Molecular studies of influenza B virus in the reverse genetics era

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Recovery of an infectious virus of defined genetic structure entirely from cDNA and the deduction of information about the virus resulting from phenotypic characterization of the mutant is the process of reverse genetics. This approach has been possible for a number of negative-strand RNA viruses since the recovery of rabies virus in 1994. However, the recovery of recombinant orthomyxoviruses posed a greater challenge due to the segmented nature of the genome. It was not until 1999 that such a system was reported for influenza A viruses, but since that time our knowledge of influenza A virus biology has grown dramatically. Annual influenza epidemics are caused not only by influenza A viruses but also by influenza B viruses. In 2002, two groups reported the successful recovery of influenza B virus entirely from cDNA. This has allowed greater depth of study into the biology of these viruses. This review will highlight the advances made in various areas of influenza B virus biology as a result of the development of reverse genetics techniques for these viruses, including (i) the importance of the non-coding regions of the influenza B virus genome; (ii) the generation of novel vaccine strains; (iii) studies into the mechanisms of drug resistance; (iv) the function(s) of viral proteins, both those analogous to influenza A virus proteins and those unique to influenza B viruses. The information generated by the application of influenza B virus reverse genetics systems will continue to contribute to our improved surveillance and control of human influenza.

Introduction

Orthomyxoviruses that infect humans can be divided into three types, influenza virus types A, B and C, based on the absence of antigenic cross-reactivity between their internal proteins. Type A influenza viruses can be further divided into subtypes depending upon the antigenic relationship of their surface glycoproteins haemagglutinin (HA) and neuraminidase (NA) (reviewed by Palese & Shaw, 2007). Two subtypes of influenza A virus, H1N1 and H3N2, have co-circulated within the human population for the past three decades. As influenza A viruses of at least 16 subtypes of HA and nine NA subtypes exist in animal species to which humans have little or no prior immunity, influenza A viruses have pandemic potential. This is realized when animal viruses acquire mutations directly or by reassortment with human viruses that adapt them for replication and transmission in human hosts. Thus in 2009, a particular genetic constellation of genes came together by reassortment of several viruses in swine that had the capacity for transmission into humans and the pandemic H1N1 virus emerged (Garten et al., 2009). Influenza virus type B does not have a natural reservoir in animal hosts and although there has been a report of influenza B virus isolation from seals, the virus showed no animal host adaptation, in fact it was identical to isolates from humans (Osterhaus et al., 2000). Moreover reassortment between influenza A and B virus RNA segments does not occur (Ghenkina & Ghendon, 1979; Gottlieb & Hirst, 1954). Thus, influenza B viruses do not have pandemic potential. On the other hand influenza B viruses cause significant disease and are the predominant circulating strain of influenza virus approximately one in every 3 years (Lin et al., 2004). Influenza B virus is therefore an essential component of the influenza vaccine administered to susceptible groups such as the elderly and asthmatic (Belshe, 2010). Influenza A and B viruses share many features; however, influenza B viruses are studied far less than their influenza A virus counterparts. Influenza B viruses harbour some interesting genetic differences (reviewed by Palese & Shaw, 2007), including additional encoded proteins like NB, lack of other proteins like PB1-F2, difference in protein length and also length of non-coding regions of the genome, which suggest both convergent and divergent evolution. They also utilize unusual alternative coding strategies that allow expansion of the genetic repertoire achieved from a relatively small RNA virus genome.

Reverse genetics is the process of understanding virus gene function by generating viruses containing specific mutations from engineered cDNAs and then performing phenotypic analysis to reveal the effects mediated by the

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genetic change(s) introduced. The term is frequently used in the modern literature to simply describe a virus generated from cDNA, whose names are often prefixed by the letter ‘RG’, for example viruses generated as vaccine seeds. For influenza viruses, the generation of recombinant virus has been achieved by transfection of mammalian cells with plasmids encoding each of the eight viral RNA (vRNA) genome segments and at the same time expressing the viral proteins required for their transcription either by transfection of four additional plasmids (Fig. 1a) or by the use of plasmids with bidirectional promoters that allow both vRNA and mRNA to be produced for the polymerase gene segments (Fig. 1b) (reviewed by Neumann & Kawaoka, 2001). This process leads to the amplification of vRNA segments through the production of replicative cRNA intermediates, as well as transcription into mRNAs leading to viral protein synthesis, all of which results in the release of infectious progeny virions from the cell (Fig. 1). The purpose of this review is to describe what is known about influenza B virus biology with particular reference to those advances recently facilitated by the advent of reverse genetics technology.

Influenza B virus RNA non-coding sequences

The genome of influenza A and B viruses consists of eight segments of negative-sense, single-stranded vRNA (Desselberger & Palese, 1978; McGeoch et al., 1976), which encode 11 or 12 known viral proteins (Fig. 2). For both virus types, the nucleotide sequences at the extreme termini of each of the eight RNA segments are completely conserved (Skehel & Hay, 1978) and display a degree of complementarity that suggests the presence of an RNA secondary structure in the vRNA (Desselberger et al., 1980; Hsu et al., 1987; Robertson, 1979). Beyond these common terminal sequences are further non-coding nucleotides which are specific for each RNA segment (Lamb & Krug, 2001). In combination these non-coding sequences were shown to represent the viral promoters for positive- and negative-RNA synthesis in early studies with influenza A virus minigenomes (Luytjes et al., 1989). Just as for influenza A viruses, these regions are absolutely essential for replication of influenza B virus-like RNAs (Barclay & Palese, 1995). Since for epidemiological purposes the exact sequence of the non-coding regions is of less interest than that of the coding regions, database entries for influenza B viral genes often lacked the nucleotide sequence of the extreme termini of the RNA segments. A report was published in 1987 which attempted to resolve this situation by cataloguing the available information at the time, but there remained a number of gaps and discrepancies amongst information available about the terminal sequences of influenza B virus RNAs (Stoeckle et al., 1987). This was a major barrier to the development of a reverse genetics system for the recovery of influenza B viruses entirely from cDNA. In such systems it is important to be able to generate full-length vRNAs with authentic 3’ and 5’ termini. Eventually, either by completing the sequence analysis themselves or by a process of mutagenesis and deduction, two groups successfully achieved the recovery of recombinant influenza B viruses in 2002 (Hoffmann et al., 2002; Jackson et al., 2002) with further groups later adding to this repertoire (Dauber et al., 2004; Hatta & Kawaoka, 2003; Imai et al., 2004) (Table 1). The non-coding sequences incorporated in the plasmids employed for virus recovery by our group are given in Table 2. The importance of certain nucleotides within these non-coding regions can be illustrated by our inability to recover viruses with a run of three rather than four U residues from positions 16–19 at the 5’ non-coding terminus of segment 2 (PB2) mRNA, nor with a G nucleotide rather than C at position 11 of the segment 5 (NP) mRNA (D. Jackson and W. Barclay, unpublished observations; sequences underlined in Table 2). Nt 11 in the 5’ non-coding region of influenza B NP mRNA represents a typical discrepancy in the sequence database; a small number of entries display this nucleotide as a G, whereas the majority display it as a C. In studies by Lee and Seong where the wild-type nucleotide at this position was mutated from C to G, transcription and translation of the downstream gene were not affected, whereas a similar mutation in an influenza A virus model RNA was detrimental to downstream gene expression (Lee & Seong, 1996, 1998). As the extreme 5’ and 3’ termini of each vRNA segment are thought to base-pair, the results of Lee and Seong suggested that base-pairing at 11:12 is not required for efficient transcription and translation of influenza B virus genes. However, our inability to recover an influenza B virus containing this mutation (D. Jackson and W. Barclay, unpublished data) suggests that in the context of virus infection this mismatch is detrimental to the virus. This could be due to the disruption of cis-acting signals required for packaging of the vRNA into the nascent virion, or another function that is not measured in in vitro transcription assays.

