Interferon induction by avian reovirus

Irene Lostalé-Seijo, José Martínez-Costas, Javier Benavente

Accepted Manuscript

How to cite:

Copyright information:

INTERFERON INDUCTION BY AVIAN REOVIRUS

AUTHORS:

Irene Lostalé-Seijo, José Martínez-Costas, and Javier Benavente*

AFFILIATION:

Centro de Investigación en Química Biológica y Materiales Moleculares (CIQUS), Universidad de Santiago de Compostela, Campus Vida, 15782-Santiago de Compostela, Spain

RUNNING TITLE:

Interferon induction by avian reovirus

KEY WORDS: avian reovirus; interferon induction; PKR; apoptosis; virus uncoating.

Abstract: 149 words

* Correspondence to: Centro de Investigación en Química Biológica y Materiales Moleculares (CIQUS), despacho D3.7. Universidad de Santiago de Compostela, 15782-Santiago de Compostela, Spain. Tel.: 34-881815734; Fax: 34-881815704; E-mail address: franciscojavier.benavente@usc.es (J. Benavente).

1

ABSTRACT

We have previously shown that the replication of avian reovirus (ARV) in chicken embryo fibroblasts (CEF) is more resistant to the antiviral action of interferon (IFN) than the replication of vesicular stomatitis virus (VSV) or vaccinia virus (VV). In this study we examined the capacity of these three viruses to induce the expression of IFN when infecting avian cells. Efficient expression of both type-α and type-β IFNs, as well as of the double-stranded RNA (dsRNA)-activated protein kinase (PKR), takes place in ARV-infected CEF, but not in cells infected with VSV or VV. PKR expression is not directly induced by ARV infection, but by the IFN secreted by ARV-infected cells. IFN induction in ARV-infected cells requires viral uncoating, but not viral gene expression, a situation similar to that reported for apoptosis induction by ARV-infected cells. However, our results demonstrate that IFN induction by ARV-infected CEF occurs by a caspase-independent mechanism.

INTRODUCTION

Interferons (IFNs) comprise a family of multifunctional cytokines that were originally discovered by their strong antiviral activity (Isaacs and Lindenmann, 1957), and which are now recognized as the first barrier that viruses have to overcome to establish a productive infection. Of the three IFN types, type I interferon-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) (reviewed in Samuel, 2001).

Successful host defense against viruses relies on early detection of intracellular virus particles followed by the rapid production of type I interferons. For this, cells contain a series of endosomal and cytosolic sensors, called pattern recognition receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPs), such as viral nucleic acids or viral intermediate products. When contacting PAMPs, PRRs become activated and transmit intracellular signaling pathways, culminating in the activation of specific transcription factors that translocate to the nucleus to stimulate type I IFN promoters (reviewed in Diebold, 2010; Edwards et al., 2007; Jefferies and Fitzgerald, 2005; Koyama et al., 2008; Yoneyama and Fujita, 2010). Newly-synthesized type I IFNs are secreted out of the cell to interact with the ubiquitously expressed IFNAR receptor complex present in neighboring cells. This interaction triggers the activation of a signal transduction pathway that leads to increased expression of the designated IFN-stimulated genes (ISGs), thus creating an antiviral state. Subsequent viral infection of IFN-primed cells induces the activation of ISG-encoded proteins; the antiviral activity of these proteins prevents further dissemination of the virus (reviewed in Doly et al., 1998; Haller et al., 2006; Sadler and Williams, 2008; Samuel, 2001; Takaota and Yanai, 2006).

3

Despite that IFN was initially discovered as a soluble chicken factor that directly interfered with influenza virus replication in chorioallontoic membranes of chicken embryos (Isaacs and Lindenmann, 1957), our understanding of the host response to pathogens in poultry is very limited, since most efforts were dedicated at characterizing the antiviral response in mammals. However, interest in IFNs of birds has recently emerged from increasing problems with viral diseases in poultry and from the observation that chickens infected with highly pathogenic avian influenza virus strains pose a high threat to human health (Fouchier et al., 2013; Karpala et al., 2012; Poovorawan et al., 2013). As in mammals, three types of chicken IFN (chIFN) have been identified in virus-infected chicken cells, and all three have been reported to display antiviral activity (reviewed in Goosens et al., 2013). Type I chIFN, which comprises multiple chIFN-α isoforms and a single chIFN-β, has the strongest antiviral activity, although chIFN-α is the dominant virus-induced IFN subtype produced by virus-infected avian cells (Schultz et al., 1995; Schwarz et al., 2004), a situation opposite to the one found in mammalian-infected cells. This, and the observations that chIFN-α exhibits stronger antiviral activity than chIFN-β against several viruses and greater induction potency on several ISGs encoding antiviral proteins (Qu et al., 2013; Schwarz et al., 2004), suggests that chIFN-α is the main defense used by chicken cells to combat viral infections.

Previous studies from different laboratories, including ours, have revealed that the replication of avian reovirus (ARV) in cultured avian cells is much more resistant to the antiviral action of chIFN than vaccinia virus (VV), vesicular stomatitis virus (VSV) or Semliki Forest virus (Ellis et al., 1983; Gonzalez-Lopez et al., 2003; Martinez-Costas et al., 2000; Sekellick et al., 1994). In this study we have examined the capacity of ARV, VV and VSV to induce IFN expression when infecting avian cells. We found that

IFN is only expressed and secreted by ARV-infected cells and that IFN induction requires virus uncoating, but not the expression of the ARV genes.

RESULTS:

IFN induction by virus-infected avian cells

In the first part of this study we sought to compare the capacity of ARV, VV and VSV to induce the production and secretion of IFN by infected avian cells. Our previous finding that VSV and VV are very sensitive to priming of CEF cells with IFN (Martinez-Costas et al., 2000) suggests that IFN should not be secreted by avian cells infected with these two viruses, otherwise the IFN present in the viral stocks used to infect the cells would block viral replication. In the case of VV, this suggestion is supported by the results of previous studies that revealed that chIFN activity was not detected upon infection of CEF cells with wild-type VV (Hornemann et al., 2003). In contrast, it has been recently reported that infection of the CEF-derived avian cell line DF1 with VSV induces increasing expression of the mRNAs coding for chIFN-α and chIFN-β (Qu et al., 2013), although the presence of IFN in the cultured medium of VSV-infected cells was not examined in this study.

To determine the capacity of the three viruses to induce chIFN expression, we first analyzed by Western blot the intracellular levels of the IFN-inducible protein PKR in virus-infected CEF cells. Since the possibility existed that IFN is produced and secreted by ARV-infected CEF, and consequently that the ARV stock used to infect the cells contains chIFN, the infection with ARV was carried out with a suspension of purified reovirions devoid of cellular proteins (Grande and Benavente, 2000). VSV and VV viruses did not require purification since these viruses were grown on BHK-21 hamster cells and therefore their stocks should not contain chIFN. The results shown in Fig. 1A revealed that PKR expression was induced when the cells were infected with ARV, but not when infected with VSV or VV, suggesting that IFN is only expressed by

6

ARV-infected cells. To confirm this suggestion, we analyzed the presence of IFN in the cultured medium (supernatant) of virus-infected CEF cells by two different approaches. In the first approach, we examined the capacity of virus-free supernatants to induce PKR expression when incubated with monolayers of uninfected CEF. Viral particles were removed from the supernatants of infected cells by precipitation with perchloric acid at 4ºC, as previously reported (Sekellick and Marcus, 1986), but similar results were obtained when the viral particles were inactivated by incubating the supernatants at 65ºC for 30 min (Liniger et al., 2012). The results shown in Fig. 1B revealed that only the supernatant from ARV-infected cells, but not those from cells infected with VSV or VV, was able to induce the expression of PKR. In the second approach, we determined the capacity of the supernatants to activate the promoter of the chicken Mx gene contained within the reporter pGL3-P-chMx-luc plasmid (Liniger et al., 2012). The supernatants were incubated with plasmid-transfected DF1 cells, since control experiments revealed that the transfection of any plasmid into CEF cells, but not into DF1 cells, already induces PKR expression (not shown). The results shown in Fig. 1C revealed that only the supernatant from ARV-infected CEF was able to activate the Mx promoter. Taken together, these results demonstrate that infection of CEF with ARV, but not with VSV or VV, induces the production and secretion of chIFN. Our results further revealed that the secreted IFN is functional in both CEF and DF1 cells, since it is able to activate the expression of those IFN-responsive genes expressing PKR and Mx.

