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Induction of Interferon and Interferon Signaling Pathways by Replication of Defective Interfering Particle RNA in Cells Constitutively Expressing Vesicular Stomatitis Virus Replication Proteins[▽]

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We show here that replication of defective interfering (DI) particle RNA in HEK293 cells stably expressing vesicular stomatitis virus (VSV) replication proteins potently activates interferon (IFN) and IFN signaling pathways through upregulation of IFN-β promoter, IFN-stimulated response element (ISRE) promoter, and NF-κB promoter activities. Replication of DI particle RNA, not mere expression of the viral replication proteins, was found to be critical for induction of IFN and IFN signaling. The stable cells supporting replication of DI RNA described in this report will be useful in further examining the innate immune signaling pathways and the host cell functions in viral genome replication.

For most of the negative-strand RNA viruses, including vesicular stomatitis virus (VSV), rescue of infectious viruses by using full-length cDNA clones, as well as examination of the roles of *cis*-acting signals and *trans*-acting functions in genome replication and transcription, has been well documented (6, 17). However, in the majority of these systems, expression of the viral proteins from support plasmids in transfected cells is driven by T7 RNA polymerase supplied by infecting the cells with the recombinant vaccinia virus expressing the T7 polymerase (vv-T7). In such systems, due to the cytopathic effects of vaccinia virus, examination of the effects of replication of viral genomes, genomic analogs, or defective interfering (DI) particle genomes on host gene expression or the effects of host cell functions on viral genome replication and transcription has been difficult. Additionally, since VSV and many other negative-strand RNA viruses exhibit overt cytopathic effects in infected cells due to the expression of viral proteins, it is not possible to study the long-term effects of viral genome replication and/or transcription on host cell functions. Therefore, development of cells capable of supporting replication and maintenance of the viral genomes on a long-term basis is highly desirable. Such systems could alleviate the limitations described above and allow us to study the interplay between the host and viral functions in the absence of the viral M and G proteins.

Efficient replication of DI particle RNA in cells constitutively expressing VSV replication proteins. The N, P, and L proteins of VSV are the replication proteins. To generate stable cell lines expressing these proteins, we constructed a plasmid (pc-NPeGFPL) in which the coding sequences for N, PeGFP (a functional P protein fused with an enhanced green fluorescent protein [eGFP] sequence) (7), and L are each placed under the control of separate cytomegalovirus (CMV) promoter and poly(A) sequences (Fig. 1A). Human embryonic kidney (HEK293) cells were transfected with the pc-NPeGFPL plasmid, and the cells were treated with G418 at 48 h posttransfection. Although several cell clones expressing all three viral proteins were isolated, we selected three cell clones (293-NPeGFPL cell clone no. 204, 206, and 211) for further studies. Immunofluorescent staining of the cells showed that all three viral proteins were expressed in the majority of the cells in the culture (Fig. 1B). Examination of expression of the viral proteins by radiolabeling, immunoprecipitation, and SDS-PAGE analyses revealed that each of these cell clones expressed readily detectable levels of the three viral proteins (Fig. 1C).

To test the functionality of the viral proteins, we infected the stable cell clones with DI particles of VSV and examined replication of DI RNA. Results showed that each of the cell clones supported efficient DI RNA replication (Fig. 2A). The levels of DI RNA replication in these cell clones (Fig. 2A, lanes 3 to 5) were comparable to that seen in the vv-T7 system (Fig. 2A, lane 1) described previously (19). We had shown previously that maintaining a certain molar ratio of the three VSV replication proteins is important for optimal replication of DI particle RNAs (19). The molar ratios of the VSV proteins in the three cell clones examined are similar to those obtained in cells infected with VSV expressing PeGFP (Fig. 1C). Furthermore, after 6 months of culture spanning over 60 passages, the cell growth characteristics were similar to those of the cells not expressing the viral proteins and the cell lines still supported efficient replication of DI RNA (Fig. 2B). The stable cells were also capable of supporting assembly and release of infectious DI particles upon coexpression of M and G proteins (Fig. 2C), confirming our previous findings obtained using the vv-T7 system (18).