There is little sequence identity between the analogous proteins of influenza A and B viruses (Table 3). The polymerase proteins, particularly PB1, show the highest identity. Several studies have used plasmid transfection to reconstitute polymerases in cells to assess the compatibility of influenza types A and B polymerase components and type-specific polymerase complexes with viral-like RNAs containing promoters derived from influenza A or B virus gene segments. Overall homologous combinations show the best activity and although the incompatibility is not absolute, it most likely contributes to the inability of viruses of different types to reassort (Crescenzo-Chaigne et al., 1999; Iwatsuki-Horimoto et al., 2008).

The available influenza B virus non-coding sequences reveal an interesting degree of complementarity that extends beyond that seen in influenza A virus RNAs (Stoeckle et al., 1987). The function of these regions within the genome are not yet defined, but it is remarkable that the 5’ non-coding regions of influenza B virus vRNA segments are much longer than those of influenza A viruses (Stoeckle et al., 1987) (Table 4). The segment-specific regions may either regulate gene expression of the different influenza proteins, or may...
Fig. 1. Schematic diagrams of reverse genetics systems for recovery of influenza viruses from plasmid DNA. (a) 293T cells are co-transfected with 12 plasmids, eight encoding each of the eight vRNA gene segments and four expressing the viral polymerase complex and nucleoprotein (NP). After transcription of the vRNA segments in the nucleus by RNA polymerase I, they associate with the polymerase complex and are encapsidated by NP to form viral ribonucleoprotein (vRNP) complexes. From this point, the situation resembles a natural infection after import of vRNPs into the nucleus. Transcription of viral mRNA, leading to viral protein synthesis and vRNP replication begin, which leads to the assembly and release of infectious virus from the cell. (b) Alternatively, 293T cells are transfected with eight plasmids in which bidirectional promoters flank the influenza genome sequences. This allows RNA polymerase I transcription of vRNAs and also RNA polymerase II driven expression of viral proteins. The resulting polymerase subunits can then drive amplification and expression of the vRNAs as in (a).
be involved in tagging segments for specific packaging into the nascent virion. In this respect, it is noteworthy that a series of mutations in which the influenza B virus HA gene-specific non-coding regions at the 5′ end of the vRNA were truncated, did not affect gene expression. However, a mutant harbouring a large deletion of 67 nt from this region could not be recovered into recombinant virus (Barclay & Palese, 1995). One explanation for this would be the loss of a segment-specific packaging signal. Recently, the existence of such signals has been demonstrated on influenza A virus RNAs where they extend over both non-coding and coding sequences (reviewed by Hutchinson et al., 2010). Computational analysis of codon conservation conducted for influenza A virus gene sequences also indicates that coding regions contain segment-specific cis-acting RNA signals (Gog et al., 2007). Similar experiments for influenza B viruses have not yet been reported.

**Reverse genetics for the generation of influenza B virus vaccines**

Like influenza A viruses, influenza B viruses are subject to selective pressure exerted by the pre-existing acquired immunity in the population. Antigenic drift and reassortment among human influenza B virus isolates drive their evolution (McCullers et al., 1999). Mutations in HA and NA genes, which aid viral evasion of pre-existing antibodies, are rapidly selected. Therefore in order for influenza vaccines to be effective, influenza A and B viruses used as vaccine strains must be continually updated. This is achieved by a concerted worldwide surveillance effort, in which meetings are held biannually to nominate a vaccine strain most likely to protect against the viruses that will circulate in the following influenza season (reviewed by Carrat & Flahault, 2007). After this selection, for influenza A viruses there follows a procedure in which the surface antigens of the selected strain are recombined by the process of gene mixing known as reassortment with the internal gene segments of the A/Puerto Rico/8/34 (PR8) influenza virus (Kilbourne, 1969). This strain has been selected as a vaccine backbone due to its high yield properties, particularly in eggs, which are currently used as the vaccine substrate. For influenza B viruses, no such recipient virus is available (Goodeve et al., 1985). As stated above influenza A/B virus reassortants are not formed, thus the PR8 influenza strain cannot be used as a backbone for influenza B virus vaccines. Therefore, the yield of influenza B virus for vaccine purposes can vary greatly each year depending on the propensity of the chosen antigenic variant to propagate in eggs. For example, when the recommended strain of influenza B virus was changed in 2001 to B/HK/330/2001 it was not apparent until late in the process that the growth of this strain in eggs was poor. Consequently, vaccine manufacturers had difficulty in achieving the necessary antigen doses required for the influenza B virus component of the vaccine. Such problems could be overcome if a high yield influenza B virus vaccine backbone can be identified into which novel HA and NA

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**Fig. 2.** Schematic diagram of the protein coding ORFs encoded on the eight segments of the influenza A and B virus genomes. Numbers in bold represent the vRNA segment number on which the proteins are encoded. The influenza A virus PB1-F2 and N40 proteins and the influenza B virus NB protein are encoded on segments two and six, respectively, as overlapping ORFs. The influenza A virus M2 protein is encoded on segment 7 via a spliced mRNA, while the influenza B virus BM2 protein is encoded on segment 7 via a translational stop-start mechanism. The NEP protein of both viruses is encoded on segment 8 via a spliced mRNA.

