Genetic Screen of a Mutant Poxvirus Library Identifies an Ankyrin Repeat Protein Involved in Blocking Induction of Avian Type I Interferon

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ABSTRACT

Mammalian poxviruses, including vaccinia virus (VACV), have evolved multiple mechanisms to evade the host type I interferon (IFN) responses at different levels, with viral proteins targeting IFN induction, signaling and antiviral effector functions. Avian poxviruses (avipoxviruses), which have been developed as recombinant vaccine vectors for permissive (i.e. poultry) and non-permissive (i.e. mammals, including humans) species, encode no obvious equivalents of any of these proteins. We show that fowlpox virus (FWPV) fails to induce chicken IFN-β (ChIFN2) and is able to block its induction by transfected poly(I:C), an analog of cytoplasmic double-strand (ds) RNA. A broad-scale loss-of-function genetic screen was used to find FWPV-encoded modulators of poly(I:C)-mediated ChIFN2 induction. It identified fpv012, a member of a family of poxvirus genes, highly expanded in the avipoxviruses (31 in FWPV; 51 in canarypox virus (CNPV), representing 15% of the total gene complement), encoding proteins containing N-terminal ankyrin repeats (ANKs) and C-terminal F-box-like motifs. Under ectopic expression, the first ANK of fpv012 is dispensable for inhibitory activity and the CNPV ortholog is also able to inhibit induction of ChIFN2. FWPV defective in fpv012 replicate well in culture and barely induce ChIFN2 during infection, suggesting other factors are involved in blocking IFN induction and resisting the antiviral effectors. Nevertheless, unlike parental and revertant viruses, the mutants induce moderate levels of expression of interferon stimulated genes (ISG), suggesting either that there is sufficient ChIFN2 expression to partially induce the ISGs or the involvement of alternative, IFN-independent pathways, that are also normally blocked by fpv012.
INTRODUCTION

The host antiviral type I interferon (IFN) system is targeted by many viruses (1), and poxviruses are no exception. The prototypic, mammalian poxvirus, vaccinia virus (VACV), encodes a number of proteins that have been shown to modulate the IFN system in diverse ways, as reviewed recently (2). Functionally they can be grouped into those that inhibit induction of IFN-β, such as NF-κB activation suppressor K1 (3) and TBK-1 adaptor binding protein, C6 (4); those that inhibit IFN signaling via the Jak/Stat pathway necessary to induce expression of IFN-stimulated genes (ISG), such as soluble IFN receptor mimic B18 (5, 6) and Stat phosphorylation inhibitor H1 (7); and those that antagonize the activity of the major antiviral ISGs, such as double-stranded (ds) RNA-binding protein E3 (8) and eIF-2α mimic K3 (9).

With the exception of H1, no orthologs (nor even any functional equivalents) of these modulators have been described in avian poxviruses. A diverse group of viruses isolated from more than 230 species of birds (10), and infecting only avian species, all avian poxviruses are grouped into the single Avipoxvirus genus of the Chordopoxvirinae sub-family (11), with Fowlpox virus (FWPV) as the type species. Like VACV, FWPV has been developed for use as a live recombinant vaccine vector, in both permissive (i.e. poultry) and non-permissive hosts (i.e. mammals, including humans, in which its replication is abortive) (12-18). Notably, a commercial FWPV recombinant vaccine (TROVACTM-H5) expressing the HA gene of H5N8 isolate A/turkey/Ireland/1378/83 has become the most extensively used live recombinant virus used in any sector, with some 2 billion doses used to counter highly pathogenic influenza H5N2 in Mexico up to 2005.
Another avipoxvirus, canarypox virus (CNPV), which is well diverged from FWPV (20), has been developed extensively for use in non-permissive mammalian hosts (21-23), with several licensed commercial vaccines available for diseases of livestock and companion animals. CNPV, as ALVACTM, was also the recombinant virus in the recent Thai HIV vaccine trial (RV144) that showed marginal indications of potential efficacy (24).

We show that, in chicken cell culture, FWPV fails to induce chicken IFN-2 (ChIFN2), believed to be the chicken equivalent of IFN-β (25, 26), and is able to block its induction by transfected poly(I:C), an analog of cytoplasmic double-strand (ds) RNA. We have used a broad-scale genetic ‘loss-of-function’ screen, involving a library of 48 FWPV in vitro-generated mutants, each defective in a single, non-essential gene, to identify a gene involved in blocking induction of the ChIFN2 promoter mediated by the dsRNA mimic, poly(I:C). The screen identified a member of a poxvirus gene family that is far more extensive in avipoxviruses than in mammalian poxviruses and has not been previously associated with IFN-modulation.

MATERIALS AND METHODS

Cells and viruses

Primary chicken embryo fibroblast cells (CEF), produced from specific pathogen free (SPF)-quality embryos (10-day old) at the Institute for Animal Health (Compton, Berkshire, U.K.) were grown in 199 medium supplemented with 10% tryptone phosphate broth (TPB), 10% newborn bovine serum, nystatin and
penicillin/streptomycin. DF-1 cells (27) obtained from the American Tissue Culture Collection (ATCC), were maintained in DMEM supplemented with 10% Foetal Bovine Serum (Autogen Bioclear) and penicillin/streptomycin.

Attenuated FWPV strain FP9, used throughout this study, as well as its pathogenic European progenitor, HP1, have been described previously (28). Infectious bursal disease virus strain PBG98, a ‘mild’ vaccine strain from laboratory stocks, was propagated in CEF (29).