These results suggest that the replication proteins of VSV

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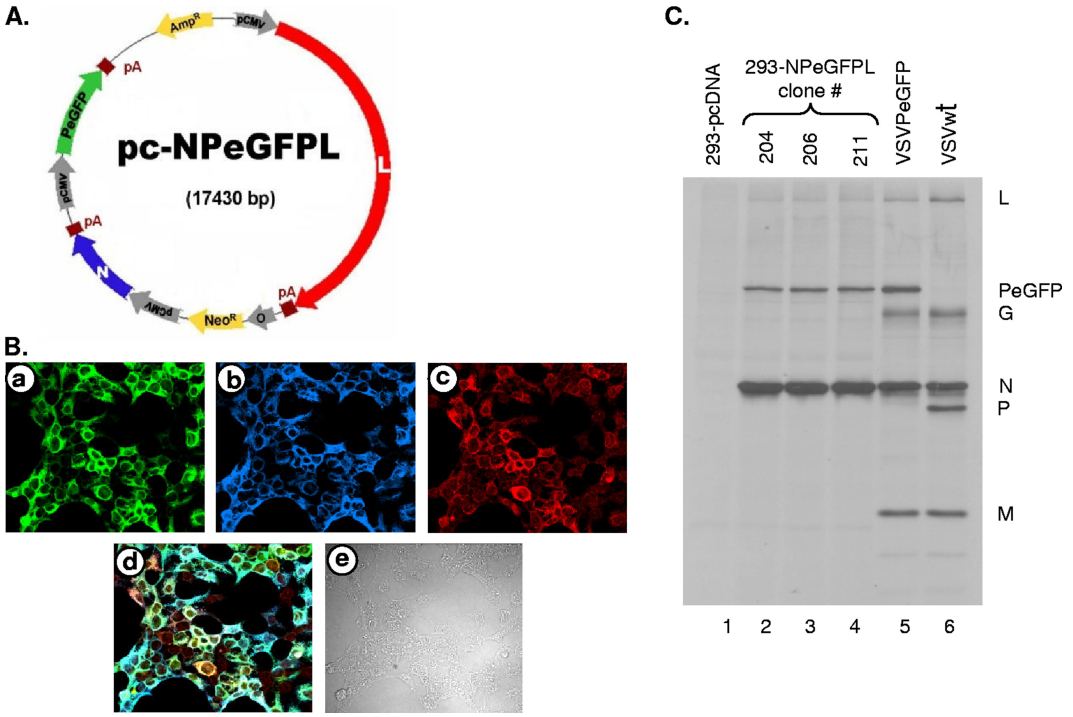

FIG. 1. (A) Schematic representation of the plasmid pc-NPeGFPL, expressing the VSV N, PeGFP, and L proteins. Coding sequences for N, PeGFP, and L were cloned under the control of a CMV immediate-early promoter and poly(A) signal in the pcDNA 3.1-neo vector backbone. The CMV promoter and poly(A) signal sequences for N and PeGFP were derived from the pHygEGFP vector. Coding sequences for N, PeGFP, and L are shown in blue, green, and red, respectively. An ampicillin resistance (Amp^r) gene, a neomycin resistance (Neo^r) gene, a simian virus 40 (SV40) promoter and origin of replication (O), the CMV promoter (pCMV), and polyadenylation signal sequences (pA) are shown. Further details of the construction of the pc-NPeGFPL plamsid can be obtained upon request. (B) Detection of VSV proteins in stable cell clones by immunofluorescence microscopy. Cells from stable cell clone no. 204 were simultaneously immunostained with anti-N monoclonal antibody (10G4) and anti-L peptide antibody and subsequently with goat anti-mouse Alexa 647 and donkey anti-rabbit Alexa 594 secondary antibodies. Cells were mounted and observed under an inverted confocal microscope. (a) Epifluorescence microscopy image of cells expressing PeGFP; (b and c) immunofluorescence images of the same cells expressing the N protein (pseudoblue color) (b) and the L protein (red) (c). Panel d shows the merged images from panels a, b, and c. A differential interference contrast (DIC) image of the cells is shown in panel e. (C) Expression of VSV proteins in different cell clones. G418-resistant stable cell clones 204, 206, and 211 (lanes 2 to 4), as well as stable 293-pcDNA cells transfected with the pcDNA 3.1 vector (lane 1), were radiolabeled with Expre^{35}S^{35}S protein labeling mix. The radiolabeled proteins were immunoprecipitated with anti-VSV antibody, analyzed by SDS-PAGE, and detected by fluorography. Radiolabeled proteins from HEK293 cells infected with VSV expressing PeGFP (lane 5) or wild-type VSV (VSVwt) (lane 6) are also shown. Viral proteins are identified to the right.

