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Packaging signals in the 5′-ends of influenza virus PA, PB1, and PB2 genes as potential targets to develop nucleic-acid based antiviral molecules

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ABSTRACT

In a previous study a 15-mer phosphorothioate oligonucleotide (S-ON) derived from the packaging signal in the 5′ end of segment 1 (PB2) of influenza A virus (designated 5–15b) proved markedly inhibitory to virus replication. Here we investigated whether analogous inhibitory S-ONs targeting the 5′ end of segments 2 (PB1) and 3 (PA) could be identified and whether viral resistance to S-ONs can be developed. Similar to our earlier result, 20-mer S-ONs reproducing the 5′ ends of segments 2 or 3 (complementary to the 3′-coding regions of PB1 and PA, respectively) exerted a powerful antiviral activity against a variety of influenza A virus subtypes in MDCK cells. Serial passage of the A/Taiwan/1/86 H1N1 strain in the presence of S-ON 5–15b or its antisense as5–15b analogue showed that mutant viruses with reduced susceptibility to the S-ON could indeed be generated, although the resistant viruses displayed reduced replicative fitness. Sequencing the resistant viruses identified mutations in the PB1, PB2, PA and M1 genes. Introduction of these changes into the A/PR/8/34 H1N1 strain by reverse genetics, suggested that alterations to RNA function in the packaging regions of segments 2 and 3 were important in developing resistance to S-ON inhibition. However, many of the other sequence changes induced by S-ON treatment were markedly deleterious to virus fitness. We conclude that packaging signals in the influenza A virus polymerase segments provide feasible targets for nucleic acid-based antivirals that may be difficult for the virus to evade through resistance mutations.

1. Introduction

Recent years have witnessed numerous attempts to develop antiviral drugs that may help in combating the emergence of seasonal and pandemic influenza viruses, especially those that have reduced susceptibility to the neuraminidase inhibitors currently available (Beigel and Bray, 2008; Boltz et al., 2010; Hayden and de Jong, 2011). A major concern regarding influenza virus is that possessing a genome consisting of seven or eight segments of negative-sense single-stranded RNA renders it prone to genetic variability and reassortment (Palese and Shaw, 2007). These features facilitate the rapid evolution of influenza virus and underpin the emergence and spread of resistance to the currently available antivirals targeting the M2 ion channel or neuraminidase (De Clercq, 2006). To circumvent these challenges, attempts have been focused on generating new therapeutic approaches with broad-spectrum activity (Das et al., 2010). The versatility and potency of nucleic acid-based antiviral molecules make them potential candidates for new tools to counteract viral transmission (Bennett and Swayze, 2010; Lyall et al., 2011; Van Aerschot, 2006). In fact, antisense oligonucleotides (with phosphorothioate or phosphorodiamidate morpholino oligomers modifications to protect them from nuclease mediated degradation) and small interfering RNAs have been used previously to inhibit viral gene expression (reviewed in Beigel and Bray, 2008; Spurgers et al., 2008). In this scenario the choice of viral targets that are as genetically conserved as possible is critical. The packaging signals in the RNA segments of the influenza virus genome should be viewed as extremely interesting targets for designing antiviral molecules thanks to their wide conservation and the critical role they play in viral assembly (Hutchinson et al., 2010).

In line with this idea, we and others recently demonstrated the antiviral activity in vitro and in vivo of phosphorothioate oligonucleotides (S-ON) derived from the conserved 5′-terminal untranslated region found in all 8 RNA segments of influenza A and from the packaging signal at the 5′ end of segment 1 complementary to the 3′ end of the PB2 coding region (Duan et al., 2008;

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Giannecchini et al., 2009). In particular, we identified a 15-mer S-ON designated 5–15b that proved markedly inhibitory for influenza virus replication in cell culture, without affecting the intracellular accumulation of viral RNA, possibly acting at a late stage of viral replication (Giannecchini et al., 2009). Here, because the packaging signals in the polymerase genes appear to play a key role in the hierarchy of segment interactions during genome packaging (Muramoto et al., 2006), we tested whether the PB1 and PA segment packaging signals also provide feasible antiviral targets for S-ON inhibition.

2. Materials and methods

2.1. Cells, viruses, plasmids and S-ON

Human embryonic kidney 293T cells and Madin-Darby canine kidney (MDCK) cells were maintained in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum. The influenza viruses used included strains of H1N1 subtype (A/Taiwan/1/86, A/PR/8/34, A/Roma/2/08, an oseltamivir resistant isolate A/Parma/24/09, and the oseltamivir sensitive new pandemic variant A/Italy/05/09), local strains of H3N2 subtype (A/Firenze/1/03, A/Firenze/5/03, A/Firenze/4/07), and two avian strains of H7N3 subtype described previously (A/Mallard/Italy/33/01, A/Turkey/Italy/214845/02; Campitelli et al., 2004). The viral stocks consisted of cell-free supernatants of infected MDCK cells. The influenza A/PR/8/34 (PR8) virus clone was an MDCK cell adapted version of the UK National Institute for Biological Standards and Control PR8 strain generated using a reverse genetics system kindly donated by Professor R. Fouchier (de Wit et al., 2004). Site-directed mutagenesis of the reverse genetics plasmids was used to introduce specific mutations. All mutated constructs were sequence confirmed. Infectivity titrations were carried out in MDCK cells either by plaque assays using an Avicel overlay (Hutchinson et al., 2008) or by 50% tissue culture infectious dose ($TCID_{50}$) measurements calculated using the Reed and Müench method (Reed and Müench, 1938). S-ON (Fig. 1a,c, and PB2 5–15b, as5–15b and scr5–15b, according to PR8 and type A consensus sequence) were synthesized and purified according to standard procedures (Roche, Milan, Italy).