ARV-infected CEF secrete type-α and type-β IFNs

To try to determine the type of chIFN (α or β) produced by ARV-infected CEF cells, antibodies to chIFN-α and/or -β were added to the culture medium of the infected cells and the intracellular levels of PKR were monitored by Western blotting. The

results revealed that each of the two antibodies was capable of inhibiting PKR induction, although maximal inhibition was only reached when the two antibodies were simultaneously present in the culture medium (Fig. 2A). These results not only indicate that ARV-infected CEF cells secrete type-α and type-β chIFNs, but also suggest that PKR is indirectly induced in these cells by the IFN secreted to the cultured medium. To confirm this suggestion we analyzed the effect of brefeldin A (BFA) on PKR expression. BFA is a macrolide antibiotic that has been shown to inhibit vesicle transport to the cell surface, by causing the resorption of the Golgi complex into the endoplasmic reticulum (Miller et al., 1992; Nebenführ et al., 2002). It has been shown that 0.25 μg/ml of BFA only caused a slight reduction of both intracellular muNS levels and the production of infectious viral particles (Bodelon et al., 2002), indicating that this antibiotic hardly affects ARV replication and assembly. On the other hand, trypan blue staining of BFA-treated CEF cells revealed that ~95% of the cells were viable after an incubation period of 18 h. The results shown in Fig. 2B revealed that PKR expression is no longer induced when ARV-infected CEF cells are incubated in the presence of 0.5 μg/ml of BFA (upper panel, compare lanes 4 and 6), suggesting that PKR is not directly induced by the viral infection, but by the IFN secreted by ARV-infected CEF cells.

IFN induction in ARV-infected cells requires viral uncoating, but not viral gene expression

Our next goal was to try to identify the stage of the ARV life cycle at which IFN expression is induced. First of all, we determined the relative production of chIFN-β mRNA in ARV-infected CEF at different infection times. We analyzed the intrcellular levels of this mRNA because it is encoded by a single gene, whereas there are multiple

8

chIFN-α genes. Real-time PCR analysis revealed that increased expression of the chIFN-β mRNA was already observed at 3 hpi and its intracellular levels increased significantly at 6 hpi (Fig. 3A), indicating that strong induction of the chIFN-β gene takes place at early infection times. To confirm this result we analyzed the intracellular PKR levels and the capacity of the supernatants to activate the Mx promoter at different infection times. Induction of PKR expression was already detected at 6 hpi and intracellular PKR levels increased with time until 12 hpi, and then declined probably because of the damage caused by the infection, as revealed by diminished actin levels (Fig. 3B). On the other hand, the capacity of the supernatant from infected cells to activate the Mx promoter was already detected at 3 hpi and steadily increased until 12 hpi (Fig. 3C). These results indicate that IFN expression is induced at an early stage of the ARV replication cycle. To try to identify that stage we first assessed whether intracellular virus uncoating is required for IFN induction. For this, we examined the effect of two lysosomotropic agents, ammonium chloride and chloroquine, that have been previously shown to prevent intraendosomal ARV uncoating by neutralizing vacuolar acidification; as a consequence virus protein synthesis and virus replication is blocked in ARV-infected CEF incubated in the presence of any of these two agents (Labrada et al., 2002). The results shown in Fig. 4A revealed that each of the two inhibitors was very effective in preventing PKR induction when added to cells at the onset of the infection (lanes 3 and 5), but not when added 3 h later (lanes 4 and 6). Furthermore, the supernatants from ARV-infected CEF that had been incubated with either of the two inhibitors from the onset of the infection, but not when added 3 h later, were no longer able to activate the Mx promoter (Fig. 4B). These results indicate that virus uncoating is required for IFN induction in ARV-infected cells.

To determine whether ARV gene expression is necessary for IFN induction, we first evaluated the effect of ribavirin, a nucleoside analog that has been reported to block ARV transcription without affecting the expression of cellular genes (Labrada et al., 2002). Our finding that the production of the ARV nonstructural muNS protein is blocked when ARV-infected cells are incubated in the presence of 100 µM ribavirin indicates that the nucleoside analog is effective in blocking ARV gene expression (compare lanes 3 and 4 in the middle panel of Fig. 5A). However, ribavirin failed to inhibit PKR expression in ARV-infected CEF (compare lanes 3 and 4 in the upper panel of Fig. 5A), and the supernatant from ribavirin-treated ARV-infected cells was still able to activate the Mx promoter (Fig. 5B). Since ribavirin has been shown to induce the expression of a subset of the IFN-responsive genes in uninfected cells (Thomas et al., 2011), we followed an alternative approach to examine whether ARV gene expression is required for IFN induction. For this, we evaluated the capacity of ultraviolet-inactivated ARV virions to induce IFN expression. As previously shown (Labrada et al., 2002), treatment of purified reovirions with ultraviolet light (UV) completely blocked ARV gene expression, as revealed by the absence of muNS production (compare lanes 2 and 3 in the middle panel of Fig. 5C), and also caused inactivation of virus infectivity as determined by plaque assay (not shown). In contrast, PKR expression was still induced when CEF cells were incubated with UV-treated virions (lane 3 in the upper panel of Fig. 5C), and the supernatant from these cells was still able to activate the Mx promoter (Fig. 5D). Taken together, these results indicate that viral gene expression is not required for IFN induction in ARV-infected CEF.

Apoptosis activation and IFN induction are unrelated events

10

The results shown so far indicate that IFN is induced in ARV-infected CEF at a stage of the virus life cycle subsequent to virus uncoating, but prior to virus gene expression. Since a similar situation has been previously reported for apoptosis induction in ARV-infected cells (Labrada et al., 2002), we next examined whether apoptosis is required for IFN induction in ARV-infected cells. For this, we examined the effect of the pancaspase inhibitor Q-VD-OPh, which has been shown to be very effective in preventing the activation of effector caspases and the conversion of muNS into muNSC in ARV-infected CEF (Rodriguez-Grille et al., 2014). The results revealed that, while the caspase inhibitor was very effective in preventing both the activation of effector caspases (Fig. 6A) and the conversion of muNS into muNSC (middle panel of Fig. 5B, compare lanes 3 and 4), Q-VD-OPh was unable to inhibit PKR expression by ARV-infected cells (upper panel of Fig. 6B, compare lanes 3 and 4). These results suggest that IFN induction in ARV-infected cells is triggered by a caspase-independent mechanism.

11

DISCUSSION

In this study we have analyzed the capacity of three different viruses to induce the expression of IFN when infecting CEF cells. The results revealed that only the infection with ARV, but not with VSV or VV, induces the expression and secretion of chIFN, which in turn indicates that signaling pathways mediating IFN gene expression are nonfunctional in VSV- or VV-infected avian cells. In the case of VV, it has been reported that the genome of this virus expresses a broad range of immune modulators, and some of them have been shown to block IFN expression by inhibiting PRR signaling pathways (Perdiguero and Esteban, 2009; Smith et al., 2013; Waibler et al., 2009). Our finding that IFN is not expressed by VV-infected CEF cells suggests that some of these viral modulators are active in avian cells. On the other hand, the absence of IFN induction that we have observed in VSV-infected CEF might be attributed to the capacity of the viral M protein to prevent IFN expression. This protein has been reported to block IFN induction in infected mammal cells by inhibiting nuclear transcription and the nucleocytoplasmic transport of cellular mRNAs (Ahmed et al., 2003; Faul et al., 2009; Ferran and Lucas-Lenard, 1997). Our unpublished observation that cellular mRNAs accumulate within the nucleus of CEF cells, together with the fact that cellular protein synthesis is drastically reduced in VSV-infected CEF (Martinez-Costas et al., 2000), suggest that the M protein also plays a key role in preventing IFN induction in VSV-infected avian cells. Other viruses, like influenza viruses A and B, Thogoto virus, herpes simplex virus type 1, human immunodeficiency virus, and Epstein-Barr virus, have also been reported to use mechanisms for blocking IFN induction in infected cells (Alcamí and Koszinowski, 2000; Weber and Haller, 2007).

