Influenza A virus utilizes a suboptimal Kozak sequence to fine-tune virus replication and host response

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The segment-specific non-coding regions (NCRs) of influenza A virus RNA genome play important roles in controlling viral RNA transcription, replication, and genome packaging. In this report, we present, for the first time to our knowledge, a full view of the segment-specific NCRs of all influenza A viruses by bioinformatics analysis. Our systematic functional analysis revealed that the eight segment-specific NCRs identified could differentially regulate viral RNA synthesis and protein expression at both transcription and translation levels. Interestingly, a highly conserved suboptimal nucleotide at the 3' position of the Kozak sequence, which downregulated protein expression at the translation level, was only present in the segment-specific NCR of PB1. By reverse genetics, we demonstrate that recombinant viruses with an optimized Kozak sequence at the 3' position in PB1 resulted in a significant multiple-cycle replication reduction that was independent of PB1-F2 expression. Our detailed dynamic analysis of virus infection revealed that the mutant virus displays slightly altered dynamics from the wild-type virus on both viral RNA synthesis and protein production. Furthermore, we demonstrated that the level of PB1 expression is involved in regulating type I IFN production. Together, these data reveal a novel strategy exploited by influenza A virus to fine-tune virus replication dynamics and host antiviral response through regulating PB1 protein expression.

INTRODUCTION

Influenza A virus is a segmented negative-sense RNA virus of the family Orthomyxoviridae (Palese & Sham, 2007). The virus genome consists of eight virion RNA (vRNA) segments that encode at least ten proteins. The coding region of each segment is flanked by the segment-specific non-coding region (NCR) and terminal promoter region at both the 3' and 5' ends. The highly conserved promoter region is formed by terminal 12 and 13 nt at the 3' and the 5' ends, respectively, which play a critical role in initiating viral RNA transcription and replication (Flick et al., 1996; Fodor et al., 1995; Kim et al., 1997). Adjacent to the promoter region, at both ends, are the segment-specific NCRs that vary in sequence and length among different segments of influenza A virus. It has previously been reported that the segment-specific NCRs of influenza A virus play multiple roles in the virus life cycle. They not only act as cis-acting signals to regulate viral transcription, replication and protein expression (Bergmann & Muster, 1996; Park & Katze, 1995; Zheng et al., 1996), but also serve as part of the packaging signals during selective virus genome packaging (Gog et al., 2007; Hutchinson et al., 2010; Zhao et al., 2014).

The viral ribonucleoproteins (RNPs) of influenza A virus are responsible for synthesizing three viral RNA species (mRNA, cRNA and vRNA) in the nucleus of infected cells. The RdRp is a heterotrimeric complex composed of three subunits (PB1, PB2 and PA) (Palese & Sham, 2007; Resa-Infante et al., 2011). The PB1 subunit, encoded by segment 2, is the core of the RdRp (Palese & Sham, 2007). It not only acts as the driving force for viral RNA polymerase assembly but also contains active sites for RNA polymerization (Biswa & Nayak, 1994; Braam et al., 1983). In addition, the PB1 subunit is responsible for binding to the viral RNA promoter to initiate viral RNA transcription and replication (González & Ortín, 1999a, b).

Upon influenza A virus infection, the synthesis dynamics of the three RNA species (mRNA, cRNA and vRNA) and all viral proteins are tightly controlled differentially (Enami et al., 1985; Hatada et al., 1989; Park & Katze, 1995; Park et al., 1999; Varich & Kaverin, 1987; Yamanaka et al., 1988, 1991). The significance of such regulation is not only to produce an appropriate amount of viral RNAs and proteins for efficient assembly of progeny virions, but also to maintain the fine balance between virus growth rate and
host antiviral status (Belicha-Villanueva et al., 2012). Both viral RNAs and viral proteins have been reported to be involved in regulation of the host antiviral status (Hale et al., 2008; Kochs et al., 2007; Rehwinkel et al., 2010; Talon et al., 2000). In addition to the main IFN antagonist NS1, RdRp was also reported to be involved in host shut-off by targeting host Pol II transcription machinery (Graef et al., 2010; Vreede et al., 2010). Moreover, a short non-structural peptide PB1-F2, expressed from the PB1 segment of influenza A virus, was reported to be involved in modulating host antiviral status (Chen et al., 2001; Wise et al., 2009). Therefore, an understanding of the mechanisms by which influenza virus controls its RNA and protein expression, and the consequences of such regulation, is of great significance in order to fully understand influenza virus replication strategies.

