus and cell control. Biochimie **89:**799–811. http://dx.doi.org/10.1016/j.biochi.2007.03.001.
12. **Munir M, Berg M.** 2013. The multiple faces of protein kinase R in antiviral defense. Virulence **4:**85–89. http://dx.doi.org/10.4161/viru.23134.
13. **Donnelly N, Gorman AM, Gupta S, Samali A.** 2013. The eIF2α kinases: their structures and functions. Cell Mol Life Sci **70:**3493–3511. http://dx.doi.org/10.1007/s00018-012-1252-6.
14. **Wek RC, Jiang HY, Anthony TG.** 2006. Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans **34:**7–11. http://dx.doi.org/10.1042/BST0340007.
15. **Baird TD, Wek RC.** 2012. Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. Adv Nutr **3:**307–321. http://dx.doi.org/10.3945/an.112.002113.
16. **Hershey JW.** 1989. Protein phosphorylation controls translation rates. J Biol Chem **264:**20823–20826.
17. **Proud CG.** 2005. eIF2 and the control of cell physiology. Semin Cell Dev Biol **16:**3–12. http://dx.doi.org/10.1016/j.semcdb.2004.11.004.
18. **Gale M, Sen GC.** 2009. Viral evasion of the interferon system. J Interferon Cytokine Res **29:**475–476. http://dx.doi.org/10.1089/jir.2009.0078.
19. **Weber F, Haller O.** 2007. Viral suppression of the interferon system. Biochimie **89:**836–842. http://dx.doi.org/10.1016/j.biochi.2007.01.005.
20. **Langland JO, Cameron JM, Heck MC, Jancovich JK, Jacobs BL.** 2006. Inhibition of PKR by RNA and DNA viruses. Virus Res **119:**100–110. http://dx.doi.org/10.1016/j.virusres.2005.10.014.
21. **Saunders LR, Barber GN.** 2003. The dsRNA binding protein family: critical roles, diverse cellular functions. FASEB J **17:**961–983. http://dx.doi.org/10.1096/fj.02-0958rev.
22. **Taylor KE, Mossman KL.** 2013. Recent advances in understanding viral evasion of type I interferon. Immunology **138:**190–197. http://dx.doi.org/10.1111/imm.12038.
23. **Ellis MN, Eidson CS, Brown J, Kleven SH.** 1983. Studies on interferon induction and interferon sensitivity of avian reoviruses. Avian Dis **27:**927–936. http://dx.doi.org/10.2307/1590194.
24. **Martinez-Costas J, Gonzalez-Lopez C, Vakharia VN, Benavente J.** 2000. Possible involvement of the double-stranded RNA-binding core protein σA in the resistance of avian reovirus to interferon. J Virol **74:**1124–1131. http://dx.doi.org/10.1128/JVI.74.3.1124-1131.2000.
25. **Sekellick MJ, Ferrandino AF, Hopkins DA, Marcus PI.** 1994. Chicken interferon gene: cloning, expression and analysis. J Interferon Res **14:**71–79. http://dx.doi.org/10.1089/jir.1994.14.71.
26. **González-López C, Martínez-Costas J, Esteban M, Benavente J.** 2003. Evidence that avian reovirus σA protein is an inhibitor of the double-stranded RNA-dependent protein kinase. J Gen Virol **84:**1629–1639. http://dx.doi.org/10.1099/vir.0.19004-0.
27. **Guardado-Calvo P, Vazquez-Iglesias L, Martinez-Costas J, Llamas-Sanz AL, Schoehn G, Fox GC, Hermo-Parrado XL, Benavente J, van Raaij MJ.** 2008. Crystal structure of the avian reovirus inner capsid protein σA. J Virol **82:**11208–11216. http://dx.doi.org/10.1128/JVI.00733-08.
28. **Rodriguez JF, Rodriguez D, Rodriguez JR, McGowan EB, Esteban M.** 1988. Expression of the firefly luciferase gene in vaccinia virus: a highly sensitive gene marker to follow virus dissemination in tissues of infected animals. Proc Natl Acad Sci U S A **85:**1667–1671. http://dx.doi.org/10.1073/pnas.85.5.1667.
