Influenza A Virus Panhandle Structure Is Directly Involved in RIG-I Activation and Interferon Induction

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ABSTRACT

Retinoic acid-inducible gene I (RIG-I) is an important innate immune sensor that recognizes viral RNA in the cytoplasm. Its nonself recognition largely depends on the unique RNA structures imposed by viral RNA. The panhandle structure residing in the influenza A virus (IAV) genome, whose primary function is to serve as the viral promoter for transcription and replication, has been proposed to be a RIG-I agonist. However, this has never been proved experimentally. Here, we employed multiple approaches to determine if the IAV panhandle structure is directly involved in RIG-I activation and type I interferon (IFN) induction. First, in porcine alveolar macrophages, we demonstrated that the viral genomic coding region is dispensable for RIG-I-dependent IFN induction. Second, using *in vitro*-synthesized hairpin RNA, we showed that the IAV panhandle structure could directly bind to RIG-I and stimulate IFN production. Furthermore, we investigated the contributions of the wobble base pairs, mismatch, and unpaired nucleotides within the wild-type panhandle structure to RIG-I activation. Elimination of these destabilizing elements within the panhandle structure promoted RIG-I activation and IFN induction. Given the function of the panhandle structure as the viral promoter, we further monitored the promoter activity of these panhandle variants and found that viral replication was moderately affected, whereas viral transcription was impaired dramatically. In all, our results indicate that the IAV panhandle promoter region adopts a nucleotide composition that is optimal for balanced viral RNA synthesis and suboptimal for RIG-I activation.

IMPORTANCE

The IAV genomic panhandle structure has been proposed to be an RIG-I agonist due to its partial complementarity; however, this has not been experimentally confirmed. Here, we provide direct evidence that the IAV panhandle structure is competent in, and sufficient for, RIG-I activation and IFN induction. By constructing panhandle variants with increased complementarity, we demonstrated that the wild-type panhandle structure could be modified to enhance RIG-I activation and IFN induction. These panhandle variants posed moderate influence on viral replication but dramatic impairment of viral transcription. These results indicate that the IAV panhandle promoter region adopts a nucleotide composition to achieve optimal balance of viral RNA synthesis and suboptimal RIG-I activation. Our results highlight the multifunctional role of the IAV panhandle promoter region in the virus life cycle and offer novel insights into the development of antiviral agents aiming to boost RIG-I signaling or virus attenuation by manipulating this conserved region.

The retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) represent two major RIG-I-like receptors (RLRs) that provide an important cytoplasmic line of defense against RNA viruses in mammalian cells. These two DEXD/H-box RNA helicases share a similar domain architecture and detect distinct but overlapping virus families (1–3). Activation of either receptor relays the antiviral signals to mitochondrial antiviral signaling protein (MAVS), interferon (IFN) regulatory factors (IRFs), and NF- κ B, leading to the production of type I IFNs (4). In recent years, extensive research has shed light on the mechanisms of action of RIG-I and its ligand characteristics. It is now widely recognized that canonical RNA elements required for optimal RIG-I activation include a blunt-ended RNA duplex and a 5'-triphosphate moiety (5–7). Additionally, some noncanonical RNA structures are also potent in RIG-I activation, such as a poly(U/UC) tract, 3'-monophosphate, AU-rich regions, and the most recently discovered 5'-diphosphates (8–13).

Recent studies on negative-strand RNA viruses spanning *Bunyaviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, and *Rhabdoviridae* have proposed that the 5'-triphosphate-containing pan-

handle structure formed from viral RNA (vRNA) is required for RIG-I activation (5, 14–17). The double-strandedness of the panhandle structure is derived from the self-complementarity of the viral genome extremities (5). This structure is also present in the defective interfering (DI) RNAs derived from aberrant viral replication (18). Unlike a fully complementary double-stranded region (such as that from Sendai virus DI RNA), the viral genomic panhandle structures are only partially complementary, contain-

Received 27 January 2015 Accepted 21 March 2015

Accepted manuscript posted online 25 March 2015

Citation Liu G, Park H-S, Pyo H-M, Liu Q, Zhou Y. 2015. Influenza A virus panhandle structure is directly involved in RIG-I activation and interferon induction. *J Virol* 89:6067–6079. doi:10.1128/JVI.00232-15.

Editor: D. S. Lyles

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doi:10.1128/JVI.00232-15

ing non-Watson-Crick base pairs, mismatches, and bulge elements.

The influenza A virus (IAV) genomic panhandle structure was visualized microscopically 2 decades ago, and its RNA secondary structure has been experimentally determined (19–22). It is well-known as the viral promoter region, and its nucleotide composition has been studied extensively with regard to the promoter activity (23–25). However, its role in RIG-I activation remains unclear. Two recent studies characterizing physiological RIG-I agonists during IAV infection demonstrated that RIG-I associates with both full-length viral genomes (preferentially shorter genome segments) and DI genomes (15, 18). Both RIG-I ligands act in concurrence with regard to the genomic panhandle structure, indicating its involvement in RIG-I activation. Compared to other viruses, the conserved IAV panhandle structure among all eight genome segments contains a relatively shorter double-stranded region (around 16 bp). It can be further divided into three elements: a proximal stem (5' positions 1 to 9 and 3' positions 1 to 9) and a distal stem (5' positions 11 to 16 and 3' positions 10 to 15) linked through an unpaired adenosine (5' position 10) (26). While the distal stem is fully complementary, the base pairing of the proximal stem is imperfect, containing two wobble base pairs (G · U) and one mismatch (A · C) (21). As such, the IAV panhandle structure appears not to be an optimal RIG-I ligand. It is therefore of great interest to analyze the impact of this imperfect complementarity on RIG-I activation.

In this study, we examined if the IAV panhandle structure is directly involved in RIG-I activation and IFN induction. We provided direct evidence that the IAV panhandle structure is sufficient for RIG-I activation *in vitro* and in primary alveolar macrophages (PAMs). To gain further insight into the contributions of the wobble base pairs and mismatch in the panhandle proximal stem and the unpaired adenosine to RIG-I activation, we eliminated these elements by introducing panhandle-stabilizing mutations into the wild-type (WT) panhandle structure. We found that panhandle variants harboring these mutations displayed stronger activity in RIG-I activation and IFN induction. Meanwhile, monitoring the impact of these mutations on viral promoter activity revealed that viral replication was moderately affected, whereas viral transcription was impaired dramatically. These results indicated that the WT panhandle promoter region adopts a nucleotide composition that is optimal for balanced RNA synthesis and sub-optimal for RIG-I activation.