**Table 1.** The strains of influenza B viruses used to generate published reverse genetics systems

| Virus       | Reference(s)                      |
|-------------|-----------------------------------|
| B/Lee/40    | Hatta & Kawaoka (2003); Dauber et al. (2004) |
| B/Beijing/1/87 | Jackson et al. (2002)           |
| B/Yamagata/1/73 | Imai et al. (2004)              |
| B/Yamanashi/166/98 | Hoffmann et al. (2002)         |
genes could be introduced. Moreover, by using a reverse genetics approach, the uncertainty of the reassortment process would be avoided. This concept was clearly demonstrated by Hoffmann et al. (2002), where 6 + 2 reassortants were generated by reverse genetics with internal genes from B/Yamanishi/166/98 and HA and NA genes from recent strains B/Victoria/504/2000, B/Hawaii/10/2001 or B/Hong Kong/330/2001. All of the recombinant 6 + 2 viruses grew at least as well as the wild-type virus in eggs, with the B/Victoria recombinant virus displaying enhanced growth (Hoffmann et al., 2002).

Vaccine manufacturers are currently developing tissue culture cell-based methods for vaccine production. Advantages over traditional egg-based processes include faster production of vaccine strains, and processes that are more adaptable to large-scale production and potentially pose a lower risk of biological contamination. In the

Table 2. The nucleotide sequences of the mRNA and vRNA 5’ non-coding regions encoded on the genome of recombinant B/Beijing/1/87 viruses generated by reverse genetics (Jackson et al., 2002)

Nucleotides in bold represent those at the extreme ends of the RNA common to all segments and viruses. Nucleotides underlined represent those discussed in the text as critical for rescue of infectious virus.

| Segment | Nucleotide sequence of the mRNA/crRNA 5’ non-coding regions | Nucleotide sequence of the vRNA 5’ non-coding regions |
|---------|------------------------------------------------------------|----------------------------------------------------|
| PB1     | AGCAGAAGCGGACGCUUUAAG                                      | AGUAGAAAAACAGGACGCUUUUCUCAUUUA                     |
|         |                                                            | AUCAUAAUGUUAUCAGACUAAUAA                              |
| PB2     | AGCAGAAGCGGACGCUUUUCUCAAG                                   | AGUAGAAAAACAGGACGCUUUUCUCAAAUUG                     |
|         |                                                            | UGUUAUUGAUUAAACAGAACAUAA                             |
| PA      | AGCAGAAGCGGAGGCUUUAUGCAUA                                       | AGUAGAAAAACAGGACGCUUUUCUCAAAUUG                     |
|         |                                                            | UCUUUAUUGAUUAAACAGAACAUAA                             |
| HA      | AGCAGAAGACGCAUUCUAAUAAACAG                                   | AGUAGAAAAACAGGACGCUUUUCUCAAAUUG                     |
|         |                                                            | AAAGCAUAAAACAGAACAUAA                                  |
| NP      | AGCAGAAGACGCAUUCUAAUAAACAG                                   | AGUAGAAAAACAGGACGCUUUUCUCAAAUUG                     |
|         |                                                            | AAAGCAUAAAACAGAACAUAA                                  |
| NA      | AGCAGAAGCGAAGAGGCUUUCUUCUUCU AAACACAG                        | AGUAGAAAAACAGGACGCUUUUCUCAAAUUG                     |
|         |                                                            | AAAGCAUAAAACAGAACAUAA                                  |
| M       | AGCAGAAGACGCAUUCUUCUCAAACAG                                   | AGUAGAAAAACAGGACGCUUUUCUCAAAUUG                     |
|         |                                                            | AAAGCAUAAAACAGAACAUAA                                  |
| NS      | AGCAGAAGCGAAGGAUUGUUGUUUGUAGCACAGUUGCAAAGAA                    | AGUAGAAAAACAGGACGCUUUUCUCAAAUUG                     |
|         |                                                            | AAAGCAUAAAACAGAACAUAA                                  |
|         |                                                            | AAAGCAUAAAACAGAACAUAA                                  |

Table 3. Comparison of length of influenza A and B virus proteins and identity between analogues

Data have been taken from A/Sydney/5/1997 (H3N2) and B/Florida/02/2006 arbitrarily nominated here as prototypic strains. Amino acid identity calculated using BLASTP 2.2.24 + on default settings for all except NS1 for which no identity was found (Altschul et al., 1997, 2005).

| ORF | Length in influenza A (aa) | Length in influenza B (aa) | Amino acid identity (%) |
|-----|-----------------------------|-----------------------------|-------------------------|
| PB1 | 757                         | 752                         | 61                      |
| PB2 | 759                         | 770                         | 37                      |
| PA  | 716                         | 726                         | 36                      |
| HA  | 566                         | 585                         | 28                      |
| NP  | 498                         | 566                         | 37                      |
| NA  | 469                         | 466                         | 30                      |
| M1  | 252                         | 248                         | 31                      |
| M2/BM2 | 97                      | 109                         | 26                      |
| NS1 | 230                         | 281                         | Too low                 |
| NEP | 121                         | 122                         | 24                      |
laboratory, reverse genetics procedures have been optimized using the highly transfectable 293T cell line. However, those cells are not useful for vaccine seed generation because they are not licensed for human use. The Madin–Darby canine kidney (MDCK) cell line is a licensed influenza vaccine substrate and one study reported the recovery of both influenza A and B viruses entirely in MDCK cells (Wang & Duke, 2007). The modified process involved the replacement of the human RNA polymerase I promoter sequence in the input DNA plasmids with that of canine RNA polymerase I. Another cell line, PER.C6, has been shown to support efficient growth of influenza A and B viruses (Pau et al., 2001) and is licensed for influenza vaccine production. Since this is a human-derived cell line, there is no need to change the human polymerase I promoter that exists on most of the rescue plasmids in use today for recovery of influenza viruses in PER.C6 cells (Koudstaal et al., 2009).