29. **Vázquez-Iglesias L, Lostalé-Seijo I, Martínez-Costas JM, Benavente J.** 2009. Avian reovirus σA localizes to the nucleolus and enters the nucleus by a nonclassical energy- and carrier-independent pathway. J Virol **83:**10163–10175. http://dx.doi.org/10.1128/JVI.01080-09.
30. **Tourís-Otero F, Martínez-Costas JM, Vakharia VN, Benavente J.** 2004. Avian reovirus nonstructural protein μNS forms viroplasm-like inclusions and recruits protein σNS to these structures. Virology **319:**94–106. http://dx.doi.org/10.1016/j.virol.2003.10.034.
31. **Knoops K, Barcena M, Limpens RW, Koster AJ, Mommaas MM, Snijder EJ.** 2012. Ultrastructural characterization of arterivirus replication structures: reshaping the endoplasmic reticulum to accommodate viral RNA synthesis. J Virol **86:**2474–2487. http://dx.doi.org/10.1128/JVI.06677-11.
32. **Welsch S, Miller S, Romero-Brey I, Merz A, Bleck CKE, Walther P, Fuller SD, Antony C, Krijnse-Locker J, Bartenschlager R.** 2009. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. Cell Host Microbe **5:**365–375. http://dx.doi.org/10.1016/j.chom.2009.03.007.
33. **Schultz U, Rinderle C, Sekellick MJ, Marcus PI, Staeheli P.** 1995. Recombinant chicken interferon from *Escherichia coli* and transfected COS cells is biologically active. Eur J Biochem **229:**73–76. http://dx.doi.org/10.1111/j.1432-1033.1995.0073l.x.
34. **Domingo-Gil E, Toribio R, Nájera JL, Esteban M, Ventoso I.** 2011. Diversity in viral anti-PKR mechanisms: a remarkable case of evolutionary convergence. PLoS One **6:**e16711. http://dx.doi.org/10.1371/journal.pone.0016711.
35. **Gerhard DS, Wagner L, Feingold EA, Shenmen CM, Grouse LH, Schuler G, Klein SL, Old S, Rasooly R, Good P, Guyer M, Peck AM, Derge JG, Lipman D, Collins FS, Jang W, Sherry S, Feolo M, Misquitta L, Lee E, Rotmistrovsky K, Greenhut SF, Schaefer CF, Buetow K, Bonner TI, Haussler D, Kent J, Kiekhaus M, Furey T, Brent M, Prange C, Schreiber K, Shapiro N, Bhat NK, Hopkins RF, Hsie F, Driscoll T, Soares MB, Casavant TL, Scheetz TE, Brown-stein MJ, Usdin TB, Toshiyuki S, Carninci P, Piao Y, Dudekula DB, Ko MS, Kawakami K, Suzuki Y, Sugano S, et al.** 2004. The status, quality, and expansion of the NIH full-length cDNA project: the Mammalian Gene Collection (MGC). Genome Res **14:**2121–2127. http://dx.doi.org/10.1101/gr.2596504.
36. **Kawakubo K, Kuhen KL, Vessey JW, George CX, Samuel CE.** 1999. Alternative splice variants of the human PKR protein kinase possessing different 5′-untranslated regions: expression in untreated and interferon-treated cells and translational activity. Virology **264:**106–114. http://dx.doi.org/10.1006/viro.1999.9995.
37. **Marissen WE, Guo Y, Thomas AA, Matts RL, Lloyd RE.** 2000. Identification of caspase 3-mediated cleavage and functional alteration of eukaryotic initiation factor 2α in apoptosis. J Biol Chem **275:**9314–9323. http://dx.doi.org/10.1074/jbc.275.13.9314.
38. **Satoh S, Hijikata M, Handa H, Shimotohno K.** 1999. Caspase-mediated cleavage of eukaryotic translation initiation factor subunit 2α. Biochem J **342:**65–70. http://dx.doi.org/10.1042/bj3420065.
39. **Walter P, Ron D.** 2011. The unfolded protein response: from stress pathway to homeostatic regulation. Science **334:**1081–1086. http://dx.doi.org/10.1126/science.1209038.