MATERIALS AND METHODS

Ethics statement. All animals used in this research project were cared for and used in accordance with the Guidelines of the Canadian Council on Animal Care, the Regulations of the University of Saskatchewan Committee on Animal Care and Supply, and in accordance with the “3R principles.” The experimental procedures were approved by the University of Saskatchewan Animal Research Ethics Board (Animal Use Protocol 20030100).

Cells and viruses. PAMs were isolated from lung lavage fluid samples from 4- to 5-week-old simian immunodeficiency virus (SIV)-seronegative piglets according to a standard protocol described previously (27). The isolated PAMs were characterized by flow cytometric analysis staining with fluorescein isothiocyanate-conjugated mouse anti-pig macrophage monoclonal antibody (MAb) clone BA4D5 (AbD Serotec) and cultivated in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS), 1% HEPES, 50 µg/ml gentamicin, and 1× Antibiotic-Antimycotic (Invitrogen). Madin-Darby canine kidney (MDCK) and human embryonic

kidney 293T (HEK293T) cells were maintained in minimal essential medium (MEM) and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, respectively. Chicken embryonic fibroblast DF-1 cells were maintained in DMEM at 39°C. Influenza A virus H1N1 strains A/Swine/Saskatchewan/18789/02 (SIV/Sk02), A/Halifax/210/09 (Halifax210) and A/Texas/36/91 (Tx91) were propagated in MDCK cells. A/Puerto Rico/8/34 (PR8) was propagated in 11-day-old embryonated chicken eggs. An NS1 mutant virus (SIV/Sk02 NS1 1-99) containing only amino acids 1 to 99 of the N-terminal NS1 was constructed by reverse genetics in the background of SIV/Sk02 and was propagated in Vero cells.

Plasmid construction. The set of pPOLI plasmids (in a pDZ vector) carrying each of the eight RNA segments of Tx91 was generously provided by Adolfo Garcia-Sastre (Mount Sinai School of Medicine, New York, NY). The pPOLI-NP-LUC plasmid was constructed by insertion of the firefly luciferase gene flanking the NP-carrying region in an antisense orientation between the human RNA polymerase I promoter and terminator (28). All panhandle derivatives were constructed in the pPOLI-NP-LUC backbone by site-directed mutagenesis (Stratagene) and confirmed by DNA sequencing. Truncations in the coding region of pPOLI-NP-LUC or pHH21-Tx91-NA were achieved by overlapping PCR.

RNA methods. *In vitro* transcription (IVT) of short hairpin RNA mimicking the authentic IAV panhandle structure and its variants was performed using the MEGAshortscript T7 transcription kit (Ambion) per the manufacturer's instructions. The DNA templates (sequences are available upon request) were modified with C-2'-methoxys at the last two nucleotides of the 5' termini and PAGE purified (Sigma) to ensure defined 3' ends of the RNA products (29). All synthesized RNA constructs were purified on 20% denaturing polyacrylamide gels and recovered by using a ZR small RNA PAGE recovery kit (Zymo Research). Recovered RNA was diluted in 1× annealing buffer (10 mM Tris-HCl [pH 7.8] and 100 mM NaCl) and reannealed at low concentration in a PCR machine by heating to 95°C for 5 min and cooling to 4°C at 0.1°C/s. Viral RNA of four H1N1 strains was extracted from sucrose gradient-purified virions by using an RNeasy minikit (Qiagen). Dephosphorylation of viral RNA was carried out using calf intestinal alkaline phosphatase (CIAP; New England BioLabs [NEB]). All purified viral RNAs were visualized on a 2.8% denaturing polyacrylamide gel and use of silver stain. Poly(I-C) was purchased from Sigma. A synthetic 19-mer 5' ppp-double-stranded RNA (dsRNA) and its control dsRNA were obtained from Invivogen. Small interfering RNA (siRNA) duplexes targeting porcine RIG-I, Toll-like receptor 3 (TLR3), and TLR7 were purchased from Qiagen. (Sequences are available upon request.) Off-target siRNA was also obtained from Qiagen. As indicated, transfection of viral RNA, reconstituted RNA, or siRNA was performed using either Lipofectamine 2000 or Lipofectamine RNAiMAX reagents (Invitrogen) per the manufacturer's instructions.

Protein expression and purification. The purification of human RIG-I was performed as described elsewhere with modification (30). Briefly, the pET-50b-hRIG-I construct (courtesy of Sun Hur, Harvard Medical School, Boston, MA) was expressed in *Escherichia coli* BL21(DE3) cells at 18°C for 20 h following induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were lysed at 35,000 lb/in² in the binding buffer (50 mM Tris [pH 7.5], 500 mM NaCl, 10% glycerol, 20 mM imidazole, 1 mM dithiothreitol [DTT]), and the protein was purified by using Ni-Sepharose 6 Fast Flow (GE Healthcare). The monomeric RIG-I was then separated from the oligomeric contaminants by using Superdex 200 10/300 GL for gel filtration, and the NusA tag was cleaved with human rhinovirus 3C protease (EMD Millipore) at 4°C for 16 h in the cleavage buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM DTT). The NusA tag was further removed by using a HiTrap heparin high-performance column (GE Healthcare). The protein was concentrated to 1 mg/ml in the storage buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM DTT, 20% glycerol), aliquoted, and stored at –80°C.

EMSA. For the electrophoretic mobility shift assay (EMSA), reannealed IAV panhandle RNA and its variants (5 pmol) were incubated with increasing amounts of purified RIG-I (0 to 15 pmol) in a total of 25 µl

helicase buffer (20 mM Tris-HCl [pH 7.8], 1.5 mM MgCl₂, 1.5 mM DTT) at 37°C for 30 min. The reaction was stopped by adding the gel loading dye Purple (6×), without SDS (NEB), and the mixture was resolved on a 4 to 20% Mini-Protein Tris-buffered EDTA gel (Bio-Rad). The gel was visualized by SYBR gold staining, and the densitometric analysis was performed using Image J (NIH). Data were fitted to the Hill slope equation for one-site specific binding, and the dissociation constant (K_d) and Hill coefficient (n) were calculated using GraphPad Prism 6.

