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The V Proteins of Simian Virus 5 and Other Paramyxoviruses Inhibit Induction of Interferon-β

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In this article we show that the paramyxovirus SV5 is a poor inducer of interferon-β (IFN-β). This inefficient induction is a consequence of the expression of an intact viral V protein. In the absence of the viral V protein cysteine-rich C-terminal domain, IFN-β mRNA is strongly induced and the transcription factors NF-κB and IRF-3 are activated significantly. The V protein can work in isolation from SV5 to block intracellular dsRNA signaling. The mechanism of block to dsRNA signaling is distinct from that previously observed for blocking IFN signaling in that proteolysis of candidate factors cannot be detected, and furthermore, the respective blocks require distinct protein domains. Blocking of the induction of IFN-β by dsRNA requires the C-terminal cysteine-rich domain, a feature that is highly conserved among paramyxoviruses. We demonstrate that the V proteins from other paramyxoviruses have equivalent functions and speculate that limiting the yield of IFN-β during infection may be a general property of paramyxoviruses.

INTRODUCTION

The type I interferons (IFNs) are secreted polypeptides that act in a pleiotropic manner to limit viral replication and spread (reviewed by Biron and Sen, 2001). In fibroblastoid cells such as bronchial epithelia the product of the single IFN-β gene is directly induced in response to viral infection and IFN-β feeds back onto cells in an autocrine fashion to set up a state that permits induction of the multigene IFN-α family if infection persists (reviewed by Taniguchi *et al.,* 2001). Since most viruses are capable of inducing IFN-β to some extent, it is generally assumed that the common inducer is intracellular double-stranded RNA (dsRNA) provided by the viral genome itself or formed as a result of replication or convergent transcription of viral genomes (reviewed by Jacobs and Langland, 1996).

To establish even transient infections *in vivo*, viruses must be able to evade the IFN response. It is well established that viruses can block the activities of enzymes involved in the IFN response; more recently it has become clear that many viruses block IFN signaling in a variety of ways (reviewed in Goodbourn *et al.,* 2000). For example, the Rubulavirus genus, including simian virus 5 (SV5) and human parainfluenza virus 2 (hPIV2) of the *Paramyxoviridae,* directs the proteolytic degradation of members of the STAT family of transcription factors in a species-specific manner. Thus, SV5 infections lead to the degradation of STAT1 in human cells but not in mouse cells (Didcock *et al.,* 1999a,b; Parisien *et al.,* 2002), whereas hPIV2 infection leads to the degradation of STAT2 in human cells (Young *et al.,* 2000; Parisien *et al.,* 2001; Nishio *et al.,* 2001; Andrejeva *et al.,* 2002). Other paramyxoviruses, for example the Respirovirus Sendai virus, interfere with IFN signaling without necessarily causing STAT degradation (Young *et al.,* 2000; reviewed in Gotoh *et al.,* 2001).

Viruses might also evade the IFN system by encoding mechanisms that limit the production of IFN. Although there is only a limited amount of information available on the molecular mechanisms of this process, there appears to be a considerable spectrum in the levels of IFN induced by individual viruses (reviewed in Marcus, 1983), suggesting that viruses have evolved mechanisms to at least limit the yield of IFN. In the case of influenza A virus, the NS1 protein may play such a role. This protein is a dsRNA-binding protein that may limit the amount of inducer presented to the cell; thus NS1 can block activation of NF-κB by synthetic dsRNA (Wang *et al.,* 2000), while an influenza strain lacking the NS1 gene induces NF-κB, IRF-3, and IFN-β (Talon *et al.,* 2000).

The induction of IFN-β expression by viral infection has been the subject of intensive research and occurs primarily at the level of transcriptional initiation. The IFN-β promoter consists of four positive regulatory domains (PRDs) that contribute to the magnitude of induction with each PRD being the target for a distinct, inde-

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pendently activated, transcription factor (reviewed in Goodbourn *et al.,* 2000; see Fig. 3A). PRD II is bound and activated by NF-κB (Lenardo *et al.,* 1989; Visvanathan and Goodbourn, 1989) and PRD IV is bound and activated by either ATF-2 homodimers or a heterodimer of ATF-2/c-Jun (Du and Maniatis, 1992; Du *et al.,* 1993). The situation with PRD I and PRD III is more complicated; these elements are related at the DNA sequence level and both bind to members of the interferon regulatory factor (IRF) family. It remains unclear whether activation through PRD I/PRD III is restricted to a specific IRF protein, but recent observations indicate that IRF-3 activation is especially important (Juang *et al.,* 1998; Lin *et al.,* 1998; Ronco *et al.,* 1998; Sato *et al.,* 1998; Schafer *et al.,* 1998; Wathelet *et al.,* 1998; Weaver *et al.,* 1998; Yoneyama *et al.,* 1998). The individually activated transcription factors that bind to PRDs I–IV are assembled in a cooperative manner on the IFN-β promoter in a multiprotein complex, called the enhanceosome, that also includes the "architectural factor" HMG-I:Y (reviewed by Merika and Thanos, 2001).

The signaling pathways between the detection of intracellular dsRNA and the activation of transcription factors are poorly understood, although it is established that the dsRNA-dependent protein kinase (PKR) plays an important role (reviewed by Williams, 2001). PKR activates the IKKβ subunit of the multicomponent IκB kinase (Chu *et al.,* 1999; Zamanian-Daryoush *et al.,* 2000) in a manner that may not require the PKR catalytic activity (Ishii *et al.,* 2001); IκB kinase phosphorylates IκB, which in turn becomes ubiquitinated by an E3 ubiquitin ligase and targeted to proteasomes for degradation. Once the inhibitory IκB is destroyed, the associated NF-κB is freed from restraint and can enter the nucleus and activate transcription (reviewed in Israel, 2000). Activation of PKR is also important in the rise in IRF-1 levels that are seen in response to viral infection or exposure to synthetic dsRNA (Kumar *et al.,* 1997), and in the activation of the PRD IV-binding ATF-2 complexes through the intermediacy of MAP kinases (Chu *et al.,* 1999; Iordanov *et al.,* 2000b). However, it has recently been shown that NF-κB may also be activated in response to dsRNA by an uncharacterized PKR-independent pathway (Iordanov *et al.,* 2000a). Thus there may be at least two distinct intracellular dsRNA signaling pathways. Furthermore, although the relocalization of IRF-3 from the cytoplasm to the nucleus in response to viral infection or synthetic dsRNA requires a cellular kinase, current experimental data are consistent with this kinase being something other than PKR (Smith *et al.,* 2001; Servant *et al.,* 2001).

In this article we show that the product of the V gene of SV5 plays an essential role in limiting the induction of IFN-β mRNA during viral infection. A recombinant SV5 virus that expresses a V protein lacking the unique C-terminus induces significant levels of IFN-β mRNA and activates NF-κB and IRF-3. These proteins are not degraded during infection by wild-type SV5, and hence, the product of the V gene can affect both arms of the IFN-mediated innate immune response using distinct mechanisms. Unlike the targeted degradation of STAT1 which requires both N- and C-terminal epitopes, only the cysteine-rich region of the V protein is required to block dsRNA signaling. This region is highly conserved among paramyxoviruses, and we show that the V gene products of hPIV2 and Sendai virus also block IFN-β induction; these observations suggest that limiting the yield of IFN-β during infection may be a general property of paramyxoviruses.