Plasmids

The ChIFN2 promoter reporter (pChIFN2lucter (30)) and the constitutive β-galactosidase reporter plasmid (pJATlacZ (31)) have been previously described. Expression plasmids were constructed by cloning FWPV or CNPV genes into pEFPlink2 or pEFPlink2Flag (32). Sequences were amplified by PCR using oligonucleotides (Table 1) containing BamHI and SpeI (NcoI in the case of fpv213) sites and cloned into pEFPlink2 or pEFPlink2Flag restricted with BamHI and SpeI (NcoI in the case of fpv213). Plasmids were sequenced to ensure no errors had been introduced and reading frame was intact.

Transfection of cells with poly(I:C) and assay of luciferase reporters

Chicken DF-1 cells in 12-well plates were transfected with the ChIFN2 promoter reporter pChIFN2lucter (167ng) and the constitutive reporter plasmid pJATlacZ (167ng), sometimes additionally with an expression plasmid driving the overexpression of viral proteins or the control empty vector pEFPlink2 (250ng). Following recovery for 24 hours, cells were either left uninfected or infected
with infectious bursal disease virus (IBDV) attenuated vaccine PBG98,
pathogenic FWPV HP1, parental or mutant attenuated FWPV FP9 (MOI 10).
Following infection for 4 hours, cells, when appropriate, were transfected with
poly(I:C) (10µg/ml) using Polyfect (Qiagen), as described by Childs et al. (30),
and incubated for 16 hours. Luciferase assays were carried out and data were
normalized using β-galactosidase measurements.

β-galactosidase assay
Cell lysate β-galactosidase concentrations were measured by incubation of 10µl
of cell lysate with ortho-nitrophenyl-β-galactoside (50µl of 0.5 mg ml⁻¹ diluted in
60mM Na₂HPO₄.7H₂O, 40mM Na₂H₂PO₄.H₂O, 10mM KCl, 1mM MgSO₄.7H₂O,
2.7ml l⁻¹ β-mercaptoethanol). The reaction was incubated at 37ºC until a yellow
coloration had appeared when A₄20 was measured using a spectrophotometer.

Analysis of gene expression by ribonuclease protection assay
Total RNA was prepared from 9cm diameter dishes of confluent cells, treated as
described above, using the acid phenol method and analyzed by ribonuclease
(RNase) protection using the method of Zinn et al. (33) as described previously
(34). To generate a probe against chicken β-actin (Ch-Act-β), a 158 bp fragment
of CEF genomic DNA was amplified by PCR using the primers 5’-
CCCATGGATGATATTGCTGCGC-3’ and 5’-
TAATACGACTCACTATAGCTGATGCTGGGCCACCCAGCA-3’; the underlined
region contains the T7 promoter allowing generation of RNA probe from the PCR
product by transcription with T7 RNA polymerase. To generate a probe for
ChIFN2, a PCR product (amplified using the primers 5’-

Laidlaw et al.
CCCAGATCTCCTCCAGTACAGCCACCACATGGT-3’ and 5’-
CCCTCTAGACAGTCACTGGGTGTTGAGAC-3’) was cloned into pCRIIBlunt.Topo (Invitrogen). Orientation of the PCR product within pCRII.Blunt.Topo was deduced by restriction mapping, it was linearized with the restriction enzyme EarI and transcribed using T7 polymerase to generate a 357bp RNA probe complementary to cellular mRNA.

**Construction of insertion knock-out mutant FWPV.**

Generation of a FWPV knockout library by insertion of the *Escherichia coli* xanthine-guanine phosphoribosyl-transferase (gpt) gene was carried out by PCR overlap extension (PCR-OE). To construct the knock-out FWPV, PCR-OE (using high fidelity Taq polymerase) was used to assemble linear recombination templates from three constituent parts: approximately 350 bp PCR fragments of the FWPV genome from either side, (i) and (ii), of the center of the target gene, disrupted in the middle by (iii) a VACV p7.5 promoter upstream of the gpt gene. For each gene, fragment (i) was amplified by primers 1 and 2, fragment (ii) by primers 5 and 6 and fragment (iii) by primers 3 and 4. Primers 2 and 3, as well as 4 and 5, had 20 bases of complementary sequence, half from the target gene sequence and half from the p7.5 gpt cassette. Details of primers used for generation of the library (data not shown) are available upon request. Following the first round of PCR, products were purified using a Qiaquick PCR purification kit (Qiagen) and combined into a PCR-OE reaction in order to amplify a product consisting of the complete gene of interest with gpt inserted into the centre of the gene. The resulting PCR product was then transfected into FWPV FP9-infected CEF and recombinant viruses were selected for the gpt gene with fresh
media containing mycophenolic acid (25µg ml⁻¹), xanthine (250µg ml⁻¹) and hypoxanthine (15µg ml⁻¹) (MXH). Recovered viruses were bulk passaged three times in CEF in MXH then plaque purified thrice. Viral genomic DNA was then extracted and analysed by PCR to confirm disruption of the target gene and loss of parental virus.