The results of this study revealed that type-α and type-β IFNs are both produced by ARV-infected CEF cells, a situation similar to that reported for CEF cells infected with MVA or with an NS1-defective influenza virus. In contrast, only IFN-α was detected during the infection of CEF with Newcastle disease virus, Rift valley fever virus, or Thogoto virus (Schwarz et al., 2004). Additional experiments were performed to assess the stage of the ARV life cycle at which the expression of IFN is induced. Our findings revealed that IFN expression is induced at an early stage of the virus replication cycle. This suggestion is supported by the fact that IFN induction does not require expression of the ARV genes, since it takes place when the cells are incubated with UV-treated virus or when ARV-infected cells are incubated in the presence of ribavirin. In both cases IFN expression, but not the expression of ARV genes, was detected. The nondependence of IFN induction on viral gene expression indicates that IFN is induced by parental viral particles at a stage prior to viral transcription and suggests that viral mRNAs and viral nonstructural proteins should not act as IFN inducers. However, the possibility still exists that abortive transcripts produced in cells incubated with UV-treated reovirions or in ribavirin-treated ARV-infected cells could act as IFN inducers. Thus, it has been reported that UV-treatment of mammalian reovirions generates particles that are able to synthesize small amounts of incomplete viral transcripts (Henderson and Joklik, 1978) and, if the same holds true for the treatment of avian reovirions, these transcripts could be the trigger for IFN induction. On the other hand, ribavirin has been shown to block transcription elongation, but not the initiation of RNA synthesis (Rankin et al., 1989), so abortive reoviral transcripts generated in ribavirin-treated cells might act as IFN inducers.

That lysosomotropic agents prevent IFN induction when added to ARV-infected cells at the onset of the infection, but not when added 3 h later, indicates both that

13

intraendosomal virus uncoating is necessary to trigger IFN expression and that the expression of IFN is induced at a post-attachment step. Our findings that IFN induction requires virus uncoating but not viral gene expression suggests that the viral PAMPs detected by the cellular PRRs should be ARV products generated or exposed upon intraendosomal uncoating, and not by intact viral particles. These products might be core proteins or viral nucleic acids, like the adenine-rich oligonucleotides or the genome segments present in ARV particles. Since reovirus cores must cross the endosomal membrane to reach the cytoplasm for expressing the viral genes, cellular PRRs for the detection of ARV infection could be endosomal-associated PRRs, such as toll-like receptors, or cytoplasmic PRRs, such as RIG-helicases. Additional studies are needed to identify both the viral trigger and the cellular sensor that are responsible for IFN induction in ARV-infected avian cells.

Data presented in this report indicates that PKR expression is induced in ARV-infected CEF cells, but not in VSV- or VV-infected cells, and that the expression of this kinase is indirectly induced by the IFN secreted by ARV-infected cells. On the other hand, in a previous report we have presented evidences that the ARV protein sigmaA exerts an anti-IFN function by preventing PKR activation (Gonzalez-Lopez et al., 2003). Although the role that PKR plays on ARV replication is the subject of another study (manuscript in preparation), we can speculate that PKR expression might benefit ARV replication by promoting the selective inhibition of cellular protein synthesis observed in ARV-infected CEF (Martinez-Costas et al., 2000; Labrada et al., 2002). Thus, as has been reported for mammalian reovirus protein sigma3 (Schmechel, et al., 1997), localization of sigmaA in and around perinuclear viral factories would prevent PKR activation and allow the translation of viral mRNAs, while PKR would be activated and the synthesis of cellular proteins blocked elsewhere in the cytosol.

14

Alternatively, the sigmaA protein expressed in ARV-infected cells might partially inhibit PKR activation, leading to intracellular levels of phosphorylated eIF-2α that would block the translation of cellular mRNAs, but not of their viral counterparts. A pro-viral effect of PKR expression has similarly been reported for the replication of mammalian reoviruses (Smith et al., 2005).

15

MATERIALS AND METHODS

Cells and viruses

Primary cultures of CEF cells were prepared from 9- to 10-day-old embryos from specific pathogenic free chickens, and grown as monolayers in medium 199 supplemented with 10% tryptose phosphate broth and 5% calf serum. Chicken fibroblast DF-1 cells and baby hamster kidney BHK-21 cells were grown as monolayers in Dulbecco's Modified Eagle's medium (D-MEM, Invitrogen). Vaccinia virus (VV; Western Reserve strain) and Vesicular Stomatitis Virus (VSV; Indiana serotype) were grown on BHK-21 cell monolayers. Avian reovirus (ARV) strain S1133 was grown on semiconfluent monolayers of CEF cells, and purified by CsCl-gradient centrifugation as previously described (Grande and Benavente, 2000), except that Freon was replaced by Vertrel-XF (Mendez et al., 2000).

For ultraviolet inactivation, purified ARV virions kept on ice were exposed to a 254 nm UV light for 15 min, as described by Tyler et al. (1995). UV-inactivated virus was devoid of infectious virus particles as determined by plaque assay.

Antibodies, plasmids and reagents

Anti-actin antibody (rabbit polyclonal, sc-1616R) was purchased from Santa Cruz Biotechnology and HRP-conjugated goat anti-rabbit IgG from Sigma. Rabbit polyclonal anti-muNS protein was raised in our laboratory (Tourís-Otero et al., 2004). Rabbit polyclonal anti-chicken PKR antibody was generated by BioSynthesis, using as immunogen a KLH-conjugated synthetic peptide comprising amino acids 527-550 of chicken PKR. Rabbit antibodies against chicken IFNs α and β were a kind gift from Dr. Peter Staeheli (Sick et al., 1996).

16

The reporter plasmid that contains the luciferase gen under the control of the chicken Mx promoter (pGL3-P-chMx-Luc) was a gift from Dr. Nicolas Ruggli (Liniger et al., 2012). Ammonium chloride, chloroquine, ribavirin and brefeldin A were purchased from Sigma, the pancaspase inhibitor Q-VD-OPh was from Calbiochem, lipofectamine from Invitrogen and the recombinant chicken IFN alpha from AbD-Serotec (#PAP004).

Western blotting

Cells were washed twice with PBS, lysed in Laemmli sample buffer (Laemmli, 1970) and boiled for 5 min. The resulting extracts were then subjected to SDS-PAGE electrophoresis, and the gel proteins transferred to PVDF membranes (Immobilon-P, Millipore) for 1 h at 100 V using a trans-blot electrophoretic transfer unit (Bio-Rad). Membranes were blocked for 1 h with PBS containing 0.05% Tween-20 and 4% non-fat dry milk, and incubated for 2 h with the primary antibodies diluted in blocking solution. After several washes, membranes were incubated for 45 min with HRP-conjugated goat anti-rabbit IgG and visualized by chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate, Millipore).

Preparation of virus-free supernatants and Mx-promoter activation assays

The virus was removed from the supernatants by perchloric acid (PCA) precipitation (Sekellick and Marcus, 1986). Briefly, foetal bovine serum in the culture medium was raised to 6% before adding 0.15 volumes of cold PCA. Samples were incubated for 24 h in a cold room and the precipitate removed by centrifugation at 2000 rpm 10 min. PCA in the supernatant was neutralized with KOH, and the pH adjusted to 6.8. The precipitate that forms after neutralization was removed by centrifugation, and the IFN-containing supernatant was filter-sterilized through a 0.22-μM Millex filter (Millipore) before further use. Alternatively, supernatants from monolayers of infected cells were

centrifugated at 4ºC for 5 min at 2000 rpm to remove cellular debris, and the resulting supernatants were incubated at 65ºC for 30 min to inactivate the virus (Liniger et al., 2012).

To determine the activation of the chicken Mx promoter, monolayers of DF-1 cells were transfected with the pGL3-P-chMx-luc plasmid and incubated 24 h later with either 50 µl of virus-free supernatants or 100 U/ml of IFN for 24 h. The cells were then harvested and lysed in CCLR buffer (Promega). The luciferase activity of the extracts was determined with a Luminoskan Ascent luminometer.

Quantitative real-time PCR of chIFN-β mRNA levels in ARV-infected CEF cells.
CEF cell monolayers were infected with 2 PFU/cell of ARV S1133, and at 0, 3 and 6 hpi cell samples were collected and their RNA was extracted using Trizol (Life Technologies). The RNA samples were treated with DNase RQ I (Promega) for 35 min at 37ºC and the enzyme was subsequently inactivated by incubation at at 65ºC for 10 min in the presence of DNase Stop Solution (Promega). Total RNA was reverse transcribed into cDNA using the iScript^{TM} cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions and the following incubation times: 25ºC for 5 min, 42 ºC for 40 min and 85ºC for 5 min. Then the exonuclease activity of the reverse transcriptase was heat-inactivated at 95ºC for 5 min. The cDNA was diluted 10 times in water, aliquoted and stored at -80ºC. For quantitative PCR amplification the following primers were used.

qPCR reactions of 10 µl contained 5 µl of IQ SYBR Green Super Mix (Bio-Rad), 2.5 µl of cDNA and 300 nM of each primer. For the detection of chicken IFN-β mRNA (NM_001024836.1), the forward primer was 5'CAACACCTCTTCAACATGCTTAG 3' and the reverse primer was 5'TGCTCAAGGTGATGGATGTAAT 3'. These primers were validated using serial dilutions of the pcDNA-I-IFNb plasmid (94.8% efficiency at

18

a Tm of 63.4ºC). An initial denaturalization step of 3 min at 95ºC was followed by 40 cycles (10 s at 95ºC, 30 s at 63.4ºC and 10 s at 72ºC) using a C1000 Touch thermocycler with a CFX96 optical module (Bio-Rad). An end-point single fluorescence was measured after each extension step. After the amplification we carried out an analysis of the dissociation curve from 65 to 95ºC by raising the temperature 0.5ºC every 5 s, to verify the specificity of the obtained products. The results were analysed and processed with the CFX Manager software (Bio-Rad). The relative amount of the mRNA was normalized to the internal β-actin control, and chicken β-actin gene expression was detected using previously described primers (Li et al., 2007).