RESULTS

Efficient induction of IFN-β by SV5 lacking the C-terminus of the V protein

We have reported previously that SV5 infection induces the expression of IFN-β in mouse and human cell lines (Didcock *et al.,* 1999a). To assess the relative efficiency of induction by SV5 with that of other known inducers, we examined the production of IFN-β mRNA in a number of cell lines. Figure 1A shows an experiment performed using MG-63 cells (a human osteosarcoma cell line widely used as a potent IFN-β producer). Although wild-type (wt) SV5 (W3)-infection induces IFN-β mRNA, the levels produced even under optimum conditions [multiplicity of infection (m.o.i.) = 5, 18 h postinfection (p.i.); Lanes 1 to 4] are barely detectable in comparison to that seen by the addition of the synthetic dsRNA [poly(I)-poly(C)] to the culture medium (Lane 7). To compare induction of IFN-β by wt SV5 (W3) with well-characterized inducers, we analyzed the behavior of Sendai virus and Newcastle disease virus (NDV) in MG-63 cells. Figure 1A (Lanes 8 and 9) shows that both of these viruses induce IFN-β at least as well as poly(I)-poly(C) and considerably better than SV5 at the same input m.o.i. The Sendai virus stock used in the experiment shown in Lane 9 was prepared by inoculation of 10-day-old embryonated chickens eggs with a high dilution of viral stock. However, the efficient induction of IFN-β normally observed with Sendai virus is associated with DI particles that are generated by serial passage in embryonated chicken eggs at low dilutions (von Magnus, 1951a,b; Johnston, 1981). When Sendai virus was prepared in this way, the induction of IFN-β was considerably greater than the high dilution inoculum stock of Sendai virus at an equivalent m.o.i. (Fig. 1A, compare Lanes 10 and 9). The relatively poor induction by SV5 in comparison to dsRNA, NDV, and the optimized Sendai virus preparation was also seen in human fibroblast 2fTGH cells, human embryonic kidney (293) cells, or mouse BALB/c cells (data not shown). These results indicate that SV5 and certain stocks of Sendai virus are intrinsically poor inducers of IFN-β in human fibroblastoid cell lines.

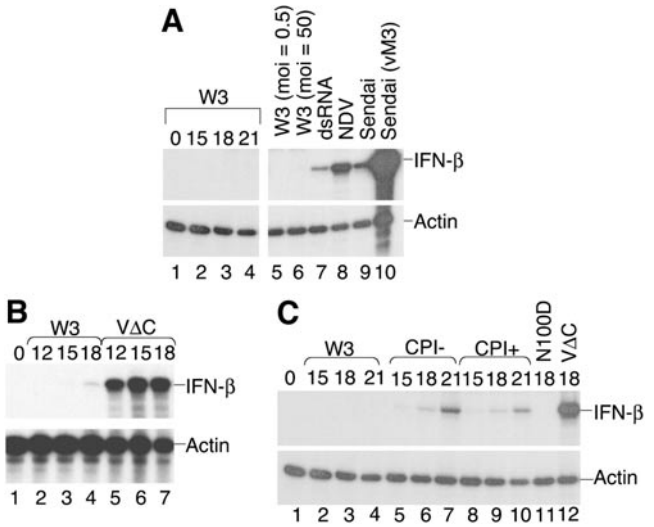


FIG. 1. SV5 is a poor inducer of IFN-β. The human osteosarcoma cell line, MG-63, was infected with viruses or treated with synthetic dsRNA, as indicated, and the IFN-β mRNA levels determined by RNase mapping. The mobilities of the IFN-β and γ-actin transcripts (internal control) are indicated to the right of each panel. (A) Cells were infected with SV5 (W3) at an m.o.i. of 5 and incubated for 0 (Lane 1), 15 (Lane 2), 18 (Lane 3), or 21 h (Lane 4) at an m.o.i. of 0.5 for 18 h (Lane 5) or at an m.o.i. of 50 for 18 h (Lane 6). Poly(I)-poly(C) (dsRNA) was added to the cell-culture media to 100 μg/ml for 4 h (Lane 7). NDV (strain Ulster 2c) was added at an m.o.i. of 5 for 18 h (Lane 8). Sendai virus was added at an m.o.i. of 5 for 18 h (Lane 9). Sendai virus (thrice passaged at 1 in 10 dilution in embryonated chicken eggs—von Magnus prep, vM3) was added at an m.o.i. of 5 for 18 h (Lane 10). (B). Cells were infected with either SV5 (W3) or rSV5VΔC (VΔC) at an m.o.i. of 5 and incubated for 0 (Lane 1), 12 (Lanes 2 and 5), 15 (Lanes 3 and 6), or 18 h (Lanes 4 and 7). (C). Cells were infected at an m.o.i. of 5 with either SV5 (W3; 15 h, Lane 2; 18 h, Lane 3; 21 h, Lane 4), CPI− (15 h, Lane 5; 18 h, Lane 6; 21 h, Lane 7), CPI+ (15 h, Lane 8; 18 h, Lane 8; 21 h, Lane 10), SV5 W3 with a single amino acid change (N100D) in the V protein (18 h, Lane 11), or VΔC (18 h, Lane 12).

Since we had previously shown that the V protein of SV5 was capable of blocking signaling in response to IFN (Didcock *et al.,* 1999b), we next investigated whether SV5 engineered to express a V gene product that is truncated so as to lack the C-terminal cysteine-rich domain (rSV5VΔC—see accompanying article, He *et al.,* 2002) was able to induce IFN-β more efficiently than wild-type SV5. Figure 1B shows that infection of MG-63 cells with the W3 strain of SV5 causes a weak induction of IFN-β mRNA that is barely detectable at 18 h p.i. (Lanes 1–4); by contrast, infection with rSV5VΔC at an equivalent m.o.i. strongly induces IFN-β mRNA at the earliest time point examined (12 h p.i.; Fig. 1B, Lanes 5–7). A similarly enhanced induction of IFN-β mRNA by rSV5VΔC relative to the W3 strain was seen in human 2fTGH cells (see Fig. 2A) and mouse BALB/c cells (data not shown). These data indicate that SV5 which lacks an intact V protein either produces an inducer of IFN-β not made by the wild-type virus and/or that the SV5 V protein is a potent inhibitor of IFN-β mRNA induction. It is also noteworthy that unlike the species-specificity of the SV5 V protein with respect to blocking IFN signaling (Didcock *et al.,* 1999b; Young *et al.,* 2001; Parisien *et al.,* 2002) expression of the intact SV5 V protein limits the production of IFN-β mRNA in both human and mouse cells.

We previously reported important differences in the ability of two closely related canine isolates of SV5, termed CPI+ and CPI−, to block IFN signaling (Chatziandreou *et al.,* 2002). CPI+ blocks IFN signaling by targeting STAT1 for degradation, whereas CPI− does not; this difference is the result of three amino acid substitutions in the common V/P N-terminal domain. Although these viruses exhibit indistinguishable growth in cells that cannot produce type I IFN it would be expected that in cells that can produce IFN, the replication of the CPI− strain would be impaired as a result of IFN sensitivity. Surprisingly, we found that CPI− infections were only cleared slowly in the latter unless the cells were treated with type I IFN (CPI+ infections which block IFN responses were resistant to exogenous IFN; Chatziandreou *et al.,* 2002). These results can be explained if CPI− and CPI+ strains are poor IFN-β inducers. To test this directly, we compared the IFN-β induction profile by CPI− and CPI+. Figure 1C shows that both CPI− (Lanes 5–7) and CPI+ (Lanes 8–10) are poor inducers in MG-63 cells when compared to rSV5VΔC (Lane 12), NDV, and the optimized Sendai virus preparation (data not shown), although they are slightly more efficient inducers than SV5 W3 (Lanes 2–4). The fact that little or no difference is seen in the early phase of IFN-β induction by CPI− and CPI+ shows that the substitutions in the CPI V protein that alter the ability to block IFN signaling are distinguishable from V protein functions that block IFN-β induction. It is interesting to note that at later periods in infection (21 h p.i.), CPI− begins to become a better inducer than CPI+ (Fig. 1C, compare Lanes 7 and 10). Although IFN-β induction occurs without the need for *de novo* protein synthesis, the yield is enhanced by a positive autoregulation loop that can involve the activation of ISGF3 or IRF-1 by IFN (Yoneyama *et al.,* 1996), or the induction of synthesis of IRF-7, which is subsequently activated by viral infection (reviewed by Taniguchi *et al.,* 2001). The ability of the CPI+ virus to block IFN signaling would prevent this virus from showing the amplification of IFN-β production seen with CPI− and thus produce the observed differences at later time points.