An alternative strategy to guarantee a consistent yield of influenza B virus antigens for vaccine purposes is to engineer high yield influenza A viruses that express influenza B virus HA antigen in place of influenza A HA. This has been achieved by generating chimeric type A/B surface antigens, which contained either full-length influenza B virus HA or the ectodomain of type B HA fused to the transmembrane domain and cytoplasmic tail of type A HA (Flandorfer et al., 2003). These chimeric viruses were found to grow as well as wild-type influenza A viruses in tissue culture. Similar chimeric A/B viruses were found to be attenuated in mice compared to wild-type virus (Horimoto et al., 2003, 2004a), but upon immunization were shown to protect mice against challenge with a lethal dose of either influenza B virus (Horimoto et al., 2003) or influenza A virus (Horimoto et al., 2004b). This demonstrated that immune responses to proteins other than HA can confer protection against influenza A virus challenge and that influenza A viruses can be used as a vehicle for presentation of influenza B virus antigen.

A further improvement of influenza B virus vaccine yield from eggs may be achieved by changing the receptor-binding properties of the influenza B virus HA protein itself. It appears that wild-type influenza B viruses often do not grow well in embryonated chicken eggs, as there is an incompatibility between the influenza B virus HA protein and the sialic acid (SA) receptors in ovo used for influenza virus binding and entry. For example, mutations at position 196–197 affecting a glycosylation site on HA near to the receptor-binding pocket are often associated with egg adaptation (Robertson et al., 1985). In fact, 6 + 2 recombinant viruses created by reverse genetics containing this glycosylation site were unable to grow in eggs and upon adaptation to eggs the glycosylation site was subsequently lost (Chen et al., 2008a). The glycosylation site was shown to adversely affect the binding of HA to α-2,3-linked SA. Another study reported that the introduction of a number of mutations into an influenza B virus HA by reverse genetics conferred high yield growth properties to the recipient strain (Lugovtsev et al., 2007). It was subsequently shown that as residue G141 in HA directly contacts SA, a G141E mutation was highly beneficial for growth in eggs, as it narrowed the range of glycans that HA was able to bind to (Lugovtsev et al., 2009). Binding to a limited subset of glycans containing α-2,3-linked SA therefore resulted in egg adaptation and high virus yields. However, with this strategy of mutagenesis caution must be exercised, as such mutations were also shown to affect the antigenicity of the virus (Chen et al., 2008a).

### Table 4. A comparison of the nucleotide lengths of the non-coding regions of the eight vRNA segments of a representative strain of influenza A (A/Udorn/72) and B (B/HK/330/01) virus

| Segment | vRNA 3' NCR (no. nucleotides) | vRNA 5' NCR (no. nucleotides) |
|---------|-------------------------------|-------------------------------|
|         | A/Udorn/72 | B/HK/330/01 | A/Udorn/72 | B/HK/330/01 |
| 1       | 27        | 21          | 34        | 89          |
| 2       | 24        | 23          | 43        | 60          |
| 3       | 24        | 29          | 58        | 94          |
| 4       | 29        | 33          | 35        | 94          |
| 5       | 45        | 60          | 23        | 101         |
| 6       | 19        | 45          | 37        | 103         |
| 7       | 25        | 24          | 20        | 90          |
| 8       | 26        | 43          | 26        | 30          |

### Using reverse genetics to understand the basis of attenuation of live vaccine strains

An alternative strategy to the high yield strains used for inactivated influenza vaccines is to produce a live attenuated influenza virus (LAIV) vaccine from strains that have attenuating traits. Indeed studies in ferrets argue strongly for the use of a LAIV vaccine for influenza B virus rather than reliance on inactivated vaccine (Huber et al., 2008). Vaccine strains using B/Ann Arbor/1/66 as the master donor virus (MDV-B) display temperature sensitivity (ts), attenuation (att) and cold adaptation (ca) (Maassab & DeBorde, 1985) and are genetically stable (Snyder et al., 1989). These strains were initially created...
through serial passages of the wild-type virus in embryonated chicken eggs and primary chicken kidney (PCK) cells at incrementally lower temperatures (Maassab, 1967). Reverse genetics has been used to identify the amino acid mutations in MDV-B responsible for these markers (Chen et al., 2006; Hoffmann et al., 2005). Sequence comparison of the progenitor and attenuated strains revealed a number of changes, which were potentially responsible for the attenuated or temperature-sensitive phenotype. These mutations were engineered singly or in combination into the relevant gene segment by reverse genetics and the resultant recombinant viruses were compared with the parental strain. This methodology indicated that two amino acid changes in NP (A114 and H410) and one in PA (M431) are responsible for the ts phenotype of the B/AA/66 strain. Furthermore, the M1 alterations at Q159 and V183 paired with the ts mutations are required to confer the att phenotype (Hoffmann et al., 2005). The ca phenotype is conferred by the changes in PA and NP plus an additional alteration to NP (T509) and one to PB2 (R630) (Chen et al., 2006). The amino acid mutations important in conferring each of the different phenotypes are summarized in Table 5. The ts mutations resulted in significantly reduced viral protein synthesis, inhibition of vRNA synthesis and impaired viral polymerase activity at the restricted temperature (37 °C) (Chen et al., 2008b). The importance of mutations in the M1 protein of the att phenotype is of interest. In the equivalent influenza A viruses developed as live attenuated vaccine backbones, analysis revealed that the phenotypes were mediated by mutations in the polymerase genes (Cox et al., 1988; Snyder et al., 1988) and although the M gene has previously been implicated in conferring the att phenotype (Snyder et al., 1988), mutations in the M1 protein did not play a role in the attenuation (Cox et al., 1988; Sweet et al., 2004). This suggested a different mode of attenuation for the influenza A and influenza B virus vaccine backbones. It was recently shown that in MDV-B the att mutations in M1 resulted in impaired multi-cycle replication, which is potentially due to the observed decrease in the incorporation of mutant M1 protein into virions (Chen et al., 2008b). Therefore, the combination of the ts and att mutations resulted in reduced polymerase function and viral assembly/release at the restricted temperature and the complexity of these mechanisms ensures the stability of the virus for vaccine purposes.