can be constitutively expressed without any adverse effects on cell growth and viability and that they can form functional polymerase complexes to support replication of RNA and assembly of infectious DI particles.

DI RNA replication activates the IFN-β promoter. Activation of double-stranded RNA (dsRNA) signaling and subsequent interferon (IFN) production by both VSV and VSV DI particles has been well documented (4, 13, 15). VSV M protein is known to shut off host cellular mRNA synthesis and nucleocytoplasmic transport of RNAs (1, 8, 10, 26) and thereby inhibit IFN production (9). The availability of a vaccinia virus-free system that supports DI RNA replication provides us a unique opportunity to examine the effects of DI RNA on host cell functions, particularly the activation of innate immune signaling pathways in the absence of the viral M protein. DI particle infection of the control cell line not expressing the viral proteins (293-pcDNA cells) yielded only basal-level induction (1.3-fold) of IFN-β promoter activity compared to that in uninfected cells. However, in cells expressing the replication proteins (293-NPeGFPL cells), DI particle infection and subsequent DI RNA replication resulted in activation of the IFN-β promoter by as much as 67-fold over that in uninfected cells and 50-fold over that in DI particle-infected control 293-pcDNA cells (Fig. 3A). Additionally, activation of the IFN-β promoter in 293-NP cells (expressing only the N and P proteins) infected with DI particles was not detected (Fig. 3B). We could demonstrate replication of DI RNA only in 293-NPeGFPL cells and not in 293-pcDNA control cells or in 293-NP cells (Fig. 3A and B). These results suggest that replication of DI RNA, not entry and uncoating of DI RNA or mere expression of the viral replication proteins *per se*, activates the IFN-β promoter.

A kinetic analysis of IFN-β promoter activation further revealed that, indeed, only replication of DI RNA induced the activation of the IFN-β promoter. In 293-pcDNA cells, DI particle infection resulted in only basal-level IFN-β promoter activity, while in 293-pcNPeGFPL cells infected with DI particles, IFN-β promoter activity increased with time postinfection (Fig. 3C). The level of DI RNA at 2 h postinfection (as determined by reverse transcription-PCR [RT-PCR] analysis) was below the level of detection (Fig. 3C, bottom), but a significant increase (7-fold) in IFN-β promoter activity over