Limited amounts of the V protein of SV5 (~350 molecules) enter the target cell as part of the virion (Paterson *et al.,* 1995). We were therefore interested to see whether small amounts of SV5 V protein would be sufficient to block IFN-β induction, as seen for the degradation of STAT1 and the inhibition of IFN signaling, and whether the induction of IFN-β seen in rSV5VΔC-infected cells could be blocked by expressing the V protein *in trans*. Coinfection of 2fTGH cells by wt SV5 (W3) and rSV5VΔC did not lead to a suppression of IFN-β induction (Fig. 2A, Lanes 1–6), perhaps suggesting that the V protein cannot

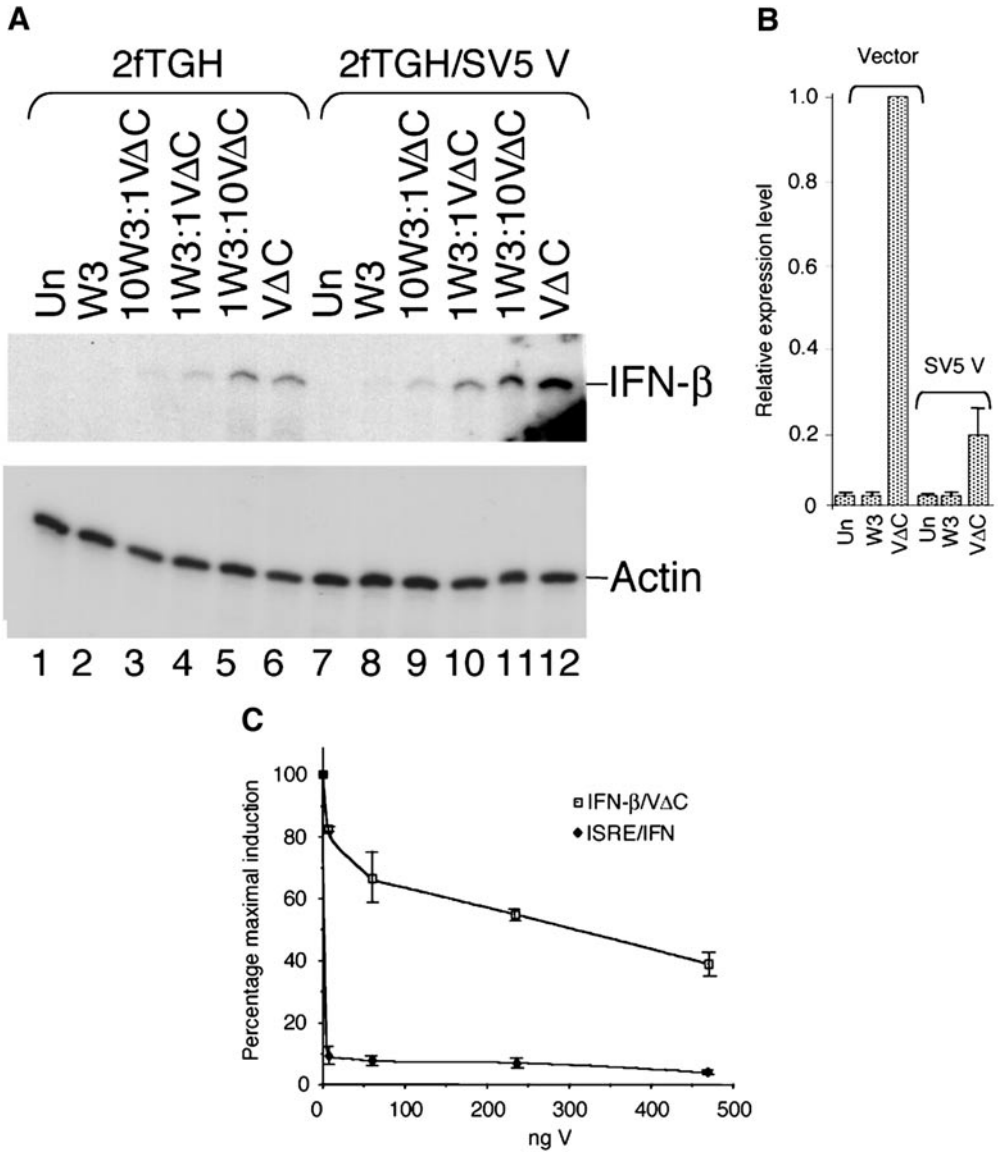


FIG. 2. The V protein of SV5 can block IFN induction by rSV5VΔC *in trans*. (A) The human diploid fibroblast cell line, 2fTGH (Lanes 1–6), and a derivative that stably expresses the SV5 V protein (Lanes 7–12) were infected with SV5 (W3) or rSV5VΔC (VΔC) at an m.o.i. of 5, or a mixture of W3 and rSV5VΔC at the indicated ratios and a total m.o.i. of 5 for 18 h, and the IFN-β mRNA levels determined by RNase mapping. The mobilities of the IFN-β and γ-actin transcripts (internal control) are indicated to the right of each panel. (B) Vero cells were transfected with a reporter for IFN-β promoter activity [pIFΔ(-125)lucter—see Materials and Methods], the β-galactosidase expression vector, pJATlac, and either a mammalian expression plasmid driving the overexpression of the SV5 V protein or a control "empty vector." Transfected cells were infected with either SV5 (W3) or rSV5VΔC (VΔC) at an m.o.i. of 5 for 18 h and then cell extracts were prepared for reporter gene assays. In each case luciferase activity was corrected to the β-galactosidase activity to normalize for variations in the transfection efficiency. Transfection experiments were repeated at least three times and averages and error bars are shown. Expression levels are relative to the rSV5VΔC-infected level of the −125 construct (=1.0). (C) Vero cells were transfected with either pIFΔ(−125)lucter as a reporter for IFN-β promoter activity or a reporter for type I IFN responsiveness [p(9–27ISRE)$_4$tkΔ(−39)lucter], the β-galactosidase expression vector, pJATlac, and a mixture of a mammalian expression plasmid driving the overexpression of the SV5 V protein and the control empty vector such that the total amount of effector plasmid was kept constant. The amount of SV5 V expressing plasmid in each transfection is indicated on the *x*-axis in nanograms. IFN responsiveness was determined by treating cells for 5 h with 1000 IU/ml of IFN. Responsiveness to rSV5VΔC (VΔC) was determined by infecting cells an m.o.i. of 5 for 18 h. In each case luciferase activity was corrected to the β-galactosidase activity to normalize for variations in the transfection efficiency. The inducibilities of the reporters in transfections with no SV5 V expressing plasmid were determined for IFN and rSV5VΔC (VΔC), respectively, and set at 100%; samples from transfections containing SV5 V protein expressing plasmids are given in percentage maximum inducibility compared to this control. Transfection experiments were performed twice and averages and error bars are shown.

work *in trans*, or that rSV5VΔC induces IFN-β sufficiently fast enough that the limited amount of V protein entering the cells in wt SV5 (W3) virions is unable to block the cellular response to SV5 infection. We next infected a 2fTGH cell line that stably expresses the V protein of SV5 (which blocks IFN signaling in these cells) (Andrejeva *et*