In the present study, we analysed the sequences of the segment-specific NCRs of all available influenza A viruses in the NCBI database and investigated the role of these NCRs in differentially regulating viral RNA synthesis and protein expression. Interestingly, we also demonstrated that influenza A virus uses a suboptimal Kozak sequence in segment 2 to fine-tune virus infection through modulating PB1 protein expression.

RESULTS

Bioinformatics analysis of the segment-specific non-coding regions of influenza A viruses

In order to obtain a full view for segment-specific NCRs of all influenza A viruses, we used bioinformatics to analyse all available NCR sequences of all the eight segments from the NCBI Influenza Virus Resource database as recently described (Zhao et al., 2014). We used H3 and N2 subtypes for the analysis of HA and NA segments. As shown in Fig. 1(a), the lengths and sequences at both 3′ and 5′ ends are segment-specific. It can be seen that, within each segment-specific NCR, most nucleotides are highly conserved, whereas, at certain nucleotide positions, the identities of nucleotides are variable (most of them are transition changes). We further validated these sequence logos by comparing them with the NCR sequence logos determined by accurate NCR sequencing methods (Park et al., 2013, 2012; Su et al., 2012; Wang & Lee, 2009; Wang & Taubenberger, 2014; Wang et al., 2014). We found that the majority of nucleotide conservation/variation at each nucleotide position was generally consistent between the sequence logos (Fig. 1b), confirming that our bioinformatics analysis of the NCR sequences was accurate and reasonable.

Segment-specific NCRs mediate the synthesis of viral RNAs and proteins at differential levels

To investigate whether these segment-specific NCRs could act as cis-acting signals to differentially regulate the syntheses of viral RNAs and proteins, we examined their effects in an RNP reconstitution system derived from the PR8 virus. To form active viral RNPs, the pPolI-HA plasmid was transfected into 293T cells to express the HA vRNA, together with four protein expression plasmids (pcDNA-PB1, pcDNA-PB2, pcDNA-PA and pcDNA-NP). The NCRs of the HA vRNA at both ends in the pPolI-HA plasmid were substituted with the corresponding NCR of the other seven segments, respectively (Fig. 2a). Then the levels of three RNA species were examined by primer extension analysis and the levels of HA protein expression were examined by Western blotting. Fig. 2(b, c) shows that the different segment-specific NCR substitutions of HA–NCR led to the synthesis of the three RNA species (mRNA, cRNA and vRNA) at different levels compared with that of the original HA template. In particular, substitutions with PB2–NCR led to increased vRNA levels, while substitutions with PB1–NCR led to increased cRNA levels and decreased vRNA levels and substitutions with NS–NCR led to increased mRNA levels (Fig. 2b, c). Meanwhile, the levels of HA protein expression were generally consistent with their corresponding mRNA levels, except for the PB1–NCR substitution (Fig. 2d, e). The mRNA levels produced from the PB1–NCR substitution were similar to those produced from other vRNA templates, but the level of protein expressed by the PB1–NCR substitution was much lower than that of the other substitutions. We further validated these results using an A/Vietnam/1194/2004 (H5N1 subtype) RNP system (data not shown). This result was also consistent with earlier observations that PB1–NCR mediated low expression level of a reporter gene in RNP reporter systems (Ma et al., 2013; Maeda et al., 2004). Together, these results suggest that the segment-specific NCRs could differentially regulate viral gene expression at transcription level. However, PB1–NCR mediated exceptional and significant downregulation at the translation level.

