Differences in Interferon Sensitivity and Biological Properties of Two Related Isolates of Simian Virus 5: A Model for Virus Persistence

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INTRODUCTION

The ability to produce and respond to interferon (IFN) is central to the immune control of virus infections in vivo. However, this is not an all or nothing response as viruses have evolved a great variety of strategies to circumvent the antiviral responses induced by IFNs. Thus, viruses may inhibit the production of IFN, block inter- and intra-cellular IFN signalling, or block the action of IFN-induced proteins, such as PKR or oligo(A) synthetase, with antiviral activity (Goodbourn et al., 2000; Levy and Garcia-Sastre, 2001). The biological activities of IFNs are initiated by binding to their cognate receptors, which leads to the activation of receptor-associated tyrosine kinase. In the case of type I IFNs (IFN-α/β), the activated kinases phosphorylate STAT1 and STAT2, which as a consequence form heterodimers that enter the nucleus and associate with p48 to form the ISGF3 complex that activates the transcription of type I IFN-responsive genes. In an analogous but distinct pathway, following the binding of type II IFN (IFN-γ) to its cognate receptor, the associated tyrosine kinases phosphorylate STAT1 (but not STAT2), which forms homodimers, termed GAF complexes, that activate type II IFN-responsive genes (for reviews see Stark et al., 1998; Goodbourn et al., 2000; Levy and Garcia-Sastre, 2001). A common strategy employed by many paramyxoviruses to circumvent the IFN response is to block the IFN-induced signal transduction pathways within infected cells, although they achieve this by distinct molecular mechanisms (Didcock et al., 1999a,b; Garcin et al., 2000; Gotoh et al., 1999; Kato et al., 2001; Parisien et al., 2001; Young et al., 2000; Kubota et al., 2001; Takeuchi et al., 2001; Komatsu et al., 2000). For example, simian virus 5 (SV5) blocks IFN signalling by targeting STAT1 for degradation (Didcock et al., 1999b), whilst human parainfluenza virus type 2 targets STAT2 for degradation (Young et al., 2000; Parisien et al., 2001). In contrast, although both Sendai virus and human parainfluenza virus type 3 block type I and type II IFN signalling they achieve this by methods that do not necessarily require the degradation of either STAT1 or STAT2 (Young et al., 2000; Garcin et al., 2000; Takeuchi et al., 2001).

The genome of SV5, a prototype species of the rubulavirus subfamily, contains seven genes: the nucleoprotein (NP), V/phosphoprotein (V/P), matrix (M), fusion (F), small hydrophobic (SH), haemagglutinin–neuraminidase (HN), and large (L) genes (for reviews on the molecular biology of Paramyxoviruses see Lamb and Kolakofsky, 2001). The V/P gene codes for two distinct proteins, V and P, which have identical amino-terminal domains but different carboxy-terminal domains. Co-transcriptional insertion of two nontemplated G residues after residue 551 in the P mRNA, but not in the V mRNA, shifts the reading frame, thereby accounting for the
unique C-terminus of the P and V proteins (Thomas et al., 1988). One of the major roles of the multifunctional V protein is to target STAT1 for degradation (Didcock et al., 1999b; Young et al., 2001). In addition, V binds the 127-kDa subunit of UV DNA damage binding protein (Lin et al., 1998), thereby delaying the cell cycle to the possible advantage of virus replication (Lin and Lamb, 2000). V also binds soluble, but not polymeric, NP and thus may have a role to play in the control of virus encapsidation (Randall and Bermingham, 1996).