2.2. Assay for influenza virus inhibition

Cultures of MDCK cells (5×10^5 cells) were exposed to 100 μl of virus at a multiplicity of infection (MOI) of 0.01 for 1 h at 37 °C and excess inoculum was removed prior to treatment with S-ON. To facilitate S-ON entry into the cells, Lipofectin reagent (Invitrogen, Carlsbad, CA) was used as a lipid-based carrier. Lipofectin was diluted in MEM (5%) and kept at room temperature for 45 min. Then, the S-ONs dissolved in MEM (the final concentration added to the cells ranged from 0.1 to 10 μM) were combined 1:1 with Lipofectin, mixed gently, and kept at room temperature for 15 min. The mixtures (200 μl) were added to cells, and incubated for 4 h. Infected MDCK cells treated with liposomes in the absence of S-ON served as a control for possible effects unrelated to antiviral molecules. Virus growth in the cultures was analyzed 24 h post-infec-

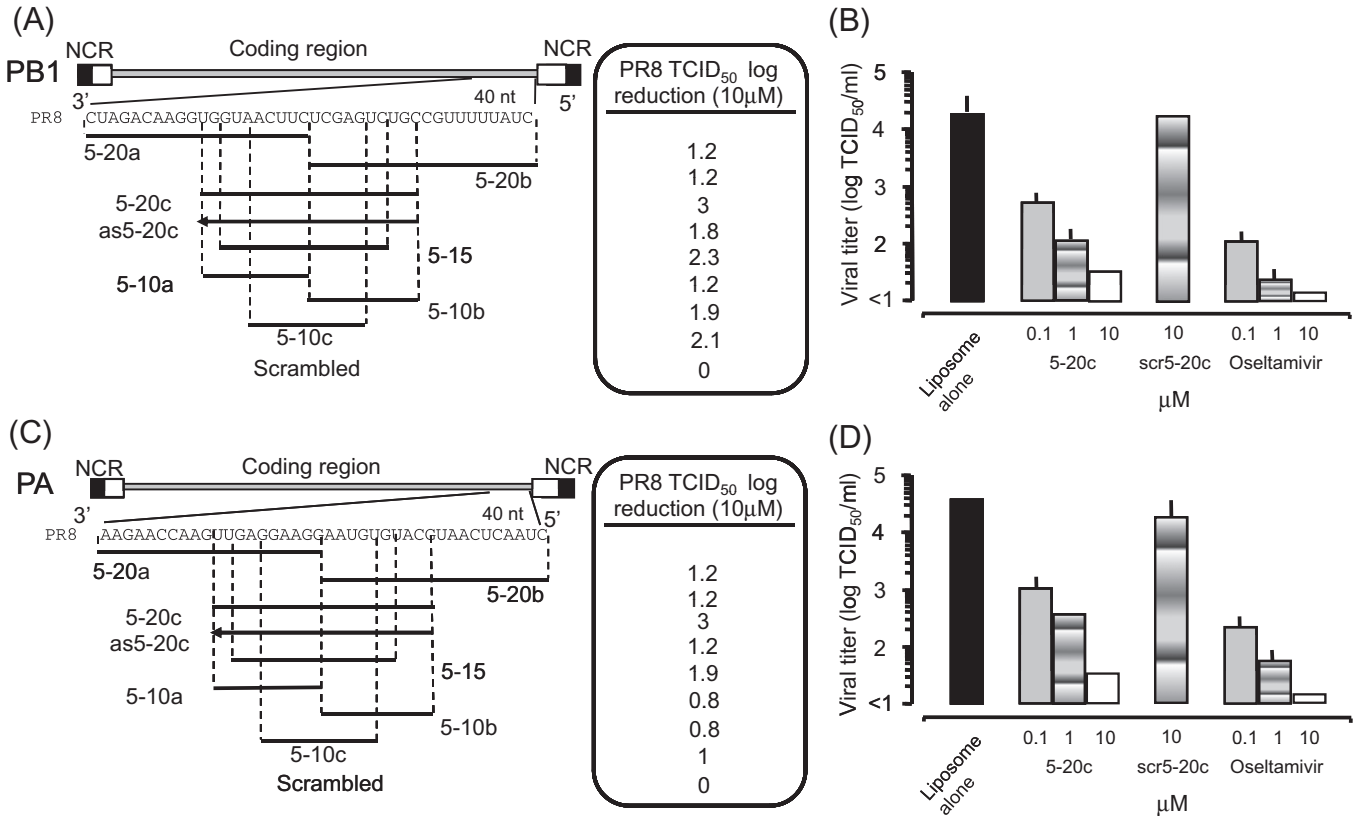

Fig. 1. Antiviral activity of PB1- and PA-derived S-ON. (A and C) Schematic representation of the PB1 and PA segments of influenza virus and the packaging signal region-derived synthetic S-ONs used in the study. Values in the boxes indicate the antiviral activity of the S-ON against influenza virus PR8 strain (Mean $\log_{10}$ reduction of viral titer compared to no treatment). S-ONs are named according to the region of vRNA from which they were derived (figure before the dash) and the number of nt (figure after the dash). An antisense S-ON reproducing the same PB1 and PA segment as 5–20c but with the complementary sequence is indicated as5–20c. NCR, non coding region. (B and D) Comparison of antiviral activity of selected S-ON PB1 and PA 5–20c with oseltamivir at different concentration. In A–D infection was carried out at an MOI of 0.01. Viral growth is expressed as $\log_{10}$ $TCID_{50}$/ml present in the supernatant fluids collected 24 h after infection. In B and D the values shown are means ± standard deviations of 3 independent experiments. In the experimental conditions used neither the S-ONs at all concentrations associated with liposomes nor liposomes alone exerted more than more than 10% reduction in cell viability.

tion by titrating the progeny virus present in the supernatants with the $CCID_{50}$ infectivity assay exactly as previously (Giannecchini et al., 2009). Cell viability was quantified using a colorimetric MTT cell proliferation assay kit (Roche, Milan, Italy) according to the Supplier's instructions.

2.3. S-ON selection in vitro

S-ON selection of the influenza A/Taiwan/1/86 H1N1 strain was performed in MDCKs. Briefly, MDCK cells were infected at an MOI of 0.01 for 1 h at 37 °C and, after washing away excess inoculum, treated with 0.1 μM S-ONs 5–15b, or its antisense (as5–15b, composed with the complementary sequence of 5–15b) or scrambled (scr5–15b, composed with the same nucleotides of 5–15b but with different sequence) forms associated with liposomes as described above for 4 h. After 4 h at 37 °C, the cells were washed and cultured in MEM supplemented with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin (2 μg/ml, Sigma). Virus variants harvested after 24 h underwent 14 additional passages on MDCK cells using an MOI of 0.01 but with increasing S-ON concentrations up to 20 μM (two additional passages at 0.1 μM, and sequential 3 serial passages at 0.5, 5, 10, 20 μM, respectively). At passages 5, 10 and 15, the variants were cloned by plaque formation in MDCK cells. Clones were picked up and propagated for one more passage to obtain virus variants for genotypic and phenotypic study.