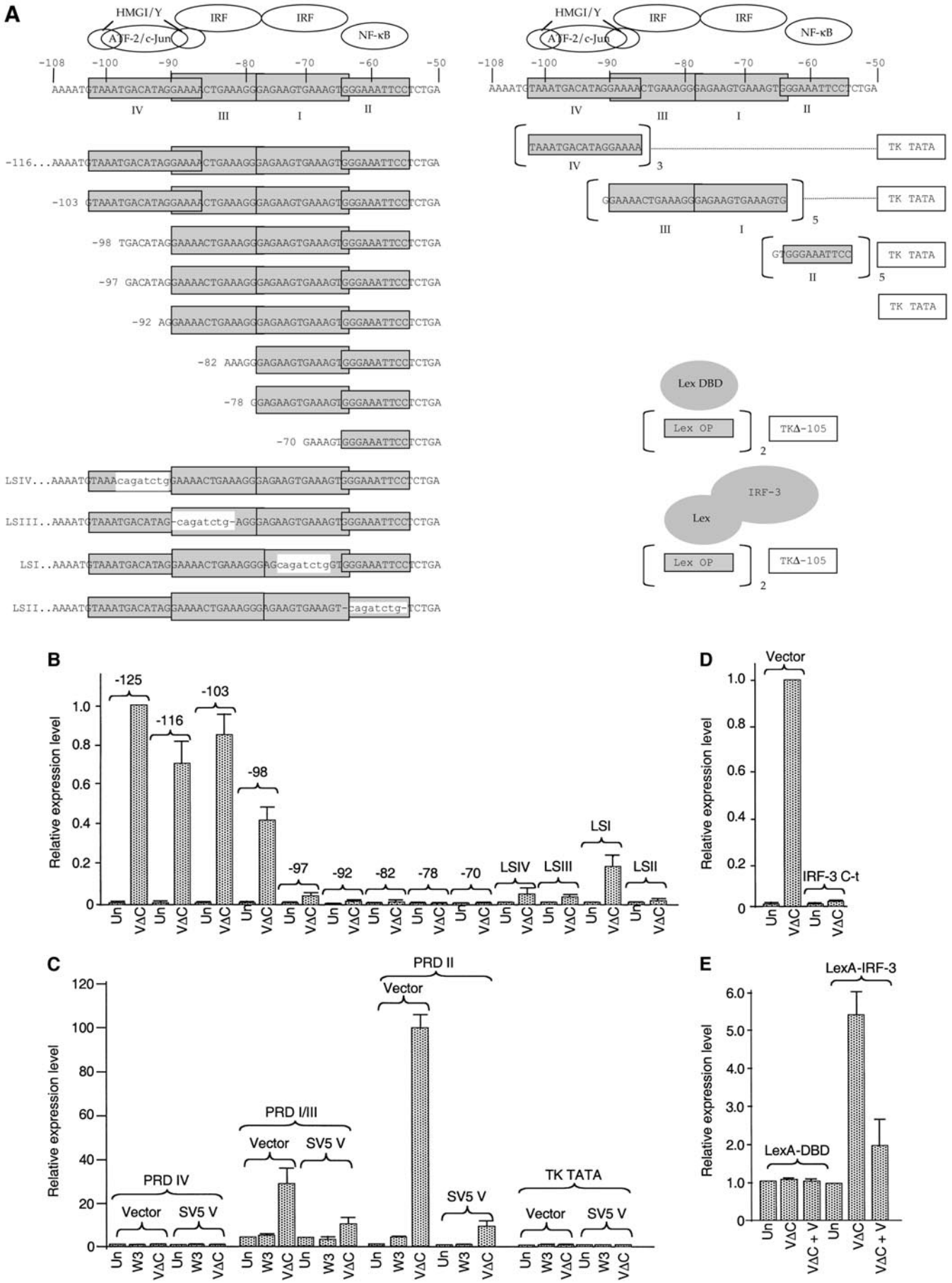
al., 2002) with the rSV5VΔC virus. Figure 2A (Lanes 7–12) shows that these cells are unable to block IFN-β induction by rSV5VΔC infection. The SV5 V protein expressing stable cell lines are characterized by levels that are at least 20-fold lower than that achieved during a viral infection (Andrejeva *et al.,* 2002), and we note that we have not succeeded in constructing cell lines that express high levels of SV5 V protein, suggesting that this may be toxic to host cells. To determine whether high-level V protein expression is capable of blocking IFN-β induction by SV5, we examined the ability of overexpressed SV5 V protein to block induction by rSV5VΔC infection of a luciferase reporter gene under the control of an IFN-β promoter in transient transfections. Figure 2B shows that induction is significantly inhibited, indicating that the viral V protein can act *in trans* to block induction if expressed to adequate levels. To test further whether the respective blocks to IFN signaling and IFN induction are differentially sensitive to expression levels of the SV5 V protein, we compared the effects of increasing levels of a plasmid expressing SV5 V on the relative inducibilities of an ISRE reporter by IFN, and of an IFN-β promoter reporter by rSV5VΔC infection. Figure 2C shows that SV5 V expression inhibits IFN responses by more than 90% even at the lowest levels tested, whereas inhibition of induction of the IFN-β promoter reporter by rSV5VΔC was barely evident at these levels and required higher levels of expression to achieve a block of greater than 50%. These data suggest that the block to IFN signaling and the block to IFN-β induction are mechanistically distinct.

NF-κB and IRF-3 activation by SV5 lacking full-length V gene product

Induction of IFN-β by viral infection requires the activation of distinct transcription factors, with considerable evidence supporting the importance of NF-κB and members of the IRF family as the key transcription factors (see Introduction). To establish the requirement for these factors for induction of IFN-β by rSV5VΔC and to determine whether the failure of wt SV5 (W3) to induce IFN-β expression could be linked to a defect in activation of one of these factors or the failure to activate several transcription factors, we transiently transfected a series of luciferase reporter constructs under the control of the human IFN-β promoter and mutant derivatives and examined their inducibility by viral infection. The constructs used in this analysis are shown in Fig. 3A. Figure 3B shows that deletion from the 5′ end of the IFN-β promoter had only a limited effect on induction by rSV5VΔC until nucleotide −98 was removed; these results demonstrate that the HMGI:Y binding sequence (TAAAT) located at residues −102 to −98 (Du *et al.,* 1993) is not required for induction of IFN-β by rSV5VΔC, in agreement with our previous observations on induction by poly(I)•poly(C) (Ellis and Goodbourn, 1994; King and Goodbourn, 1994). Further deletion from the 5′ end of the IFN-β promoter led to a stepwise loss in inducibility with removal of the PRD III, PRD I, and PRD II elements. None of these constructs were responsive to wt SV5 (W3) infection (data not shown). To evaluate the individual contribution of the PRD elements, we examined the inducibility of reporter constructs under the control of the IFN-β promoter containing linker scan mutations. Figure 3B shows that disruption of any of the four PRD elements severely impairs induction by rSV5VΔC. Since each of the individual PRD elements has been shown to be independently inducible in certain cell/inducer combinations, we tested the responsiveness of reporter constructs containing multimers of either PRD IV, PRD III/PRD I, or PRD II placed upstream of a minimal HSV-1 thymidine kinase TATA box. Figure 3C shows that the NF-κB-responsive PRD II construct and the IRF-responsive PRD I/PRD III construct respond dramatically to infection by rSV5VΔC, whereas expression driven by the PRD IV construct is not responsive. Taken together, the insensitivity to deletion of the HMGI:Y site between −103 and −97 and the lack of inducibility of the PRD IV reporter construct demonstrate that activation of the ATF-2-c-Jun/HMGI:Y module (Du and Maniatis, 1992; Du *et al.,* 1993) is unimportant for IFN-β induction by rSV5VΔC, and we have not considered it further.

In contrast to the induction of both the PRD II and the PRD I/III reporters by rSV5VΔC, neither reporter is activated by wt SV5 (W3) infection (Fig. 3C), suggesting that infection by wt SV5 (W3) virus fails to activate a signaling pathway that is common to the induction of factors that stimulate transcription through the unrelated PRD II and PRD I/III elements. Consistent with this, the overexpression of the SV5 V protein significantly diminishes the induction of both reporters by rSV5VΔC (Fig. 3C).

Although the PRD I/III site of the IFN-β promoter can bind to several members of the IRF family, it is believed that IRF-3 activation plays a key role in IFN-β induction in response to viral infection (see Introduction). To test whether IRF-3 activation plays a role in IFN-β induction by rSV5VΔC, we examined the effect of expressing a dominant-interfering form of IRF-3 (Lin *et al.,* 1998). Figure 3D shows that induction by rSV5VΔC is completely abolished in the presence of the interfering form of IRF-3. To examine the behavior of IRF-3 in isolation from other members of the IRF family, we constructed a fusion to the bacterial DNA-binding protein, lexA, and examined the ability of this fusion to stimulate transcription from a luciferase reporter gene under the control of a lexA operator site. Figure 3E shows that this construct is responsive to infection by rSV5VΔC [but not by infection by wt SV5 (W3)—data not shown]. The induction in response to rSV5VΔC is significantly inhibited by the coexpression of the SV5 V protein (Fig. 3E). These data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Lex
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-116
-103
-98
-97
-92
-82
-78
-70
LSIV
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TK TATA
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SV5 V
W3
VΔC
D
IRF-3 C-t
E
LexA-IRF-3
LexA-DBD
VΔC + V

show that IRF-3 activation is required for IFN-β induction by rSV5VΔC and that the activation of IRF-3 can be blocked by the SV5 V protein.

The data above suggest that NF-κB and IRF-3 are the key transcription factors involved in IFN-β induction in response to rSV5VΔC infection; these factors are not induced in the presence of the full-length V protein expressed during either a wild-type infection or expressed *in trans* during infection by rSV5VΔC. We have confirmed the activation of both NF-κB and IRF-3 by rSV5VΔC infection, but not wt SV5 (W3) infection, by gel-retardation assays and immunofluorescence experiments; also note that rSV5VΔC infection leads (indirectly) to IRF-1 production and ISGF3 activation (He *et al.,* 2002 and data not shown). The failure of wt SV5 (W3) infection to activate these responses could be due to a virally induced degradation of transcription factors in a manner analogous to the degradation of STAT1. Western blot analysis using antibodies specific for either the p65 or the p50 subunits of NF-κB subunit, or for IRF-3, demonstrated that neither of these proteins are targeted for degradation by SV5 infection, in contrast to the consequences of SV5 infection on STAT1 (Fig. 4).