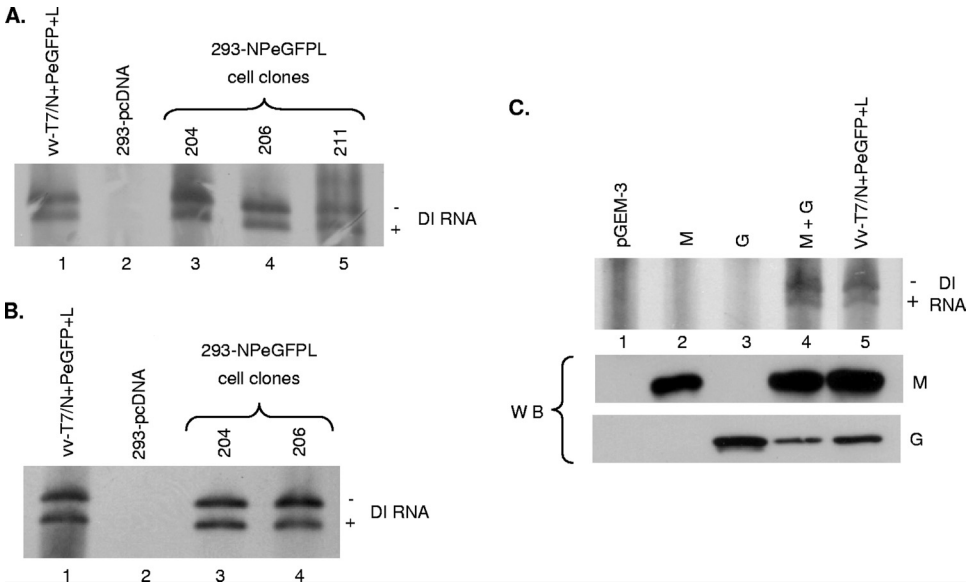

FIG. 2. Replication of DI particle RNA genomes in 293-NPeGFPL cell clones. (A) Cell clones 204, 206, and 211 (lanes 3, 4, and 5, respectively) or 293-pcDNA control cells (lane 2) grown in 60-mm dishes were infected with DI particles and radiolabeled with [^{3}H]uridine. Radiolabeled DI RNA was recovered from cell extracts by immunoprecipitation with anti-N antibody, analyzed by agarose-urea gel electrophoresis, and detected by fluorography as described previously (19). Lane 1 shows DI RNA replication products obtained from BHK-21 cells by using the vv-T7 system. DI RNAs (negative and positive sense) are identified to the right. (B) DI RNA replication in stable cell clones after 60 passages (approximately 6 months) in culture. The experimental protocol was the same as that described in the legend to panel A. (C) Rescue of DI particles from cells supporting DI RNA replication following expression of M and G proteins. Cells from clone 206 were infected with DI particles. After 6 h of infection, the cells were infected with vaccinia virus vTF7-3 and were subsequently transfected with the pGEM-3 vector (lane1), the plasmid encoding the M protein (lane 2), the plasmid encoding the G protein (lane 3), or the plasmids encoding the M and G proteins (lane 4) as described previously (18). Supernatants from cells collected 12 to 14 h posttransfection were used to infect fresh cells of stable 293-NPeGFPL clone 206. The cells were radiolabeled with [^{3}H]uridine and processed as described in the legend to panel A to detect DI particles by using an RNA replication assay. Lane 5 shows the DI RNA replication products obtained from BHK-21 cells by using the vv-T7 system as described in the legend to panel A. DI RNAs (negative and positive sense) are identified to the right. Parallel samples were analyzed for expression of M protein and G protein by Western blotting (WB) with appropriate antibodies, and results are shown in the bottom panels.

that in similarly infected control cells could be readily seen. As the time postinfection with DI particles increased, IFN-β promoter activity also increased, concomitant with increased DI RNA replication (Fig. 3C, bottom). Since in cells expressing only the viral N and P proteins and infected with DI particles (a condition under which DI RNA replication does not occur), activation of the IFN-β promoter was not observed, the results of our studies demonstrate that IFN-β promoter activation requires DI RNA replication. Together, the findings that the mere expression of the three replication proteins did not activate the IFN-β promoter and that we could not detect RNP formation with cellular RNAs in the presence of N, PeGFP, and L proteins (data not shown) strengthen our conclusion that only replication of DI RNA induces IFN-β.