ATPase activity assay. A RIG-I ATPase activity assay was performed as previously described with minor modifications (31). Purified RIG-I (10 pmol) was incubated with increasing amounts of reannealed RNA (0 to 2.5 pmol) or purified viral RNA (with or without CIAP treatment) in a total of 25 µl helicase buffer at 37°C for 30 min. ATP (Invitrogen) was then supplemented to a final concentration of 1 mM. Reaction mixtures were further incubated at 37°C for 15 min and developed with 100 µl of Biomol Green reagent (Enzo Life Sciences) for 5 min at room temperature. Free phosphate quantitation was obtained via a standard curve by measuring the optical density at 620 nm. Data were fitted to the Michaelis-Menten equation, and the Michaelis-Menten constant (K_m) was calculated using GraphPad Prism 6.

Luciferase reporter assay. The luciferase reporter assay was performed as previously described with minor modifications (32). Briefly, UMNSAH/DF-1 cells, which do not express RIG-I endogenously, were grown in a 6-well plate (2.5×10^5 cells/well) and transfected with 0.5 µg of pcDNA-dRIG-I or empty vector, 1 µg of pGL3-chIFN-β (courtesy of Katharine E. Magor, University of Alberta, Edmonton, Canada), and 0.05 µg of pTK-rLuc for 30 h. The transfected cells were then lifted and seeded into a 48-well plate at a density of 6×10^4 cells/well. After 16 h, reannealed panhandle RNA (20 pmol) or the synthetic 19-mer 5' ppp-dsRNA and 5' OH-dsRNA (Invivogen) were transfected using Lipofectamine RNAiMAX reagent (Invitrogen); the relative luciferase activity was determined at 20 h posttransfection (p.t.) by using the Dual-Luciferase Reporter assay system (Promega).

RNP reconstitution and strand-specific RNP pulldown. RNP reconstitution was performed as described elsewhere (15). Briefly, 1 million HEK293T cells were cotransfected with four RNA polymerase II (Pol II)-driven plasmids expressing each of the viral RNA-dependent RNA polymerase (RdRp) subunits (PB2, PB1, and PA) and NP, as well as a Pol I-driven plasmid expressing either wild-type mimetic vRNA or derivatives by using TransIT-LT1 reagent (Mirus). Cells were harvested at 48 h p.t., and total RNA was extracted using an RNeasy Plus minikit (Qiagen). Strand-specific RNP isolation was performed as described elsewhere with minor modifications (33). Briefly, the pPOLI-NA (A/WSN/33) plasmid carrying a vRNA-PP7 tag (courtesy of Ervin Fodor, Sir William Dunn School of Pathology, University of Oxford) was used in place of pPOLI-NP-LUC for RNP reconstitution. Plasmid expressing the PP7 coating protein (pcDNA-PP7CP-Strep; courtesy of Ervin Fodor; permission obtained from J. Robert Hogg, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) was cotransfected to capture the reconstituted vRNP. Cells were harvested at 60 h p.t. and subjected to affinity purification using Strep-Tactin Superflow resin (IBA). The purified RNP complex was confirmed by immunoblotting for viral RdRp and NP, and RNA was extracted, quantified by quantitative reverse transcription-PCR (qRT-PCR), and tested for immunostimulatory activity in PAMs.

Enzyme-linked immunosorbent assay (ELISA). Supernatants from transfected PAMs were harvested at 24 h p.t., and IFN-α production was detected as previously described with modifications (34). Briefly, 96-well Immulon 2HB microtiter plates (Thermo) were coated with mouse anti-recombinant porcine IFN-α K9 Mab (2700-1; R&D Systems) at a concentration of 1 µg/ml in coating buffer overnight at 4°C and blocked with blocking buffer (Tris-buffered saline containing 0.05% Tween 20 and 1% nonfat milk) for 1 h. An IFN-α standard and samples diluted in blocking buffer were applied into each well and incubated for 2 h. Biotinylated mouse anti-recombinant porcine IFN-α F17 Mab (27105-1) was then

added to wells at a concentration of 0.25 µg/µl and the mixture was incubated for 1 h. After subsequent incubation with streptavidin-alkaline phosphatase (Jackson) for 1 h, plates were developed by adding 1 mg/ml PNPP in PNPP buffer. Reactions were stopped by the addition of 0.3 M EDTA, and the optical density at 405 nm with reference at 490 nm was measured with an xMark microplate reader (Bio-Rad). The IFN-α concentrations in the samples were determined from the standard curve.

Strand-specific quantitative real-time PCR. Absolute quantitation of the viral mimetic vRNA, cRNA, and mRNA in the total reconstituted RNA was performed using the HotStart RT with tagged primer method previously described (35). Briefly, RNA standards for viral mimetic vRNA, cRNA, and mRNA were synthesized by *in vitro* transcription using T7 promoter-containing PCR products amplified from pPOLI-NP-LUC plasmid (MEGAscript T7 transcription kit; Ambion). The numbers of molecular copies of synthetic RNA standards were determined by using the corresponding size of the RNA transcripts (i.e., 1,747 nucleotides [nt] for vRNA and cRNA; 1,742 nt for mRNA). Two hundred nanograms of total reconstituted RNA was reverse transcribed using 10 pmol of 5'-end-tagged RT primers in the presence of 32.5% saturated trehalose and using SuperScript III reverse transcriptase (Invitrogen) at 60°C for 1 h. Real-time PCR was performed in a 20-µl reaction mixture consisting of 2 µl of 10-fold-diluted cDNA, 500 nM primers, and 1× Power SYBR green PCR master mix (Applied Biosystems) and a StepOnePlus real-time PCR system (Applied Biosystems). Ten-fold serial dilutions of synthetic RNA standards over 8 orders of magnitude (10^{10} to 10^3 copies/µl) were used to generate a standard curve. All primer sequences are available upon request.

RNA modeling. *In silico* RNA secondary structure prediction was conducted using the Mfold Web server with default settings as previously described (36). A pool of 20 predicted structures for each panhandle variant was examined manually, and the predominant terminal structure with minimal free energy was selected for illustration.

Immunoblotting. Viral PB2 and NP proteins were probed with homemade rabbit antisera. Standard immunoblotting protocol was performed as previously described (37) and the nitrocellulose membranes were visualized with an Odyssey infrared imaging system (LI-COR).

Statistical analysis. The statistical significance of differences was calculated using Prism 6 (GraphPad Software, Inc., USA) with one-way or two-way analysis of variance followed by the Bonferroni posttest to obtain the *P* value. Data are reported as means ± standard errors of the means (SEM) of three independent experiments performed in triplicate, unless otherwise indicated. Significant differences between groups are denoted by an asterisk ($P < 0.05$), two asterisks ($P < 0.01$), or three asterisks ($P < 0.001$).