2.4. Sequencing

Viral RNA was extracted and purified using an RNAeasy mini Kit (Qiagen). RNA was reverse-transcribed, and PCR-amplified employing terminal segment specific primers of 21–29 nucleotides, as one single amplicon, or as two overlapping amplicons using an additional pair of internal primers for the PB2, PB1, PA and HA segment (primer sequences are available upon request). The PCR products were purified using the PCR purification Kit (Qiagen) and sequenced using the BigDye Terminator Cycle-Sequencing Ready Reaction (Applied Biosystems, CA). Sequences were analyzed and edited using Bioedit 5.0.9.

2.5. Reverse genetics

Wild type and mutagenized virus clones made using the 8 plasmid PR8 reverse genetics constructs described by de Wit et al. (2004) were rescued in 293T cells by transfecting 10^6 cells with 250 ng of each plasmid and 4 μl of lipofectamine 2000. Supernatants were harvested 48 h later, and virus was amplified and plaque titred on MDCK cells (Hutchinson et al., 2008; Wise et al., 2009).

2.6. Minireplicon assay

Viral RNPs were reconstituted by transfecting 50 ng each of plasmids encoding the three polymerase proteins (3P) and NP from the PR8 strain into adherent 293T cells along with 20 ng of a plasmid that expresses a synthetic segment 8-based vRNA encoding luciferase in antisense (Mullin et al., 2004; Wise et al., 2009). Forty-eight hours post transfection, luciferase activity in cell lysates was measured using a Promega Glomax luminometer.

2.7. Quantitative PCR

Quantitative PCR was performed on RNA extracted from equal plaque forming units (PFU) of virus using a QiaSymphony extractor (Qiagen). RT-PCR was performed by using the SuperScript IIIPlatinum one-step qRT-PCR system in a Rotor-gene 3000 real-time thermal cycler. Segment concentration was calculated with respect to plasmid standards as described previously (Hutchinson et al., 2008, 2009).

3. Results

3.1. Effect of type A influenza PB1- and PA-based S-ONs on H1N1 influenza virus replication

We first explored whether virus sequences from the packaging signals at the 5′ end of the PB1 and PA genes, complementary to the 3′ end of their coding region, could be exploited to design new antiviral molecules as previously demonstrated for the PB2 gene (Giannecchini et al., 2009). For this purpose, S-ONs of different

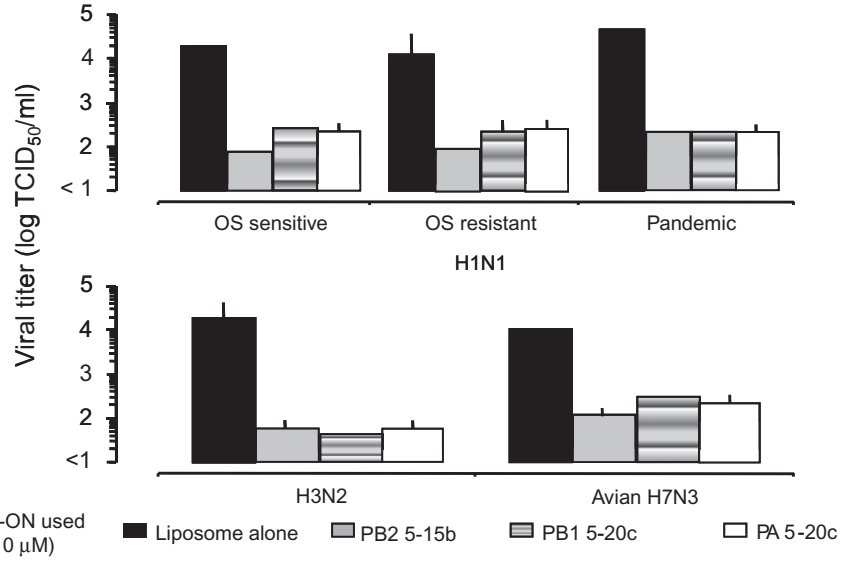

Fig. 2. Inhibition of different influenza virus strains by PB1 and PA 5–20c and PB2 5–15b S-ON. Virus used: subtype H1N1 isolates included seasonal oseltamivir (OS) sensitive and resistant strains as well as H1N1pdm pandemic strain; subtype H3N2 isolates; avian subtype H7N3 isolates. The 5–15b sequence is 3′ GGUUUUCUUAAGCCU 5′. Infection was carried out at an MOI of 0.01. Viral growth is expressed as log $TCID_{50}$/ml present in the supernatant fluids collected 24 h after infection. The values shown are means ± standard deviations of 3 independent experiments.

lengths (Fig. 1a,c) were synthesized based on known packaging signal regions of segments 2 and 3 of type A influenza virus (Liang et al., 2005, 2008; Marsh et al., 2008). We targeted areas that showed a high level of functional constraint for both protein coding and *cis*-acting RNA elements (Gog et al., 2007). The S-ON were tested for their impact on influenza virus H1N1 replication using the experimental procedures established previously for S-ON derived from PB2 (Giannecchini et al., 2009). Overall, at 10 μM concentration, the S-ONs with highest antiviral activity were the 5–20c ones from the terminal regions of both PB1 and PA vRNAs, showing a reduction in virus yield of 3 $\log_{10}$ (Fig. 1a,c). The antisense S-ONs composed with the complementary sequence of the PB1- and PA-derived 5–20c (as5–20c) also proved inhibitory. Conversely, a scrambled S-ON molecule was totally devoid of inhibitory activity. The PB1- and PA-derived 5–20c molecules showed inhibitory activities that were clearly dose- and sequence-dependent but somewhat lower than that of the same concentrations of oseltamivir (Fig. 1b,d). The PB1- and PA-derived S-ON also exerted effective antiviral activity against a variety of H1N1 subtype human influenza virus isolates (including both oseltamivir susceptible and resistant seasonal viruses as well as the 2009 pandemic strain), H3N2 and avian H7N3 strains, comparable to that exerted by the PB2-derived S-ON 5–15b (reducing virus yields between 1.8 and 2.2 $\log_{10}$; Fig. 2).