DI RNA replication activates the ISRE promoter and the NF-κB promoter. In contrast to the IFN-β promoter, the IFN-stimulated response element (ISRE) promoter contains two ISREs which can be activated by IFN regulatory factor 3 (IRF3), IRF7, or both (22). To elucidate the effect of DI RNA replication upon IRF3-mediated signaling, we used an ISRE promoter-driven luciferase reporter plasmid. DI particle infection of 293-pcDNA control cells led to a meager 1.7-fold increase in luciferase activity over that in the corresponding uninfected cells (Fig. 4A). However, DI particle infection of 293-NPeGFPL cells resulted in greater than 200-fold induction of luciferase activity over that in the uninfected 293-NPeGFP cells (Fig. 4A). Activation of IFN-β gene transcription requires coordinate actions of IRF3, NF-κB, and ATF-2/c-Jun transcription factors (27). Nearly 40-fold induction of NF-κB promoter activity in 293-NPeGFPL cells infected with DI particles compared to that in similarly infected 293-pcDNA cells was observed (Fig. 4B). For direct verification of IFN-stimulated gene expression, we examined the expression of IFN-stimulated gene 56 (ISG56), one of the viral stress-inducible genes that are induced by IFNs, dsRNA, and virus infections (24). Results (Fig. 4C) showed strong induction of ISG56 protein expression in 293-NPeGFPL cells infected with DI particles (lane 4), whereas in uninfected 293-NPeGFPL cells and in 293-pcDNA cells with or without DI particle infection, ISG56 protein was undetectable. In each of the above-described studies, DI RNA replication products were readily detected (data not shown). Taken together, the results from the above-described studies show that replication of DI RNA in 293-NPeGFPL cells potently activates IFN and IFN signaling.

In summary, we report here the establishment of stable cells expressing the VSV replication proteins. Although a stable cell line expressing Sendai virus replication proteins has been described previously (28), this is the first description of a cell line constitutively expressing the VSV replication proteins. Using this cell line, we have shown that replication of DI RNA activates IFN and IFN signaling pathways. It has been reported previously that snap-back DI (±) particles of VSV activate