**Reverse genetics to analyse the potential for the emergence of antiviral resistance**

Anti-influenza drugs can complement vaccine use for control of influenza B viruses. There are currently four licensed antiviral drugs available for the treatment and prophylaxis of influenza virus infections. Amantadine and rimantadine (adamantamines) have been widely studied for many years and are known to inhibit influenza A viruses by blocking the M2 ion channel protein (Hay, 1992) that mediates efficient entry of the viral genome into host cells. The HXXXW amino acid motif in the transmembrane domain of the tetrameric M2 protein is responsible for allowing the passage of protons across the channel and for gating it. M2 possesses a large apolar cavity containing hydrophobic pore residues (Ala30 and Gly34) within its transmembrane domain. The adamantamine drugs interact with M2 at high affinity inside the channel pore as well as at a low affinity-binding site at the base of the pore near the helix–helix interface of the tetramer (Cady et al., 2010; Schnell & Chou, 2008; Stouffer et al., 2008). However, neither rimantadine nor amantadine inhibit the replication of influenza B viruses (Davies et al., 1964). Although influenza B viruses encode a homologue of M2, known as BM2 (Mould et al., 2003; Paterson et al., 2003) (see below), the BM2 channel is not blocked by adamantane drugs as the residues lining the ion channel pore are polar residues (Ser9, Ser12, Ser16 and Phe 13) that decrease the cavity size and occlude access of the drug to the HXXW motif (Ma et al., 2008). A recent study reported the creation of an amantadine-sensitive BM2 protein, in which the transmembrane domain of the influenza A virus M2 protein was fused to the cytoplasmic tail of BM2 (Ohigashi et al., 2009). However, a virus containing this chimeric protein could not be recovered by reverse genetics.

The two remaining licensed anti-influenza compounds, zanamivir (Relenza; GlaxoSmithKline) and oseltamivir (Tamiflu; Roche) are members of a class of drugs known as neuraminidase inhibitors (NAIs). The first NAI (DANA) was described in 1969 (Meindl & Tuppy, 1969). Following the elucidation of the crystal structure of influenza A virus subtype 9 NA in 1983 (Varghese et al., 1983), structural-based drug design was employed to improve upon the interactions of the NAIs with the enzyme and resulted in the development of first zanamivir and then oseltamivir (Kim et al., 1997; von Itzstein et al., 1993). As the structure of the active site of their NA enzymes are very similar, both

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**Table 5. Mutations within each of the eight segments of the influenza B virus genome involved in conferring the temperature-sensitive (ts), attenuated (att) and cold-adapted (ca) phenotypes to the B/Ann Arbor/1/66 virus**

Information from Chen et al. (2006); Hoffmann et al. (2005).

| Gene segment | ts  | att | ca  |
|--------------|-----|-----|-----|
| PB1          | None| None| None|
| PB2          | None| None| R630|
| PA           | M431| M431| M431|
| HA           | None| None| None|
| NP           | A114, H410 | A114, H410 | A114, H410, T509 |
| NB/NA        | None| None| None|
| M1/BM2       | None| M1: Q159, V183 | None|
| NS1/NEP      | None| None| None|
influenza A and B viruses are susceptible to the NAIs which are currently licensed for use in the treatment and prophylaxis of both virus types (Hayden et al., 1999, 2000; Monto et al., 2002). The natural substrate of NA is SA, the terminal sugar molecule found on cell surface glycans utilized as receptors by the virus to enter the host cell. NA cleaves SA from these receptors to allow the efficient release of progeny virions from the infected cell. NAIs are SA analogues that competitively inhibit the cleavage of SA from the infected cell upon viral release, leading to the aggregation of viral particles at the cell surface and ultimately preventing viral spread (Luo et al., 1999).

As with all antiviral drugs, there is the potential that drug-resistant variants may exist naturally or may emerge during treatment. In vitro acquired mutations associated with resistance to NAIs predominantly appear in the HA protein prior to the generation of NA mutations (Baum et al., 2003; reviewed by McKimm-Breschkin, 2000; Smee et al., 2001). The HA mutations tend to reduce the SA receptor affinity of HA and allow the virus to be released from infected cells even in the presence of NAIs (reviewed by Hayden et al., 2000). However, the relevance of these mutations in vivo is questionable. Rather mutations in NA, which prevent the binding of the inhibitor in the enzymic site, are observed in natural isolates (Sheu et al., 2008; Zambon & Hayden, 2001).

There have been numerous studies published that address the impact of HA and NA mutations on the viability of viruses and the level of NAI resistance they confer (Gubareva et al., 2001a, b; reviewed by McKimm-Breschkin, 2000). However, most of the work has focused on influenza A virus resistance, with only limited work on influenza B viruses. In 2005, we described the use of the reverse genetics system in order to create a number of recombinant influenza B viruses containing mutations in the B/Beijing/1/87 virus NA protein at positions corresponding to the known influenza A virus resistance mutations (Jackson et al., 2005). These included mutations at the ‘framework’ residue E116 (corresponding to residue E119 in influenza A virus N2 NA) and at two ‘functional’ residues, R149 and R291. The mutations resulted in varying levels of resistance to zanamivir, oseltamivir and a third currently unlicensed NAI, peramivir. The variation in resistance could be explained by analysis of the predicted interaction of each drug with the NA enzymic site. The study demonstrated a high level of resistance to oseltamivir and peramivir for an influenza B virus containing an R291K (R292K in N2 numbering) mutation in NA. This suggests that a reorientation event occurs in the influenza B virus NA active site upon binding of both oseltamivir and peramivir, similar to that observed of influenza A virus NA. We also showed that mutations at ‘framework’ residues of the influenza B virus NA are not the only mutations that can cause reduced stability of the protein, as the two mutated ‘functional’ residues also resulted in significant reductions in NA protein stability. Overall the drug resistance profile for each of the mutant influenza B viruses was similar to those of the reported influenza A viruses containing the corresponding amino acid changes in NA. As some of the mutations rendered the viruses resistant to more than one NAI, it is possible that the emergence of influenza B viruses resistant to all currently available NAIs could occur. This highlights the need for continued surveillance and drug susceptibility profiling of current circulating influenza A and B viruses.

Recently, it has been found that circulating influenza B viruses are less susceptible than expected to oseltamivir (Kawai et al., 2006; Sugaya et al., 2007). Moreover, in Japan where the NAIs are used extensively, a number of influenza B viruses displaying oseltamivir resistance have been isolated from children. Interestingly, these viruses do not harbour the previously described mutations associated with NAI resistance but two new mutations at D198N and I222T appear to be responsible (Hatakeyama et al., 2007). It is not yet clear how transmissible or virulent these newly described drug-resistant influenza B viruses are, but clearly a reverse genetics approach could be utilized to analyse the effect of the mutations on such properties. The observations are of considerable concern in the light of recent findings that the H1N1 influenza A viruses that circulated in 2008 had also naturally acquired oseltamivir resistance and their prevalence increased during the season, suggesting little or no compromise in fitness or transmissibility as a result of the NA mutation (Lackenby et al., 2008).