**Construction of deletion knock-out mutant FWPV.**

An fpv012 deletion mutant, FP9Δ012TD (trans dominant), was generated independently of the FWPV FP9 knockout mutant library by the transient dominant selection (TDS) method of Falkner (35), as described previously (36). Briefly, two 0.6kbp regions of the FP9 genome, comprising (i) 500bp upstream of fpv012 plus 100bp from the 5’ end of the ORF and (ii) 100bp from the 3’ end of fpv012 plus 500bp downstream, were amplified by PCR using oligonucleotides 5’-ATCGGGATCCCTTTAGTATTAGTTAAACCCGG and 5’-CATTCTGTATTTAACGATGGAATCTACGTTCGGTGTATTAGGATTTACACC for amplicon (i) and 5’-CCTAATACACCGAACGTAGATTCCATCGTTAAATACAGAATGGTGTTTACTTCC and 5’-ATCGGACGTCCTTAGCAGTGCAGAAGAATTTATC for amplicon (ii). The two amplicons were joined by PCR-OE (deleting about 800 bp from the fpv012 ORF), digested with BamHI and AatII then ligated into pGNR (36) to give pGNRdel012. CEF were infected with FP9 (MOI 0.1) and then transfected with pGNRdel012 using Lipofectin (Life Technologies), according to the manufacturer’s instructions. Twenty-four hours following transfection the media was replaced with fresh media containing MXH then infection was allowed to proceed for a further 72 hours. Progeny virus was harvested and plaque purified thrice on
CEFs in the presence of MXH and then resolution was accomplished by plaque purification in the absence of MXH. The resolved viruses were tested for loss of resistance to mycophenolic acid and the genotype of these gpt- viruses were established by PCR (using internal and flanking primers) following DNA isolation from infected cells using Wizard SV genomic DNA purification (Promega). In this way, FP9Δ012TD was isolated.

Construction of knock-in mutant and revertant FWPV.

To construct knock-in viruses expressing TAP-tagged fpv012, transient dominant (TD) plasmid pUC13-FL012TAP was constructed in two steps. Firstly, the 400bp 3’ flanking sequence from fpv012 was amplified by PCR and cloned into the poxvirus transient dominant TAP vector pUC13TAP (from Prof. G. Smith), such that the 3’ flanking sequence was downstream of the TAP tag, generating the intermediate plasmid pUC13TAP012-3’. Subsequently the open reading frame (ORF), minus the stop codon, and 400 bp of upstream sequence was amplified by PCR and cloned into pUC13TAP012-3’ so that the virus ORF was fused in-frame with the TAP tag. Constructs were sequenced to ensure no errors had been introduced. Recombinant TAP-tagged fpv012 virus was generated by transfection of the construct into CEFs infected with FP9Δ012TD. The knock-in mutants were then isolated by the TDS method, as described above.

The TDS method described above was also used to construct a revertant virus from mutant FP9Δ012TD. The fpv012 ORF was amplified, with 500 bp flanking regions, using primers 5’-ATCGGGATCCCTTTAGTATTAGTTATTAAACCGG and
5'-ATCGGACGTCCTTAGCAGTGCAGAAGAATTTATC, digested with *Bam*HI and *Aat*II then ligated into pGNR to generate pGNR012REV. This was used to generate revertant virus, 012REV, with a reconstituted *fpv012* locus by the TDS method described above.

**RNA extraction and processing of samples**

RNA was extracted from cells using an RNeasy kit (Qiagen) according to the manufacturer's instructions. On-column DNA digestion was performed using RNase-free DNase (Qiagen) to remove contaminating genomic DNA. RNA samples were quantified using a Nanodrop Spectrophotometer (Thermo Scientific) and checked for quality using a 2100 Bioanalyzer (Agilent Technologies). All RNA samples had an RNA integrity number (RIN) ≥ 9.6.

**Reverse transcription (RT) PCR and quantitative real-time RT PCR**

qRT-PCR was performed using MESA GREEN qPCR MasterMix Plus for SYBR® Assay I dTTP (Eurogentec) according to the manufacturer's instructions. A final volume of 10μl per reaction was used, with 1μl cDNA diluted 1:10 in nuclease-free H₂O as a template. Primers (Table 2) were used at a final concentration of 300nM. qPCR was performed on an ABI-7900HT Fast Real-Time PCR System (Applied Biosystems) using the following programme: 95°C for 5 minutes; 40 cycles of 95°C for 15 seconds, 57°C for 20 seconds, 72°C for 20 seconds; 95°C for 15 seconds; and 60°C for 15 seconds.

The qRT-PCR primer pairs (Table 2) were validated by generating standard curves using PCR products corresponding to each gene. A 10-fold dilution series was made for each PCR product and 1 μl was used with the MESA green qPCR.
mastermix. Ct values were analysed using SDS2.3 (Applied Biosystems). The slopes of the standard curves were used to identify the amplification efficiencies (E) of the qRT-PCR primer pairs, using the equation $E = 10^{(-1/slope)} - 1$. Only qRT-PCR primer pairs with efficiencies of 90-110% were used further. The linear correlation coefficient (R²) was used to assess the linearity of the standard curve. Standard curves with an R² value of >0.985 were used.

Data were analysed using SDS 2.3 and RQ Manager 1.2 software (Applied Biosystems). All target gene expression levels were calculated relative to expression levels of the reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which were shown to remain constant over 24 h in uninfected and FP9-infected cells, and the target gene expression level in control CEF, using the comparative $C_T$ method (also referred to as the $2^{-\Delta\Delta C_T}$ method).

**RESULTS**

**Fowlpox virus fails to induce ChIFN2 in chicken DF-1 cells.**

Infection of immortalized chicken fibroblast DF-1 cells with FWPV FP9 led to no detectable induction in the expression of ChIFN2 mRNA, as determined by RNase protection assay. In contrast, infection with the chicken birnavirus, infectious bursal disease virus (IBDV) attenuated vaccine strain PBG98 (29) showed clear induction of ChIFN2 (Figure 1A).
To test whether the failure of FWPV to induce ChIFN2 was reflected by a failure to activate the ChIFN2 promoter, the effects of infection on expression of a luciferase reporter gene under the control of the ChIFN2 promoter (30), transfected into chicken DF-1 cells, was examined. Compared to poly(I:C) transfection or IBDV infection, FWPV infection did not induce the ChIFN2 promoter (Figure 1B and 1D). This effect was seen whether the highly passaged and attenuated FP9 strain of FWPV or its pathogenic precursor strain (HP1) were used (Fig. 1B), despite the fact that the former has undergone numerous mutations including the loss of 22 kbp of genomic sequence (28).