RESULTS

The IAV genomic coding region is dispensable for IFN stimulatory activity. It has been shown that RIG-I binds to IAV genomic RNA *in vitro* (38), although the conclusion that RIG-I associates with 5'-triphosphorylated single-stranded RNA (ssRNA) was proved to be inappropriate, because a dsRNA motif is also required for RIG-I activation (5, 6). However, it remains unknown if the panhandle structure within the genomic RNA meets the dsRNA requirement. Therefore, we sought to delineate which specific region within the viral genomic RNA is responsible for RIG-I activation. To this end, we first confirmed the immunostimulatory activity of virion-derived vRNA in primary PAMs. We chose these immune cells because they respond rapidly to RNA virus infection in the airway and robustly produce type I IFNs (39). More importantly, PAMs are essential in controlling IAV infection in pigs (40). To establish the PAM system, we examined the ability of PAMs to be infected by IAV and to produce type I IFNs. Infection with SIV/Sk02 significantly elevated mRNA levels of IFN-α/β over the mock controls at 4 h postinfection (h p.i.) (Fig. 1A). In parallel, infection with an isogenic NS1 mutant virus (SIV/

FIG 1 RIG-I-dependent IFN production in PAMs induced by virion-derived vRNA. (A) PAMs were infected with SIV/Sk02 and its isogenic NS1 mutant virus. SIV/Sk02 NS1 1-99 at a multiplicity of infection of 1 for 4 h. The levels of IFN- α / β mRNA were quantified by qRT-PCR. (B) PAMs were transfected with increasing amounts of virion-derived vRNA from three IAV H1N1 strains (SIV/Sk02, Halifax210, and Tx91). IFN- α production was determined at 24 h p.t. via an ELISA. The integrity and identity of these vRNA were examined on 2.8% polyacrylamide-7 M urea gels and visualized by silver staining. (C) PAMs were transfected with virion-derived vRNA from PR8 (50 ng) with or without CIAP treatment. IFN- α production was determined at 24 h p.t. The vRNA integrity was examined on a 2.8% denaturing gel. (D) ATPase activity of purified hRIG-I was determined in the presence of increasing amounts of either untreated or CIAP-treated PR8 vRNA. (E) PAMs were left untreated or pretreated with 10 μ M or 20 μ M chloroquine for 1 h, followed by transfection with PR8 vRNA (50 ng) or poly(I:C) for 8 h in the presence of inhibitor. IFN- α production was determined in an ELISA. (F) PAMs pretreated with chloroquine were stimulated with LPS (1 μ g/ml) for 8 h, and IL-1 β production was determined by ELISA. (G) PAMs were transfected with 20 nM off-target siRNA (siOT) or siRNA against porcine RIG-I, TLR3, or TLR7 for 24 h, followed by transfection with PR8 vRNA (50 ng) for 24 h. IFN- α production was determined in an ELISA. (H) The knockdown efficiency of siRNA was examined by qRT-PCR. Data were normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase, and expression was calculated by using the $\Delta\Delta C_T$ method relative to the result with siOT.

Sk02 NS1 1-99) induced higher IFN- α/β mRNA levels than the WT virus, confirming the IFN-antagonizing role of NS1. We next extracted viral genomic RNA from purified virions of three H1N1 strains (i.e., SIV/Sk02, Halifax210, and Tx91) and tested their immunostimulatory activities in PAMs. As expected, transfection of viral genomic RNA induced strong IFN- α production in PAMs in a dose-dependent but virus strain-independent manner (Fig. 1B).

To determine if RIG-I is dominant in mediating the IFN response to IAV genomic RNA in PAMs, we tested the dependency of 5'-triphosphate for vRNA-induced IFN- α production. Treatment of vRNA with CIAP largely abrogated its IFN stimulatory activity in PAMs (Fig. 1C). We also examined the effect of CIAP treatment on vRNA-stimulated ATPase activity of purified RIG-I. As expected, the dephosphorylated vRNA was unable to activate RIG-I ATPase activity (Fig. 1D). Moreover, we ruled out the involvement of endosomal TLR3/7 in vRNA-induced IFN- α production by using chloroquine, which prevents endosomal acidification thereby inhibiting TLR enzymatic activity (41). Chloroquine treatment exerted a dose-dependent inhibitory effect on poly(I:C)-induced IFN- α production, which requires TLR3 in macrophages (42), but not that induced by vRNA (Fig. 1E). The specificity of chloroquine for endosomal TLRs was also confirmed by the unchanged interleukin-1 β production induced by bacterial lipopolysaccharide (LPS) (Fig. 1F), which is known to be mediated by TLR4 on the cell surface (43). Finally, we utilized siRNA to specifically knock down RIG-I or TLR3/7 and found that only siRNA against RIG-I was able to significantly dampen IFN- α induction by vRNA (Fig. 1G). It is noteworthy that primary macrophages are difficult to knock down, and we could only achieve around 50% gene silencing efficiency as examined by qRT-PCR (Fig. 1H). We were unable to determine the protein levels due to a lack of porcine antibodies. Nonetheless, our complementary experimental approaches were sufficient to demonstrate that RIG-I plays a dominant role in mediating the IFN response to viral genomic RNA in PAMs.

To further probe the specific region within viral genomic RNA that is responsible for IFN induction in PAMs, we utilized the RNA Pol I-based RNP reconstitution system to produce structurally defined IAV genomic RNA *in vivo* (44) (Fig. 2A). We tested this system by reconstituting each of the eight viral segments, followed by transfection of PAMs with reconstituted RNA. Similarly to the virion-derived vRNA, reconstituted viral RNA was also potent in stimulating RIG-I-dependent IFN- α production in PAMs (Fig. 2B and C). The NP construct induced more IFN, owing to the higher abundance of the vRNA species in the total RNA (data not shown). Next, we examined the requirement of the viral coding region for IFN induction. Serial truncation of the NA coding sequence to 1/2 (loop810) or 1/16 (loop96) its original full length (FL) did not affect its IFN stimulatory activity (Fig. 2D). In addition, exclusion of RNA species smaller than 200 nt by fractionation largely diminished IFN- α induction by loop96 RNA (146 nt in length), but not loop810 RNA (860 nt) or FL RNA (1,463 nt) (Fig. 2D). This demonstrated that the IFN response to reconstituted RNA can be attributed specifically to the viral RNA of the corresponding size, but not other cellular RNA species. Comparable results were also obtained from a similar set of truncation variants in the backbone of the pPOLI-NP-LUC construct (data not shown), demonstrating that this effect is not segment specific. Taken together, these results suggest that the viral coding region is

dispensable for the IFN stimulatory activity and that the IFN stimulatory region most likely resides in the 5' and 3' noncoding regions (NCRs) of vRNA.