SV5 has been isolated from a number of species, including man, monkey, and dog (for reviews see Hsiung, 1972; Randall and Russell, 1991; Southern et al., 1990). Furthermore, the ability of SV5 to block IFN signalling is also species-specific and indeed this may be one constraint which prevents viruses crossing species barriers (Didcock et al., 1999a). Thus, whilst SV5 blocks IFN signalling in monkey, human, and dog cells, natural isolates do not block IFN signalling in murine cells, and the virus is nonpathogenic in mice (Didcock et al., 1999a; Young et al., 2001). An isolate, termed SV5-mci2, was generated by continuous passage of SV5 in murine cells. This virus had mutations including an asparagine (N) to aspartic acid (D) substitution at position 100 in the V protein, which resulted in the ability of the virus to block IFN signalling in murine cells. Furthermore, a recombinant virus was constructed that differed from the parental W3 strain only by the N to D substitution in P/V, termed rSV5-V/P N100D, and that also blocked IFN signalling in murine cells (Young et al., 2001). The N to D substitution also abolished the binding of the SV5-P-k monoclonal antibody (mAb) to the P or V proteins of mci2 and rSV5-V/P N100D. Intriguingly, the same mAb was the only mAb, in a panel of over 50, that could distinguish between two related canine isolates of SV5, termed CPI′ and CPI″ (Southern et al., 1990).

The CPI″ strain of SV5 was isolated from the cerebrospinal fluid of a dog with temporary posterior paralysis and has been further studied as a neurotropic strain of SV5 (Evermann et al., 1980; Baumgärtner et al., 1981, 1982). The CPI′ strain of SV5 was isolated from a gnotobiotic dog experimentally infected with CPI′ 12 days p.i. (Baumgärtner et al., 1987). Characterisation of CPI″ and CPI′ revealed biological and phenotypic differences between these two isolates. Thus, for example, CPI″ is attenuated in ferrets compared to CPI′ (Baumgärtner et al., 1991) and in tissue culture cells more readily establishes persistent infections (Baumgärtner et al., 1987). Here we demonstrate that whilst CPI″ degrades STAT1 and blocks IFN signalling in human and canine cells, CPI′ does not. We discuss how CPI″ may have been selected in vivo and present a general model of how paramyxoviruses may establish persistent infections in vivo.

**RESULTS**

CPI″ fails to degrade STAT1 and block IFN signalling

We have previously shown that a single N to D substitution at position 100 in the V protein of the W3 strain of SV5 differentiates its ability to block IFN signalling in human and murine cells (Young et al., 2001); since this amino acid substitution mapped to the binding site of the mAb SV5-Pk that distinguished between the CPI″ and CPI′ strains of SV5 (Southern et al., 1990), we examined the ability of CPI″ and CPI′ to degrade STAT1 in human, canine, and murine cells. CPI″ behaved exactly like the W3 strain, inducing the degradation of STAT1 in human and canine cells but not in murine cells (Fig. 1, left). In striking contrast, CPI″ failed to target STAT1 for degradation in human, dog, or murine cells.

Comparative sequence analysis of the V genes of W3, CPI′, and CPI″ showed that there are three amino acid differences between the V proteins of W3 and CPI″ that all reside in the P/V N-terminal common domain; CPI′ has three additional amino acid substitutions, also all
Relative contribution of the three amino acid substitutions in the N-terminal domain of the V protein of CPI<sup>−</sup> to loss of function

To compare the relative contributions of the three amino acid substitutions in the failure of CPI<sup>−</sup> to block IFN signalling, the CPI<sup>−</sup> mutations were reintroduced individually or in pairwise combinations into the V gene of CPI<sup>+</sup> by site-directed mutagenesis, generating the plasmids pEFW3/CPI<sup>−</sup>/V-(Y26H), -(L50P), -(L102P), -(Y26H/L50P), -(L50P/L102P), and -(Y26H/L102P). V proteins expressed from the resulting plasmids were tested for their ability to block IFN signalling in the type I IFN luciferase reporter assays using 2fTGH cells. Of the single amino acid changes, only the L to P mutation at position 50 had any effect on V function (Fig. 3). However, this single amino acid substitution only partially abolished the ability of V to block IFN signalling. When placed in combination with second amino acid changes at either position 26 or position 102, the block to signalling was further impaired, but still not as disabled as that seen for pEFW3/CPI<sup>−</sup>/V, suggesting that all three amino acid changes are required to abolish IFN signalling in the context of the CPI<sup>−</sup> V protein. Interestingly, when the amino acids 26 and 102 in CPI<sup>−</sup> were changed to CPI<sup>+</sup>.