40. **Welnowska E, Sanz MA, Redondo N, Carrasco L.** 2011. Translation of viral mRNA without active eIF2: the case of picornaviruses. PLoS One **6:**e22230. http://dx.doi.org/10.1371/journal.pone.0022230.
41. **Ingrand S, Barrier L, Lafay-Chevassier C, Fauconneau B, Page G, Hugon J.** 2007. The oxindole/imidazole derivative C16 reduces in vivo brain PKR activation. FEBS Lett **581:**4473–4478. http://dx.doi.org/10.1016/j.febslet.2007.08.022.
42. **Jammi NV, Whitby LR, Beal PA.** 2003. Small molecule inhibitors of the RNA-dependent protein kinase. Biochem Biophys Res Commun **308:**50–57. http://dx.doi.org/10.1016/S0006-291X(03)01318-4.
43. **Perdiguero B, Esteban M.** 2009. The interferon system and vaccinia virus evasion mechanisms. J Interferon Cytokine Res **29:**581–598. http://dx.doi.org/10.1089/jir.2009.0073.
44. **Moss B, Shisler JL.** 2001. Immunology 101 at poxvirus U: immune evasion genes. Semin Immunol **13:**59–66. http://dx.doi.org/10.1006/smim.2000.0296.
45. **Langland JO, Jacobs BL.** 2002. The role of the PKR inhibitory genes, E3L

and K3L, in determining vaccinia virus host range. Virology **299:**133–141. http://dx.doi.org/10.1006/viro.2002.1479.
46. **Smith GL, Benfield CTO, Maluquer de Motes C, Mazzon M, Ember SWJ, Ferguson BJ, Sumner RP.** 2013. Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. J Gen Virol **94:**2367–2392. http://dx.doi.org/10.1099/vir.0.055921-0.
47. **Beattie E, Paoletti E, Tartaglia J.** 1995. Distinct patterns of interferon sensitivity observed in cells infected with vaccinia K3L$^-$ and E3L$^-$ deletion mutants. Virology **210:**254–263. http://dx.doi.org/10.1006/viro.1995.1342.
48. **Beattie E, Kauffman EB, Martinez H, Perkus ME, Jacobs BL, Paoletti E, Tartaglia J.** 1996. Host-range restriction of vaccinia virus E3L-specific deletion mutants. Virus Genes **12:**89–94. http://dx.doi.org/10.1007/BF00370005.
49. **Chang HW, Watson JC, Jacobs BL.** 1992. The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. Proc Natl Acad Sci U S A **89:**4825–4829. http://dx.doi.org/10.1073/pnas.89.11.4825.
50. **Xiang Y, Condit RC, Vijaysri S, Jacobs B, Williams BR, Silverman RH.** 2002. Blockade of interferon induction and action by the E3L double-stranded RNA binding protein of vaccinia virus. J Virol **76:**5251–5259. http://dx.doi.org/10.1128/JVI.76.10.5251-5259.2002.
51. **Romano PR, Zhang F, Tan SL, Garcia-Barrio MT, Katze MG, Dever TE, Hinnebusch AG.** 1998. Inhibition of double-stranded RNA-dependent protein kinase PKR by vaccinia virus E3: role of complex formation and the E3 N-terminal domain. Mol Cell Biol **18:**7304–7316. http://dx.doi.org/10.1128/MCB.18.12.7304.
52. **Pennington TH.** 1974. Vaccinia virus polypeptide synthesis: sequential appearance and stability of pre- and post-replicative peptides. J Gen Virol **25:**433–444. http://dx.doi.org/10.1099/0022-1317-25-3-433.
53. **Kazemi S, Papadopoulou S, Li S, Su Q, Wang S, Yoshimura A, Matlashewski G, Dever TE, Koromilas AE.** 2004. Control of α subunit of eukaryotic translation initiation factor 2 (eIF2α) phosphorylation by the human papillomavirus type 18 E6 oncoprotein: implications for eIF2α-dependent gene expression and cell death. Mol Cell Biol **24:**3415–3429. http://dx.doi.org/10.1128/MCB.24.8.3415-3429.2004.