Reverse genetics to determine the functions of the influenza B virus NS1 protein

The 281 aa NS1 protein of influenza B viruses (B/NS1) is encoded on an unspliced mRNA derived from segment eight of the viral genome (Briedis & Lamb, 1982). Like the influenza A virus NS1 protein (A/NS1), the 32 kDa B/NS1 protein is thought to be a virally encoded interferon (IFN) antagonist and has been shown to share many functions with its influenza A virus counterpart (Wang & Krug, 1996) (Fig. 3). These similarities include the formation of NS1 dimers within infected cells, conservation of an N-terminal RNA-binding domain that binds dsRNA, and the inhibition of activation of the antiviral mediator protein kinase R (PKR). However, the C terminus of B/NS1 shares little sequence or functional homology with the C-terminal ‘effector domain’ of A/NS1. For influenza A virus, this domain has many functions (Fig. 3) (reviewed by Hale et al., 2008), including binding to and inhibiting two cellular proteins, the 30 kDa subunit of cleavage and polyadenylation specificity factor (CPSF30) and poly(A)-binding protein II (PAPBII). Thus, A/NS1 expression results in the nuclear retention of host poly(A)-containing mRNAs and prevention of host pre-mRNA splicing (Chen & Krug, 2000; Chen et al., 1999; Nemeroff et al., 1998; Qiu & Krug, 1994). In contrast, influenza B virus NS1 proteins do not inhibit the expression of recently induced host mRNAs (W. Barclay, unpublished data). While residues 1–
93 of B/NS1 have been shown to form the RNA-binding domain, residues 1–103 have also been shown to bind to the IFN-induced protein ISG15 (interferon stimulated gene 15) (Yuan et al., 2002; Yuan & Krug, 2001) (Fig. 3). ISG15 is a ubiquitin-like protein that has potent antiviral activity against influenza and other viruses (Lenschow et al., 2007). B/NS1 expressed independently of viral infection binds to ISG15 and prevents the conjugation of ISG15 to particular target proteins (Yuan & Krug, 2001). This B/NS1-mediated inhibition of ISGylation appears specific to human and non-human primate cells, a limiting factor that restricts the host range of influenza B viruses (Sridharan et al., 2010; Versteeg et al., 2010).

In 2004, reverse genetics was used to create a recombinant influenza B virus based on the laboratory strain B/Lee/40 that lacked the coding capacity for B/NS1 (ΔNS1-B) (Dauber et al., 2004), which was similar to the mutant of influenza A virus known as delNS1 (Garcia-Sastre et al., 1998). Recovery and growth of ΔNS1-B was performed in 6 day-old embryonated chicken eggs as they lack IFN production (Isaacs & Baron, 1960) and the recombinant virus was predicted to be extremely IFN-sensitive. Both the ΔNS1-A (delNS1) and ΔNS1-B viruses induced high levels of IFN-β mRNA in infected cells and prior expression of either wild-type A/NS1 or B/NS1 protein abrogated the increase in IFN-β synthesis (Dauber et al., 2004). This strongly supports the hypothesis that B/NS1 is the functional homologue of A/NS1 and possesses IFN antagonistic activity. As nuclear translocation (activation) of IRF-3, a transcription factor required for the induction of IFN-β synthesis, was prevented by expression of either the N- or C-terminal domains of wild-type B/NS1 (Donelan et al., 2004), inhibition of IFN-β promoter activation is mediated by activities held in both domains (Fig. 3). Like the ΔNS1-A virus, ΔNS1-B virus was severely attenuated in IFN-competent systems. However, in contrast to ΔNS1-A, the growth of ΔNS1-B was also attenuated in IFN-deficient Vero cells by up to 40,000-fold. This suggested that B/NS1 must have additional functions within infected cells that are either absent or not essential for efficient replication of influenza A virus.

dsRNA-binding by basic residues in the N terminus of B/NS1 (Fig. 3) prevents activation of PKR and this function is crucial for influenza B viruses. It was recently shown that the cytoplasmic accumulation of influenza B virus vRNPs acts as a potent activator of PKR. However, the dsRNA-binding ability of B/NS1 allows it to sequester PKR via an interaction with RNA, which subsequently prevents vRNP-mediated PKR activation (Dauber et al., 2009). This explains why recombinant influenza B viruses encoding mutant B/NS1 proteins that were unable to bind dsRNA strongly activated PKR and were highly attenuated even though they could control IFN activation (Dauber et al., 2006).

In contrast to the severe attenuation seen for ΔNS1-B virus or viruses compromised in N-terminal dsRNA-binding, a recombinant virus encoding a C-terminally truncated B/NS1 protein (NS1–104) that retained the dsRNA- and ISG15-binding domains, grew to similar titres as wild-type virus in IFN-competent 11 day-old embryonated chicken eggs, despite its compromised ability to control IFN induction. Indeed this recombinant virus induced twice as much

Fig. 3. Schematic diagram of the A/NS1 and B/NS1 proteins, highlighting the two distinct domains and the known protein interactions/functions of each. Numbers represent amino acid positions. NLS, Nuclear localization sequence; NES, nuclear export sequence.
secreted IFN-α/β as did ΔNS1-B virus indicating that, in the context of virus infection, the C terminus of B/NS1 protein is essential to control IFN induction. However, the mechanism by which this is achieved and the reason for the relative IFN insensitivity are as yet unknown.

One functional difference between A/NS1 and B/NS1 is that the latter does not bind to and activate the signalling intermediate phosphatidylinositide kinase 3 (PI3K) (Ehrhardt et al., 2007a). During influenza A virus infection A/NS1 activates the PI3K signalling pathway (reviewed by Hale et al., 2008) and failure to do so results in attenuation of the virus (Hale et al., 2006). A/NS1-mediated PI3K activation was initially thought to prevent apoptosis (Ehrhardt et al., 2007b); however, this has recently been disputed (Jackson et al., 2010). Regardless of the biological significance of A/NS1-mediated PI3K activation it appears that influenza B viruses do not have this function.