Located within the N-terminal V/P common domain (tyrosine to histidine at residue 26 and leucine to proline at residues 50 and 102; Fig. 2). In order to confirm that the amino acid differences in the V proteins of CPI<sup>+</sup> and CPI<sup>−</sup> were responsible for their differing abilities to block IFN signalling, the appropriate regions containing the mutations of V/P genes were cloned into the W3 backbone of V by generating the plasmids pEFW3/CPI<sup>−</sup>/V and pEFW3/CPI<sup>+</sup>/V, using the strategy outlined in Fig. 2. Human (2fTGH), canine (MDCK), and murine (BF) cells were cotransfected with the appropriate plasmids together with a type I IFN-responsive (ISRE) luciferase reporter plasmid. At 44 h posttransfection, the cells were induced with type I IFN and lysed 4 h later and the relative luciferase activities determined. These results clearly demonstrated that the V protein of CPI<sup>−</sup> blocks IFN signalling in human and canine cells, but not in murine cells. However, CPI<sup>−</sup> V failed to block IFN signalling in any of the cells (Fig. 1, right).

![FIG. 2.](image)

![FIG. 3.](image)
[i.e., -Y26H/L102P], which is the same thing as changing the amino acid at position 50 in CPI\(^+\) (P) to CPI\(^+\) (L)]. The resultant V protein was able to efficiently block IFN signalling. Similar results were obtained using MDCK cells (data not shown), indicating that the various mutations did not have clear differential effects in human and canine cells.

**Relative rates of virus protein synthesis in canine cells infected with CPI\(^+\) and CPI\(^-\)**

Given that CPI\(^+\) blocks IFN signalling but CPI\(^-\) fails to, it was of interest to compare the efficiency of replication of CPI\(^+\) and CPI\(^-\) in cells that produce and respond to IFN. Canine (MDCK) cells were infected with CPI\(^+\) or CPI\(^-\) at a high m.o.i. and metabolically labelled with \([^{35}S]\)methionine at 10–12, 16–18, 22–24, and 46–48 h p.i. The cells were then lysed and HN, NP, F, P, and M were immune precipitated with a pool of mAbs specific for these proteins. The proteins in the total cell extracts (Fig. 4a) and the immune precipitates (Fig. 4b) were subsequently analysed by SDS–PAGE and the profiles visualised by phosphoimager analysis. There was a small but significant increase in the amount of virus protein synthesis in cells infected with CPI\(^+\) compared to CPI\(^-\) at 18 and 24 h p.i. However, no significant differences were observed at 12 or 48 h p.i.

**Cell-to-cell spread of CPI\(^+\) and CPI\(^-\) in presence and absence of IFN following a low m.o.i.**

The above experiments were carried out on cells that had been infected at high m.o.i. and thus relied on the cells producing and responding rapidly to IFN. To examine the effects of IFN on the cell-to-cell spread of CPI\(^-\) or CPI\(^+\) following low m.o.i., MDCK cells were infected at 0.01 pfu/cell and the culture medium was or was not supplemented with IFN at 12 h p.i. The addition of IFN was delayed until 12 h p.i. so as not to interfere with the initial replication of virus. At 24 and 72 h p.i., virus-infected cells were examined by immunofluorescence using a pool of anti-NP and anti-P mAbs (Fig. 5). In the absence of exogenous IFN, both CPI\(^+\) and CPI\(^-\) spread rapidly through the monolayer such that all the cells were infected by 72 h p.i. The addition of exogenous IFN completely blocked the spread of CPI\(^-\). In contrast, IFN delayed but did not prevent the spread of CPI\(^+\) from cell to cell, such that by 72 h p.i. approximately 30% of the cells in monolayers infected with CPI\(^+\) were infected.