The IAV panhandle structure is sufficient for RIG-I activation. The NCRs of the eight viral segments differed in length, but all contained conserved nucleotides (12 and 13 nt) at the 3' and 5' terminals, respectively (45). The dsRNA formed by the partial complementarity of these conserved nucleotides was further extended by a fully complementary region (2 to 3 bp) whose sequence is segment specific among all IAV subtypes (45). To provide direct evidence that this panhandle structure is able to activate RIG-I and relay the signal for IFN induction, we synthesized a structurally well-defined hairpin RNA that mimicked the authentic panhandle structure by IVT. This 35-nt-long hairpin RNA (designated WT-hp) contained the conserved 3' and 5' nucleotides followed by the segment-specific region from the NP segment, except that the first conserved base pair was switched from A:U to G:C for a better IVT yield (Fig. 3A). We also paid particular attention to ensure its blunt-ended and double-stranded nature, which was achieved by using DNA template with 2'-O-methyl modification and connecting the RNA 3' and 5' arms at the distal ends with a stabilizing UUCG tetraloop (29). We first analyzed the RIG-I binding property of the panhandle RNA (WT-hp) via EMSA. We found that WT-hp was able to form a high-molecular-weight complex with RIG-I (Fig. 3B). In addition, because the EMSA only revealed one shifted band of the complex, this interaction most likely reflects a binding scenario with 1:1 stoichiometry, as previously reported for synthetic RNA of similar size (46). By holding the concentration of WT-hp constant (0.2 μ M), titration of the RIG-I concentration over a 0 to 0.6 μ M range revealed a K_d value of 312.5 ± 13.7 nM, with a Hill coefficient (n) of 5.41 (Fig. 3C). Next, we determined if the binding of WT-hp to RIG-I activates its ATPase activity. A constant amount of RIG-I (0.4 μ M) was incubated with increasing concentrations of WT-hp (0 to 0.1 μ M) and saturating ATP (1 mM). We found that WT-hp was able to stimulate RIG-I ATPase activity, with a Michaelis constant (K_m) of 42.6 ± 5.1 nM (Fig. 3D). Last, we confirmed the ability of WT-hp to induce a RIG-I-dependent IFN response in chicken DF-1 cells, which are deficient in endogenous RIG-I expression (32). Only when cotransfecting duck RIG-I did WT-hp stimulate the chicken IFN- β promoter activity (Fig. 3E). Taken together, these results show a clear association between the WT panhandle RNA and RIG-I-mediated IFN induction.

Panhandle-stabilizing mutations promote RIG-I activation and IFN induction. The panhandle structures of most negative-strand RNA viruses are only partially complementary (5, 47). Very limited data obtained with authentic viral sequences, such as those of rabies virus and vesicular stomatitis virus, led to the conclusion that RIG-I tolerates partial complementarity in initiating the IFN response (5, 48). Indeed, we have demonstrated that the partially complementary IAV panhandle structure is able to bind to and activate RIG-I. However, whether this partial complementarity contributes as efficiently as full complementarity to RIG-I activation and IFN induction is unknown. The proximal stem of the panhandle structure contains two wobble base pairs (G · U) at positions 3 and 5 and one mismatch (A · C) at position 8. This region is followed by an unpaired adenosine at 5'-end position 10 that together with the A · C mismatch at position 8 results in a bulge structure (25) (Fig. 3A). We synthesized two sets of panhandle-stabilized variants to address the contribution of these ele-

FIG 2 The IFN stimulatory region lies within the vRNA noncoding region. (A) Schematic diagram of the RNA Pol I-based RNP reconstitution system used to synthesize the structurally defined panhandle vRNA *in vivo*. The viral coding region (CR, in an antisense orientation) flanked by the 5' and 3' NCR of the IAV segment was inserted between the RNA Pol I promoter and terminator. Detailed nucleotide composition of the panhandle structure is illustrated according to a previous report (25). Symbols for base pairs: solid line, Watson-Crick base pair; broken line, mismatch; dot, wobble base pair. (B) PAMs were transfected with RNA (100 ng) extracted from reconstituted vRNP representing each of the eight IAV segments for 24 h. Reconstituted RNA from the Pol I vector (vec) and poly(I:C) were used as negative and positive controls, respectively. IFN-α production was determined in an ELISA. (C) PAMs were transfected with 20 nM off-target siRNA (siOT) or siRNA against porcine RIG-I, TLR3, or TLR7 for 24 h, followed by transfection with reconstituted RNA from the NP segment for 24 h. IFN-α production was determined in an ELISA. (D) PAMs were transfected with either total or fractionated (>200 nt) reconstituted RNA (100 ng) derived from Tx91-NA constructs containing different lengths of the coding region, FL, loop810, or loop96, for 24 h. IFN-α production was determined in an ELISA.

ments to RIG-I activation. In one set, transition mutations were introduced from either the 5' end (5'Complete-hp) or 3' end (3'Complete-hp) of the panhandle to eliminate the wobble base pairs and the mismatch at position 3/5 and position 8, respectively (Fig. 3A). In the other set, the unpaired adenosine was either deleted from the 5' end (5'dA10-hp) or paired with a uridine inserted from the 3' end (3'i10U-hp) (Fig. 3A). Compared to the WT-hp RNA, two variants with fully complementary proximal stems (5' and 3'Complete-hp) exhibited higher affinities to RIG-I ($K_d = 195.3 \pm 3.9$ nM and 245.3 ± 10.5 nM, respectively) than did WT-hp (Fig. 3B and C). In contrast, the 5'dA10 and 3'i10U mutants had K_d values comparable to that of the WT-hp (276.2 ± 8.2 nM and 291.2 ± 17.9 nM, respectively), indicating similar affinities (Fig. 3B and C). We further determined the ATPase activity of RIG-I stimulated by these panhandle variants. Remarkably, all variants stimulated RIG-I ATPase activity more efficiently than WT-hp (Fig. 3D). Moreover, in line with their RIG-I binding properties, the 5' and 3'Complete variants had much lower Michaelis constants than the WT-hp ($K_m = 8.1 \pm 0.8$ nM and $14.4 \pm$

2.8 nM, respectively), and the 5'dA10 and 3'i10U mutants displayed K_m values similar to that of the WT-hp (33.1 ± 4.1 nM and 42.1 ± 3.4 nM, respectively) (Fig. 3D). We further compared the IFN stimulatory activity of these panhandle variants to that of the WT-hp in DF-1 cells. While the Complete variants were superior to the WT in IFN-β induction, the debulged mutants (5'dA10 and 3'i10U) displayed IFN stimulatory activity comparable to that of the WT (Fig. 3E). Taken together, these results demonstrated that the degree of complementarity of the panhandle proximal stem plays a critical role in activating RIG-I and that the internal bulge structure has a minimal effect on RIG-I activation.