ACKNOWLEDGMENTS

REFERENCES

Ahmed, M., McKenzie, M. O., Puckett, S., Hojnacki, M., Poliquin, L., and Lyles, D. S. (2003). Ability of the matrix protein of vesicular stomatitis virus to suppress beta interferon gene expression is genetically correlated with the inhibition of host RNA and protein synthesis. J. Virol. 77, 4646-4657.

Alcamí, A., and Koszinowski, U. H. (2000). Viral mechanisms of immune evasion. Immunol. Today 21, 447-455.

Bodelón, G., Labrada, L., Martínez-Costas, J. M., and Benavente, J. (2002). Modification of late membrane permeability in avian reovirus-infected cells: viroporin activity of the S1-encoded nonstructural p10 protein. J. Biol. Chem. 277,17789-17796.

Diebold, S. (2010). Innate recognition of viruses. Immunol. Let. 128, 17-20.

Doly, J., Civas, A., Navarro, S., and Uze, G. (1998). Type I interferons: expression and signalization. Cell Mol. Life Sci. 54, 1109-1121.

Edwards, M. R., Slater, L., and Johnston, S. L. (2007). Signalling pathways mediating type I interferon gene expression. Microbes Infect. 9, 1245-1251.

Ellis, M. N., Eidson, C. S., Brown, J., and Kleven, S. H. (1983). Studies on interferon induction and interferon sensitivity of avian reoviruses. Avian Dis. 27, 927-936.

Faul, E. J., Lyles, D. S., and Schnell, M. J. (2009). Interferon response and viral evasion by members of the family rhabdoviridae. Viruses 1, 832-851.

Ferran, M. C., and Lucas-Lenard, J. M. (1997). The vesicular stomatitis virus matrix protein inhibits transcription from the human beta interferon promoter. J. Virol. 71, 371-377.

Fouchier, R. A., and Kawaoka, Y. (2013). Transmission of influenza A/H5N1 viruses in mammals. Virus Res. 178, 15-20.

González-López, C., Martínez-Costas, J., Esteban, M., and 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.

Goossens, K. E., Ward, A. C., Lowenthal, J. W., and Bean, A. G. D. (2013). Chicken interferons, their receptors and interferon-stimulated genes. Dev. Comp. Immunol. 41, 370-376.

Grande, A., and Benavente, J. (2000). Optimal conditions for the growth, purification and storage of the avian reovirus S1133. J. Virol. Methods 85, 43-54.

Haller, O., Kochs, G., and Weber, F. (2006). The interferon response circuit: induction and suppression by pathogenic viruses. Virology 344, 119-130.

Henderson, D. R., and Joklik, W. K. (1978). The mechanism of interferon induction by UV-irradiated reovirus. *Virology*, 91, 389-406.

Hornemann, S., Harlin, O., Staib, C., Kisling, S., Erfle, V., Kaspers, B., Häcker, G., and Sutter, G. (2003). Replication of modified vaccinia virus Ankara in primary chicken embryo fibroblasts requires expression of the interferon resistance gene E3L. J. Virol. 77, 8394-8407.

Isaacs, A., and Lindenmann, J. (1957). Virus interference. I. The interferon. Proc. R. Soc. London Ser. B 147, 258-267.

Jefferies, C. A., and Fitzgerald, K. A. (2005). Interferon gene regulation: not all roads lead to Tolls. Trends Mol. Med. 11, 403-411.

Karpala, A. J., Lowenthal, J. W., and Bean, A. G. D. (2012). Identifying innate immune pathways of the chicken may lead to new antiviral therapies. Vet. Immunol. Immunopathol. 148, 100-109.

Koyama, S., Ishii, K. J., Coban, C., and Akira, S. (2008). Innate immune response to viral infection. Cytokine 43, 336-341.

Labrada, L., Bodelon, J., Viñuela, J., and Benavente, J. (2002). Avian reoviruses cause apoptosis in cultured cells: viral uncoating, but not viral gene expression, is required for apoptosis induction. J. Virol. 76, 7932-7941.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Li, Y. P., Handberg, K. J., Juul-Madsen, H. R., Zhang, M. F., and Jørgensen, P. H. (2007). Transcriptional profiles of chicken embryo cell cultures following infection with infectious bursal disease virus. Arch. Virol. 152, 463-478.

Liniger, M., Summerfield, A., Zimmer, G., McCullough, K. C., and Ruggli, N. (2012). Chicken cells sense influenza A virus infection through MDA5 and CARDIF signaling LPG2. J. Virol. 86, 705-717.

Martinez-Costas, J., Gonzalez-Lopez, C., Vakharia, V. N., and Benavente, J. (2000). Possible involvement of the double-stranded RNA-binding core protein sigmaA in the resistance of avian reovirus to interferon. J. Virol. 74, 1124-1131.

Mendez, I. I., Hermann, L. L., Hazelton, P. R., and Coombs, K. M. (2000). A comparative analysis of freon substitutes in the purification of reovirus and calicivirus. J. Virol. Methods 90, 59-67.

Miller, S. G., Carnell, L., and Moore, H. H. (1992). Post-Golgi membrane traffic: brefeldin A inhibits export from distal Golgi compartments to the cell surface but not recycling. J. Cell Biol. 118, 267-283

Nebenführ, A., Ritzenthaler, C., and Robinson, D. G. (2002). Brefeldin A: deciphering an enigmatic inhibitor of secretion. Plant Physiol. 130, 1102-1108.

Perdiguero, B., and Esteban, M. (2009). The interferon system and vaccinia virus evasion mechanisms. J. Interferon Cytokine Res. 29, 581-598.

22

Poovorawan, Y., Pyungporn, S., Prachayangprecha, S., and Makkoch, J. (2013). Global alert to avian influenza virus infection: from H5N1 to H7N9. Pathog. Glob. Health 107, 217-223.

Qu, H., Yang, L., Meng, S., Xu, L., Bi, Y., Jia, X., Li, J., Sun, L., and Liu. W. (2013). The differential antiviral activities of chicken interferon α (ChIFNα) and ChIFNβ are related to distinct interferon-stimulated gene expression. PLoS One 8, e59307.

Rankin, J. T., Eppes, S. B., Antczak, J. B. and Joklik, W. K. (1989). Studies on the mechanism of the antiviral activity of ribavirin against reovirus. Virology, 168, 147-58.

Rodríguez-Grille, J., Busch, L. K., Martínez-Costas, J. M., and Benavente, J. (2014). Avian reovirus-triggered apoptosis enhances both virus spread and the processing of the viral nonstructural muNS protein. Virology, 462-463, 49-59.

Sadler, A. J., and Williams, D. J. (2008). Interferon-inducible antiviral effectors. Nat. Rev. Immunol. 8, 559-568.

Samuel, C. E. (2001). Antiviral actions of interferons. Clin. Microbiol. Rev. 14, 778-890.

Schmechel, S., Chute, M., Skinner,, P., Anderson, R., and Schiff, L. (1997). Preferential translation of reovirus mRNA by a sigma3-dependent mechanism. Virology 232, 62-73.

Schultz, U., Rinderle, C., Sekellick, M. J., Marcus, P. I., and Staeheli, P. (1995). Recombinant chicken interferon from Escherichia coli and transfected COS cells is biologically active. Eur. J. Biochem. 229, 73-76.

Schwarz, H., Harlin, O., Ohnemus, A., Kaspers, B., and Staeheli, P. (2004). Synthesis of IFN-β by virus-infected chicken embryo cells demonstrated with specific antisera and a new bioassay. J. Interferon Cytok. Res. 24, 179-184.