**FWPV actively blocks dsRNA induction of the ChIFN2 promoter.** We next examined whether the failure of FWPV to activate the ChIFN2 promoter represented a failure to generate a PAMP or the production of an FWPV-encoded inhibitor of IFN induction. Fig. 1C and 1D show that FWPV-infected DF-1 cells exhibited a block to subsequent induction of the ChIFN2 promoter by transfected poly(I:C). In contrast, not only did the positive control IBDV PBG98 fail to block poly(I:C)-mediated induction of the promoter, it actually enhanced the induction. The FWPV-mediated block to induction of the ChIFN2 promoter upon virus infection alone or after subsequent poly(I:C) transfection was confirmed by qRT-PCR in parallel with luciferase reporter assays (Fig. 1 D and E). Infection of appropriate samples with FWPV was demonstrated by qRT-PCR for FWPV early and late genes (Fig. 1F). These data indicated that FWPV encodes an inhibitor of transfected poly(I:C)-mediated ChIFN2 induction.
Identification of a knockout mutant of FWPV partially defective in the ability to block induction of ChIFN2.

A loss-of-function approach was adopted to identify a FWPV gene(s) that blocked the induction of ChIFN2 by poly(I:C). To facilitate this, a panel of FWPV non-essential gene \textit{gpt}-insertion knockout mutants (48 single genes and one double gene; see Table 3), was screened by luciferase reporter assay for complete or partial loss of the ability of FP9 to block induction of ChIFN2 by transfected poly(I:C).

One \textit{gpt}-insertion knockout mutant consistently demonstrated higher levels of transfected poly(I:C)-induced luciferase activity compared to parental FP9 and to the other mutants (see Fig. 2a). This mutant, FP9\_012::\textit{gpt}, had a copy of the \textit{gpt} gene inserted in the centre of \textit{fpv012}. An independent deletion mutant, FP9\Delta012TD, generated using the transient dominant method (35, 36) displayed the same phenotype (Fig. 2b). Multi-step growth curves of the replication of parental FWPV FP9 and FP9\Delta012TD in CEF, infected at a multiplicity of infection (MOI) of 0.01, showed no significantly adverse affect of the \textit{fpv012} disruption on the production of intra- and extra-cellular virus up to 72 h.p.i. (Fig. 3).

Expression kinetics of \textit{fpv012}.

Analysis of the kinetics of expression of \textit{fpv012} by qRT-PCR revealed that it was expressed early during FWPV infection, but only weakly so (Fig. 4A). Levels of \textit{fpv012} RNA, relative to GAPDH, were below those observed for control genes \textit{fpv094} (an ortholog of VACV E9L encoding DNA polymerase) and \textit{fpv100} (an
ortholog of VACV E4L encoding RNA polymerase subunit RPO30). Both of the
VACV orthologs showed early expression kinetics (37).

As expected, no fpv012 expression was detected in cells infected with the fpv012
deletion mutant FP9Δ012TD (Fig. 4B) but it was evident in cells infected with the
revertant (Fig. 4C). Analysis of the same data plotted on a linear scale (data not
shown) revealed that in cells infected with the mutant, there appeared to be a
slight delay (1.5 to 2.5 h), relative to those infected with parental or revertant
viruses, in the kinetics of expression of FWPV genes that in this assay appear to
be expressed late (fpv165, fpv168 and fpv176), with half maximal expression at
12 rather than 10 hpi. Expression of the early genes (fpv094 and fpv100)
appeared to be delayed by 3 to 4 h in FP9Δ012TD (with half maximal expression
at 7 rather than 3 to 4 hpi).

Antibodies for fpv012 are unavailable but a recombinant ‘knock-in’ virus, with a
TAP-tagged version of fpv012 inserted into the native locus under control of the
resident promoter, was constructed, allowing demonstration of the expression of
the tagged protein by western blotting of lysates with anti-FLAG antibodies (Fig.
4D).

**Expression of ISGs is elevated in CEF infected with the fpv012 deletion mutant.**

Complex viruses often encode multiple antagonists of IFN-mediated responses,
targeting IFN induction, signalling and antiviral activities of IFN-stimulated
genomes (ISG). Releasing the viral block to induction of ChIFN2 might not,
therefore, lead to expression of ISG if robust blocks to IFN signaling remain intact. To investigate whether this might be the case for FWPV, CEF were infected with parental FP9 or the fpv012 deletion mutant and the induction, by virus infection alone, of selected ISGs was analysed by qRT-PCR (Table 2). Significant, moderate-level induction of Mx1 by infection with the fpv012 deletion mutant alone, and lower (but highly significant) level induction of ZC3HAV1, was observed (Fig. 5A) but no significant induction of mda-5/IFIH1 of ChIFN2 was observed. No significant induction, by virus infection alone, of any of the ISGs or ChIFN2 was observed for parental FP9. Expression of Mx1, but not ZC3HAV1, induced by the mutant alone appeared highest at 8hpi. The experiment (Fig. 5B) was therefore repeated to include an earlier time point (4hpi), additional ISGs (IFIT5 and ISG12-2) and the revertant virus. This confirmed that the fpv012 deletion mutant is phenotypically distinct from parental FP9 and the revertant in terms of ISG expression following virus infection alone (Fig. 5B). It is clear that Mx1 shows a biphasic response with an immediate peak of expression at 4hpi, a trough at 8 to 16hpi then increased expression at 24hpi. A similar profile is shared by IFIT5, though it showed much higher induction at 24hpi. Another ISG (ISG12-2) showed a different profile, with no immediate peak at 4hpi but increased expression at 16 and especially 24hpi. This profile actually resembles that for ChIFN2, which showed trace induction (2 fold) at 24hpi.