Unique, suboptimal Kozak sequence in PB1–NCR mediates low expression at the translation level

It is known that the Kozak sequence has a significant effect on protein expression in eukaryotic cells at the translation level (Kozak, 1986). It has been previously reported that the NCR at 3′ end of the PB1 segment of influenza virus strain A/PR/8/34 contains a suboptimal nucleotide (A) at the −3 position (Chen et al., 2001; Kozak, 1991). Our bioinformatics analysis confirmed the observation that the PB1 segments of all influenza A viruses contain this −3(A) Kozak sequence in their 3′ NCR (Fig. 3a), suggesting that it might be important for virus replication. To confirm the low expression level of the HA protein was mediated by the suboptimal Kozak sequence of the PB1–NCR in our RNP reconstitution systems, we created an optimal Kozak sequence by introducing a U-to-A mutation at position −3 in the positive sense of plasmid pPolI-PB1–NCR–HA (Fig. 3b). The effects of the −3A mutation on viral RNA synthesis and protein expression were then examined. The results showed that the level of HA protein expressed from PB1(−3A)–NCR substitution was about six times higher than the levels of the protein expressed from PB1–NCR containing the wild-type PB1 sequence [PB1(WT)–NCR]...
mRNA at the 22 growth properties of recombinant virus WSN–PB1(–3A) in both MDCK cells and A549 cells (at m.o.i. 0.001). The results showed that the maximum virus titre of WSN–PB1(–3A) was about 1 log lower than that of WSN–WT in both cell types (Fig. 4a, b). We further confirmed this result in a seasonal influenza A virus [A/Hong Kong/1968(H3N2)] background, in which the maximum virus titre of HK68–PB1(–3A) was about 0.7 log lower than that of HK68–WT (Fig. 4c). These data indicated that the U-to-A mutation in PB1 result in virus attenuation.

Since it has been proposed that the suboptimal Kozak consensus of PB1, with the unique ‘U’ nucleotide in the –3 position, is involved in regulating the expression of PB1–F2 protein by leaky ribosomal scanning (Chen et al., 2001; Kozak, 1991; Wise et al., 2011), the U-to-A mutation at the –3 position might be expected to result in defective expression of PB1–F2 protein. In order to examine whether the attenuation of WSN–PB1(–3A) was caused by defective expression of PB1–F2, we made three mutations (T120C, C153G and G291A) (Ma et al., 2013; Su et al., 2012) in the coding region of both the wild-type and the U-to-A mutant virus to completely eliminate PB1–F2 expression in both viruses (Fig. 4d) (Le Goffic et al., 2010; Tauber et al., 2013; Su et al., 2010). The results demonstrated that the nucleotide U at the –3 position of the PB1 mRNA, which weakened the strength of the Kozak consensus of PB1, with the unique ‘U’ nucleotide in the –3 position, was rescued in an influenza A virus background, in which the maximum virus titre of HK68–PB1(–3A) was about 0.7 log lower than that of HK68–WT (Fig. 4c). These data indicated that the U-to-A mutation in PB1 result in virus attenuation.

Recombinant viruses containing U-to-A mutation at position –3 in PB1 are attenuated, independently of the presence or absence of PB1–F2

In order to study the effect of the U-to-A mutation in the context of virus infection, a recombinant influenza A virus, WSN–PB1(–3A), bearing a U-to-A mutation in PB1 mRNA at the –3 position, was rescued in an influenza A/WSN/33 background (Hoffmann et al., 2000). The growth properties of recombinant virus WSN–PB1(–3A) and the wild-type virus were first assessed in both MDCK cells and A549 cells (at m.o.i. 0.001). The results showed that the maximum virus titre of WSN–PB1(–3A) was about 1 log lower than that of WSN–WT in both cell types (Fig. 4a, b). We further confirmed this result in a seasonal influenza A virus [A/Hong Kong/1968(H3N2)] background, in which the maximum virus titre of HK68–PB1(–3A) was about 0.7 log lower than that of HK68–WT (Fig. 4c). These data indicated that the U-to-A mutation in PB1 result in virus attenuation.