Sekellick, M. J., Ferrandino, A. F., Hopkins, D. A., and Marcus, P. I. (1994). Chicken interferon gene: cloning, expression and analysis. J. Interferon Res. 14, 71-79.

23

Sekellick, M. J., and Marcus, P. I. (1986). Induction of high titer chicken interferon. Methods Enzymol. 119, 115-125.

Sick, C., Schultz, U., and Staeheli, P. (1996). A family of genes coding for two serologically distinct chicken interferons. J. Biol. Chem. 271, 7635-7639.

Smith, G. L., Benfield, C. T. O., Maluquer de Motes, C., Mazzon, M., Ember, S. W. J., Ferguson, B. J. and Sumner, R. P. (2013). Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. J. Gen. Virol. 94, 2367-2392.

Smith, J. A., Schmechel, S. C., Williams, B. R. G., Silverman, R. H., and Shiff, L. A. (2005). Involvement of the interferon-regulated antiviral proteins PKR and RNase L in reovirus-induced shutoff of cellular translation. J. Virol. 79, 2240-2250.

Takaoka, A., and Yanai, H. (2006). Interferon signaling network in innate defence. Cell. Microbiol. 8, 907-922.

Thomas, E., Feld, J. J., Li, Q., Hu, Z., Fried, M. W. and Liang, T. J. (2011). Ribavirin potentiates interferon action by augmenting interferon-stimulated gene induction in hepatitis C virus cell culture models. Hepatology 53, 32-41.

Tourís-Otero, F., Martínez-Costas, J. M., Vakharia, V. N., and Benavente, J. (2004). Avian reovirus nonstructural protein muNS forms viroplasm-like inclusions and recruits protein sigmaNS to these structures. Virology 319, 94–106.

Tyler, K. L., Squier, M. K. T., Rodgers, S. E., Schneider, B. E., Oberhaus, S. M., Grdina, T. A., Cohen, J. J., and Dermody, T. S. (1995). Differences in the capacity of reovirus strains to induce apoptosis are determined by the viral attachment protein σ1. J. Virol. 69, 6972–6979.

Waibler, Z., Anzhage, M., Frenz, T., Schwantes, A., Pöhlmann, C., Ludwig, H., Palomo-Otero, M, Alcami, A., Sutter, G., and Kalinke, U. (2009). Vaccinia-virus-mediated inhibition of type I interferon responses is a multifactorial process involving

the soluble type I interferon receptor B18 and intracellular components. J. Virol. 83, 1563-1571.

Weber, F., and Haller, O. (2007). Viral suppression of the interferon system. Biochimie 89, 836-842.

Yoneyama, M., and Fujita, T. (2010). Recognition of viral nucleic acids in innate immunity. Rev. Med. Virol. 20, 4-22.

Figure 1. IFN induction by virus-infected CEF cells. (A) CEF monolayers were mock-infected (U) or infected with 2 PFU/cell of the viruses indicated on top. At 18 hpi the cells were lysed and the resulting extracts were analyzed by Western blot with anti-chPKR (top panel) and anti-actin (bottom panel) antibodies. **(B)** Virus-free supernatants from the same cells shown in Fig. 1A were added to the culture medium of CEF cell monolayers and 24 h later the cells were lysed and the intracellular PKR and actin levels were compared by Western blotting. A Western blot analysis of extracts from uninfected CEF cells that were incubated with 1000 U/ml of chIFN for 24 h is shown in lanes 5 of Figs. 1A and 1B. **(C)** Virus-free supernatants from the same cells shown in Fig. 1A were added to the culture medium of DF1 cells that had been transfected with the pGL3-P-chMx-luc plasmid. These cells, as well as IFN-primed uninfected cells, were lysed 24 h later and the luciferase activity of the extracts was determined with a luminometer. The induction of the Mx promoter-dependent firefly luciferase was expressed as fold induction compared to that of unstimulated cells. The data are representative of three independent experiments. The error bars indicate the standard deviation of three measurements for each experiment.

Figure 2. ARV-infected CEF cells express type-α and type-β IFNs. (A) The cultured medium of CEF cells infected with 2 PFU/cell of ARV S1133 was supplemented at the onset of the infection with antibodies against type-α IFN (lane 3), type-β IFN (lane 4), each at the concentration of 2%, or with the two antibodies (lane 5) and incubated for 18 h. These cells, as well as mock-infected nontreated cells (lane 1) and mock-infected cells primed with IFN for 24 h (lane 6), were lysed and the extracts analyzed by Western blotting with antibodies against the proteins indicated at the right of the figure.

26

(B) Mock-infected CEF cells, either untreated (lanes 1-3) or primed with IFN for 24 h (lane 7), as well as CEF cells infected with 2 PFU/cell of ARV, were incubated from the onset of the infection with the concentrations of brefeldin A (BFA) indicated on top. The cells were lysed at 18 hpi and the resulting extracts analyzed by Western blotting with antibodies against the proteins indicated on the right. The sample in lane 7 was run on the same gel, but two internal lanes were removed.

Figure 3. IFN is induced at early infection times in ARV-infected CEF. CEF cell monolayers were infected with 2 PFU/cell of ARV for the hours indicated. **(A)** The cells were harvested for RNA isolation, and virus-induced expression of chIFN-β was determined by real-time PCR. The data are representative of three independent experiments, with the error bars indicating the standard deviations of the mean. **(B)** The infected cells, as well as IFN-primed noninfected cells (lane 7), were lysed and the extracts subjected to Western blot analysis with the antibodies indicated on the right. **(C)** The capacity to activate the Mx promoter of virus-free supernatants from the cells shown in Fig. 3A was determined as for Fig. 1C. The data are representative of three independent experiments, with the error bars indicating the standard deviations of the mean.

Figure 4. Induction of IFN expression requires ARV uncoating. (A) CEF cells infected with 2 PFU/cell of ARV (lanes 2-6) were treated with 100 μM chloroquine (lanes 3 and 4) or with 10 mM ammonium chloride (lanes 5 and 6), from the onset of the infection (lanes 3 and 5) or from 3 hpi (lanes 4 and 6). At 18 hpi these cells, as well as mock-infected nontreated cells (lane 1) and mock-infected cells primed with IFN for 24 h (lane 7), were lysed and the extracts analyzed by Western blotting with antibodies

against the proteins indicated at the right of the figure. **(B)** The capacity to activate the Mx promoter of virus-free supernatants from the cells shown in Fig. 4A was determined as for Fig. 1C. The data are representative of three independent experiments, with the error bars indicating the standard deviations of the mean.

Fig. 5. ARV gene expression is not required for IFN induction. (A) Mock-infected CEF (lanes 1 and 2) and ARV-infected CEF (lanes 3 and 4) were incubated from the onset of the infection with 100 μM ribavirin (lanes 2 and 4). At 18 hpi these cells, as well as IFN-primed uninfected cells (lane 5) were lysed and the extracts subjected to Western blot analysis with antibodies against the proteins indicated on the right. **(B)** The capacity to activate the Mx promoter of virus-free supernatants from the cells shown in Fig. 5A was determined as for Fig. 1C. The data are representative of three independent experiments, with the error bars indicating the standard deviations of the mean. **(C)** Monolayers of CEF were mock infected (lane 1) or infected with purified ARV virions that had been inactivated (lane 3) or not (lane 2) by UV light treatment. At 18 hpi the cells were lysed and the extracts analyzed by Western blotting with antibodies against the proteins indicated on the right. **(D)** The capacity to activate the Mx promoter of virus-free supernatants from the cells shown in Fig. 5C was determined as for Fig. 1C. The data are representative of three independent experiments, with the error bars indicating the standard deviations of the mean.