A further difference between CPI\(^+\) and CPI\(^-\) was noted in the distribution of the NP and P proteins. In the absence of exogenous IFN at 72 h p.i., the NP and P proteins were primarily located in cytoplasmic inclusion bodies with very little diffuse cytoplasmic fluorescence. In contrast, in the majority of cells infected with CPI\(^-\) in the presence of IFN there was both diffuse cytoplasmic distribution of the NP and P proteins and inclusion bodies (data not shown; see also below).

**Characterisation of cells persistently infected with CPI\(^+\) and CPI\(^-\)**

The W3 strain of SV5 does not block IFN signalling in murine cells (Didcock et al., 1999a). Nevertheless, following infection of murine cells with W3 at high m.o.i., although all the cells initially make large amounts of virus protein, once the cells begin to produce and respond to IFN the majority of cells clear the virus infec-
tion. Thereafter, in those cells which remain infected, the virus fluxes between active and repressed states in response to cell passage and the local production of IFN (Young et al., 1997). Since CPI− also fails to block IFN signalling, a series of experiments was carried out to determine whether CPI−behaved in canine cells similar to the W3 strain in murine cells. MDCK cells were infected at a high m.o.i. and passaged. At 1 and 20 days p.i. monolayers were immunostained with a pool of anti-NP and anti-P mAbs (Fig. 6). At 1 day p.i. all the cells were infected with either CPI+ or CPI−. However, by 20 days p.i., whilst all the cells remained infected with CPI+, a significant proportion (approx 40%) of cells infected with CPI−had cleared the infection. Again there were marked differences between the distribution of NP and P in cells infected with CPI+ and CPI−. Thus, whilst there was primarily a diffuse cytoplasmic distribution of NP and P in cells infected with both CPI+ and CPI−at 1 day p.i., by 20 days p.i. in the majority of cells infected with CPI−the NP and P proteins were located in tight cytoplasmic inclusion bodies with little, if any, diffuse cytoplasmic distribution of the proteins (compare magnified Figs. 6A and 6B). In contrast, although inclusion bodies could be visualised in cells infected with CPI+, at 20 days p.i. the NP and P proteins were primarily diffusely distributed throughout the cytoplasm.

Another characteristic of murine cells infected with the W3 strain of SV5 is that once the cells begin to produce and respond to IFN, the cells rapidly become negative for the virus glycoproteins whilst remaining positive for nucleocapsid proteins (Young et al., 1997). To determine whether this was the case for cells infected with CPI−, monolayers of MDCK cells were infected at 0.5 to 1.0 pfu/cell and at 12 h p.i. exogenous IFN was, or was not, added to the culture medium. At 24 and 72 h p.i. the cells were fixed and stained either with a mixture of anti-NP and anti-P mAbs or with an anti-HN mAb. Although cells infected with CPI+, monolayers of MDCK cells were infected at 0.5 to 1.0 pfu/cell and at 12 h p.i. exogenous IFN was, or was not, added to the culture medium. At 24 and 72 h p.i. the cells were fixed and stained either with a mixture of anti-NP and anti-P mAbs or with an anti-HN mAb. Although cells infected with CPI+ and CPI−were positive for NP/P and HN at 24 h p.i. (data not shown) the addition of exogenous IFN to the medium of cells infected with CPI−dramatically reduced the surface expression of the HN protein by 72 h p.i. In contrast, exogenous IFN had a much less dramatic effect on the levels of expression of HN in CPI+ -infected cells at 72 h p.i. (Fig. 7).