3.2. S-ON in vitro selection of H1N1 influenza virus variants

We next investigated whether virus resistance could be induced as a consequence of S-ON treatment. To this end, the influenza A virus strain A/Taiwan/1/86 (H1N1), previously used to investigate in vitro virus selection of resistance to the neuraminidase inhibitors (Giannecchini et al., 2007), was chosen. We serially passaged this highly S-ON sensitive H1N1 strain (Fig. 3a) in the presence of increasing concentrations of the PB2-derived S-ON molecules 5–15b, the antisense analogue as5–15b, or its scrambled control form scr5–15b. At passage number 5, 10, and 15 virus variants were cloned by plaque formation in MDCK cells for phenotypic and genotypic analysis. After 10 passages only the virus variants

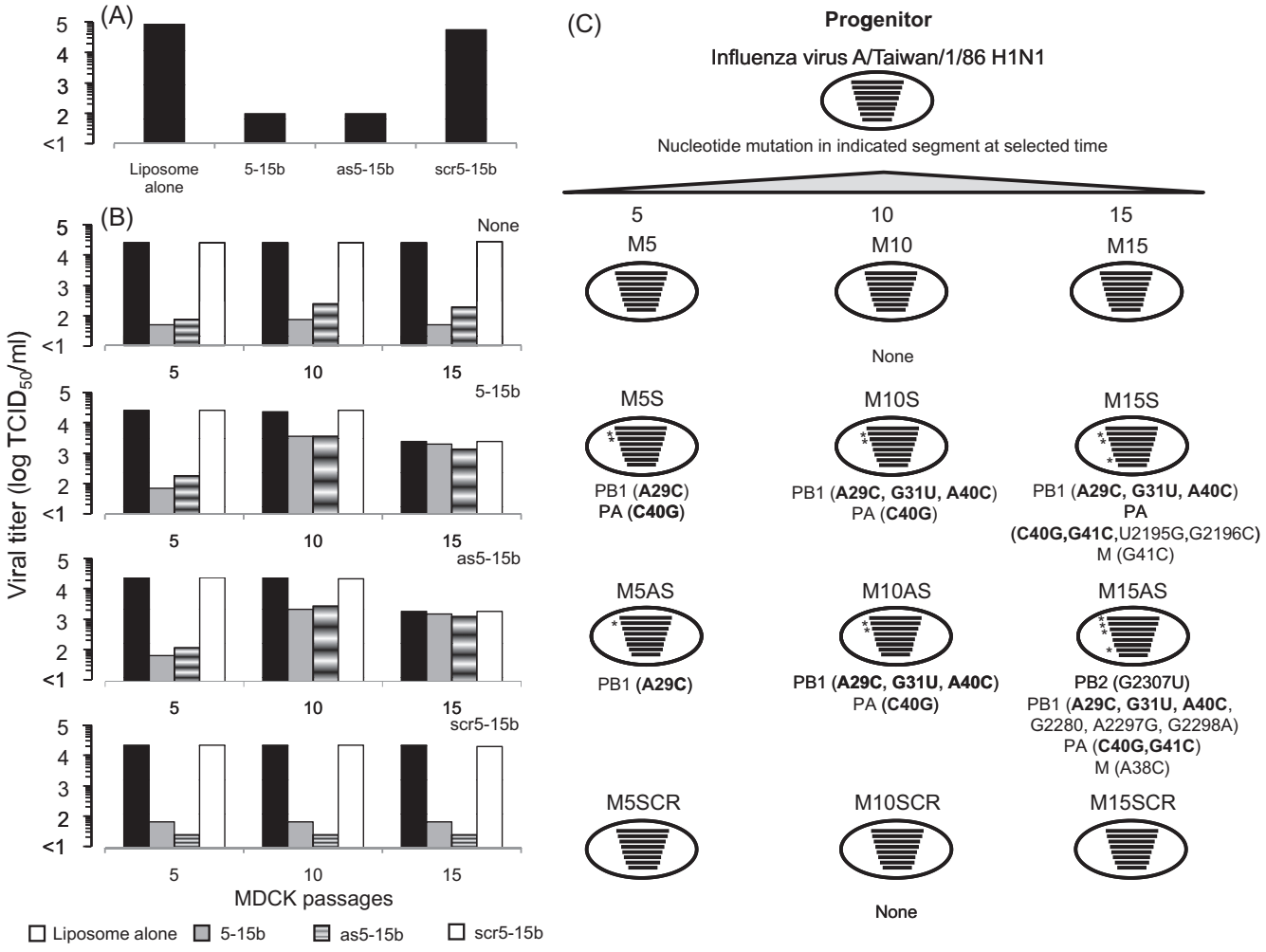

Fig. 3. Effect of the S-ON in vitro selection of an H1N1 influenza virus. (A) Progenitor virus A/Taiwan/1/86 H1N1 susceptibility to indicated S-ON at 10 μM (final concentration). (B) Virus variants were collected after 5, 10 and 15 passages on MDCK cells in the absence (M5, M10 and M15) or in the presence of PB2-derived 5–15b (M5S, M10S an M15S), as5–15b (M5AS, M10AS and M15AS), scr5–15b (M5SCR, M10SCR and M15SCR) selecting factors at concentration indicated in Section 2, and genotypes and phenotypes were characterized. The S-ON used to select virus variant is indicated at the top right of each graphic. None, liposome alone. All variants were assayed for their susceptibility to S-ON of PB2 at 10 μM (final concentration). In A and B infection was carried out at an MOI of 0.01 using S-ON at 10 μM. Viral growth is expressed as log $TCID_{50}$/ml present in the supernatant fluids collected 24 h after infection. The values shown are means of 3 independent experiments. (C) Difference of genotype of each variant compared to the progenitor. The eight segments are indicated by solid bars. Segments carrying mutations are indicated by asterisk. Coordinates of the mutations observed in the virus variants compared to A/Taiwan/1/86 are given in mRNA sense (Genebank accession numbers are JF816562–JF816657). In bold are indicated common mutation.

passaged in the presence of 5–15b (M10S) or as5–15b (M10AS) S-ON exhibited reduced sensitivity to either S-ON, acquiring complete resistance at the 15th passage (variant M15S and M15AS, Fig. 3b). Of note, the variants M15S and M15AS, also resistant to the PB1- and PA-derived 5–20c S-ON (data not shown), showed a decreased replication ability compared to the virus selected in the absence of S-ON.