**Ectopic expression of fpv012 blocks induction of ChIFN2**

Ectopic expression of native or N-terminally FLAG-tagged fpv012, but not of control fpv155 (N1R/p28-like) nor fpv213, following transfection of the
pEFPlnk2-derived expression plasmid, blocked poly(I:C)-mediated induction of
the ChIFN2 promoter in DF-1 cells when expressed in the absence of other FWPV
proteins (Fig. 6B), offering the opportunity for ready analysis of its activity.

**IFN modulator fpv012 is a member of the ANK/PRANC poxvirus gene family.**

The IFN modulator identified by this study, fpv012, is encoded by a member of
the largest gene family in poxviruses (Fig. 6A), representing those proteins
containing multiple copies of the ankyrin repeat (ANK; INTERPRO IPR002110).
The majority of poxviral ANK proteins, including fpv012, appear to be of a
particular type, ANK/PRANC, with an N-terminal domain (INTERPRO
IPR020683) containing multiple ANKs (4 in the case of fpv012) and a C-terminal
F-box like motif in what has been described by Mercer and colleagues (38) as a
PRANC domain (Pox protein Repeats of ANkyrin – C terminal; INTERPRO
IPR018272). Deletion analysis was therefore used to define whether both of
these domains were required for activity.

**Inhibition of the ChIFN2 promoter is not dependent on fpv012 ankyrin repeat 1.**

In an attempt to analyze the relative importance of the ANK domain and the C-
terminal F-box like motif in the ability of fpv012 to inhibit the induction of
ChIFN2 by poly(I:C), a panel of fpv012 variants with domain deletions (Fig. 6B)
was generated and examined in transient transfections. Unfortunately, the levels
of expression of wild-type and mutant fpv012 from the constructs were too low
to allow investigation of expression and/or stability of the mutated proteins by
western blot analysis using anti-FLAG antibody. Nevertheless, the first ANK
appears to be dispensable for the inhibitory activity of fpv012, with Mut1
displaying as much inhibitory activity as full-length or N-terminally Flag-tagged
fpv012 (Fig. 6B). Removal of additional ANKs from the N-terminus (in Mut2 and
Mut3) or deletion of just 13 amino acids from the C-terminus (Mut4),
downstream of the acknowledged F-box motif (38), completely abrogated the
inhibitory effect (Fig. 6B) but we cannot eliminate the possibility that the loss of
inhibitory activity is due to reduced stability of the truncated proteins.

cnpv030, the canarypox virus ortholog of fpv012, also inhibits poly(I:C)
induction of the ChIFN2 promoter.

Although both CNPV and FWPV are members of the Avipoxvirus genus, they are
considerably diverged, they are found in different major clades of the genus (39)
and they display significant differences in gene complement (20, 40).
Comparisons between ANK proteins can be problematic but extensive
phylogenetic analysis (41) revealed that fpv012 is most similar (45% amino acid
identity) to an ANK protein, cnpv030, from CNPV. Genes fpv012 and cnpv030
appear to be in relatively syntenic, yet diverged, locations within their respective
genomes (Fig. 7), in that cnpv030 is flanked by orthologs of fpv011 (cnpv025) and
fpv016 (cnpv032). Moreover, ectopic expression of cnpv030 appeared to be as
effective as fpv012 at blocking the transfected poly(I:C)-mediated induction of
the ChIFN2 promoter (Fig. 6B), indicating that the possession of genes for
modulating the induction of the avian equivalent of IFN-β is probably conserved
across the avipoxvirus genus.
Although poxviruses have dsDNA genomes, they have long been known to produce dsRNA by production of convergent, heterogeneous length, complimentary late transcripts (42). Indeed a VACV mutant temperature sensitive in transcription factor A18 produces more dsRNA, which leads to increased activation of 2’, 5’-oligoadenylate synthetase (OAS) (43). VACV E3 has long been known to play a major role in resisting the antiviral effects of IFN, sequestering dsRNA and thereby preventing allosteric activation of the dsRNA-dependent protein kinase, PKR, and OAS (44). More recently, E3 was also shown to inhibit activation of IRF3 (45), mediated via PKR and IPS-1 (46), thereby interfering with induction of IFN-β. Although avipoxviruses lack an ortholog of E3, parental FP9 is able to block transfected poly(I:C)-mediated induction of ChIFN2 (Fig. 1) so we sought to identify proteins involved.

The genetic strategy employed identified fpv012*, a member of a large avipoxvirus gene family encoding ANK proteins, most of which have C-terminal F-box motifs in so called PRANC domains. Extensive passage (more than 430 times) of pathogenic FWPV HP1 through CEF culture by Anton Mayr, with concomitant attenuation (47), led to the loss or disruption in FP9 of 12 of the 31 FWPV ANK genes, but fpv012 was not affected (28).