Fig. 6. Effect of Q-VD-OPh on caspase activity and IFN induction. (A) CEF monolayers were mock-infected (U) or infected with 2 PFU/cell of ARV in the absence (lanes -) or presence (lanes +) of 10 μM Q-VD-OPh. At 18 hpi caspase activity was determined with the Caspase-Glo 3/7 Assay kit (Promega) following the manufacturer's

28

instructions, and the results were expressed as arbitrary RLU units. Each value is the mean of three independent experiments. **(B)** A replica of the cells shown in Fig. 6A were lysed at 18 hpi and the cell extracts were subjected to Western blot analysis with antibodies against the proteins indicated on the right.
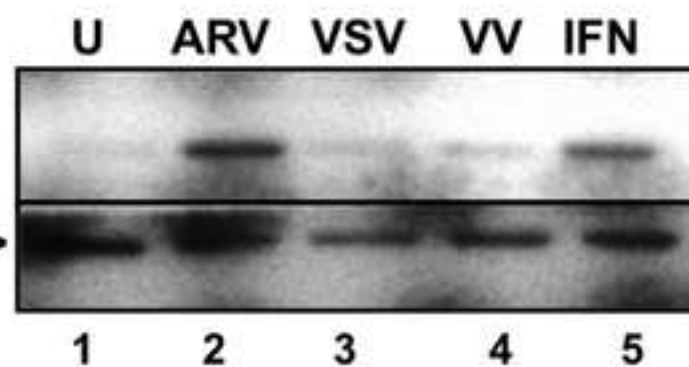
Figure 1

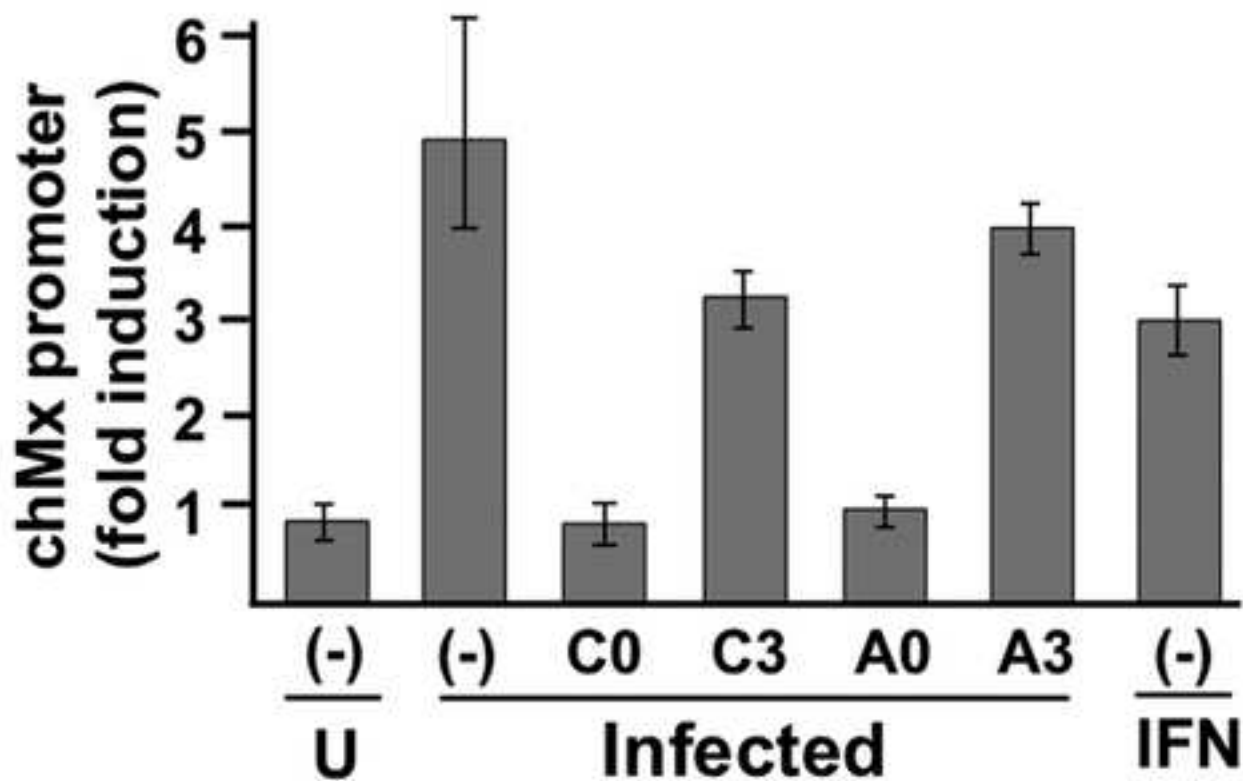**Figure 2**
Click here to download high resolution image
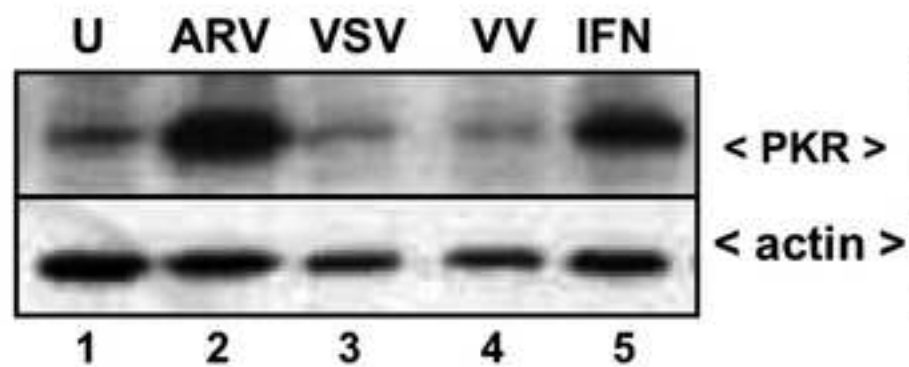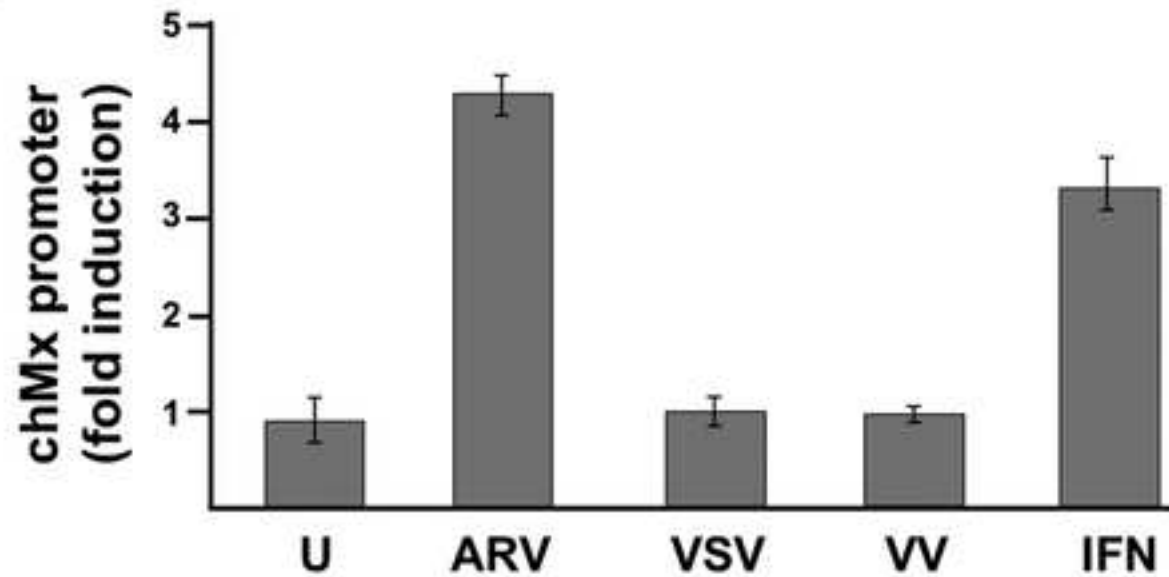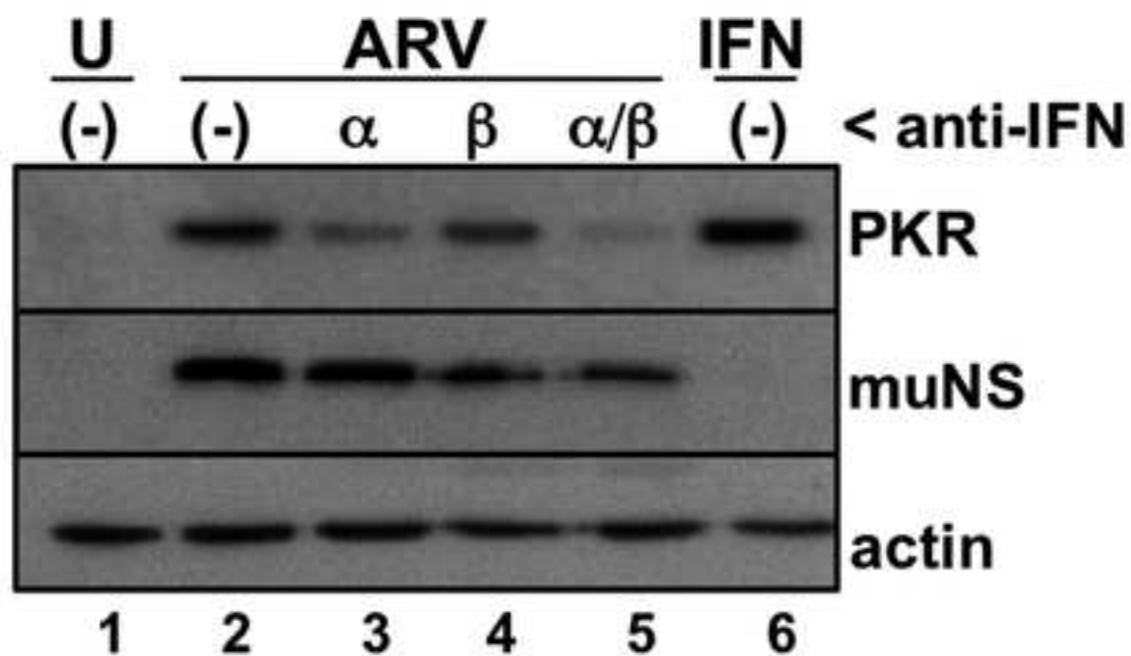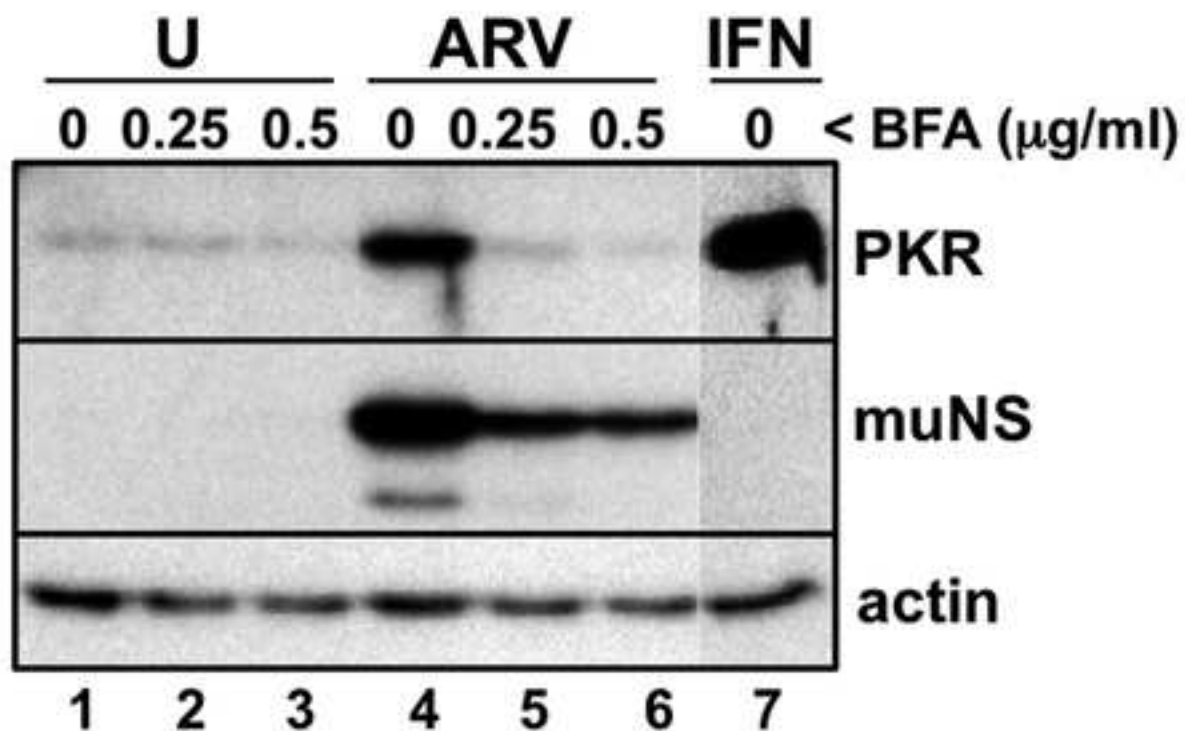
Figure 2