Sequencing the virus variants obtained at 5, 10 and 15 passages in the presence of the inhibitory oligonucleotides identified nucleotide changes in a total of 13 positions located in the 3′ and 5′ ends of the PB2, PB1, PA and M segments. Certain mutations in the PB1 and PA segments were acquired early during selection and were also common to viruses selected with sense and antisense S-ON inhibitors (Fig. 3c; highlighted in bold). All changes were within regions defined as containing the packaging signals of the four segments (Hutchinson et al., 2010), and 5 of the 7 mutations within coding regions affected highly conserved codons (Table 1). The single mutation seen in segment 1 did not map to the S-ON binding site. Instead, a G2307U change was identified in the 5′ end of the PB2 ORF, which removed the stop codon leading to a predicted coding extension of 4 amino acids (Table 1). Changes in the 3′ end of the PB1 gene were seen with both the sense and antisense S-ON treated viruses (A29C, G31U and A40C in mRNA sense), and these introduced amino acid changes D2A, V3F and T6P respectively. With the as5–15b S-ON, additional changes were seen after 15 passages in the 5′ end of the vRNA, where G2280C introduced the amino acid change E752D, and A2297G and G2298A were seen in the NCR (Non Coding Region). Overlapping nucleotide changes also occurred in segment 3 following passage in the presence of either inhibitory S-ON. C40G and G41C substitutions were seen in the 3′ end of vRNA, leading to an R6A change in the PA protein. Two other mutations were identified in the non coding region at the 5′ end of segment 3 vRNA following passage with 5–15b (U2195G and G2196C). Single changes in the 3′ end of segment 7 vRNA were seen following serial passage with 5–15b and as5–15b, but only after 15 passages. Both of these were non-synonymous: A38C seen with as5–15b led to a T5P substitution, whereas the G41C change seen in the presence of 5–15b lead to a V6L change. No changes were found in the viruses passaged in the absence of S-ON or in the presence of the scrambled control (Fig. 3c).

3.3. Effect of mutations on viral genome packaging and on susceptibility to the S-ON

To test the role of the mutations in mediating S-ON resistance, we used a PR8 virus-reverse genetics system (de Wit et al., 2004) to produce virus clones with subsets of the changes, taking the strategy of altering individual termini of one vRNA at a time (Table 1). This approach took advantage of a robust and readily available reverse genetics system and was valid because the regions targeted by the S-ON, and changed by the potential resistance mutations, were well conserved between the two virus strains. Wild type PR8 was rescued as a positive control, and one of the polymerase segments was omitted as a negative control. Viruses were amplified by a single passage in MDCK cells and endpoint titers determined by plaque assay. Initially attempts to rescue a virus containing all of the sequence changes failed (data not shown), suggesting that some of the changes might be deleterious to virus viability. We therefore used the strategy of rescuing viruses with alterations to one vRNA terminus at a time. Of the seven rescues attempted, 4 viable viruses were obtained, even after multiple attempts: PB1 5′, PA 3′, PA 5′ and M 3′b (Table 1). Of these, PA 3′, PA 5′ and M 3′b were attenuated to varying extents, showing replication deficits of between ~4 and 10-fold compared to WT PR8, while PB1 5′ grew comparably (Fig. 4a).

To try to understand the reason for these defects, the ability of the mutant polymerase proteins to support viral gene expression was analysed (again, on a PR8 background) using a minireplicon system that recreates functional virus RNPs encoding luciferase by plasmid transfection (Mullin et al., 2004; Wise et al., 2009). The wild type genes were transfected as a positive control (3PNP) and used as a baseline to which the activities of the mutant proteins were normalized, while one of the polymerase plasmids was substituted with empty vector as a negative control (2PNP). The two polymerase mutants that could not be rescued (PB2 5′ and PB1 3′) supported levels of transcription less than 5% of the wild type segment (Fig. 4b). Polymerase mutations (mutants PB1 5′ and PA 5′ and 3′) that could be rescued into viable viruses all produced higher levels of transcriptional activity; PB1 and PA 5′ supported similar levels of transcription to the WT polypeptides, although PA 3′ had only 9.3% of the activity of wild type PA. Thus, alterations to polymerase function resulting from non-synony-

Table 1
Characteristic of the construct carrying the mutations in 3′- or 5′-ends of selected segment.

Name	Segment	Mutation[a]	MPD score[b]	Coding[c]	S-ON used[d]	Viable virus[e]	Resistance[f]
PB2 5′	1	G2307U	N/A	Stop760Y	AS	0/2	N/A
PB1 3′	2	A29C	0.012	D2A	S and AS	0/2	N/A
		G31U	0.008	V3F			
		A40C	0.29	T6P			
PB1 5′	2	G2280C	1	E752D	AS	5/5	Yes
		A2297G	N/A				
		G2298A	N/A				
PA 3′	3	C40G	0.03	R6A	S and AS	4/4	Yes
		G41C		R6A			
PA 5′	3	U2195G	N/A	None	S	2/2	No
		G2196C					
M 3′a	7	A38C	0.23	T5P	AS	0/2	N/A
M 3′b	7	G41C	0.018	V6L	S	2/2	No

[a] Coordinates of the mutations in A/Taiwan/1/86 are given in mRNA sense.
[b] Normalized mean pairwise difference (MPD) scores of codons altered by S-ON resistance mutations. Low values (<0.1) indicate codons that show high levels of conservation above that expected for protein-coding capacity, consistent with the presence of RNA packaging signals (Gog et al., 2007). N/A: not applicable.
[c] Predicted coding changes resulting from the S-ON resistance mutations.
[d] Mutations seen after passage in the presence of S-ON 5–15b (S) or as5–15b (AS).
[e] Number of times an A/PR/8/34 virus containing the indicated mutation was successfully rescued/number of independent attempts.
[f] Summary of whether an A/PR/8/34 virus containing the indicated changes was resistant to S-ON treatment. N/A: not applicable.