* FWPV gene names are italicized, the equivalent protein names are not.
Many studies with mammalian poxviruses have demonstrated the role of multiple viral modulators in controlling host IFN responses at various levels. Data shown in Fig. 2 indicate that fpv012 is responsible for blocking about 20% of expression from the ChIFN2 promoter induced by transfected poly(I:C), suggesting that the deletion mutant still has another mechanism(s) to control induction of ChIFN2. The additional mechanisms for controlling ChIFN2 induction appear to be sufficient, even in the absence of fpv012, to control the bulk of the residual ChIFN2 induction stimulated by virus infection as infection alone by the fpv012 deletion mutant virus stimulated only trace increase (not more than 2 fold at 24hpi) induction of ChIFN2 over that observed for FP9 infection alone (Fig. 5). Nevertheless, infection alone with the fpv012 deletion mutant, but not with FP9, led to significant, moderate-level induction of the ISGs Mx1, ISG12-2 and especially IFIT5 and lower level (but highly significant) induction of ZC3HAV1 (Fig. 5A and B). Several studies have described induction of such IFN-induced downstream effectors by avian viruses in avian systems (48, 49). Increased expression of the effectors in cells infected by the fpv012 deletion virus might be attributable to the induced trace level expression of ChIFN2. We cannot exclude the possible involvement of alternative, IFN-independent pathways in induction of the ISG, especially given the different kinetic expression profiles observed for the various ISGs, although it should be stressed that any such pathways are also clearly normally blocked by fpv012. Clarifying this issue will need extensive study of the chicken innate responses, which remain relatively poorly characterized, but fpv012 should prove a useful tool in those studies.
Despite moderate level induction of ISGs, and some delay in expression of viral genes, the fpv012 deletion virus did not appear to be significantly compromised in its replicative capacity in tissue culture (Fig. 3). This indicates that virus mechanisms for subverting the antiviral effectors must exist in FWPV and must remain intact in the fpv012 deletion mutant. We have identified fpv014 as a contributor to chicken type I IFN resistance in VACV MVA/FWPV chimaeras (see accompanying manuscript by Buttigieg et al.; JVI02738-12) but, based on the VACV paradigm, it is likely that more genes will be involved.

Ectopic expression of fpv012 leads to inhibition of transfected poly(I:C)-mediated induction of the ChIFN2 promoter (Fig. 6). This phenotype offered the ready opportunity to analyse the viral determinants of modulation. Unfortunately, the levels of expression of wild-type and mutant fpv012 from the constructs were too low to allow investigation of expression and/or stability of the mutated proteins by western blot analysis using anti-FLAG antibody. Nevertheless, the data shown in Fig. 6 demonstrate that the first ANK is dispensable for the inhibitory activity of fpv012. Although removal of further ANKs or just 12 residues from the C-terminus of fpv012 disrupted its function when expressed ectopically (Fig. 6B), we were not able to confirm stability of the truncated protein so detailed mutagenic analysis of fpv012 must await an extensive study using expression of mutated FLAG-tagged proteins expressed from knock-in virus mutants.

In an accompanying manuscript (Buttigieg et al.; JVI02378-12), we demonstrated interaction between the fpv014 ANK/PRANC protein involved in ChIFN1 (chicken IFN-α) resistance and proteins of the SCF (Skp-1, Cullin-1, F-box) ubiquitin ligase complex, as has been observed for a number of mammalian
poxvirus ANK proteins (50-54). It is postulated (38) that such interactions allow ANK/PRANC proteins to act as adapters, targeting for ubiquitination (and probable proteasomal degradation) ligands captured by the N-terminal ANK domains. Despite considerable effort, probably because of the low levels of expression of \textit{fpv012} in the various systems, we have so far been unable to identify interaction of \textit{fpv012} with the SCF complex. We have also been unable to identify any cellular proteins captured by the N-terminal ANK domain of \textit{fpv012}, but ligands for this family of proteins have proved generally elusive. Thus far, the only ligands identified are Akt, for myxoma virus MT-5 (55), and NF-κB, for variola virus G1 (56) and its cowpox virus (CPXV) ortholog CPXV006 (57), as well as for CPXV CP77, encoded by \textit{CPXV025} (51).

It is not clear why so many ANK proteins are encoded by the avipoxviruses. The difference in numbers of ANK protein genes carried by relatively closely related viruses (\textit{e.g.} 31 in FWPV and 51 in CNPV) suggests that gene expansion involves duplication and subsequent evolution. Such expansion, driven by IFN, has recently been demonstrated (using deep sequencing) for VACV in cell culture (58). In a genetic background defective for E3L, the copy number of the K3L gene was observed to expand allowing it to mutate and evolve so that it was more effective at inhibiting PKR. The authors coined the term “viral gene-accordions” for this mechanism, as selection of an advantageous allele allowed subsequent contraction in copy number (58). They also recognised that the expansion of the ANK genes in avipoxviruses is a “particularly clear example of such adaptive gene expansions” and, moreover, that it represents “an exceptional example” whereby multiple functional variants have been generated so that the accordion...
has not collapsed to a single copy. Our demonstration that fpv012 and cnpv030, which are an orthologous pair (41), share the ability to block induction of avian IFN-β provides a strong indication that ANK gene expansion in the avipoxviruses pre-dates speciation of FWPV & CNPV, particularly as fpv012 and cnpv030 are far from basal to the avipoxvirus ANK phylogenetic tree (see supplemental figure of Sonnberg et al. (41)).

Expansion of such a large complement of related genes to the extent seen for this “viral gene accordion” might initially have facilitated high-level expression of inhibitors of a limited number of targets by a gene dosage effect (58). Subsequent selection and evolution might have allowed the targeting of a limited number of host factors in a redundant manner, as seen for binding of NF-κB by both CPXV006 and CPXV025 (51, 57). Ultimately, the accordion might have become capable of targeting multiple, distinct, cellular proteins. Our demonstration of different functions for two avipoxvirus ANK proteins (fpv012 here and fpv014 in the accompanying manuscript Buttigieg et al.; JVI02378-12), both of which fall in the same larger phylogenetic cluster of avipoxvirus ANK genes (see supplemental figure of Sonnberg et al. (41)), hints at a complex and highly dynamic evolutionary picture. There is clearly wide scope for evolutionary selection of functions for the avipoxvirus ANK genes - it is notable that avipoxviruses lack equivalents of mammalian poxvirus genes encoding a family of related proteins with structural homology to cellular Bcl-2 (note that fpv039, an antiapoptotic, Bcl-2-like FWPV protein (59), appears to be unrelated (60)). In VACV, these proteins (A46, A52, B14, C1, C6, C16/B22, K7 and N1 (60-66)) target host factors involved in immunomodulation and the control of apoptosis. It is
likely that these genes represent another “viral gene-accordion”. It will be interesting to discover whether the avipoxvirus ANK protein family has evolved to recognize a range of targets similar to that of the VACV Bcl-2-like proteins.