54. **Li Y, Zhang C, Chen X, Yu J, Wang Y, Yang Y, Du M, Jin H, Ma Y, He B, Cao Y.** 2011. ICP34.5 protein of herpes simplex virus facilitates the initiation of protein translation by bridging eukaryotic initiation factor 2α (eIF2α) and protein phosphatase 1. J Biol Chem **286:**24785–24792. http://dx.doi.org/10.1074/jbc.M111.232439.
55. **Reineke LC, Lloyd RE.** 2011. Animal virus schemes for translation dominance. Curr Opin Virol **1:**363–372. http://dx.doi.org/10.1016/j.coviro.2011.10.009.
56. **Tourís-Otero F, Martínez-Costas J, Vakharia VN, Benavente J.** 2005. Characterization of the Nucleic acid-binding activity of the avian reovirus non-structural protein sigmaNS. J Gen Virol **86:**1159–1169. http://dx.doi.org/10.1099/vir.0.80491-0.
57. **Yin HS, Shien JH, Lee LH.** 2000. Synthesis in *Escherichia coli* of avian reovirus core protein σA and its dsRNA-binding activity. Virology **266:**33–41. http://dx.doi.org/10.1006/viro.1999.0020.
58. **Bialy HS, Colby C.** 1972. Inhibition of early vaccinia virus ribonucleic acid synthesis in interferon-treated chicken embryo fibroblasts. J Virol **9:**286–289.
59. **Esteban M, Metz DH.** 1973. Inhibition of early vaccinia virus protein synthesis in interferon-treated chicken embryo fibroblasts. J Gen Virol **20:**111–115. http://dx.doi.org/10.1099/0022-1317-20-1-111.
60. **Grün J, Kroon E, Zöller B, Krempien U, Jungwirth C.** 1987. Reduced steady-state levels of vaccinia virus-specific early mRNAs in interferon-treated chick embryo fibroblasts. Virology **158:**28–33. http://dx.doi.org/10.1016/0042-6822(87)90234-0.
61. **Degen HJ, Blum D, Grün J, Jungwirth C.** 1992. Expression of authentic vaccinia virus-specific and inserted viral and cellular genes under control of an early vaccinia virus promoter is regulated post-transcriptionally in interferon-treated chick embryo fibroblasts. Virology **188:**114–121. http://dx.doi.org/10.1016/0042-6822(92)90740-G.
62. **Beattie E, Denzler KL, Tartaglia J, Perkus ME, Paoletti E, Jacobs BL.** 1995. Reversal of the interferon-sensitive phenotype of vaccinia virus lacking E3L by expression of the reovirus S4 gene. J Virol **69:**499–505.
63. **Guerra S, Abaitua F, Martinez-Sobrido L, Esteban M, Garcia-Satre A, Rodriguez D.** 2011. Host-range restriction of vaccinia virus E3L deletion mutant can be overcome in vitro, but not in vivo, by expression of the influenza virus NS1 protein. PLoS One **6:**e28677. http://dx.doi.org/10.1371/journal.pone.0028677.
64. **Ball LA, White CN.** 1979. Nuclease activation by double-stranded RNA and by 2′,5′-oligoadenylate in extracts of interferon-treated chick cells. Virology **93:**348–356. http://dx.doi.org/10.1016/0042-6822(79)90239-3.
65. **Beattie E, Tartaglia J, Paoletti E.** 1991. Vaccinia virus-encoded eIF2α homolog abrogates the antiviral effect of interferon. Virology **183:**419–422. http://dx.doi.org/10.1016/0042-6822(91)90158-8.
66. **Carroll K, Elroy-Stein O, Moss B, Jagus R.** 1993. Recombinant vaccinia virus K3L gene product prevents activation of double-stranded RNA-dependent, initiation factor 2α-specific protein kinase. J Biol Chem **268:**12837–12842.
67. **Rothenburg S, Seo EJ, Gibbs JS, Dever TE, Dittmar K.** 2009. Rapid evolution of protein kinase PKR alters sensitivity to viral inhibitors. Nat Struct Mol Biol **16:**63–70. http://dx.doi.org/10.1038/nsmb.1529.