Full-length vRNA containing panhandle-stabilizing mutations promotes IFN induction in PAMs. We further analyzed the IFN stimulatory activity of the panhandle-stabilized variants in the context of the full-length genome. To this end, we synthesized full-length panhandle variants *in vivo* via the RNP reconstitution system and selectively isolated the vRNA species from the reconstituted RNA by using a recently developed strand-specific RNA-tagging approach (33). This approach relies on introducing a PP7

FIG 3 The panhandle structures bind to and activate RIG-I *in vitro*. (A) *In silico*-predicted hairpin WT panhandle structure and its variants. Transition and deletion/insertion mutations in the Complete and debulged variants are highlighted in yellow and green, respectively. (B) Representative gels showing EMSA results for hRIG-I with the WT and mutant panhandle structures without ATP. (C) Summary of EMSA results, fitted into the specific binding with Hill slope function (one site). (D) Effects of WT and mutant panhandle RNA binding on the ATPase activity of hRIG-I. Data fitting was performed with the Michaelis-Menten function. (E) Stimulation of the chicken IFN- β promoter in DF-1 cells by the WT and mutant panhandle RNA with or without duck RIG-I (dRIG-I) cotransfection. A synthetic 19-mer 5'ppp-dsRNA and its control dsRNA (5'OH-dsRNA) were used as controls. Data were normalized to the internal *Renilla* luciferase activity, and statistical significance was determined relative to the WT panhandle.

tag into the vRNA coding region and the high-affinity binding of a PP7 coating protein (PP7CP) to the PP7 tag. We first verified this system by reconstituting the NA segment with a PP7 tag inserted in the NA stalk region (33). As previously reported, The PP7CP

pulldown fraction from the reconstituted cells contained exclusively vRNA in the form of vRNP (Fig. 4A, left panels, and B). The identity of the purified reconstituted NA vRNA was further confirmed to match the size of the virion-derived NA segment based

FIG 4 Panhandle-stabilizing mutations promote IFN stimulatory activity in the context of full-length vRNA in PAMs. (A) The PP7CP pull-down fraction was examined by Western blotting against viral NP and PB2 proteins. (B) The relative levels of vRNA, cRNA, and mRNA in the PP7CP pull-down fraction from the A/WSN/33 NA segment were quantified by qRT-PCR. Data were normalized to 18S rRNA and are expressed based on the $\Delta\Delta C_T$ method relative to the result with the Pol I vector control. (C) RNA extracted from the PP7CP pull-down fraction was resolved on a 4% polyacrylamide-7 M urea gel and visualized by SYBR Gold staining. Virion-derived PR8 vRNA was loaded in parallel as a size marker. Of note, the NA segments of WSN33 and PR8 are of similar sizes (1,409 versus 1,413 nt). (D) PAMs were transfected with PP7CP pull-down vRNA or PR8 vRNA (50 ng) for 24 h. IFN- α production was determined in an ELISA. (E) PAMs were transfected with an equal molar amount (3×10^7 copies) of purified vRNA harboring panhandle-stabilizing mutations for 24 h. IFN- α production was determined in an ELISA and is expressed as the fold change over the WT vRNA-induced level.

on results with denaturing polyacrylamide gels (Fig. 4C). Remarkably, the reconstituted NA vRNA stimulated IFN- α production in PAMs as efficiently as the virion-derived vRNA (Fig. 4D). These results reinforced the notion that the reconstituted vRNA recapitulates the virion-derived vRNA in IFN stimulatory activity (15). We next introduced the PP7 tag into the Pol I-driven plasmids carrying the panhandle-stabilizing mutations in the backbone of the NP-luciferase (NP-LUC) construct, in which the NP coding region is replaced by a firefly luciferase gene while the 3' and 5' NP NCRs are retained. We chose this construct because it provides a reporter system to examine the effect of panhandle mutations on viral promoter activity (discussed below). We then isolated mutant vRNPs from reconstituted cells and extracted full-length vRNAs harboring the panhandle-stabilizing mutations (Fig. 4A, right panel). Notably, transfection of PAMs with equal molar amounts of these panhandle variants stimulated significantly higher levels of IFN- α production than did the WT (Fig. 4E). Interestingly, we consistently observed that the superiority of the full-length panhandle-stabilizing variants in IFN stimulation in PAMs was more pronounced than that of the hairpin RNA in DF-1 reporter cells, particularly for the debulged mutants (Fig. 3E and 4E). Nonetheless, our results clearly demonstrate that the

panhandle-stabilizing mutations conferred enhanced IFN stimulatory activity to the WT panhandle structure existing either alone or in the context of the full-length viral genome.

Panhandle-stabilizing mutations do not impair viral replication. The IAV panhandle structure serves as the viral promoter region that is crucial for viral transcription and replication. We demonstrated that the panhandle structure is a RIG-I agonist and could be modified to possess enhanced IFN stimulatory activity. To gain further insight into the impact of the panhandle-stabilizing mutations on the viral promoter activity, we examined the promoter activity of the panhandle-stabilizing variants in the backbone of the NP-LUC construct. By directly measuring firefly luciferase expression in reconstituted cells, we found that the Complete mutants showed reduced luciferase activity, indicating impaired viral mRNA synthesis (Fig. 5A). In comparison, viral mRNA synthesis was abrogated in the debulged mutants, which is in line with previous reports (24, 49). To more comprehensively monitor the influence of these mutations on viral RNA synthesis, we quantified the levels of viral vRNA, cRNA, and mRNA in the total reconstituted RNA from each panhandle variant. Consistent with the luciferase reporter results, the 5'dA10/3'i10U mutations abolished mRNA synthesis, whereas the 5'Complete/3'Complete