ACKNOWLEDGEMENTS

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ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific

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immunization with DNA, recombinant fowlpox virus and VLP(SHIV) elicit
both neutralizing antibodies and IFNgamma-producing T cells against the
HIV-envelope protein in mice that control env-bearing tumour cells.
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vaccines against liver-stage P. falciparum malaria in non-immune
volunteers. Vaccine. **24**:3026-3034.

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FIGURE LEGENDS

Figure 1. Induction of the ChIFN2 promoter upon transfection with poly(I:C) and/or infection with viruses. (A) RNase protection analysis of expression of ChIFN2 in DF-1 cells upon virus infection. DF-1 cells, mock treated or infected with virus (FWPV FP9 or IBDV strain PBG98) for the times stated, were analysed for expression of ChIFN2 by RNase protection using 10 μg RNA. The levels of control Chβ-actin are also shown. (B to D) Luciferase reporter analysis of expression of ChIFN2 in DF-1 cells. DF-1 cells were transfected with the ChIFN2 promoter luciferase reporter plasmid (pChIFN2lucter) and plasmid pJATlacZ, constitutively expressing β-galactosidase from the rat β-actin promoter (31). Following recovery for 24 hours cells were either left uninfected or infected (at an MOI of 10) with poxviruses (attenuated FWPV FP9 or its progenitor HP1) or attenuated IBDV vaccine strain PBG98. Following infection for four hours, cells were either left untreated or transfected with poly(I:C) (10 μg ml⁻¹) and incubated for 16 hours. Luciferase expression values were normalised to those of β-galactosidase. ChIFN2 expression levels were compared to the uninduced control to calculate the fold induction. (B) Induction of the ChIFN2 promoter by transfected poly(I:C), as a positive control, or by infection alone. (C) Modulation of induction of the ChIFN2 promoter, mediated by transfected poly(I:C), following infection with FWPV (FP9 or HP1) or IBDV PBG98. (D and E) Modulation of induction of the ChIFN2 promoter, mediated by virus infection or by transfected poly(I:C), following infection with FWPV FP9 or IBDV PBG98. Samples from the same experiment were split for luciferase assay (D) and for qRT-PCR (E). ChIFN2 expression levels were calculated relative to
GAPDH and the untreated control. (F) The same samples as in (D) and (E) were tested for expression of early (fpv094) and late (fpv176) genes by qRT-PCR. Their expression was normalized against GAPDH and is presented relative to that in the FP9 sample.

Figure 2. Screening FWPV FP9 mutants for reduced ability to block poly(I:C)-mediated induction of the ChIFN2 promoter. (A) Chicken DF1 cells were transfected with the ChIFN2 promoter reporter (pChIFN2luc)er and the constitutive LacZ reporter plasmid pJATlacZ. Following recovery for 24 hours, cells were either left uninfected or infected with parental FWPV FP9 or single-gene mutants of FP9, at an MOI of 10. Following infection for 4 hours, cells were either left untreated or were transfected with poly(I:C) (10µg ml⁻¹) and incubated for 16 hours. Luciferase assays were carried out and data were normalised using β-galactosidase measurements. Each sample was compared to the uninfected, poly(I:C)-treated control to calculate percent induction. Results show the mean (n=3) ± SD. (A) The results of an experiment screening 15 single-gene insertion mutants. (B) Blocking of induction of the ChIFN2 promoter by two independently isolated fpv012 mutants, created by two different methods (gpt selection cassette insertion, FP9_012::gpt, or trans-dominant selection-mediated deletion, FP9Δ012TD), is compared with that observed for parental FP9.

Figure 3. Multi-step growth kinetics of parental FWPV FP9 and transient dominant selection deletion mutant FP9Δ012TD. Chick embryo fibroblasts (CEF) were infected with FP9 or FP9Δ012TD at an MOI of 0.01. Extracellular (supernatant) and intracellular (cells) samples were harvested at 2, 10, 24, 48
and 72 hours post infection. Samples were freeze thawed three times and virus titre determined in triplicate using plaque assay.

**Figure 4. qRT-PCR analysis of FWPV gene expression by parental, 012 mutant and revertant viruses.** Expression of mRNA specific for fpv012 was assayed by qRT-PCR, using as controls FWPV genes: fpv094 (ortholog of VACV E9L; DNA polymerase), fpv100 (ortholog of VACV E4L; RNA polymerase subunit RPO30), fpv165 (ortholog of VACV A2L; VLTF-3 late transcription factor), fpv168 (39K core protein (67, 68); ortholog of VACV A4L) and fpv176 (ortholog of VACV A12L). Expression was standardized against that for ChGAPDH. Graphs show the results for parental FP9 (A), FP9Δ012TD (B) and revertant (C) viruses from two experiments (mean +/- SD). (D) Expression of TAP-tagged fpv012 (with TAP-tagged fpv014 as a control) inserted back into the native locus in FWPV FP9 under control of its cognate promoter, in infected CEF and DF-1 cells. TAP-tagged proteins were detected by immunoblotting of SDS polyacrylamide gels with anti-FLAG antibody (Sigma) and anti-mouse secondary antibody (LI-COR) as per the manufacturers’ protocols. The immunoblots were imaged using a LI-COR Odyssey infrared imaging system. Samples were obtained at 24 hpi (MOI=3).