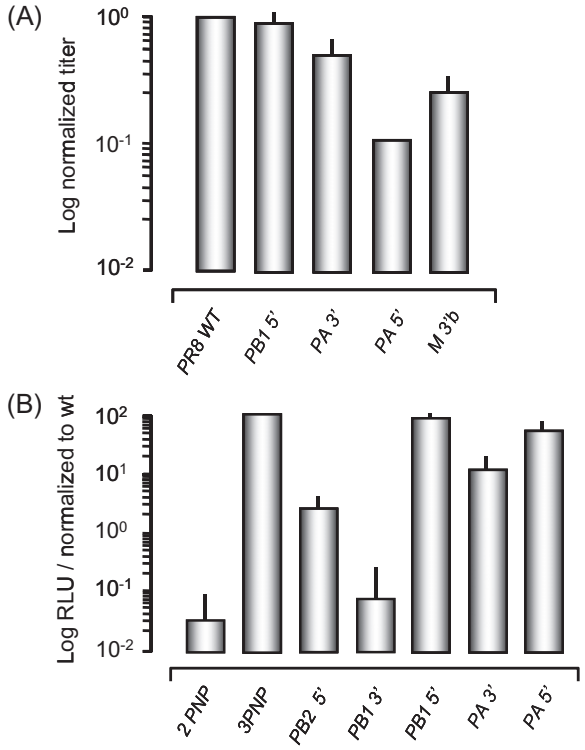

Fig. 4. Characterization of mutant segments used in reverse genetics. (A) Normalized titers for successfully rescued viruses. The mean ± standard error relative to wild type PR8 virus is plotted ($n \geqslant 2$). (B) Transcriptional activity of the mutant polymerase proteins. RNPs were reconstituted by transfection of plasmids expressing PR8 PB2, PB1, PA and NP and a vRNA encoding luciferase into 293T cells (3PNP denotes the full wild type PR8 polymerase, 2PNP indicates samples where PB2 was replaced with empty vector as a negative control and the mutants are as indicated), and luciferase activity was measured at 48 h post transfection. The mean ± standard error ($n \geqslant 3$) is plotted, normalized to the wild type activity (100%).

mous mutations provided a plausible partial explanation for the changes in virus fitness seen, with the exception of PA 5′ where near normal polymerase activity was seen despite a 10-fold drop in virus titer.

Because the S-ONs mapped to regions implicated in genome packaging it was possible that resistance mutations might affect vRNA incorporation. We therefore used quantitative RT-PCR to examine the vRNA content of equal PFU of the viruses, since previous work has shown that influenza packaging mutants often have a higher particle:PFU ratio and that this is reflected in a higher segment copy number:PFU ratio (Hutchinson et al., 2008, 2009). As targets, we chose the three vRNA segments (2, 3 and 7) where we could successfully introduce resistance mutations as well as the unaltered segment 5. The only virus with an obvious difference in genome packaging was the segment 3 mutant, PA 5′ (U2195G, G2196C), which in comparison to WT virus, showed underincorporation of segments 3 and 5 whilst overincorporating segments 2 and 7 (Fig. 5a). An apparent link between segments 3 and 5 during genome packaging has been previously reported (Hutchinson et al., 2009; Marsh et al., 2008). The packaging defect displayed by the PA 5′ virus also provides an explanation for its reduced replication, despite normal polymerase activity.

Next, the susceptibility of the A/PR/8/34 mutants to the PB1 (5–20c), PA (5–20c) and PB2 (5–15b and as5–15c) S-ON was tested. The WT virus was sensitive to all S-ON treatments, displaying a 1–2 $\log_{10}$ reduction in $TCID_{50}$ in the presence of the inhibitors (Fig. 5b). Similarly, the PA 5′ and M 3′b mutants remained sensitive to all the S-ON molecules. In contrast, the virus with mutations in the 5′ end of segment 2 vRNA, PB1 5′, was markedly resistant to the presence of any of the S-ON tested. A more modest reduction in susceptibility to the S-ON was also seen for the PA 3′ mutant.

Both resistant viruses that were generated by reverse genetics contained more than one nucleotide change. Therefore to determine if individual mutation(s) were critical for resistance, viruses with single nucleotide changes were generated and characterized. Where necessary, to determine whether S-ON resistance resulted from changes to protein sequence and/or alterations to overlapping RNA signals, additional mutations were made that were either synonymous or altered the codon via a different nucleotide change (Table 2). For PB1 5′, where three nucleotide changes were found to generate S-ON resistance, the following series of constructs were generated. PB1 5′a contained a single G2280C mutation (within the PB1 5–20c S-ON target sequence), leading to a predicted E752D change in PB1. To ascertain if the amino acid or the RNA change was important for S-ON resistance, PB1 5′b was made. This mutant had a G2280U change, also leading to E752D in the protein. In contrast, in PB1 5′c, G2280 was mutated to A, which was synonymous in PB1. PB1 5′d and 5′e contained the single changes A2297G and G2298A, respectively, while PB1 5′f contained both alterations. In the case of PA 3′, the individual C40G and G41C nucleotide changes lead to non-synonymous changes to codon 6 different from the parental double mutation (R6G and R6P respectively, compared to R6A), potentially complicating interpretation. We therefore created two additional mutants: PA 3′c with a silent C40A alteration; and PA 3′d, that was also silent but had C40A and A42G changes flanking an unaltered G41 residue (Table 2).

All mutant PB1 constructs and the two PA clones with synonymous nucleotide changes supported efficient viral gene expression (Table 2). However, non synonymous changes to PA R6 were not well tolerated, with both PA 3′a and b having less than 10% of the transcriptional activity of the wild type protein, consistent with the transcription defect previously seen with the PA 3′ clone. Notwithstanding these results, it was possible to generate most viruses twice, with the exception of PA 3′b, which rescued only once from two attempts and could not be titered due to its failure to form plaques. PA 3′a had a 60% reduction in titer compared to wild type. However, all the other mutants grew comparably to wild type (Fig. 6a).

These mutants were then tested for their sensitivity to S-ON treatment. In the case of the PB1 mutants, 4 out of 6 (PB1 5′a, c, e and f) were resistant to growth inhibition by all the S-ON (Fig. 6b; data summarized in Table 2). The two remaining mutants, PB1 5′b and d, were resistant to the PB2 S-ON, but sensitive to the PB1 and PA-derived S-ON. Therefore the identity of the nucleotide at G2280 of segment 2 was important for resistance, rather than the identity of PB1 residue 752. When the significance of the segment 2 changes at A2297 and G2298 were investigated, both the PB1 5′f mutant, with both changes, and the PB1 5′e mutant, with G2298A, were resistant to all four S-ON, while a virus with the single A2297G change remained sensitive to the PB1 and PA-derived S-ON molecules (Table 2). This suggested that segment 2 nucleotide G2298 was the more important determinant of S-ON resistance. Understanding the effects of the individual segment 3 mutations was more difficult because of the confounding effects of the differing but equally deleterious (to polymerase activity) effects of the non-synonymous changes to PA codon 6. Of the original PA mutations, only the PA 3′a virus (C40G) grew well enough to test but this change was unable to confer resistance to the S-ON tested. This could indicate that the original PA R6A substitution was important for resistance, and that an R6G change could not compensate. Alternatively, the G41C change could be crucial for altering packaging signal function (and thus generating resistance), but require a simultaneous C40G change to produce a less harmful coding change in PA. Consistent with the latter hypothesis, the PA 3′c virus with a silent C40A change remained sensitive to S-ON