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et al., 2012), and designated these viruses WSN–WT-del F2 and WSN–PB1(−3A)-del F2, respectively. The growth curve of the viruses was then measured with infection at m.o.i. 0.001 in MDCK cells. Consistent with results previously reported (Le Goffic et al., 2010; Wise et al., 2009), there were no significant differences in the growth of WSN–WT and WSN–WT-del F2 virus (Fig. 4e). Similarly, the WSN–PB1(−3A)-del F2 virus showed similar growth properties to WSN–PB1(−3A) virus, indicating that eliminating PB1–F2 expression did not alter virus replication of the WSN–PB1(−3A) virus (Fig. 4e). This result suggested that the presence or absence of PB1–F2 was not responsible for attenuation of the WSN–PB1(−3A) virus.

**WSN–PB1(−3A) virus shows a slightly different protein expression profile from that of the WSN–WT virus**

In order to investigate the mechanism(s) by which the U-to-A mutation in the PB1 mRNA affected the efficiency of virus replication, we examined the effects of the optimized PB1 Kozak sequence in the course of a single-cycle infection. We first examined the viral protein expression profiles in A549 cells infected with WSN–PB1(−3A) and WSN–WT virus at m.o.i. 3 (Fig. 5a, b). It can be seen that the level of PB1 in the WSN–PB1(−3A) virus infected cells was significantly higher than that in the wild-type virus infected...
cells at early times post-infection (p.i.; 4 h p.i.). However, the difference could not be detected at later time points (8 and 12 h p.i.). Meanwhile, we also found that the expression of N40, a shorter form of PB1 protein that could be produced from the PB1 segment (Wise et al., 2009), was decreased in WSN–PB1(−3A) virus infected cells. It has been reported that overexpression of N40 had a detrimental effect on virus replication in cell culture (Tauber et al., 2012). Therefore, reduced expression of N40 was unlikely to contribute to the reduction in replication observed with the WSN–PB1(−3A) virus. In addition, we also detected significantly higher levels of PA and NP in the

![Diagram](image)
WSN–PB1(−3A) virus infected cells than those in wild-type virus infected cells at early times post-infection (4 h p.i.; Fig. 5a, b).

To further investigate the replication dynamics of the two viruses, we also performed immunofluorescence assay with anti-NP antibody during the course of their single-cycle infection (at m.o.i. 2). It showed that the nuclear export of NP occurred earlier in the WSN–PB1(−3A) virus infected cells than that in the WSN–WT virus infected cells (data not shown). These results suggested that the replication cycle of the WSN–PB1(−3A) virus was slightly accelerated compared to that of the WSN–WT virus during a single-cycle infection.

Enhanced PB1 expression alters the RNA synthesis dynamics of the WSN–PB1(−3A) virus

Since PB1 is the core subunit of the viral RNA polymerase, the U-to-A mutation in PB1 mRNA could alter the level of PB1 expression and subsequently could affect secondary viral RNA syntheses. To further demonstrate the possible mechanism(s) that could cause the attenuation of WSN–PB1(−3A) virus, we performed primer extension analysis to measure RNA synthesis dynamics of the three viral RNA species of the NA segment in a single-cycle infection of the two viruses (Fig. 5c). Marking 6 h p.i. as a turning point, the rate of synthesis of mRNA, cRNA and vRNA in cells infected with WSN–PB1(−3A) virus was slightly higher than that of the wild-type before 6 h p.i. (Fig. 5d). But after that, we found that the levels of mRNA and cRNA fell faster in cells infected with WSN–PB1(−3A) virus than those infected with WSN–WT virus (Fig. 5d). Meanwhile, although the vRNA level in the two virus infected cells continued to increase after 6 h p.i., the rate of vRNA synthesis in cells infected with WSN–PB1(−3A) virus also declined faster than that in WSN–WT infected cells (Fig. 5d). Overall, these data demonstrated that enhanced expression of PB1 protein in the early stage of infection resulted in slightly different RNA synthesis dynamics of WSN–PB1(−3A) virus from that of the WSN–WT virus.