Another difference between A/NS1 and B/NS1 is that only the latter has been shown to localize to nuclear speckle domains early in infection (Schneider et al., 2009). B/NS1 co-localizes with SC35, a serine/arginine-rich splicing factor, but not with promyelocytic leukaemia protein-containing bodies (PML bodies), which is in contrast to A/NS1 (Sato et al., 2003). The N-terminal domain of B/NS1 mediates nuclear speckle association and a monopartite nuclear localization signal (NLS) in this domain was shown to be required for this function (Schneider et al., 2009). The NLS (residues 46–56) was shown to mediate binding to importin α3 and when removed led to a slight attenuation of the virus. However, although required for optimal virus replication, the functional significance of B/NS1 nuclear speckle association is not yet known. Recent observations that binding of B/NS1 to ISG15 leads to relocalization of ISG15 to nuclear speckles (Sridharan et al., 2010) suggests the possibility that nuclear speckle association of B/NS1 is involved in antagonizing cellular antiviral responses.

In spite of the differences between the functional domains of influenza A and B virus NS1 proteins, the fact that both proteins act as potent suppressors of innate immunity has led to the development of recombinant viruses that either encode truncated NS1 proteins or lack the coding capacity for NS1 altogether for testing as live attenuated vaccine backbones (Hai et al., 2008; Wressnigg et al., 2009a, b). Such viruses induce moderate levels of IFN that compromise their replication in vivo and also act to stimulate adaptive immunity to protect against wild-type virus challenge. In order to achieve a good balance of immune stimulation and attenuation, a thorough understanding of the effects of such mutations using the reverse genetics approach will be important.

Reverse genetics to describe functions of proteins unique to influenza B virus

The influenza B virus genome encodes two small hydrophobic type III integral membrane proteins (Fig. 4). The first is unique to influenza B viruses and is encoded on RNA segment 6 from an initiation codon just 4 nt upstream of the start of the NA ORF (Shaw et al., 1983; Williams & Lamb, 1986). The continuous ORF that follows spans 100 codons and encodes a protein known as NB (Fig. 2). NB is completely conserved in all sequenced influenza B virus strains. It has two N-linked glycosylation sites in the ectodomain on which the sugars are further modified to contain poly lactosaminoglycans (Williams & Lamb, 1988, 1989). For many years it was hypothesized that the NB protein was the functional analogue of the influenza A virus M2 ion channel protein. Since it was known that uncoating of the influenza B virus genome is also pH-dependent and requires an acidification step in the endosome (Zhirnov, 1992), it was reasonable to hypothesize that there was an analogous gene to M2 in influenza B virus. Indeed the NB protein was shown to be incorporated into virus particles (Betakova et al., 1996; Brassard et al., 1996) in similar levels to M2 (Zebedee & Lamb, 1988). NB has a similar domain distribution to M2 (Fig. 4), although the two proteins show no sequence homology. Some studies have reported ion channel activity for NB although the specificity of ions was quite different from that of the M2 channel (Fischer et al., 2001; Sunstrom et al., 1996).

Using reverse genetics it was shown that NB is not essential for replication of influenza B viruses in cell culture (Hatta & Kawaoka, 2003). Viruses lacking the coding capacity for NB replicated as well as wild-type controls in MDCK cells.

**Fig. 4.** Schematic diagram of the influenza A virus M2 protein and the influenza B virus NB and BM2 proteins. All three proteins are type III integral membrane proteins consisting of an N-terminal extracellular domain (ECD), a single transmembrane domain (TMD) and a C-terminal cytoplasmic domain (CD). The known functions of each domain are indicated, including the HXXXW ion channel motif of M2 and BM2. Numbers represent amino acid positions.
This made it very unlikely that NB was the true analogue of the influenza A M2 ion channel. Although the NB deletant virus was attenuated for replication in mouse lungs, it was impossible to exclude that the mutations engineered to abrogate NB expression had not affected expression of the NA gene that is translated from the same mRNA. This would result in virus attenuation through a loss of NA activity, which is required for the spread of virus in vivo. Thus, NB is an interesting additional protein conserved in influenza B viruses with a currently unknown function.

It is now clear that the analogous gene for M2 in influenza B viruses is the other small type III transmembrane protein known as BM2. BM2 is a 109 aa protein that, like M2 of influenza A virus, is encoded on RNA segment 7, although their coding strategies are different (Briedis et al., 1982; DeBorde et al., 1988; Hiebert et al., 1986; Horvath et al., 1990). Due to phosphorylation, two species of BM2 can be detected and both forms of the protein are transported to the cell surface for incorporation into progeny virions (Odagiri et al., 1999). The first 50 residues of BM2 are critical for its cell surface transport (Watanabe et al., 2003). Residues 7–25 are sufficiently hydrophobic to act as the transmembrane anchor and contain an HXXXW motif reminiscent of that found in the influenza A virus M2 protein and known to be responsible for conducting protons across the channel (Fig. 4) (Paterson et al., 2003). This led to speculation that BM2 is the influenza B virus analogue of the M2 protein, and this was demonstrated using electrophysiology experiments (Mould et al., 2003). Structurally the tetrameric transmembrane channels formed by M2 and BM2 are very similar (Fig. 5), which further adds to the hypothesis that these proteins share function. Confirmation of the functional homology between the influenza A and B virus ion channel proteins was achieved by the creation of chimeric proteins with N-terminal and/or cytoplasmic domains from one protein and the transmembrane domain of the other. These chimeric proteins were able to form oligomers that acted as functional ion channels (Balannik et al., 2008).

The mechanism for translation of BM2 is very unusual; a coupled termination/reinitiation event was described and characterized in an exogenous system some years ago (Horvath et al., 1990). The termination codon of M1 and the initiation codon of BM2 overlap in a pentanucleotide UAAUG motif. Interestingly, although this AUG was shown to be the site for BM2 initiation, the ORF is also conserved upstream for a further 85 codons. Thus, it could be that another gene product is encoded from this area of the genome and expressed by an as-yet unidentified mechanism, although no evidence for the existence of such a protein has yet been presented. Moreover the conservation of sequence in this region may be accounted for by the presence of a cis-acting RNA signal that enhances translation of the BM2 ORF (see below).