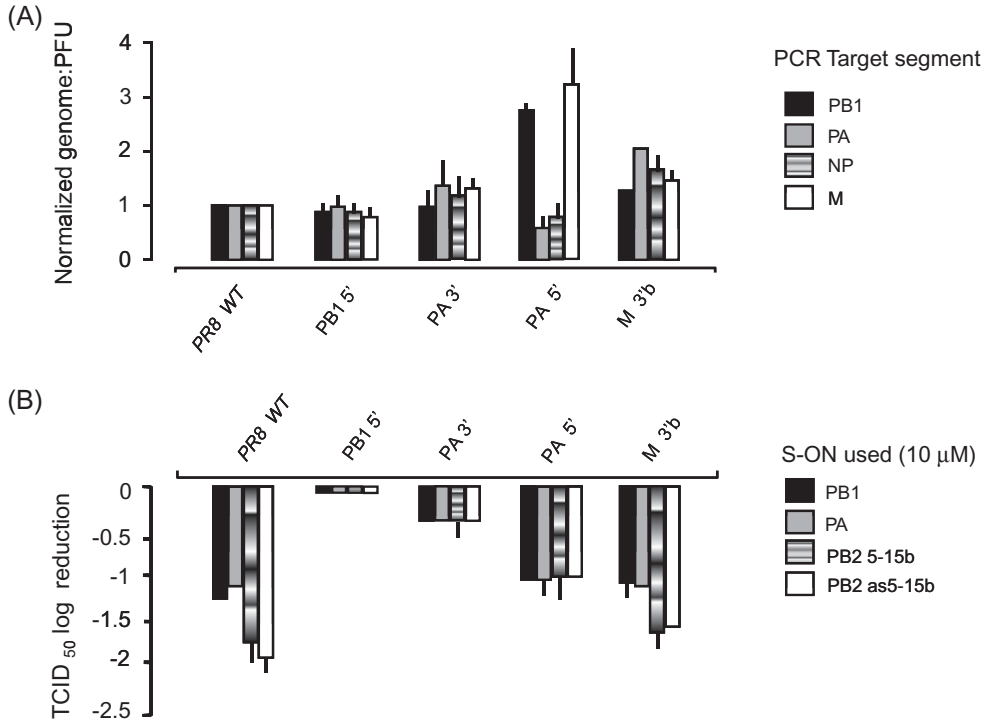

Fig. 5. Effect of mutations in selected segments on viral packaging and sensitivity to PB2-, PB1- and PA-derived S-ON. (A) RNA was extracted from equal PFU of cell free viral supernatants, and the genome content of the viruses was determined using qRT-PCR for segments 2, 3, 5 and 7. The copy number was normalized to wild type in each case. The mean ± standard error relative to wild type PR8 is plotted ($n > 2$ in each case). (B) Viral titer reduction of indicated molecular clones after treatment with PB1- and PA- 5–20c, PB2 5–15b or as5–15b S-ON. No antiviral activity was observed with the scrambled forms of the S-ON (data not shown). Infection was carried out at an MOI of 0.01. Viral growth is expressed as log $TCID_{50}$/ml present in the supernatant fluids collected 24 h after infection. Values shown are mean ± standard deviation of 3 independent experiments.

Table 2
Characteristic of construct carrying single mutations in 3′- or 5′-ends of selected segment.

Name	Mutation[a]	Coding[b]	Minireplicon % WT[c]	Viable virus[d]	Resistance[e]
PB1 5′a	G2280C	E752D	120	2/2	Yes
PB1 5′b	G2280U	E752D	119	2/2	Partial
PB1 5′c	G2280A	None	139	2/2	Yes
PB1 5′d	A2297G	Stop753W	131	2/2	Partial
PB1 5′e	G2298A	None	111	2/2	Yes
PB1 5′f	A2297G G2298A	None	171	2/2	Yes
PA 3′a	C40G	R6G	6	2/2	No
PA 3′b	G41C	R6P	2	1/2[f]	N/D
PA 3′c	C40A	None	150	2/2	No
PA 3′d	C40A+A42G	None	131	2/2	Yes

[a] Coordinates of the mutations are given in mRNA sense. The PB1 5′ virus had G2280C/A2297G/G2298A changes and the PA 3′ virus had C40G/G41C changes.
[b] Predicted coding changes resulting from the mutations.
[c] Transcriptional activity assessed by minireplicon assay. Results are mean of at least 2 independent experiments performed in duplicate, and values are given as % WT.
[d] Number of times an A/PR/8/34 virus containing the indicated mutation was successfully rescued/number of independent attempts.
[e] Summary of whether a virus containing the indicated changes was resistant to S-ON treatment. Partial: resistant to PB2 S-ON only. N/D: not done.
[f] This virus rescued once from 2 attempts but produced cytopatic effect rather than plaques.

inhibition, while a virus with two silent alterations C40A and A42G (PA 3′d) was resistant (Fig. 6b, Table 2). Overall, these data identify individual mutations that confer resistance to the S-ON molecules, and show that resistance can, but is not always, achieved to multiple inhibitory oligonucleotides. The data also suggest that the resistance mutations act by altering RNA function rather than by changing the sequence/activity of the viral polypeptides. In turn, this suggests that the inhibitory activity of the S-ON molecules targets viral RNA.

4. Discussion

Here we investigated whether it was possible to identify analogous S-ON derived from the packaging signals in the 5′ end of PB1 and PA genes as previously shown for PB2, and whether influenza virus resistance to S-ON can develop. In particular, a 5–20c 20-mer inhibitory molecule targeted against the PB1 and PA packaging signals in the 5′ end of the vRNA was identified. The S-ON antiviral activity, similar to that of PB2 5–15b molecule described