 PB1 U-to-A mutant virus can induce type I IFN production at a higher level than wild-type virus

While our research was in progress, Belicha-Villanueva et al. (2012) reported that enhanced expression of PB1 increased the number of immunostimulatory RNAs and the production of type I IFN during virus infection. Therefore, we speculated that the PB1 U-to-A mutant virus may cause higher levels of type I IFN production than the wild-type virus during infection. To answer this question, we first
tested the replication properties of WSN–WT/WSN–PB1(−3A) and HK68–WT/HK68–PB1(−3A) viruses in Vero cells (an IFN secretion defective cell line). Interestingly, the results showed that virus titre differences between the wild-type and the PB1 U-to-A mutant virus in Vero cells became much smaller than that in MDCK or A549 cells (Fig. 6a, 6b). These results suggested that the infection with PB1(−3A) viruses may induce more type I IFN than that with wild-type viruses, which could account for the attenuation of the PB1(−3A) viruses. As the replication efficiency of the PB1 U-to-A mutant virus was increased in Vero cells, we excluded the possibility that the U-to-A mutation, located in the packaging signal of PB1, might affect virus replication efficiency in MDCK or A549 cells by affecting PB1 segment packaging efficiency.

To further investigate the properties of the two viruses, 293T cells transfected with an IFN-β luciferase reporter plasmid, were infected by WSN–WT and WSN–PB1(−3A) virus at m.o.i. 1, and the luciferase activity of cells was
measured at 12 h p.i. The results showed that infection with WSN–PB1(−3A) virus increased IFN-β promoter activity compared to that with WSN–WT virus infection (Fig. 6c). We also confirmed this result by measuring endogenous IFN-β mRNA levels in the two virus infected cells by real-time PCR (data not shown). In addition, to elucidate the above result, the IFN-β luciferase promoter assay was performed in 293T cells transfected with the RNP reconstitution system with gradually increasing concentrations of PB1 expression plasmids. Meanwhile, the levels of the three RNA species synthesized by corresponding RNP were also examined by primer extension assay. It could be seen that the IFN-β promoter was activated in a PB1 dose-dependent manner, whereas no activation of the IFN-β promoter was observed when the PB1 expression plasmid or HA RNA expression plasmid was omitted (Fig. 6d). Accordingly, the levels of the three RNA species were also increased with the increasing concentrations of the transfected PB1 expression plasmid (Fig. 6e, f), suggesting that viral RNAs act as immunostimulatory factors to

**Fig. 6.** The PB1 U-to-A mutant virus induced more type I interferon than wild-type virus. (a) Growth curves of WSN–WT and WSN–PB1(−3A) virus in Vero cells. (b) Growth curves of HK68–WT and HK68–PB1(−3A) virus in Vero cells. (c) The infection with WSN–PB1(−3A) virus induced more IFN-β than that with WSN–WT virus. The IFN-β level induced by WSN–WT and WSN–PB1(−3A) infection was measured by IFN-β luciferase promoter assay in 293T cells. Data are mean ± SD; n=3. Significance was analysed with two-tailed Student’s t-test: *, P<0.05. (d) The level of PB1 determines the amount of IFN-β production in the RNP reconstitution system. 293T cells were transfected with the PR8 RNP reconstituting plasmids, with or without the PR8 RNP reconstituting plasmids and with increasing dose of the pcDNA-PB1-PR8 (0.5, 1, 2 or 3 μg). Then luciferase activity was measured at 24 h post-transfection. Data represent the mean ± SD of three independent experiments. (e) The increasing amount of PB1 expression led to increasing amounts of RNA synthesized in the RNP reconstitution system. RNP was reconstituted in 293T cells as described in (d), synthesized levels of mRNA, cRNA and vRNA were detected by primer extension assay after transfection for 24 h. The results are representative of three independent experiments. (f) Quantitative analysis of viral RNAs in (e). Values of viral RNAs are expressed as fold change relative to the viral RNA levels synthesized from the RNP system with 0.5 μg pcDNA-PB1 plasmid. Data represent the mean ± SD of three independent experiments.
induce type I IFN production in RNP reconstituted cells. Together, these data demonstrated that enhanced PB1 expression in infected cells could induce more IFN-β production.

DISCUSSION

It is known that the segment-specific NCRs of influenza A virus contain critical signals required for transcription, replication and packaging of each viral gene segment. In this report, through our systematic bioinformatics analysis, we present, for the first time to our knowledge, a full view of the segment-specific NCR of all influenza A viruses, which not only fully summarizes the genetic characteristics of the segment-specific NCR, but also directly shows the nucleotide conservations/variations at each nucleotide position as a result of natural selection during long-term virus evolution.