A role for the unique cysteine-rich C-terminal domain of the V proteins of rubulaviruses in blocking IFN signalling is suggested by the observation that the C-terminal domain alone of mumps virus can prevent IFN-induction of an antiviral state (Kubota et al., 2001). Furthermore, a recombinant hPIV2 virus that expresses a truncated version of V that lacks the C-terminal domain appears to be sensitive to IFN (Kawano et al., 2001). On the other hand, we have also demonstrated a role for the P/V (N-terminal) common domain of V in blocking IFN signalling by demonstrating that the recombinant virus rSV5-V/P N100D, with an asparagine to aspartic acid substitution at position 100 in the V protein of the W3 strain, differentiates its ability to block IFN signalling in human and murine cells (Young et al., 2001). Here we demonstrate that mutations in the N-terminal domain can also abolish the ability of the V protein to block IFN signalling in cells of the natural host species, thereby establishing a clear role for the N-terminus of V in targeting STAT1 for degradation. In the case of CPI−, for complete loss of the ability of V to block IFN signalling, it appears that all three mutations are necessary. Thus, although the single L to P mutation at position 102, although in the same epitope recognised by the mAb SV5-Pk (which distinguishes W3 from the murine-adapted virus mci2 and rSV5-V/P N100D), on its own had no clear effect on V function. However, given that it clearly contributes to the loss of function when in combination with the other mutations, it again points to this region being critical for V function.

Whilst increased protein synthesis was observed in cells that produce and respond to IFN infected at high m.o.i. with CPI+ compared to CPI−, the difference was not as great as might have been expected. Thus, by 48 h p.i. similar but reduced levels of virus protein synthesis were occurring in both CPI+ - and CPI−-infected cells (Fig. 4). The reason(s) for this late reduction in virus protein synthesis in CPI+ -infected cells remains unclear, but these observations are similar to those in mouse cells infected with rSV5-V/P N100D (which blocks IFN signalling) in which there was a marked reduction in

FIG. 5. Photomicrographs demonstrating the cell-to-cell spread of CPI+ and CPI− in MDCK cells in the presence or absence of exogenous IFN. Cells were infected at 0.01 pfu/cell and 12 h later IFN was or was not added to the culture medium. Monolayers were fixed at 24 or 72 h p.i. and infected cells visualised by immunofluorescence using a pool of mAbs to the NP and P proteins. For reference, some individual cells have been highlighted with white arrows.

FIG. 6. Photomicrographs showing MDCK cells infected with CPI+ and CPI− at 1 and 20 days following a high m.o.i. Magnified images of highlighted areas of cells infected with CPI− at 1 (A) and 20 (B) days p.i. (computer-generated conversion of red image to green for ease of visualisation) have been included to better illustrate the accumulation of the NP and P proteins into large cytoplasmic inclusion bodies at later times p.i. in CPI−-infected cells. For reference a single cell has been highlighted with a white arrow, and in the expanded images the dark areas of two nuclei have been labelled.

FIG. 7. Photomicrographs in which CPI+ - (a) and CPI− - (b) infected MDCK cells have been stained with either a mixture of mAbs to the NP and P or a mAb to the HN protein. Cells were infected at an m.o.i. of 0.5 to 1.0 and at 12 h p.i. IFN was (+IFN) or was not (−IFN) added to the culture medium. Monolayers were fixed at 72 h p.i. prior to immunofluorescence. For reference a single cell has been highlighted with a white arrow.
virus protein synthesis between 48 and 72 h p.i.. Thus, it seems unlikely that the reduction in CPI\textsuperscript{+} virus protein synthesis was due to the actions of IFN but rather the induction of an antiviral state within infected cells either by some other cell signalling pathway, e.g., through the activation of IRF-1, or by virus-induced inhibition (maybe as a result of the buildup of intracellular concentration of NP and the switch from virus transcription to replication). However, clear biological differences in the way CPI\textsuperscript{+} and CPI\textsuperscript{-} respond to IFN were observed in that CPI\textsuperscript{+} spread from cell to cell much more readily than CPI\textsuperscript{-} in the presence of exogenous IFN. Nevertheless even though CPI\textsuperscript{+} blocked IFN signalling, exogenous IFN significantly reduced the speed of spread of CPI\textsuperscript{+} from cell to cell. This is because cells, once in their IFN-induced antiviral state, clearly suppress CPI\textsuperscript{+} replication. However, we have previously shown that even in cells in an IFN-induced antiviral state STAT1 is degraded and eventually the cells go out of their antiviral state and normal levels of virus proteins are synthesised (Didcock et al., 1999b).