68. **Basu M, Maitra RK, Xiang Y, Meng X, Banerjee AK, Bose S.** 2006. Inhibition of vesicular stomatitis virus infection in epithelial cells by alpha interferon-induced soluble secreted proteins. J Gen Virol **87:**2653–2662. http://dx.doi.org/10.1099/vir.0.82039-0.
69. **Belkowski LS, Sen GC.** 1987. Inhibition of vesicular stomatitis viral mRNA synthesis by interferons. J Virol **61:**653–660.
70. **Marcus PI, Sekellick MJ.** 1978. Interferon action III. The rate of primary transcription of vesicular stomatitis virus is inhibited by interferon action. J Gen Virol **38:**391–408.
71. **Thacore HR.** 1978. Effect of interferon on transcription and translation of vesicular stomatitis virus in human and simian cell cultures. J Gen Virol **41:**421–426. http://dx.doi.org/10.1099/0022-1317-41-2-421.
72. **Weidner JM, Jiang D, Pan XB, Chang J, Block TM, Guo J.** 2010. Interferon-induced cell membrane proteins, IFITM3 and tetherin, inhibit vesicular stomatitis virus infection via distinct mechanisms. J Virol **84:**12646–12657. http://dx.doi.org/10.1128/JVI.01328-10.
73. **Zhou A, Paranjape JM, Der SD, Williams BR, Silverman RH.** 1999. Interferon action in triply deficient mice reveals the existence of alternative antiviral pathways. Virology **258:**435–440. http://dx.doi.org/10.1006/viro.1999.9738.
74. **Balachandran S, Roberts PC, Brown LE, Truong H, Pattnaik AK, Archer DR, Barber GN.** 2000. Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. Immunity **13:**129–141. http://dx.doi.org/10.1016/S1074-7613(00)00014-5.
75. **Connor JH, Lyles DS.** 2005. Inhibition of host and viral translation during vesicular stomatitis virus infection. eIF2 is responsible for the inhibition of viral but not host translation. J Biol Chem **280:**13512–13519. http://dx.doi.org/10.1074/jbc.M501156200.
76. **Jin HK, Takada A, Kon Y, Haller O, Watanabe T.** 1999. Identification of the murine *Mx2* gene: interferon-induced expression of the Mx2 protein from the feral mouse gene confers resistance to vesicular stomatitis virus. J Virol **73:**4925–4930.
77. **Lostalé-Seijo I, Martínez-Costas J, Benavente J.** 2016. Interferon induction by avian reovirus. Virology **487:**104–111. http://dx.doi.org/10.1016/j.virol.2015.10.009.
78. **Martinez-Sobrido L, Zúñiga EI, Rosario D, Garcia-Sastre A, de la Torre JC.** 2006. Inhibition of the type I interferon response by the nucleoprotein of the prototypic arenavirus lymphocytic choriomeningitis virus. J Virol **80:**9192–9199. http://dx.doi.org/10.1128/JVI.00555-06.
79. **Alff PJ, Gavrilovskaya IN, Gorbunova E, Endriss K, Chong Y, Geimonen E, Sen N, Reich NC, Mackow ER.** 2006. The pathogenic NY-1 hantavirus G1 cytoplasmic tail inhibits RIG-I- and TBK-1-directed interferon responses. J Virol **80:**9676–9686. http://dx.doi.org/10.1128/JVI.00508-06.
80. **Lyles DS.** 2000. Cytopathogenesis and inhibition of host gene expression by RNA viruses. Microbiol Mol Biol Rev **64:**709–724. http://dx.doi.org/10.1128/MMBR.64.4.709-724.2000.
81. **Pichlmair A, Buse J, Jennings S, Haller O, Kochs G, Staeheli P.** 2004. Thogoto virus lacking interferon-antagonistic protein ML is strongly attenuated in newborn Mx1-positive but not Mx1-negative mice. J Virol **78:**11422–11424. http://dx.doi.org/10.1128/JVI.78.20.11422-11424.2004.
82. **Holcik M.** 2015. Could the eIF2α-independent translation be the Achilles heel of cancer? Front Oncol **5:**264. http://dx.doi.org/10.3389/fonc.2015.00264.