We also studied the roles of the segment-specific NCRs in the differential regulation of both viral RNA synthesis and protein production in influenza RNP reconstitution systems. At the viral RNA transcription and replication level, we observed that the segment-specific NCR of NS segment mediated a particularly high level of mRNA production (Fig. 2b, c). This is consistent with the fact that a high level of NS mRNA is naturally required because it not only acts as a template directly to produce the NS1 protein, but it is also used as pre-mRNA to be spliced into nuclear export protein mRNA (Hale et al., 2008). In addition, we found that the NCR of PB2 mediated high levels of vRNA production and the NCR of PB1 mediated relatively low levels of vRNA production but higher levels of cRNA production (Fig. 2b, c). It would be interesting to further elucidate whether these regulations have biological significance during viral replication.

Interestingly, at the level of protein translation, we found that a highly conserved nucleotide at position −3 of the Kozak sequence in the PB1 segment uniquely mediated a significant downregulation of protein expression at the translation level (Fig. 3). Although Maeda et al. (2004) have previously made an effort to study the biological function of the U nucleotide at position −3 of the PB1 vRNA, together with the −3U of the NA vRNA in the context of the WSN virus, they showed negative effects in general (Maeda et al., 2004). However, in the present study, we found that the replication level of the PB1 U-to-A mutants in both a laboratory adapted WSN virus [WSN–PB1(−3A)] and a seasonal influenza virus [HK68–PB1(−3A)] were reduced about 7–10-fold compared with wild-type viruses in MDCK cells (Fig. 4a–c). The reason for the discrepancy is unknown. Nevertheless, our in-depth study further revealed that the U-to-A mutation enhanced PB1 expression at an early stage of infection and resulted in not only an alteration in RNA synthesis dynamics of WSN–PB1(−3A) virus from that of the WSN–WT virus (Fig. 5), but also induction of a higher type I IFN level than did WSN–WT during infection (Fig. 6c).

It was reported that the viral single-stranded RNA genomes bearing 5′ triphosphates can be recognized by RIG-I in influenza A virus infected cells, triggering cell-intrinsic innate immune response (Davis et al., 2012; Rehwinkel et al., 2010). Our study showed that RNPs with an increasing amount of PB1 expression result in increased levels of three species of RNA and increased type I IFN production in a PB1 dose-dependent manner (Fig. 6d–f). Therefore, minor changes in viral RNA synthesis dynamics would affect host innate immunity responses in the course of virus infection. These observations are also consistent with a study by Belicha-Villanueva et al. (2012), which showed that enhanced PB1 protein expression resulting from G3A/C8U mutation in the PB1 gene promoter disrupts the stoichiometry of viral RNA synthesis and/or protein expression, and consequently increases the number of immunostimulatory RNAs to induce type I IFN production. Therefore, we propose that the suboptimal Kozak sequence is used by the influenza A virus PB1 gene to not only fine-tune virus replication kinetics, but also to optimize the balance between virus replication rate and host antiviral status to make sure viruses are produced in the most efficient manner.

In summary, our results have revealed that segment-specific NCRs play important roles in the differential regulation of viral RNA synthesis and protein expression. Intriguingly, in the course of viral evolution the PB1 segment of influenza A virus has specifically selected a relatively weak Kozak sequence in its segment-specific NCR, which mediates a relatively low expression of the PB1 protein. We report here that the suboptimal Kozak sequence is specifically used by the PB1 segment of influenza A virus to fine-tune virus replication efficiency and host responses. This finding provides new insights into the regulatory mechanisms of NCRs of the influenza A virus genome.

METHODS

Cells and antibodies. 293T, MDCK, A549 and Vero cells were purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium (Gibco) with 10% FBS (Gibco) and penicillin–streptomycin. The rabbit anti-PA polyclonal antibody was kindly provided by Dr Ervin Fodor (University of Oxford). Goat polyclonal antibody against PB1 was purchased from Santa Cruz Biotechnology, mouse anti-influenza NP monoclonal antibody from Millipore (MAB8251), mouse anti-β-actin monoclonal antibody from Cell Signaling Technology, and rabbit monoclonal anti-influenza virus HA from Sino Biological (86001-RM01).