FIG 5 Panhandle-stabilizing mutations do not impair viral replication. (A) Firefly luciferase expression from the NP-LUC constructs was measured directly from reconstituted HEK293T cells and normalized to internal *Renilla* luciferase activity. (B, C, and D) The absolute levels of viral mimetic mRNA (B), vRNA (C), and cRNA (D) in total reconstituted RNA from the Complete and debulged panhandle variants were quantified by strand-specific qRT-PCR. Of note, the number of copies of vRNA template produced from cellular RNA Pol I transcription was determined by omitting the PB2 subunit in reconstitution. This number was subtracted from the total RNA copy number.

mutations reduced viral mRNA levels (Fig. 5B). Moreover, while both the 5'dA10 and 3'i10U mutations augmented vRNA synthesis (Fig. 5C), the 3'i10U variant showed a moderate reduction in cRNA synthesis (Fig. 5D). The cRNA levels for the 5' end variants were not determined due to primer incompatibility with mutations in the vRNA 5'-NCR. For the Complete variants, we revealed distinct impacts of 3' and 5' complementary mutations on vRNA synthesis. While the 3' Complete mutant showed increased vRNA synthesis, the 5' Complete mutant was largely impaired in vRNA production (Fig. 5C). This difference could result from the critical role of the 5'-end guanosine at position 5 in viral polymerase binding (24). Moreover, the 3' Complete mutant produced levels of cRNA comparable to those of the WT (Fig. 5D). Taken together, we found that most panhandle-stabilizing mutations (except for the 5' Complete mutation) did not dramatically affect viral replication, although the transcription levels were all impaired.

The segment-specific region within the panhandle distal stem contributes little to IFN induction. We further extended our study to analyze the contribution of the panhandle distal stem to IFN induction. This region is fully complementary and contains a 3-bp-long conserved promoter region (5' positions 11 to

13 and 3' positions 10 to 12) and a contiguous 3-bp-long segment-specific region (5' positions 14 to 16 and 3' positions 13 to 15). Because alteration of the conserved promoter region significantly impacts viral promoter activity, as previously reported (50), we only examined the segment-specific region by introducing transversion mutations to sequentially disrupt the base pairing (Fig. 6A). Compared to the WT panhandle, variants carrying successive transversion mutations in the 3' end of the segment-specific region (3'U15C, UA15-14CC, and UAC15-13CCA) had sequential impairment in vRNA and cRNA synthesis (Fig. 6B and C). The 5' variants (5'A16C, UA15-16GC, and GUA14-16UGC) also showed reduced vRNA levels, but a sequential reduction was not seen (Fig. 6B). Moreover, successive mutations in either strand resulted in a sequential reduction in viral mRNA levels (Fig. 6D). To further address the IFN stimulatory activity of these panhandle variants, we transfected PAMs with equal molar amounts of vRNA from these variants. It is noteworthy that here we used total reconstituted RNA for transfection, and any difference in the RNA mass amount was compensated by supplementing control 293T total RNA to ensure consistent transfection efficiency. We found that all variants harboring segment-specific mutations induced IFN- α

FIG 6 Minor contribution of the distal segment-specific region to vRNA-induced IFN production. (A) *In silico*-predicted WT panhandle structure. The segment-specific region is boxed, and sequential introduction of transversion mutations is illustrated by an arrow. (B, C, and D) The absolute levels of viral mimetic vRNA (B), cRNA (C), and mRNA (D) in total reconstituted RNA from panhandle variants carrying sequential segment-specific mutations were quantified by strand-specific qRT-PCR. (E and F) PAMs were transfected with an equal molar amount of vRNA (4×10^7 copies) (E) or combined vRNA and cRNA (total, 4×10^7 copies) (F) from panhandle variants carrying sequential segment-specific mutations for 24 h. IFN- α production was determined in an ELISA, and results are expressed as the fold change over the WT panhandle-induced level.

production comparable to that of the WT panhandle (Fig. 6E). Of note, we also took into account the inclusion of viral cRNA and mRNA in the total reconstituted RNA. It has been shown that in RNP reconstitution settings, viral genomic or antigenomic RNA, but not mRNA, is responsible for RIG-I-mediated IFN induction (15). We were also able to demonstrate that viral mRNA is dispensable for IFN induction because transfection with reconstituted RNA from 5'dA10 and 3'i10U mutants, both of which did not produce mRNA, showed it was competent in IFN induction (data not shown). Interestingly, transfection of PAMs with an equal molar amount of combined vRNA and cRNA from the segment-specific variants induced a pattern of IFN- α production similar to that from transfection with vRNA alone (Fig. 6F versus E), suggesting a minor contribution of cRNA to IFN induction. Taken together, these results demonstrate that the base pairing of the vRNA segment-specific region plays a minor role in RIG-I activation and IFN induction.

DISCUSSION

The indispensable role of RIG-I in IFN response to IAV infection has long been observed (1, 51); however, the nature of the RIG-I ligands from IAV is not well understood. In accordance with the well-accepted criteria for optimal RIG-I activation, it has been speculated that the panhandle structure formed from the viral genome extremities meets the dsRNA requirement in addition to the 5'-triphosphates (5, 15, 18, 38, 52). In the present study, we employed multiple approaches to determine if the IAV panhandle

structure is directly involved in RIG-I activation and IFN induction. We first established a physiologically relevant IFN production platform in PAMs and demonstrated that that vRNA-induced IFN response is highly dependent on RIG-I (Fig. 1). Using the well-established RNP reconstitution system, we synthesized truncated viral RNA *in vivo* and demonstrated that the genomic coding region is dispensable for IFN induction (Fig. 2). We do not preclude the possibility that the presence of a full-length viral coding sequence may enhance IFN stimulatory activity, although this possibility is very slim, as the viral coding region is encapsidated by NP through the virus life cycle. The observation that internally truncated vRNA is competent in IFN induction is also in agreement with previous deep sequencing results that IAV DI genomes constitute one arm of RIG-I ligands in addition to full-length genomic RNA during infection (15, 18). We further synthesized the authentic IAV panhandle structure *in vitro* and provided direct evidence that the panhandle structure is able to bind to and activate RIG-I with a 1:1 stoichiometry (Fig. 3). These results clearly demonstrate that the IAV panhandle structure is competent in, and sufficient for, RIG-I activation.