It seems reasonable to assume that one of the reasons that CPI\textsuperscript{+} is more pathogenic than CPI\textsuperscript{-} (Baumgartner et al., 1991) is its ability to block IFN signalling. Thus, the isolation of the IFN-sensitive virus (CPI\textsuperscript{+}) from an animal 12 days after infection with CPI\textsuperscript{+} seems counterintuitive but may reflect an evolutionary adaptation by the virus to the development of acquired immune responses. Whereas cells infected with the IFN-resistant CPI\textsuperscript{-} would continue to synthesise virus proteins in the presence of IFN and become ready targets for killing by cytotoxic T cells or antibody-mediated killing, cells infected with the IFN-sensitive CPI\textsuperscript{-} would exhibit impaired viral protein synthesis. Thus in cells infected with CPI\textsuperscript{-}, viral nucleocapsids together with aggregates of NP and P would accumulate in stable cytoplasmic inclusion bodies, virus glycoproteins would be lost from the surface of infected cells, and consequently the cells may become hidden from adaptive immune responses (Fears et al., 1994; Precious et al., 1995; Young et al., 1997).

If the selection of IFN-sensitive viruses, such as CPI\textsuperscript{-}, generally occurs as the adaptive immune response develops, this may be one way in which certain paramyxoviruses establish persistent infections in vivo. Even if most cells harbouring IFN-sensitive viruses were eliminated, a few cells harbouring IFN-sensitive virus in a repressed state may survive until the acute immune response and inflammation dampens down. Presumably, and as we have previously shown (Young et al., 1997), the IFN-sensitive viruses would then flux between active and repressed states in response to local production of IFN. During active replication of IFN-sensitive viruses revertant viruses could once again be generated which are resistant to IFN, and in the absence of an acute immune reaction, these IFN-resistant revertants may then replicate and spread to a limited extent (before being con-trolled by a rapid secondary immune response but perhaps in the absence of overt disease) such that enough virus might be liberated into the environment to infect susceptible individuals. Alternatively, small amounts of IFN-sensitive viruses that might occasionally be liberated by persistently infected individuals may infect non-immune individuals and establish limited infections. Subsequently, IFN-resistant revertants may be selected leading to increased virus spread within these individuals. This may or may not result in clinical disease, depending upon the speed of induction of the adaptive immune response compared to that of virus dissemination.

Whilst this model is dependent upon the selection of point mutations in the V gene, it is worth noting that it is already accepted that the survival of many RNA viruses in nature is dependent upon the selection of point mutations, e.g., to generate antigenic variation to escape from neutralising antibodies. The model is also consistent with the known ability of SV5 to establish persistent infections in monkeys, with the virus regularly recoverable from kidney cell cultures generated long after the animals were infected (Tribe, 1966; Atoynatan and Hsiung, 1969; Hsiung, 1972; Randall and Russell, 1991). Thus, specific selection of IFN-resistant and IFN-sensitive viruses depending on the state of the adaptive immune response offers one possible way in which RNA viruses could establish persistent infection; in this model viruses not only circumvent the IFN response, but also use it to their own advantage.