Figure 3
[Click here to download high resolution image](#)

Figure 4
Click here to download high resolution image
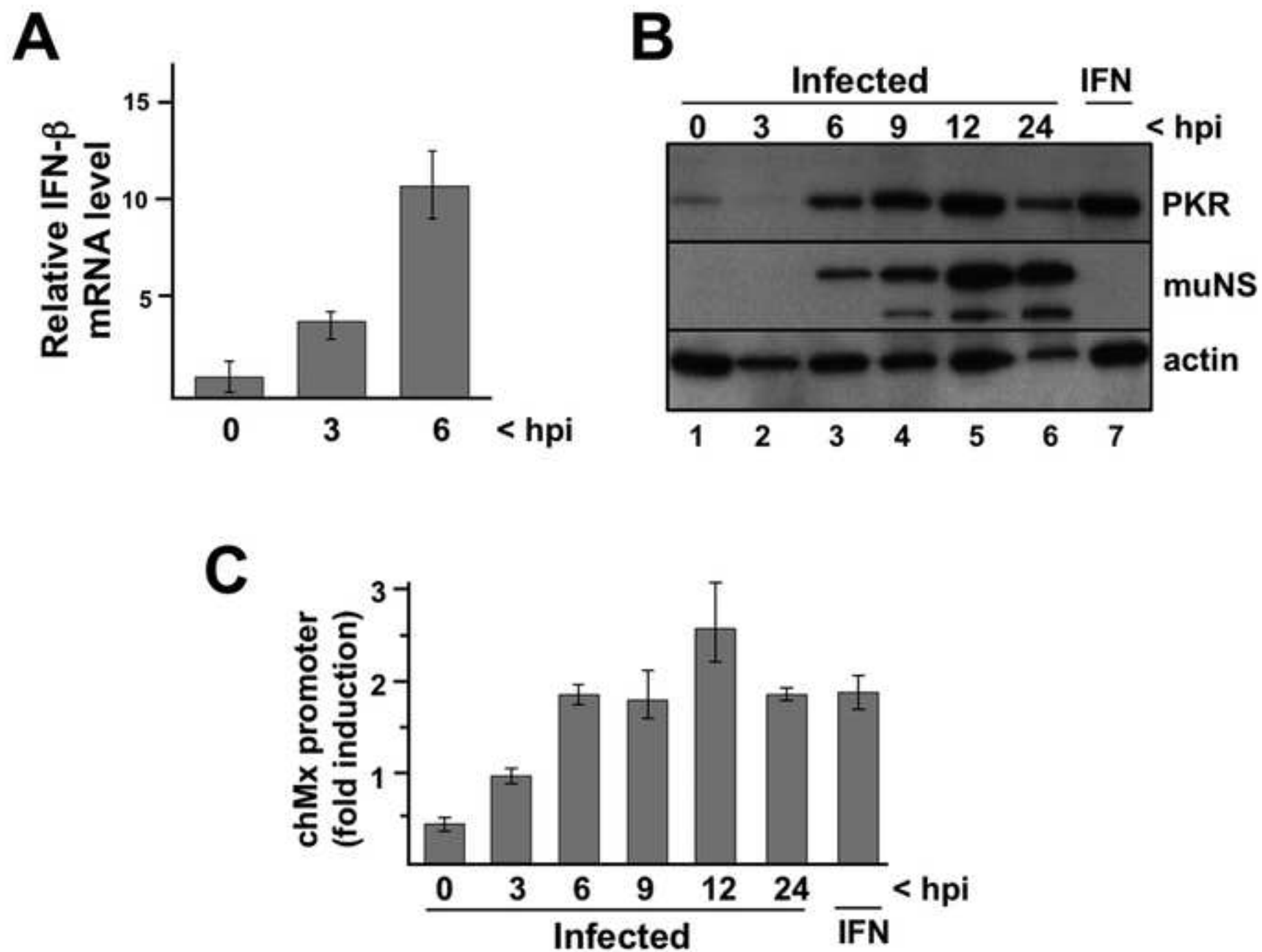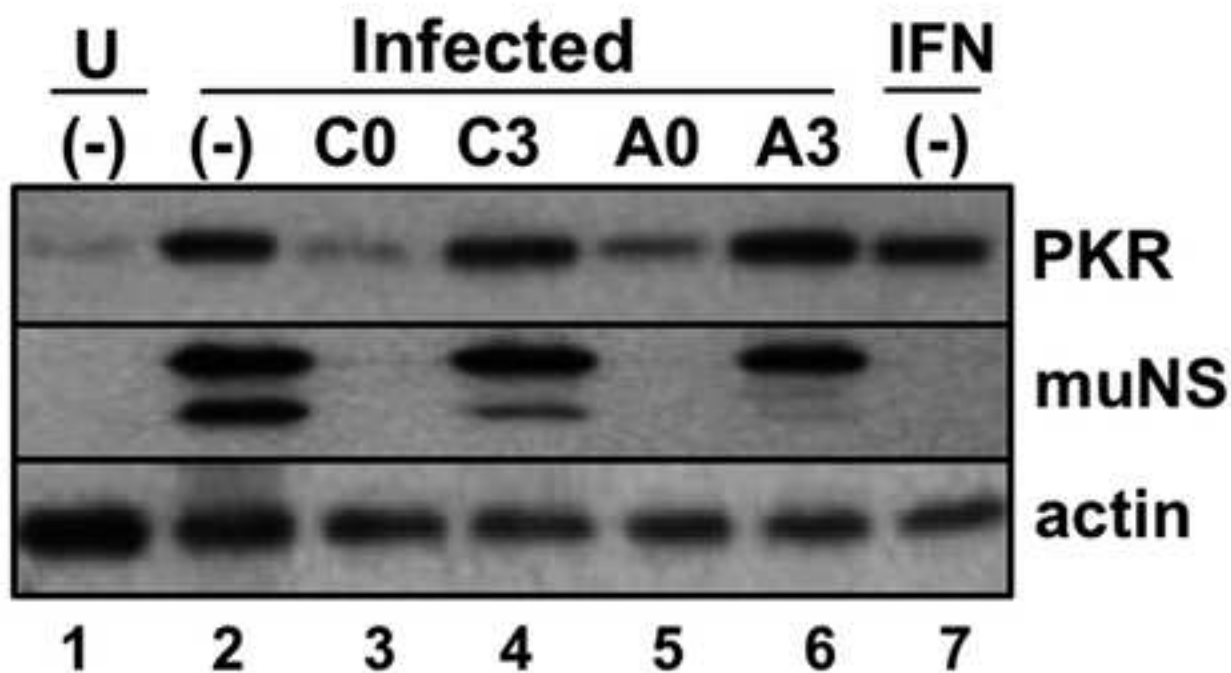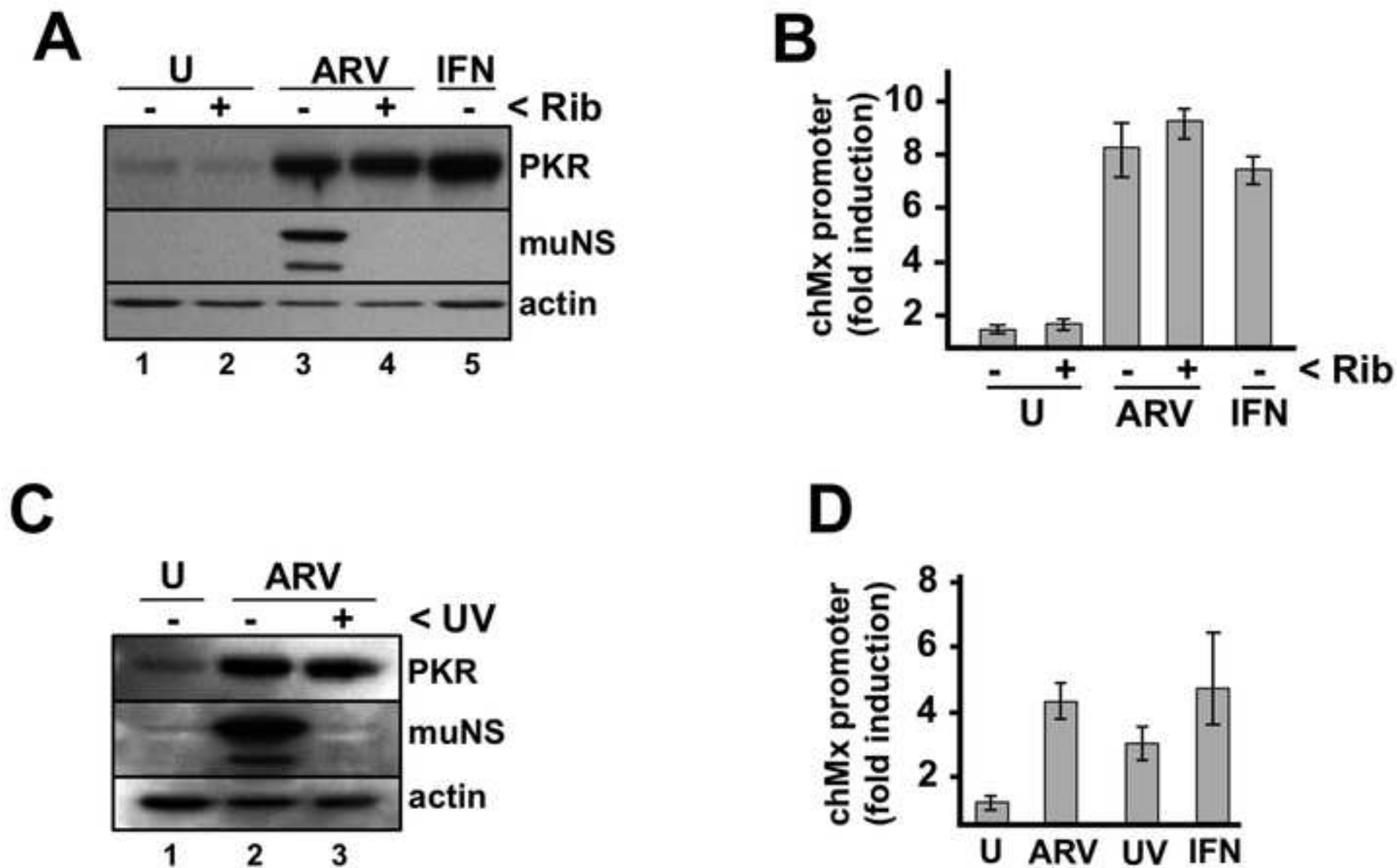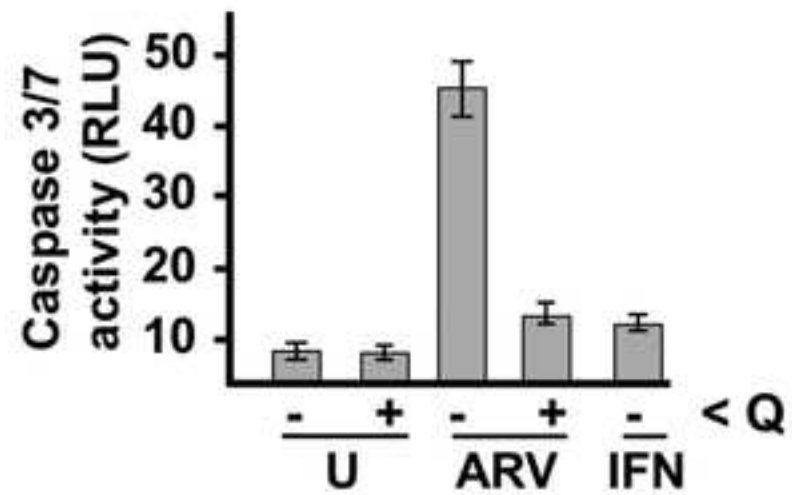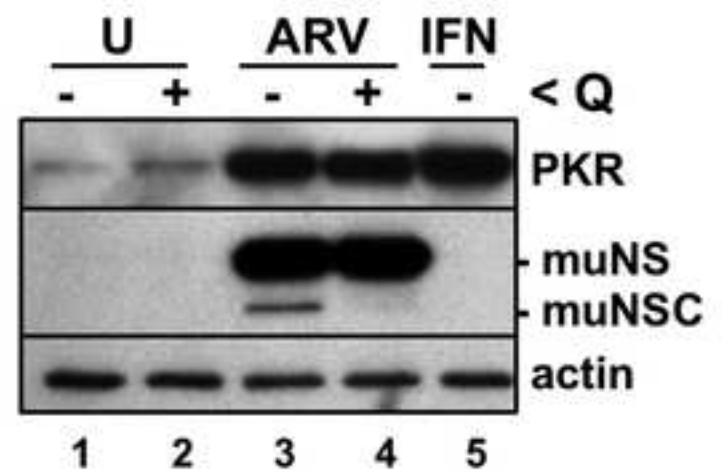
Figure 5
Click here to download high resolution image

Figure 6
Click here to download high resolution image

Supplementary Material (Not to be Published)

Click here to download Supplementary Material (Not to be Published): BFA.pptx