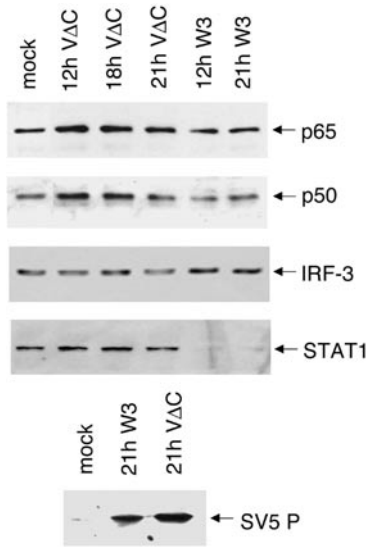

FIG. 4. STAT1, but not NF-κB subunits or IRF-3, is degraded by SV5 (W3) infection. MG-63 cells were infected with either SV5 (W3) or rSV5VΔC (VΔC) at an m.o.i. of 5 and incubated for the indicated times, and cell extracts were analyzed by Western blotting using antisera specific for p65, p50, IRF-3, STAT1, or the P protein of SV5.

The V protein of SV5 can block the induction of IFN-β by dsRNA

The difference in the IFN-inducing properties of wild-type SV5 (W3) and rSV5VΔC could be due either to the V product having a specific function that directly blocks IFN-β production or to an indirect effect such that the viruses have different innate IFN inducing capacities; for example, rSV5VΔC infections could generate more intracellular dsRNA than wt SV5 (W3). To distinguish these possibilities, we investigated whether overexpression of the SV5 V protein could block induction by synthetic dsRNA of a luciferase reporter gene under the control of an intact IFN-β promoter in transient transfections. Figure 5A shows that SV5 V protein expression can limit induction of the IFN-β promoter by about 90%; we performed similar experiments in 293 cells and obtained a similar inhibition of IFN-β induction by the SV5 V protein (Fig. 5B). In addition to the ability of the SV5 V protein to block dsRNA induction of the IFN-β promoter, blockage was also seen in response to Sendai virus infection (Fig. 5C). Since dsRNA can activate individual transcription factors that are involved in IFN-β induction, we examined the ability of the SV5 V protein to block NF-κB induction by dsRNA. Figure 5D shows that the induction of reporter activity by dsRNA is down-regulated by about 80% when

FIG. 3. NF-κB and IRF-3 are required for IFN-β induction by rSV5VΔC. (A) Reporter gene constructs. The architecture of the IFN-β promoter is indicated at the top of the panel and described in the text. The 5′ deletion series is shown in the left panel; the bottom four constructs are the linker scan mutations which are all based on the −116 deletion but which have an 8–10 base-pair region of the IFN-β promoter replaced by a *Bgl*II linker (lower case). Each linker scan mutation inactivates one of the PRDs. The top right panel shows the construction of the individual PRD reporters; the IFN-β promoter sequences indicated are multimerized and inserted 5′ to the minimal (−39) TATA box of the HSV-1 thymidine kinase promoter. The bottom right panel shows the constructs used to analyze the role of IRF-3; the reporter gene is a dimerized lex operator sequence placed upstream of the HSV-1 thymidine kinase promoter and the effectors are either the DNA-binding domain of lexA alone (Lex-DBD) or the DNA-binding domain of lexA fused to IRF-3 (Lex-IRF-3). (B) Vero cells were transfected with the indicated reporter and the β-galactosidase expression vector, pJATlac, and subsequently mock-infected or infected with rSV5VΔC (VΔC) at an m.o.i. of 5 for 18 h. Expression levels are relative to the rSV5VΔC-infected level of the −125 construct (=1.0). (C) Vero cells were transfected with the indicated reporter, pJATlac, and either a mammalian expression plasmid driving the overexpression of the SV5 V protein or a control empty vector, and subsequently mock-infected or infected with SV5 (W3) or rSV5VΔC (VΔC) at an m.o.i. of 5 for 18 h. Expression levels are relative to the mock-infected level of the TK TATA box only construct (=1.0). (D) Vero cells were transfected with pIFΔ(−125), pJATlac, and either a mammalian expression plasmid driving the overexpression of the C-terminus of IRF-3 (amino acids 132–427; IRF-3C-t) or the control empty vector and subsequently mock-infected or infected with rSV5VΔC (VΔC) at an m.o.i. of 5 for 18 h. Expression levels are relative to the rSV5VΔC-infected level of the −125 construct (=1.0). (E) Vero cells were transfected with the lexA-responsive luciferase reporter, p(lexOP)2TKLuc, pJATlac, a mammalian expression plasmid driving the overexpression of either the DNA binding domain (DBD) of lexA or the fusion between the lexA DBD, and IRF-3, and a mammalian expression plasmid driving the overexpression of the SV5 V protein or the control empty vector, and subsequently mock-infected or infected with rSV5VΔC (VΔC) at an m.o.i. of 5 for 18 h. Expression levels are relative to the mock-infected level of the lex DBD construct (=1.0). For each of (B) to (E) luciferase and β-galactosidase activities were determined from cellular extracts and relative expression values calculated accordingly. Transfection experiments were repeated at least three times and averages with error bars are shown.

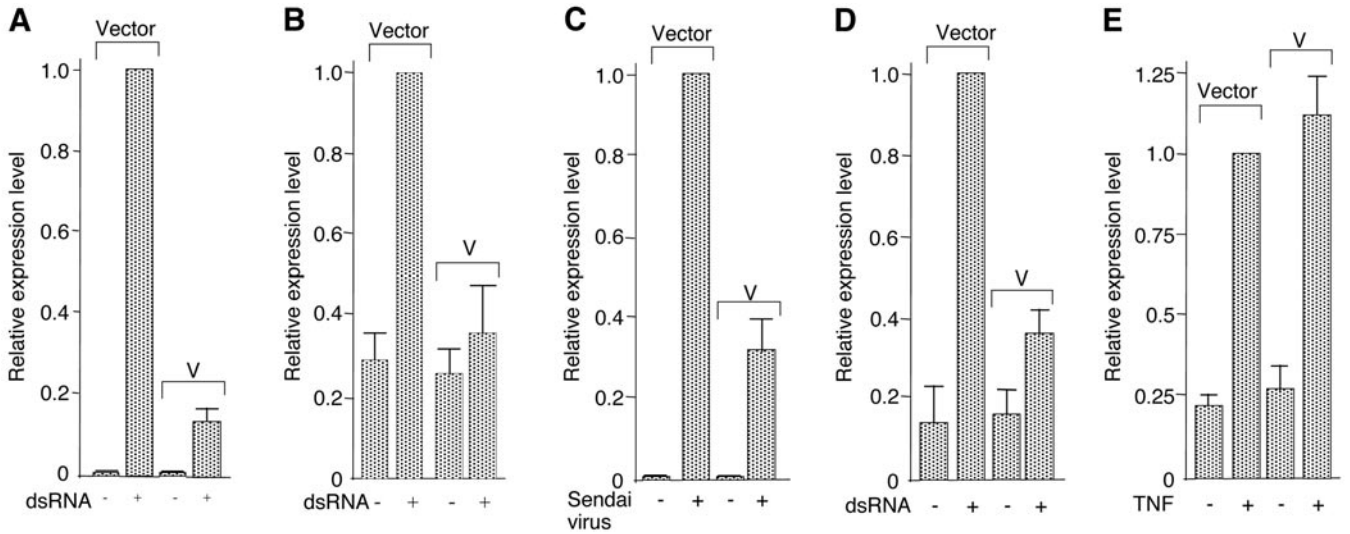

FIG. 5. The V protein of SV5 can block signaling in response to dsRNA. (A) Vero cells were transfected with pIFΔ(−125)lucter, pJATIac, and either a mammalian expression plasmid driving the overexpression of the SV5 V protein or the control empty vector. Transfected cells were either mock-treated or treated with poly(I)-poly(C) (dsRNA) and cell extracts prepared. (B). Experiments were performed identically to (A) except that 293 cells were used. (C). Experiments were performed identically to (A) except that Sendai virus (vM3 preparation) was used as an inducer instead of dsRNA. (D). Vero cells were transfected with a reporter for NF-κB activity [p(PRD II)5tkΔ(-39)lucter], pJATIac, and either a mammalian expression plasmid driving the overexpression of the SV5 V protein or the control empty vector. Transfected cells were either mock-treated or treated with poly(I)-poly(C) (dsRNA) and cell extracts prepared. (E) Experiments were performed identically to (D) except that TNFα was used as an inducer of NF-κB instead of dsRNA. For each of (A)–(E) luciferase and β-galactosidase activities were determined from cellular extracts and relative expression values calculated accordingly (expressed relative to the induced level of the vector-only sample = 1.0). Transfection experiments were repeated at least three times and averages with error bars are shown.

cells were cotransfected with the SV5 V protein expressing plasmid; by contrast, we observed a small but reproducible enhancement of induction by an alternative NF-κB inducer, TNFα (Fig. 5E). These data indicate that the SV5 V protein can block intracellular dsRNA signaling in the absence of other viral products.