83. **Andreev DE, O'Connor PB, Fahey C, Kenny EM, Terenin IM, Dmitriev SE, Cormican P, Morris DW, Shatsky IN, Baranov PV.** 2015. Translation of 5′ leaders is pervasive in genes resistant to eIF2 repression. eLife **4:**e03971. http://dx.doi.org/10.7554/eLife.03971.

84. **Shabman RS, Hoenen T, Groseth A, Jabado O, Binning JM, Amarasinghe GK, Feldmann H, Basler CF.** 2013. An upstream open reading frame modulates Ebola virus polymerase translation and virus replication. PLoS Pathog **9:**e1003147. http://dx.doi.org/10.1371/journal.ppat.1003147.
85. **Garcia-Moreno M, Sanz MA, Pelletier J, Carrasco L.** 2013. Requirements for eIF4A and eIF2 during translation of Sindbis virus subgenomic mRNA in vertebrate and invertebrate host cells. Cell Microbiol **15:**823–840. http://dx.doi.org/10.1111/cmi.12079.
86. **Garrey JL, Lee YY, Au HH, Bushell M, Jan E.** 2010. Host and viral translation mechanisms during cricket paralysis virus infection. J Virol **84:**1124–1138. http://dx.doi.org/10.1128/JVI.02006-09.
87. **Jan E, Kinzy TG, Sarnow P.** 2003. Divergent tRNA-like element supports initiation, elongation, and termination of protein biosynthesis. Proc Natl Acad Sci U S A **100:**15410–15415. http://dx.doi.org/10.1073/pnas.2535183100.
88. **Pestova TV, de Breyne S, Pisarev AV, Abaeva IS, Hellen CU.** 2008. eIF2-dependent and eIF2-independent modes of initiation on the CSFV IRES: a common role of domain II. EMBO J **27:**1060–1072. http://dx.doi.org/10.1038/emboj.2008.49.
89. **Redondo N, Sanz MA, Steinberger J, Skern J, Kusov Y, Carrasco L.** 2012. Translation directed by hepatitis A virus IRES in the absence of active eIF4F complex and eIF2. PLoS One **7:**e52065. http://dx.doi.org/10.1371/journal.pone.0052065.
90. **Redondo N, Sanz MA, Welnowska E, Carrasco L.** 2011. Translation without eIF2 promoted by poliovirus 2A protease. PLoS One **6:**e25699. http://dx.doi.org/10.1371/journal.pone.0025699.
91. **Sanz MA, Castello A, Ventoso I, Berlanga JJ, Carrasco L.** 2009. Dual mechanism for the translation of subgenomic mRNA from Sindbis virus in infected and uninfected cells. PLoS One **4:**e4772. http://dx.doi.org/10.1371/journal.pone.0004772.
92. **Terenin IM, Dmitriev SE, Andreev DE, Shatsky IN.** 2008. Eukaryotic translation initiation machinery can operate in a bacterial-like mode without eIF2. Nat Struct Mol Biol **15:**836–841. http://dx.doi.org/10.1038/nsmb.1445.
93. **Ventoso I.** 2012. Adaptive changes in alphavirus mRNA translation allowed colonization of vertebrate hosts. J Virol **86:**9484–9494. http://dx.doi.org/10.1128/JVI.01114-12.
94. **White JP, Reineke LC, Lloyd RE.** 2011. Poliovirus switches to an eIF2-independent mode of translation during infection. J Virol **85:**8884–8893. http://dx.doi.org/10.1128/JVI.00792-11.
95. **Yamamoto H, Unbehaun A, Loerke J, Behrmann E, Collier M, Bürger J, Mielke T, Spahn CM.** 2014. Structure of the mammalian 80S initiation complex with initiation factor 5B on HCV-IRES RNA. Nat Struct Mol Biol **21:**721–727. http://dx.doi.org/10.1038/nsmb.2859.
96. **Montero H, Rojas M, Arias CF, López S.** 2008. Rotavirus infection induces the phosphorylation of eIF2α but prevents the formation of stress granules. J Virol **82:**1496–1504. http://dx.doi.org/10.1128/JVI.01779-07.
97. **Benavente J, Martínez-Costas J.** 2007. Avian reovirus: structure and biology. Virus Res **123:**105–119. http://dx.doi.org/10.1016/j.virusres.2006.09.005.