Plasmids. The pcDNA and pPoll-HA plasmids of RNP reconstitution system of influenza A/Puerto Rico/8/34 (PR8) and A/Viet Nam/1194/2004 (H5N1) were provided by Dr Ervin Fodor. The series of plasmids pPoll-NCR(1–7)–HA (where 1–7 represents PB2, PB1, PA, NP, NA, M and NS), containing HA ORF (PR8) flanked by NCRs from other segments, were constructed as follows: at both ends of HA ORF, NCRs of the other seven segments were extended with the primers containing flu segment-specific NCR sequences and HA-specific sequences by two-step PCR; the second-step PCR primers contained a SapI restriction site. The PCR products were then digested with SapI and cloned into pPoll-SapI-Rib empty plasmid. The pGL3-INF/β-Luc and pGL3-RLuc plasmids were gifts from Professor Zhenzong Zhao in our institute. The eight pHW plasmids for generation of recombinant A/WSN/33 (H1N1) viruses have been
described previously (Hoffmann et al., 2000). The eight pLLB plasmids for generating recombinant A/Hong Kong/1968(H3N2) viruses were kindly provided by Professor Earl G Brown (University of Ottawa). The −3 U-to-A mutation in both pHW-PB1(−3A)–WSN and pLLB-PB1(−3A)–HK68 was generated by site-directed mutagenesis using a QuikChange Site-Directed Mutagenesis kit (Stratagene).

Bioinformatics analysis of NCRs of influenza A viruses. The same bioinformatics analysis was performed as previously described (Zhao et al., 2014). The sequences of all segments (PB2, PB1, PA, H3, NP, N2, M and NS) were obtained from NCBI Influenza Virus Resource database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) on 2 April 2013 (Bao et al., 2008).

Primer extension and Western blot analyses. Primer extension analysis was carried out as described previously (Fodor et al., 2002). The primer sequences used to detect HA(PR8) vRNA and mRNA/cRNA were 5′-AGTTCACTGGTGCTTTTGGTC-3′ and 5′-TGTCACATCTGAGGCAC-3′. The primer sequences used to detect NA(WSN) vRNA and mRNA/cRNA were 5′-TCCAGTATGGTTTGATTTCGG-3′ and 5′-TGACGAGTTGGGACCATACT-3′. The primer sequence used to detect 55 RNA was 5′-TCCACGGGCTTCCTCCATCC-3′. Western blotting was carried out by standard procedures with IRDye secondary antibodies (LI-COR Biosciences). Protein expression levels were visualized with an Odyssey Infrared Imaging System (LI-COR Biosciences). Relative protein expression level was analysed using the integrated software of the Odyssey system.

Virus rescue and viral growth kinetics. Recombinant viruses (the wild-type and mutant viruses) were rescued by the previously described eight-plasmid rescue system (Hoffmann et al., 2000; Liu et al., 2009). The rescued viruses were plaque purified and fully sequenced. The viral growth kinetics of wild-type and mutated viruses were performed in various types of cells (A549, MDCK and Vero cells) at m.o.i. 0.001 and p.f.u. titres were determined by a standard plaque assay in MDCK cells.

IFN-β luciferase promoter assay. 293T cells (2 × 10^5) were transfected using Lipofectamine 2000 (Invitrogen) with 200 ng pGL3-IFNβ-Luc and 5 ng pGL3-RLuc plasmids. Cells were co-transfected with indicated RNP reconstitution plasmids or infected with WSN–WT or WSN–PB1(−3A) virus, respectively, for 24 h. Cells were lysed and luciferase activity was measured by a Dul-Luciferase Reporter Assay System (Promega).

ACKNOWLEDGEMENTS

We thank Professor George Brownlee (University of Oxford) for critically reading the manuscript. This work is supported by grants from the National Natural Science Foundation of China (grant no. 31070152) and Chinese Science and Technology Key Projects (grant nos 2013ZX10004601 and 2013ZX10004611).

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