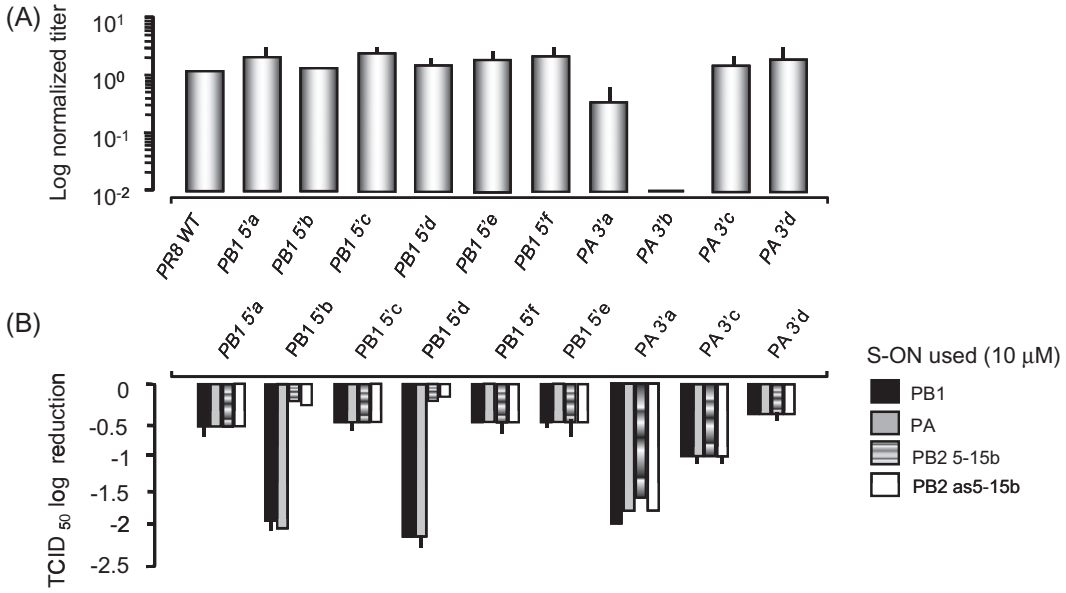

Fig. 6. Role of single mutation in segments PB1 and PA on viral sensitivity to PB2-, PB1- and PA-derived S-ON. (A) Normalized titers for successfully rescued viruses. The mean ± standard error titer relative to wild type PR8 is plotted ($n \geqslant 2$ in each case). (B) Viral titer reduction of indicated molecular clones after treatment with PB1- and PA-5–20c, PB2 5–15b or as5–15b S-ON. Infection was carried out at an MOI of 0.01. Viral growth is expressed as log $TCID_{50}$/ml present in the supernatant fluids collected 24 h after infection. The values shown are the means of 3 independent experiments.

previously (Giannecchini et al., 2009), was maintained in the 5–20c antisense analogues. As judged by the reduced sensitivity to the S-ON exerted by the selected variants obtained after 15 passages, cross-resistance to the specific PB2 S-ON and also to the other S-ON derived from PB1 and PA can be induced. During in vitro selection 13 mutations were identified in segments 1, 2, 3 and 7, which appeared at different times during the passage, but which became fixed in the viral population. Several changes were seen with both the sense and antisense S-ON used. None of the mutations were localized in the PB2 S-ON sequence, although the mutation G2280C was located in the PB1-derived 5–20c region. The functional consequence of this change is unclear. The mutations were located within the coding and NCR sequences of the segments, in regions identified as containing conserved packaging signals (Gog et al., 2007; Liang et al., 2008; Marsh et al., 2008; Muramoto et al., 2006). Several of the mutations in the coding sequences introduced amino acid changes predicted to affect polymerase function (Das et al., 2010; Tarendeau et al., 2007). After introducing the mutations into PR8 virus clones by reverse genetics, three groups of mutations were identified. The first group included mutations that affected polymerase activity or other features of the M segment that prevented virus rescue (mutation 2307 in PB2, mutations 29, 31 and 40 in PB1, mutation 41 in PA and mutation 38 in M), thus making analysis of their role in S-ON resistance impossible. The apparently lethal nature of these mutations is puzzling, given their occurrence in the passaged resistant viruses, but may be related to differences between the A/Taiwan/1/86 and PR8 strains, or perhaps simply reflect inefficiencies in virus rescue methodology. The second group included mutations that generated resistance to S-ON treatment (mutations 2280, 2297, and 2298 in PB1) that could be not attributed to amino acid changes in the viral protein either because synonymous mutations of the nucleotide were protective, or because the mutations were in the NCR. The last group included mutations that produced viable virus but maintained S-ON susceptibility, showing no role in the resistance (mutations 40, 2195 and 2196 in PA and mutation 41 in M). Of note however, the change 40 in PA could be involved in compensating for a harmful coding change during generation of resistance.

Collectively, these findings suggest that the resistance to S-ON 5–15b and its antisense analogue were induced by alterations to packaging signals rather than the viral proteins. In this context, in the absence of mutations in the PB2 S-ON derived region, we speculate that the mutations appeared elsewhere in the genome. This suggests a compensatory mechanism involving base-pairing interactions among these different regions of segment 2 and 3 packaging signals in order to evade the specific PB2 S-ON pressure. A possible analogous precedent for this has been observed in West Nile virus (Zhang et al., 2008). Also of note, the resistance mutations we observed often induced cross-resistance to S-ON derived from other segments, suggesting a common functional target, plausibly involving, but not necessarily limited to (given the effectiveness of both sense and anti-sense oligonucleotides), the packaging mechanism. The investigation of such specific segment interactions could be of interest in order to shed light on a packaging mechanism that is not yet properly understood (Hutchinson et al., 2010).

To date, the use of several nucleic acid-based molecules targeting PB2, PB1 and PA genes have been reported for influenza virus in vivo and in vitro (Abe et al., 2001; Gabriel et al., 2008; Ge et al., 2003, 2006; Kwok et al., 2009; Mizuta et al., 1999; Zhang et al., 2011). It is worth noting that there have been no reports that investigate whether influenza viral resistance can occur during such treatment. A major concern in the use of antiviral molecules to counteract influenza virus transmission is the development of resistance. Moreover, in recent years mutations conferring amantadine and oseltamivir virus resistance have emerged during treatment or in the absence of drug pressure that do not diminish virus replication or transmissibility (Hayden and de Jong, 2011). This highlights the need to understand the molecular biology of the influenza virus in order to select better conserved antiviral targets that will potentially reduce this phenomenon. Here we show that the constellation of mutations selected by serial passage of virus in the presence of a PB2 S-ON targeted to the segment packaging

signal induced cross-resistance to all S-ON derived from polymerase segments, likely due to a common functional activity exerted by their target genes (Muramoto et al., 2006). However, the mutations also had a profoundly detrimental effect on virus replication thus probably complicating the development of fully transmissible drug-resistant virus. The possibility that these changes might emerge in clinical setting altering viral transmissibility and/or pathogenicity awaits further investigation. However, these results demonstrate that developing new nucleic-acid based molecules targeting the packaging signal in the 5′ end of influenza virus polymerase segments could be pursued to provide effective countermeasures against influenza virus, especially those with pandemic potential.

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