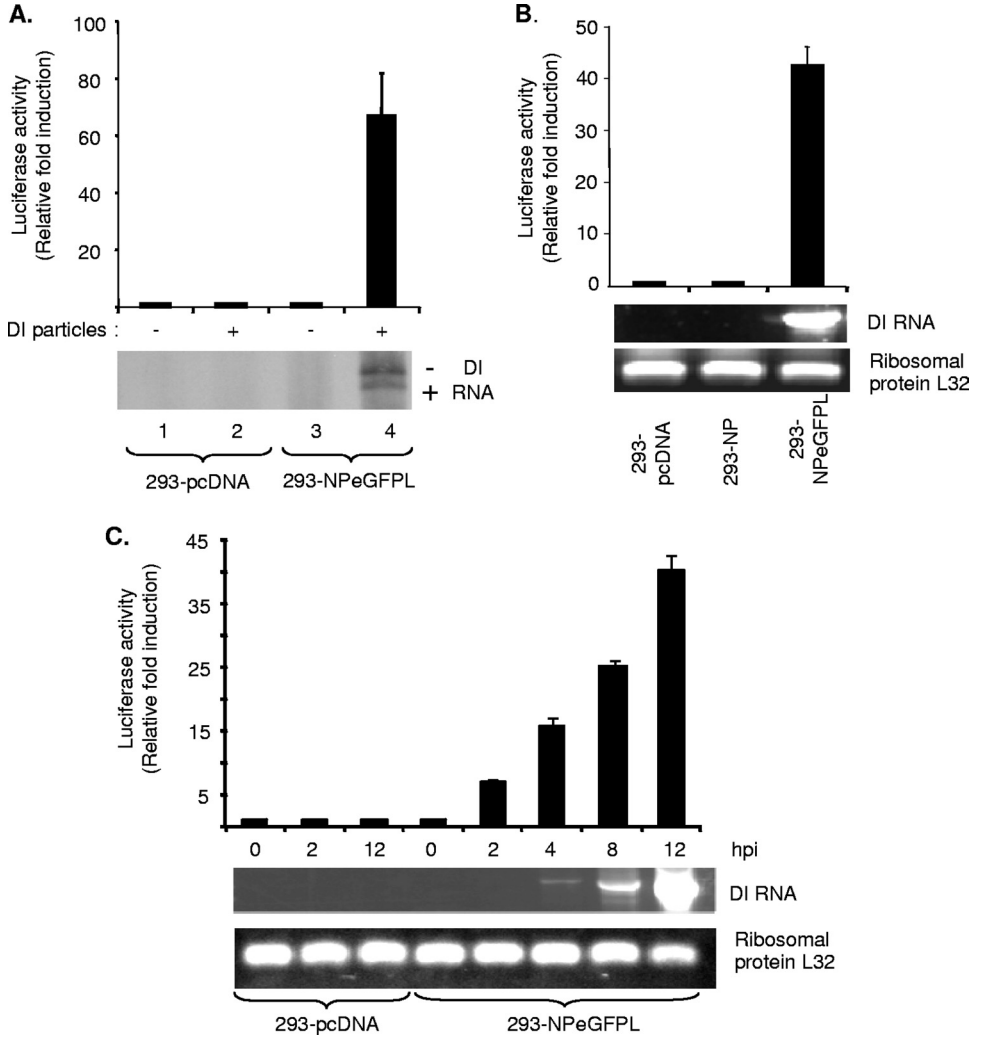

FIG. 3. Induction of IFN by replication of DI RNA. (A) Stable 293-NPeGFPL cell clone 206, expressing N, PeGFP, and L proteins, or 293-pcDNA control cells, not expressing the viral proteins, were cotransfected with 0.4 μg of IFN-β–Luc along with 10 ng of pRL-TK (a *Renilla* luciferase plasmid from Promega, which served as an internal control for transfection efficiency). At 24 h posttransfection, the cells were either mock infected or infected with DI particles. At 16 h postinfection, the cells were lysed in buffer (Promega), and a luciferase assay was performed using a Dual-Luciferase assay kit (Promega). Luciferase activity results were expressed as the relative induction (*n*-fold) over the level of activity in uninfected 293-pcDNA control cells after normalization with respect to *Renilla* luciferase activity. Data from three independent experiments are presented, with standard deviations indicated by error bars. [^{3}H]uridine-radiolabeled samples from a similar experiment were also analyzed for replication of the DI RNA genome as described in the legend to Fig. 2A, and results are shown in the lower panel. DI RNAs (negative and positive sense) are identified to the right. (B) (Top) HEK293 cells stably expressing N and P proteins or N, PeGFP, and L proteins were transfected with luciferase reporter plasmids as described in the legend to panel A and then infected with DI particles. Luciferase activity in the samples was analyzed at 12 h postinfection with DI particles as described in the legend to panel A and expressed as the change (*n*-fold) from that in 293-pcDNA control cells. Data from three independent experiments are presented, with standard deviations indicated by error bars. (Bottom) Parallel sets of samples were analyzed for DI RNA replication products by RT-PCR. As a control for similar amounts of total RNA being used in the analysis, RT-PCR products for ribosomal protein L32 are also shown. Representative data for DI RNA replication and ribosomal protein L32 mRNA quantitation are shown. (C) Kinetics of IFN-β promoter activation. The experimental protocol was the same as that described in the legend to panel B, except that the samples were collected at various times (0, 2, and 12 h for 293-pcDNA cells and 0, 2, 4, 8, and 12 h for 293-NPeGFPL cells) postinfection with DI particles. Luciferase activity in the samples was measured and expressed as the change (*n*-fold) from that in 293-pcDNA control cells. Replication of DI RNA was measured as described in the legend to panel B. hpi, hours postinfection.