Inhibition of induction of IFN-β by SV5 V protein is a property of the cysteine-rich C-terminus

To map the regions of the SV5 V protein that block the induction of IFN-β by synthetic dsRNA, we first determined whether the viral polymerase P protein had similar properties, as the V and P proteins share 164 N-terminal residues. As shown in Fig. 6A the SV5 P protein is unable to block the induction of IFN-β by synthetic dsRNA. Thus, these data indicate that the unique cysteine-rich C-terminus of V plays an important role in this process.

A panel of mutations in the SV5 V gene was constructed and the effect of expression of these proteins on IFN-β induction by dsRNA was examined. As shown in Fig. 6B deletion of N-terminal sequences up to amino acid 125 had no effect on inhibition. Deletion of the C-terminal 48 amino acids inactivated the inhibition, indicating that the C-terminal region of the protein is important for blocking IFN-β induction, in contrast to the block to IFN signaling which requires sequences at the N-terminus as well as the C-terminus (Young *et al.,* 2001; Chatziandreou *et al.,* 2002; Andrejeva *et al.,* in press).

The C-terminal region of the SV5 V protein is characterized by the presence of several cysteine residues. To investigate the importance of these cysteine residues in blocking IFN-β induction, we changed several of them by site-directed mutagenesis, cloned the mutant genes into a mammalian expression vector, and then examined their effects in response to synthetic dsRNA. As shown in Fig. 6C changes at cysteines 193, 207, and 214 individually inactivated the ability of the viral V protein to block dsRNA signaling, despite the proteins being expressed at equivalent levels as assessed by immunofluorescence (data not shown). These data show that inhibition of induction of IFN-β requires a cysteine-rich region of no more than 97 amino acids (amino acids 126 to 222) at the C-terminal end of the SV5 V protein.

Inhibition of induction of IFN-β is a property of other paramyxovirus V proteins

The importance of the cysteine-rich C-terminus of SV5 in blocking the induction of IFN-β is particularly interesting since this region is the most conserved domain among V proteins of other *Paramyxovirinae*, including viruses from all three genera, Respiroviruses, Rubulaviruses, and Morbilliviruses. To investigate whether V proteins from viruses other than SV5 could block IFN-β induction, we constructed mammalian expression vectors for the V proteins of hPIV2 and Sendai virus. Both of these constructs blocked IFN-β induction by dsRNA to

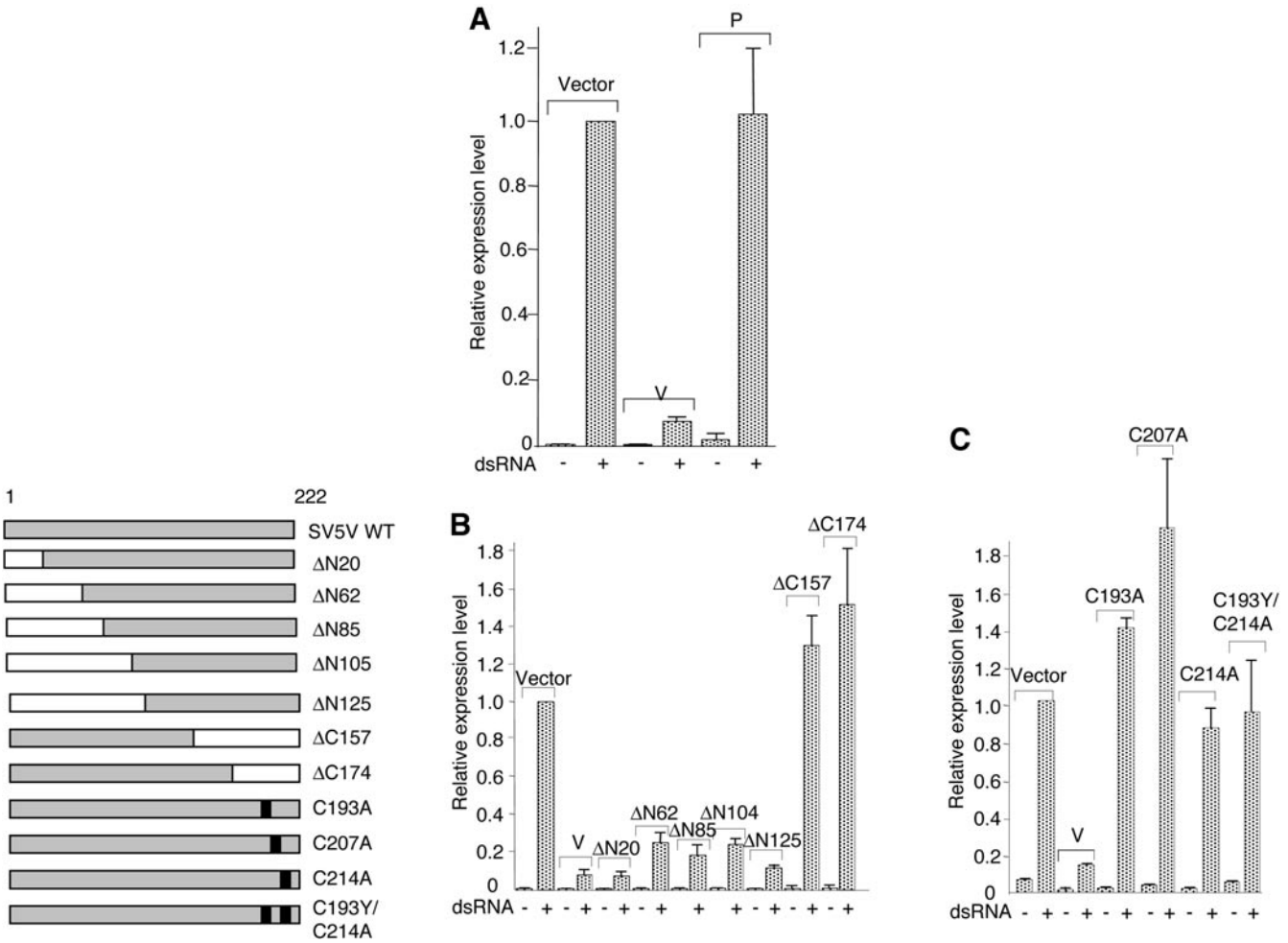

FIG. 6. The C-terminal cysteine-rich region of the SV5 V protein is essential for blocking dsRNA signaling. (A) Vero cells were transfected with pIFΔ(−125)lucter, pJATIac, and either a mammalian expression plasmid driving the overexpression of SV5 V protein, a mammalian expression plasmid driving the overexpression of SV5 P protein, or the control empty vector. Transfected cells were either mock-treated or treated with poly(I)-poly(C) (dsRNA) and cell extracts prepared. (B) and (C) Vero cells were transfected with pIFΔ(−125)lucter, pJATIac, and either a mammalian expression plasmid driving the overexpression of wt or mutant forms of SV5 V protein (see table at the left of figure), or the control empty vector. Transfected cells were either mock-treated or treated with poly(I)-poly(C) (dsRNA) and cell extracts prepared. For each of (A)–(C) luciferase and β-galactosidase activities were determined from cellular extracts and relative expression values calculated accordingly (expressed relative to the induced level of the vector-only sample = 1.0). Transfection experiments were repeated at least three times and averages with error bars are shown.

an equivalent extent to the V protein of wt SV5 (W3) (Fig. 7). We also examined the properties of V proteins of distinct isolates of SV5. Figure 7 shows that an N100D change (from a virulent mouse-adapted strain) (Young *et al.,* 2001), and the limited substitutions observed in the CPI+ and CPI− strains (Chatziandreou *et al.,* 2002) had no effect on the blockage to IFN-β induction by dsRNA; these results were expected since these amino acid changes lie outside of the conserved cysteine-rich C-terminus.