Recognition of dsRNA ligands by RIG-I is sequence independent and does not require full complementarity (53). The panhandle proximal stem and the unpaired adenosine are 10 bp in length, which exactly fits in the coverage range of monomeric RIG-I (30, 46). In an attempt to scrutinize the effect of this imperfect complementarity on RIG-I activation, we analyzed two sets of panhan-

dle variants carrying panhandle-stabilizing mutations. Strikingly, these variants had enhanced or comparable RIG-I binding and activating abilities *in vitro* (Fig. 3). Moreover, in the context of the full-length viral genome, these variants exhibited IFN stimulatory activity superior to that of the WT panhandle, demonstrating that the WT panhandle structure has the potential to be more immunostimulatory (Fig. 4). In support of our observation, a recombinant virus harboring a G3A/C8U double mutation in the panhandle 3' arm stimulated higher levels of IFN production than the WT virus, and this was attributable to the generation of more potent RIG-I-associated immunostimulatory RNA species (54). Considering the basic function of the panhandle structure as the viral promoter, we extended our analysis to assess the influence of these panhandle mutations on viral promoter activity. By accurately quantifying the levels of viral RNA species produced by these panhandle-stabilized variants, we found that most of these mutations promoted viral vRNA synthesis while they impaired mRNA production to various extents (Fig. 5). This imbalanced RNA synthesis, coupled with the enhanced IFN stimulatory activity of these variants compared to the WT panhandle, is obviously detrimental to virus survival from the perspective of the virus and the host as well. This indicates that the WT panhandle promoter region adopts a nucleotide composition that is optimal for balanced viral transcription and replication and that is suboptimal for RIG-I activation. Moreover, adoption of the bulge element (5' unpaired adenosine) in the WT panhandle structure has other impacts on the virus life cycle, such as the specificity in packaging of vRNP, but not cRNP, into the virion (55).

Compared to the proximal stem, the distal stem of the panhandle structure is fully complementary. By sequentially disrupting the base pairing of the distal segment-specific region, we demonstrated that the complementarity of this region plays a minor role in RIG-I activation (Fig. 6). This observation, in conjunction with the significant impact of the panhandle-stabilizing mutations on RIG-I activation, highlights a dominant role of the panhandle proximal stem in RIG-I activation. In the context of virus infection, viral genomic RNA exists in the form of vRNP, in which the genome extremities are covered by the viral polymerase. Researchers over the past decades have proposed alternative models for the viral polymerase-bound promoter region. These include the "corkscrew" and the "fork" conformations (26, 56). Most recently, the crystal structures of the complete viral polymerase bound to the vRNA promoter revealed the presence of a 5' intrastand "hook" structure. In contrast, no internal structure was seen in the 3' strand (57, 58). Nonetheless, these models concur on the double-strandedness of the panhandle distal stem and only differ in the configuration of the proximal strands, indicating that in the context of vRNP, it is the configuration of the promoter-proximal strands that determines RIG-I recognition. Remarkably, a recent study taking advantage of the three-dimensional stochastic optical reconstruction microscopy (STORM) technique clearly visualized an association of RIG-I with incoming IAV vRNP at the mitochondrion (59). This is also in line with a previous report demonstrating that RIG-I recognizes incoming bunyavirus vRNP (16). These results imply that RIG-I is able to capture a certain RNA configuration imposed by the promoter-proximal strands, which is most likely the panhandle configuration. While the manuscript was in preparation, Weber et al. reported that RIG-I binds the panhandle promoter of incoming influenza A viruses and directly inhibits the onset of infection by destabilizing nucleocapsids (60),

reinforcing the role of the panhandle structure, not only in the context of purified RNA but also within vRNP, in RIG-I activation and IFN induction. However, the exact mechanism by which RIG-I gains access to the panhandle structure, particularly if RIG-I could displace or compete with viral polymerase for dsRNA binding, is still unclear. Of note, the formation of a panhandle configuration does not overturn the potential of other alternative models. Indeed, it has been suggested that the panhandle model serves as the initial RNA configuration for viral polymerase binding, while the corkscrew/fork conformations only represent the promoter structure during certain stages of a virus life cycle (22). Furthermore, a dynamic equilibrium between the panhandle and the corkscrew conformation may also exist to support efficient viral transcription and replication (61). Overall, it would be rational to expect that recognition of IAV genome termini by RIG-I occurs during a dynamic conformational change of the promoter region. This recognition might be coupled with viral transcription and replication that involve active viral polymerase and other host factors. This is of particular interest since RIG-I has been observed to partially relocate into the cell nucleus during late stages of infection (62); however, whether this relocation event correlates with IFN induction requires further investigation.

In addition to the full-length and DI genome in the form of vRNP, recent attempts to identify authentic RIG-I ligands during IAV infection have raised the possibility that some aberrant viral RNA species contribute to IFN induction (63). Although the nature of these RNA species remains to be determined, it is plausible that some of these RNA species may have panhandle-forming potential. In fact, deep sequencing analyses have identified that IAV produces high levels of small viral RNA (svRNA) from early stages of infection. These svRNAs were 18 to 27 nt in size and their sequences were mapped to the exact 5' end of vRNA (64, 65). Remarkably, even in the presence of 5'-triphosphates, transfection with chemically synthesized svRNA failed to induce an IFN response (64). This implies that the contribution of svRNA to the IFN response, if any, requires binding to a complementary strand to gain the dsRNA structure required for RIG-I activation. Interestingly, deep sequencing results have also revealed a modest enrichment of small viral RNA species derived from 3' ends of vRNA and cRNA (65), which appear to be good candidates in forming panhandle structures with the 5' svRNA.

In summary, despite the controversy over the nature of the authentic RIG-I ligand(s) from IAV, we assert that the panhandle structure constitutes one of the fundamental attributes of these ligands. The fact that the panhandle-stabilizing mutations promote RIG-I activation and dampen viral transcription indicates that the WT panhandle promoter region adopts a nucleotide composition that is suboptimal for RIG-I activation and optimal for balanced viral RNA synthesis.

ACKNOWLEDGMENTS

We thank Ervin Fodor for sharing the PP7-based RNP pulldown system, Adolfo García-Sastre for providing pDZ plasmids carrying Tx91 segments, Sun Hur for providing the hRIG-I plasmid, and Katharine E. Magor for providing the dRIG-I and chicken IFN- β luciferase reporter plasmids. We are also grateful to Will Deck at the VIDO-InterVac for his excellent technical assistance in RIG-I purification.

This work was supported by a CIHR grant to Y.Z. G.L. is partially supported by the Vaccinology and Immunotherapeutics (V&I) Scholarship from the School of Public Health, University of Saskatchewan.

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