**Figure 5. qRT-PCR analysis of IFNb and ISG mRNA expression after infection with parental, fpv012 deletion mutant or revertant FP9.** CEF were infected (MOI = 5) with parental FP9, Δ012TD or 012REV (B only) and harvested for RNA extraction at 0, 4 (B only), 8, 16 and 24 h post infection. Total RNA was extracted
from samples, cDNA was synthesised and qRT-PCR was performed in triplicate to quantify relative expression kinetics. Panels (A) and (B) show the results from 2 different experiments, using completely different batches of CEF. Bar graphs show the results from three experiments (mean +/- SD), each performed with a different batch of CEF. Asterisks indicate probability (2-way ANOVA followed by Bonferroni post-hoc test: * P < 0.05, ** P < 0.01, *** P < 0.001). Data were normalized against ChGAPDH and are presented relative to mock infected (A) or parental FP9-infected (B).

**Figure 6. Domain structure and deletion mutant analysis of fpv012.**

(A) Domain structure of fpv012 showing N-terminal ankyrin (ANK) repeats as well as the C-terminal F-box motif and the larger, encompassing PRANC domain. To scale. (B) Schematic illustrating the position of deletions of fpv012 in pEF012Flag mutants 1 to 4 (Mut1(Δaa1-42), Mut2(Δaa1-74), Mut3(Δaa1-108), Mut4 (Δaa319-331) with comparison of the ability of the FLAG-tagged parental (pEF012Flag) and mutant forms (pEF012FlagMut1 to 4) of fpv012 to block poly(I:C)-mediated induction of the ChIFN2 promoter. Empty pEFPlink2 vector, vector expressing native or FLAG-tagged fpv012 (pEF012 and pEF012FLAG, respectively) or vectors expressing other FWPV genes (fpv155 or fpv213; pEF155, pEF213, respectively) served as controls. The ability of the CNPV ortholog of fpv012, (CNPV030) expressed from pEFPlink2 (pEFCNPV030), to block the ChIFN2 promoter was also assayed.

**Figure 7. Comparative genomic organization of the fpv010 to fpv016 locus in FWPV and CNPV.** Genes conserved between FWPV and CNPV are shown as
filled boxes; non-conserved genes are shown as open blocks. The arrowhead at the end of each block shows the direction of transcription. Reading frames (RF) are indicated. Short and long dashed lines correlate the left and right ends of the genes, respectively, in the two avipoxviral genomes. To scale.
Table 1. Primers used for constructing expression clones.

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<th>Vector</th>
<th>Fwd/Rev</th>
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<tr>
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<td>Rev</td>
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<td>pEFPlink2</td>
<td>Fwd</td>
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<td>pEFPlink2</td>
<td>Rev</td>
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<td>Fwd</td>
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<td>Rev</td>
<td>GATGGATCGTTATCCTGTCAAGAGATAC</td>
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Table 2. Primers used to quantify gene expression in real-time qRT-PCR

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<th>Reverse PCR primer 5'-3'</th>
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<td>ChGAPDH</td>
<td>NM_204305.1</td>
<td>GGCACTGTCAAGGCTGAGAA</td>
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<td>ChIFN2</td>
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<td>CAAGGCAAGCGCTCCCAGAG</td>
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<td>ChIFIT5</td>
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<td>CCACCTCATCTAACAAACA</td>
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<td>AGTGTCCCTTACCTCC</td>
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Table 3. FWPV FP9 gene knockouts screened for loss of ability to block poly(I:C) induction of ChIFN2luc reporter

<table>
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<tr>
<th>Targeted FWPV FP9 gene (Gene prediction and/or VACV ortholog)</th>
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<tr>
<td>006 (C4/C10L)</td>
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<tr>
<td>010 (SERPIN)</td>
<td>092 (E11L)</td>
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<tr>
<td>011 (SNAP)</td>
<td>097 / 098 (VARV B22R)</td>
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<td>012 (ANK)</td>
<td>099 (VARV B22R)</td>
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<td>014 (ANK)</td>
<td>105 (F15L)</td>
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<td>016 (IFN-γ-binding protein)</td>
<td>107 (VARV B22R)</td>
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<td>017 (V Ig domain)</td>
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Gene assignments as described previously [28]. ‘K/O’, knockout.
% Poly(I:C)-induced ChIFN2luc expression relative to uninfected

A

Uninfected
Wild type FP9
FP9Δ012TD
FP9_012::gpt
FWPV gene mutated by gpt insertion

B

% Poly(I:C)-induced ChIFN2luc expression relative to uninfected

Virus
Uninfected
FP9
FP9_012::gpt
FP9Δ012TD
Number of plaques

Time post-infection (hours)

- FP9 supernatant
- FP9 cells
- FP9Δ012TD supernatant
- FP9Δ012TD cells
Gene expression compared to GAPDH

Time (hours post-infection)

D

<table>
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<th>CEF</th>
<th>DF1</th>
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<td>41 &gt; TAP-fpv012</td>
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</table>
αSNAP - blocks ChIFN2 induction
fpv011
fpv012
fpv013

fpv014 - ChIFN1 resistance
≡ cnpv019
fpv015
fpv016

Ig domain ChIFNγ binding

C4L/C10L-like ANK

Consensus

RF4
RF5
RF6

RF1
RF2

C-type lectin

fpv010 serpin

fpv013

C10

rfv022
cnv023

rfv025
cnv026

rfv027
cnv028

rfv029
cnv030

cnv031

C-type lectin

C4L/C10L-like