DISCUSSION

We have reported previously that the V protein of SV5 plays an essential role in evading the IFN system by targeting the host cells STAT1 protein for proteosomal degradation (Didcock *et al.,* 1999b). Similar results have been reported for the V protein of hPIV2, except that STAT2 is preferentially degraded (Parisien *et al.,* 2001; Nishio *et al.,* 2001; Andrejeva *et al.,* 2002). In this article, we show that the V protein of SV5 has an additional distinct effect on the IFN system, namely that of acting to minimize the yield of IFN-β produced by an infected cell. While this property is dispensable for growth in Vero cells that cannot produce IFN, it appears to be essential for efficient growth in other cell lines in which the IFN system is intact (see He *et al.,* 2002); consistent with this revertants are generated at high frequency during passage of the SV5VΔC mutant virus. Since the SV5 V protein blocks transcriptional activation of the IFN-β gene by dsRNA, it is also probable that the direct viral activation (i.e., through the production of dsRNA) of other genes

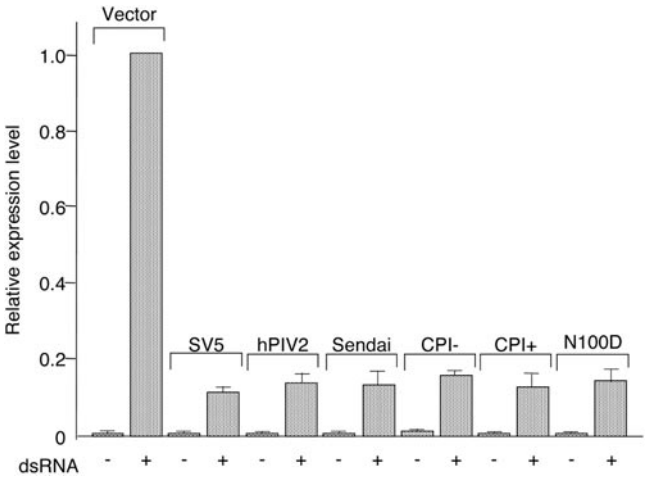

FIG. 7. The V proteins of other paramyxoviruses block dsRNA signaling. Vero cells were transfected with pIFΔ(−125)lucter, pJATIac, and either a mammalian expression plasmid driving the overexpression of the V protein of the indicated paramyxovirus or the control empty vector. Transfected cells were either mock-treated or treated with poly(I)-poly(C) (dsRNA) and cell extracts prepared. Luciferase and β-galactosidase activities were determined from cellular extracts and relative expression values calculated accordingly (expressed relative to the induced level of the vector-only sample = 1.0). Transfection experiments were repeated at least three times and averages with error bars are shown.

involved in the antiviral response, such as Mx, are also blocked.

Our analysis of the V proteins of hPIV2 and Sendai virus show that they also block induction of IFN-β, and since the domain that is required for blockage by the SV5 V protein is the C-terminal cysteine-rich domain that is highly conserved among paramyxoviruses, we think it likely that the ability to block IFN-β production may be a general property of paramyxovirus V proteins. Indeed, this property may be a requirement for efficient growth in the face of a functional IFN system. The V protein is often described as a "luxury function" in that it is not required for growth in culture for rinderpest (Baron and Barrett, 2000), Sendai virus (Delenda *et al.,* 1997, 1998), or measles virus (Schneider *et al.,* 1997). Nevertheless, pathogenicity has been shown to be impaired for Sendai virus (Kato *et al.,* 1997a,b; Huang *et al.,* 2000) or measles virus (Tober *et al.,* 1998; Valsamatis *et al.,* 1998; Mrkic *et al.,* 2000; Patterson *et al.,* 2000) lacking an intact V protein and we speculate that this is at least in part due to the loss of ability to block IFN production. It is interesting to note that measles virus has been shown to block IFN production (Naniche *et al.,* 2000), although this property has yet to be ascribed to the V protein.

Although the V proteins of the Rubulaviruses have the ability to block both the induction of IFN and the downstream signaling in response to IFN, this function has been separated in the Respirovirus, Sendai virus (Garcin *et al.,* 1999; reviewed in Gotoh *et al.,* 2001). Thus the C protein(s) of Sendai virus is responsible for blocking IFN signaling, while the V protein blocks IFN induction. Nevertheless, the fact that both Rubulavirus and Respirovirus genera of paramyxoviruses have acquired the ability to block both arms of the IFN system demonstrates the importance of avoiding this aspect of innate immunity to establish a productive infection. It should be emphasized that the blocks are probably operating at different stages during the course of an infection. We have shown previously that although SV5 can degrade the antiviral state established in a cell by previous exposure to IFN (Didcock *et al.,* 1999b), this process takes several hours and hence the rate of viral spread would be much enhanced if IFN production was limited during the first round of viral infection.

The role of the V protein in limiting the yield of IFN could act in two fundamentally distinct ways. First, it could act to limit the generation of an inducer produced during the viral lifecycle. Second, the V protein could act to prevent the downstream action of a virally generated inducer. Since the V protein can inhibit the signaling response to synthetic dsRNA in activating NF-κB, this second role for the V protein would appear to be of major importance. However, we are struck by our observation that none of the paramyxovirus V proteins we have examined are capable of inhibiting the induction of IFN-β by dsRNA more than 80–90%. This contrasts with the extremely limited induction of IFN-β in the context of viral infection, where wt SV5 (W3) (which expresses a wt V protein) barely induces IFN-β in any cell line tested (less than 1% of the levels seen in rSV5VΔC infections), strongly suggesting that a function of the V protein (e.g., in the control of transcription or replication—see below) also limits the generation of the viral inducer of IFN. This is consistent with our observations that inhibition of dsRNA signaling requires significant levels of V protein expression that might only be achieved relatively late in viral infection and perhaps after the start of generation of the viral signal.

This virally induced signal could be dsRNA, produced during the viral lifecycle. It is well established that V proteins help to regulate viral replication. Sendai virus deficient in V protein synthesis shows increased antigenome synthesis and transcription (Kato *et al.,* 1997a), and expression of the V protein decreased replication by 60–70% in a Sendai virus DI RNA assay (Curran *et al.,* 1991), suggesting that V protein may negatively regulate viral replication. Studies on rinderpest virus lacking expression of the V protein show a rise in synthesis of genome and antigenome RNAs, as well as an increase in the cytopathic effect (Baron and Barrett, 2000). Although a role for the SV5 V protein in regulating RNA replication has not been shown, the SV5 V protein is known to have many functions. The shared N-terminal domain of V and P proteins binds single-stranded RNA through a basic region (Lin *et al.,* 1997) and the SV5 V protein interacts with soluble but not polymeric NP protein and thus may

act as a chaperone to keep NP soluble prior to encapsidation of vRNA (Randall and Bermingham, 1996). Taken together these observations suggest that the V protein may perhaps indirectly limit viral dsRNA levels. It is also possible that the virally induced signal could be some component of the viral lifecycle other than dsRNA. In this context, a recent article suggesting that the measles N gene product can function as an activator of IRF-3 (tenOever *et al.,* 2002) is particularly interesting. The interaction of SV5 V protein with NP might inhibit an equivalent activation by IRF-3.

The target for the V protein in blocking dsRNA signaling is not known. An obvious target would be the dsRNA-dependent protein kinase, PKR. This enzyme has been shown to be important for the activation of NF-κB by dsRNA (Yang *et al.,* 1995; Kumar *et al.,* 1997), although recent results suggest that there is a second PKR-independent pathway (Abraham *et al.,* 1999; Iordanov *et al.,* 2000a). Our observation that the V protein cannot block dsRNA signaling completely may reflect expression levels, or it may reflect the possibility that the V protein can only block one of the alternative dsRNA signaling pathways. It is also unclear whether PKR can function as an upstream kinase for IRF-3 activation; although IRF-3 nuclear translocation in response to either Sendai virus or NDV is not dependent upon PKR (Servant *et al.,* 2001; Smith *et al.,* 2001), it is not clear whether PKR can perform this function in a redundant pathway. Since the SV5 V protein can block activation of both NF-κB and IRF-3, it promises to be a useful reagent for analyzing signaling events that occur during paramyxovirus and other viral infections.