AUG shows complementarity to a region of 18S rRNA and this recruits ribosomes for internal initiation (Powell et al., 2008). This mechanism is similar to that used by some caliciviruses, but has not been previously described for an orthomyxovirus. In fact insertion of the BM2 pentanucleotide motif with the upstream 45 nt of the M1 ORF between two non-influenza ORFs allowed expression of both foreign proteins, highlighting the potential use of this system in molecular biology for expressing multiple proteins from a single mRNA (Hatta et al., 2009).

A mutant influenza B virus altered in the pentanucleotide motif surrounding the BM2 initiation site from UAAUG to UAGUG showed a loss of BM2 expression, a decrease in BM2 incorporation into virions and an attenuated growth phenotype (Jackson et al., 2004). The mutant virus particles had lower infectivity than those of wild-type virus, which can be accounted for by an inability to efficiently uncoat when less ion channel is incorporated in the virion. A reduction of BM2 expression led to attenuated replication.

It has not been possible to recover a virus which lacked BM2 altogether, unless exogenous BM2 was provided.
(Hatta et al., 2004; Imai et al., 2004; Jackson et al., 2004). In fact recombinant viruses with BM2 proteins truncated by greater than two amino acids at the C-terminal region of the cytoplasmic tail, could only be generated when BM2 was supplemented from an exogenous source (Imai et al., 2008). Alanine scanning mutagenesis and truncation mutants suggest that the length of this region is crucial, although some substitutions between residues 86 and 97 resulted in attenuation despite retention of full-length protein; therefore, the primary sequence also plays a role in its function. This region of the BM2 cytoplasmic tail appears to aid recruitment of M1 to the site of virus assembly at the plasma membrane (Imai et al., 2008). The non-infectious virus-like particles formed in the absence of BM2 were enlarged spherical or amorphous particles, with reduced levels of NP and M1 protein incorporation when compared with the wild-type virus. However, a direct interaction between BM2 and M1 has not yet been demonstrated. Nonetheless, these data further reinforce the idea that BM2 is the true analogue of the influenza A virus M2 protein, as evidence suggests that the M2 cytoplasmic tail also plays a role in virion assembly. Mutants of influenza A virus whose M2 proteins are truncated at the C terminus are also deficient in viral ribonucleoprotein (vRNP) incorporation and display altered morphology (Iwatsuki-Horimoto et al., 2006; McCown & Pekosz, 2005, 2006).

Reverse genetics to create a mouse-adapted influenza B virus

A useful mutation in the influenza B virus M1 protein was generated by passing the avirulent B/Memphis/12/97 virus repeatedly in mice, which selected for a lethal mouse adaptation (McCullers et al., 2005). This genotype was mapped to a single N221S amino acid change in M1. Using reverse genetics mouse adaptation was conferred to a second strain of virus, B/Yamanashi/166/98, either by transfer of the B/Memphis/12/97 virus RNA-segment 7 or by introducing the N221S mutation directly into the B/Yamanashi/166/98 M1 gene. This reinforces the notion that for influenza B viruses a virus–host interaction, mediated by the M1 protein, plays an important role in replication efficiency.

Genetic differences between influenza A and B viruses that have not yet been investigated by reverse genetics

Comparison of influenza A and B virus genes indicates that the B virus orthologues are often longer than those encoded by influenza A viruses. One example is the NP protein of influenza B virus that contains an additional 50 aa at the N terminus. Using a reconstituted polymerase complex to drive replication of influenza B virus-like RNAs, it was shown that this N-terminal extension was not required to support virus replication (Stevens & Barclay, 1998). It is likely that the role of this novel domain could be addressed by a reverse genetic approach; however, interpretation may be confounded by the likelihood that cis-acting packaging signals for segment 5 may exist within this coding region of the RNA.

Segment 2 of the influenza A virus genome encodes the PB1 subunit of the viral polymerase complex; however, it was recently shown to contain a small overlapping ORF (Fig. 2), which encodes the PB1-F2 protein (Chen et al., 2001) and an N-terminally truncated PB1 protein known as N40 (Wise et al., 2009). The roles of PB1-F2 and N40 for influenza A viruses are still not completely clear. Although neither protein is absolutely required for replication, PB1-F2 may enhance virulence of influenza A viruses by either enhancing replication (Mazur et al., 2008) or affecting immune functions predisposing hosts to secondary bacterial infections (Macculey et al., 2007). At present the influenza B virus genome appears devoid of PB1-F2 or N40 orthologues. It is possible that neither gene is conserved between influenza types. Indeed, some influenza A viruses also contain natural truncations in the PB1-F2 ORF.

Concluding remarks

Increasing our knowledge of the biology of influenza viruses is paramount for our efforts to control them. Influenza B viruses are no less important than their influenza A virus counterparts as aetiological agents of seasonal influenza epidemics and their infections result in hospitalization of at-risk patients such as the young and elderly. The advent of reverse genetics technology allowing the recovery of infectious influenza B viruses entirely from cDNA containing any mutation of choice has significantly increased our ability to study all aspects of their biology. Perhaps one of the most useful and important aspects of influenza B virus reverse genetics is in the design of high-growth vaccine strains. This could revolutionize the current method of vaccine production, making it more efficient, reliable and used together with tissue culture cell-based systems could significantly reduce vaccine production time. Recent evolution of influenza B viruses has taken a bifurcated path and two separate lineages of influenza B viruses currently circulate (McCullers et al., 2004; Rota et al., 1990). Vaccine strains may display low efficacy against strains of the opposite lineage (Belshe et al., 2010; Camilloni et al., 2009). If this situation continues, decisions have to be made to include both lineages into vaccine formulations, or choose a single strain (Belshe, 2010). Reverse genetics does offer an opportunity to address these issues by understanding the genetic and structural basis of the lineages. For example, in the future viruses might be engineered that display antigenic epitopes of both lineages. Influenza B viruses differ from influenza A viruses in a number of aspects that can be dissected using reverse genetics technology. The role of the accessory protein NB is not yet clear but provides a prime example of a viral protein that has remained refractory to study for years until the advent of the reverse genetic technique. The ability to
generate viruses altered at will in the B/NS1 gene demonstrated a strategy to produce a high growth strain that lacks efficient IFN-antagonistic properties. This could potentially aid in the creation of future live attenuated vaccine candidate strains or the development of new antiviral drugs.

There is no doubt that reverse genetics technology for both influenza A and B viruses has ignited a new era of exciting research when influenza viruses are at the forefront of scientific and public attention.

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