METHODS

Cells, viruses, and interferon

Human 2fTGH cells (Pellegrini et al., 1989), canine MDCK cells, and murine BF cells (Balb/c mouse embryob fibroblasts) were cultured as monolayers in 25- or 75-cm\textsuperscript{2} tissue culture flasks, in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (growth medium). All cell lines were negative for mycoplasmas as screened by DAPI staining. Human cells and murine cells were treated with recombinant human interferon-\alpha/V (rHuIFN-\alpha/V) (Rehberg et al., 1982) at 1000 IU/ml. Canine cells were treated with interferon produced by infecting MDCK cells with SV5, collecting the medium at 24 h p.i., and inactivating the virus with UV light prior to pelleting of the virus by centrifugation at 100,000 g for 30 min. The amount of canine IFN in the medium was quantitated by a luciferase reporter assay, rather than by its ability to inhibit EMC virus. The amount of IFN used in the assays described was 16 times greater than the minimum amount which would activate the IFN-responsive plasmid. In the equivalent tests with human IFN-\alpha this would be equivalent to 100 IU/ml. SV5 strains W3A (Choppin, 1964), CPI\textsuperscript{+} (78-238), and CPI\textsuperscript{-} (Baumgartner et al., 1981, 1982, 1987) were grown and titrated under appropriate conditions in Vero cells.
Plasmid DNAs and site-directed mutagenesis

The IFN-α/β-responsive plasmid [termed p(9-27)-4tkΔ(−39)luc] contained four tandem repeat sequences of the ISRE from the IFN-inducible gene, 9-27, fused to the firefly luciferase gene (King and Goodbourn, 1998). pJATlacZ, a plasmid used as a transfection standard, contains a β-galactosidase gene under the control of the rat β-actin promoter (Masson et al., 1992). The construction of the plasmid pEF.SV5-V (here termed pEF.W3/V as the V gene was derived from the W3 strain of SV5) has been reported elsewhere (Didcock et al., 1999b) The pEFW3/CPI+/V and pEFW3/CPI−/V plasmids were constructed by PCR amplification of the P/V genes from CPI− and CPI+-infected cells, cleaving the products with BamHI, and using the respective fragments to replace the BamHI fragment in pEFW3/V as outlined in Fig. 2. The single and double point mutations that differentiate CPI− from CPI+ were reintroduced into pEFW3/CPI+/V by PCR mutagenesis. For transfections, monolayers of cells grown in six-well plates to 50−70% confluence were transfected with 1 μg of the appropriate DNA and 1.5 μl of Eugene 6 (Roche) according to the manufacturer’s instructions.

Preparation of radiolabelled antigen extracts, immunoprecipitation, and SDS–PAGE

MDCK cell monolayers in six-well tissue culture plates were infected with CPI+ or CPI− (or mock infected) and the inoculum was adsorbed for 1 h. At various times p.i. (see text) cells were metabolically labelled with L-[35]Smethionine (500Ci/mmol; Amersham International Ltd., UK) in methionine-free tissue culture medium for 2 h. At the end of the labelling interval, the cells were washed in ice-cold PBS and lysed into immune precipitation buffer (10 mM Tris–HCl, pH 7.8, 5 mM EDTA, 0.5% Nonidet P-40, and 0.65 M NaCl; 4 × 10⁶ to 6 × 10⁶ cells per millilitre of buffer) by sonication with an ultrasonic probe. Soluble antigen extracts were obtained after pelleting the particulate material from the total cell antigen extracts by centrifugation at 400,000 g for 30 min. Immune complexes were formed by incubating (for 2 h at 4 °C) 0.2- to 1-ml samples of the soluble antigen extracts with an excess of anti-SV5 mAbs to the HN, F, P, M, and NP proteins (1 μl of concentrated tissue culture fluid of mAbs SV5-HN-4a, NP-a, P-e, M-h, or F-1a; Randall et al., 1987). The immune complexes were treated and stained with specific primary antibodies used to detect the HN, P, and NP were SV5-HN-4a, SV5-P-e, and SV5-NP-a, respectively (Randall and Dinwoodie, 1986). The antibody–antigen interactions were detected by indirect immunofluorescence using a secondary antimouse Ig Texas red-conjugated antibody (Seralab, UK; Cat. No. SBA 1010-02). The primary antibodies used to detect the HN, P, and NP were SV5-HN-4a, SV5-P-e, and SV5-NP-a, respectively (Randall et al., 1987). The treated, stained monolayers were examined for immunofluorescence using a Nikon Microphot-FXA fluorescence microscope.

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