MATERIALS AND METHODS

Plasmids

The reporter plasmids with the firefly luciferase gene under the control of the human IFN-β promoter, pIFΔ(−125)lucter and the 5′ deletion mutants, pIFΔ(−116)lucter, pIFΔ(−103)lucter, pIFΔ(−98)lucter, pIFΔ(−97)lucter, pIFΔ(−92)lucter, pIFΔ(−82)lucter, pIFΔ(−78)lucter, and pIFΔ(−70)lucter have been previously described (King and Goodbourn, 1994). The linker scan mutants were constructed by generating a series of 3′ deletion mutants flanked by *Bgl*II linkers starting from the *Nco*I site in the human IFN-β promoter of pIFΔ(−116)lucter at −12 using the method of King and Goodbourn (1992). These mutants were characterized by sequencing and then recombined via their *Bgl*II linkers with the appropriate 5′ deletion mutants from our extensive collection (King and Goodbourn, 1992). The synthetic PRD multimer reporters, p(PRD IV)$_3$tkΔ(−39)lucter, p(PRD I/PRD III)$_5$tkΔ(−39)lucter and p(PRD II)$_5$tkΔ(−39)lucter, the IFN-responsive ISRE reporter p(9–27ISRE)$_4$tkΔ(−39)lucter, and the TK TATA only reporter, ptkΔ(−39)lucter, have been previously described (Visvanathan and Goodbourn, 1989; King and Goodbourn, 1994, 1998) as has the constitutive β-galactosidase transfection control reporter plasmid, pJATlac (Masson *et al.,* 1992). The lex-responsive luciferase reporter, p(lexOP)$_2$TKΔ(−105)luc, was a kind gift of Dr. Richard Treisman (Cancer Research, U.K.).

All expression in mammalian cells was achieved by subcloning into pEF.plink2 (Dr. Richard Treisman, Cancer Research, U.K.), a vector in which the test transcript is placed under the control of the mammalian EF1α promoter. The plasmids directing the expression of the V or P proteins of SV5, pEF.SV5-V, and pEF.SV5-P have been previously described (Didcock *et al.,* 1999b). Equivalent plasmids expressing the V genes of hPIV2, CPI−, CPI+, mci-2 (Young *et al.,* 2001), and Sendai virus were obtained using the same strategy [plasmids pEF.hPIV2-V, pEF.SV5-V(CPI−), pEF.SV5-V(CPI+), pEF.SV5-V(mci-2), and pEF.SeV-V, respectively]. pEF− plasmids directing the expression of C-terminal truncations of SV5 V protein were obtained by cloning *Nco*I-*Pst*I (amino acids 1–56; pEF.SV5-V[1–56]), *Nco*I-*Cla*I (amino acids 1–157; pEF.SV5-V[1–157]), or *Nco*I-*Sca*I (amino acids 1–174; pEF.SV5-V[1–174]) fragments between the *Nco*I site and a filled-in *Eco*RI site of pEFplink2. pEF− plasmids directing the expression of N-terminal truncations of SV5 V protein were obtained by cloning PCR-amplified fragments (incorporating *Nco*I and *Eco*RI sites into the 5′ and 3′ ends, respectively, of the SV5 V fragment) directly between the *Nco*I and *Eco*RI sites of pEFplink2 to generate pEF.SV5-V[20–222] (amino acids 20–222), pEF.SV5-V[85–222] (amino acids 85–222), and pEF.SV5-V[104–222] (amino acids 104–222). Point mutants were introduced into pEF.SV5-V using recombinant PCR to create pEF.SV5-V[C193A], pEF.SV5-V[C207A], and pEF.SV5-V[C214A]. All V mutated genes were sequenced (Lark Technologies) and a double-mutant pEF.SV5-V[C193Y/C214A] was obtained as a by-product of one of the mutagenesis experiments. The lexA DBD vector, pEF.mlexA, was constructed by recombinant PCR so as to obtain a DNA-binding-domain fragment (amino acids 1 to 202) of the bacterial lexA transcription factor containing a mutation in the cryptic nuclear localization sequence (Rhee *et al.,* 2000) and inserted into the *Nco*I site of pEF.plink2. The full-length ORF of IRF-3 flanked by *Nco*I (5′) and *Xba*I (3′) sites was obtained from MG-63 osteosarcoma cells by RT-PCR and was cloned in-frame to the lexA DBD of pEF.mlexA to give pEF.mlexA.IRF-3. The full-length cDNA of IRF-3 was also used to generate the C-terminal dominant interfering form of IRF-3 by subcloning the *Sca*I-*Xba*I fragment in-frame with the polylinker of pEF.plink2 to give pEF.IRF-3(132–427).

Viruses and cells

The W3 strain of SV5 (Choppin, 1964), rSV5VΔC (see accompanying article, He *et al.,* 2002), N100D (Young *et*

al., 2001), and canine parainfluenza virus strains, CPI+ and CPI− (Chatziandreou *et al.,* 2002), were propagated and titered in Vero cells. Sendai virus (strain Z) and NDV (Ulster 2c strain) were a kind gift from Dr. J. W. McCauley (Institute of Animal Health, Compton, U.K.) and were prepared by inoculation of 10-day-old embryonated chickens eggs with a high dilution of viral stock, followed by 48 h incubation at 35°C. The efficient IFN-β inducer stock of Sendai virus used in Fig. 1A was prepared by three serial passages of the above Sendai virus stock at a 1:10 dilution each passage (von Magnus, 1951a,b). Viral titers for Sendai virus and NDV were established by egg-infectious dose.

MG-63 human osteosarcoma cells (ATCC CRL 1427), human diploid fibroblast 2fTGH cells (Pelligrini *et al.,* 1989), 2fTGH/V cells (Andrejeva *et al.,* 2002), 293 transformed primary human embryonic kidney cells (ATCC CRL 1573), and mouse BALB/c cells (ATCC CCL 163) were maintained in Dulbecco's modified Eagle's medium (Biowhittaker Europe) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 100 U/ml penicillin/streptomycin and were routinely screened for mycoplasma contamination.

Inductions

Six-well dishes containing in excess of 10^6 cells per well (determined by trypan blue exclusion) were washed twice with PBS and then inoculated with virus at the given multiplicity of infection in 1 ml of DME + 2% FBS. Cells were incubated with gentle rocking for 1 h at 37°C and then the virus was removed and the medium replaced with 5 ml DME + 2% FBS until harvesting. Cells were either harvested for RNA analysis by acid-phenol/guanidinium isothiocyanate extraction or were scraped into 1 ml ice-cold PBS and pelleted for nuclear extract preparation. For induction by synthetic dsRNA, poly(I)-poly(C) (Amersham Biosciences) was either added directly to cells at a final concentration of 100 μg/ml or was transfected into cells using Lipofectamine (Invitrogen) under conditions specified by the manufacturer. TNFα (Preprotech) was added to cells at 10 ng/ml. Type I IFN (Intron A; Schering-Plough) was added to cells at 1000 IU/ml.

Protein analysis

To analyze protein levels by Western blotting, cells were washed twice with PBS, disrupted into Laemmli's loading buffer, sonicated, and boiled for 5 min. Polypeptides were separated by SDS–PAGE using 10% gels and transferred to PVDF membranes using semidry blotting. Filters were blocked and incubated with primary antibody under conditions specified by the manufacturers, and proteins detected by enhanced chemiluminescence using horseradish peroxidase conjugated sheep anti-mouse or donkey anti-rabbit IgG (Amersham Biosciences) as secondary antibodies. Primary antibodies used were as follows: SV5 P/V protein–anti-Pk mAb (Randall *et al.,* 1987); p65 and p50 (Mellits *et al.,* 1993); IRF-3 (Santa Cruz Biotechnology, Inc., catalog number sc-9082); STAT1 mAb (Transduction Laboratories, catalog number G16920).

Analysis of gene expression

Total cellular RNA was prepared using the acid phenol method from 9-cm-diameter dishes of confluent cultures of cells treated as indicated and analyzed by RNase protection as described previously using probes for human IFN-β and γ-actin (Goodbourn *et al.,* 1986). For reporter gene assays, lysates were prepared and analyzed for luciferase and β-galactosidase activity as previously described (King and Goodbourn, 1994).

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