IFN signaling. Furthermore, a preexisting molecule in the snap-back DI genome (presumably a dsRNA structure) has been proposed to be responsible for induction of IFN, as heat inactivation or UV treatment of DI particles did not inhibit IFN induction in chicken embryo fibroblasts and mouse L cells (15, 23). Contrary to these findings, our results revealed that replication of DI particle T RNA with a panhandle-type DI genome (16) is required for IFN-β activation as well as IFN signaling. Mere entry and uncoating of DI particles or expression of only the viral replication proteins was not sufficient to induce IFN-β or IFN signaling. VSV DI genomes are synthesized in the form of nucleocapsid, and possible formation of dsRNA structures in infected cells has not been reported. Viral replication intermediates like dsRNA or single-stranded RNAs

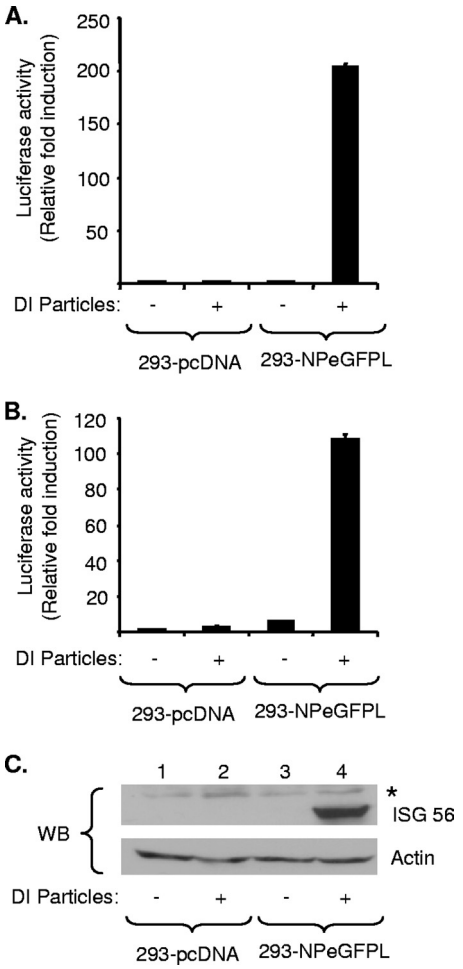

FIG. 4. (A) Activation of the ISRE promoter by DI particle infection. The experiment was conducted as described in the legend to Fig. 3A but using the ISRE promoter-driven luciferase gene. (B) Activation of the NF-κB promoter by DI particle infection. The experiment was conducted as described in the legend to Fig. 3A but using the NF-κB promoter-driven luciferase gene. (C) For detection of endogenous ISG56 protein, cell lysate was prepared in lysis buffer. Equal amounts of total protein were separated by SDS–10% PAGE, transferred onto polyvinylidene difluoride membrane, and probed with polyclonal rabbit anti-ISG56 antibody. A nonspecific band detected with anti-ISG56 antibody is identified with an asterisk. To serve as a loading control, the level of actin in each sample was obtained after stripping and reprobing the same membrane with antiactin antibody.

(ssRNAs) with triphosphorylated 5′ ends are sensed by both cytoplasmic sensors, e.g., RIG-I, MDA-5, and Nod2 (5, 21), and endosomal receptors (Toll-like receptor 3 [TLR3], TLR7/TLR8, and TLR9) (25). However, HEK293 cells are deficient in TLRs and Nod2. Therefore, in these cells, IFN induction and signaling may be mediated by RIG-I and/or MDA-5 through recognition of DI RNA. RIG-I has been shown previously to be involved in detection of rhabdoviruses and paramyxoviruses, resulting in subsequent induction of IFN responses (11, 14, 20). MDA-5 has also been shown to be involved in recognition of measles virus (12) and Sendai virus (29) DI particles. It would therefore be interesting to examine whether VSV DI RNA replication activates IFN through involvement of one or more of these cytoplasmic sensors.

Since infection of cells with VSV and many other negative-strand RNA viruses results in cytopathogenesis and cell death, it is difficult to study the long-term effects of virus replication on host cell functions in the context of virus infection. VSVs encoding mutant M proteins have been used to study IFN activation (2, 3, 14), but these viruses are also cytopathic. Therefore, results from studies of the effects of VSV RNA replication on IFN activation and signaling in the context of VSV infection are difficult to assess. Our system will provide the opportunity to examine the effects of viral genome replication on host cell functions in the absence of the cytopathogenic effects of VSV M and G proteins.

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