Journal of General Virology (2011), **92**, 1–17 DOI 10.1099/vir.0.026187-0

Review

Molecular studies of influenza B virus in the reverse genetics era

David Jackson,[1] Ruth A. Elderfield[2] and Wendy S. Barclay[2]

Correspondence
Wendy S. Barclay
w.barclay@imperial.ac.uk

[1]Centre for Biomolecular Sciences, University of St Andrews, St Andrews, Fife KY16 9ST, UK
[2]Section of Virology, Faculty of Medicine, Imperial College, London W2 1PG, UK

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; Gotlieb & 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

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

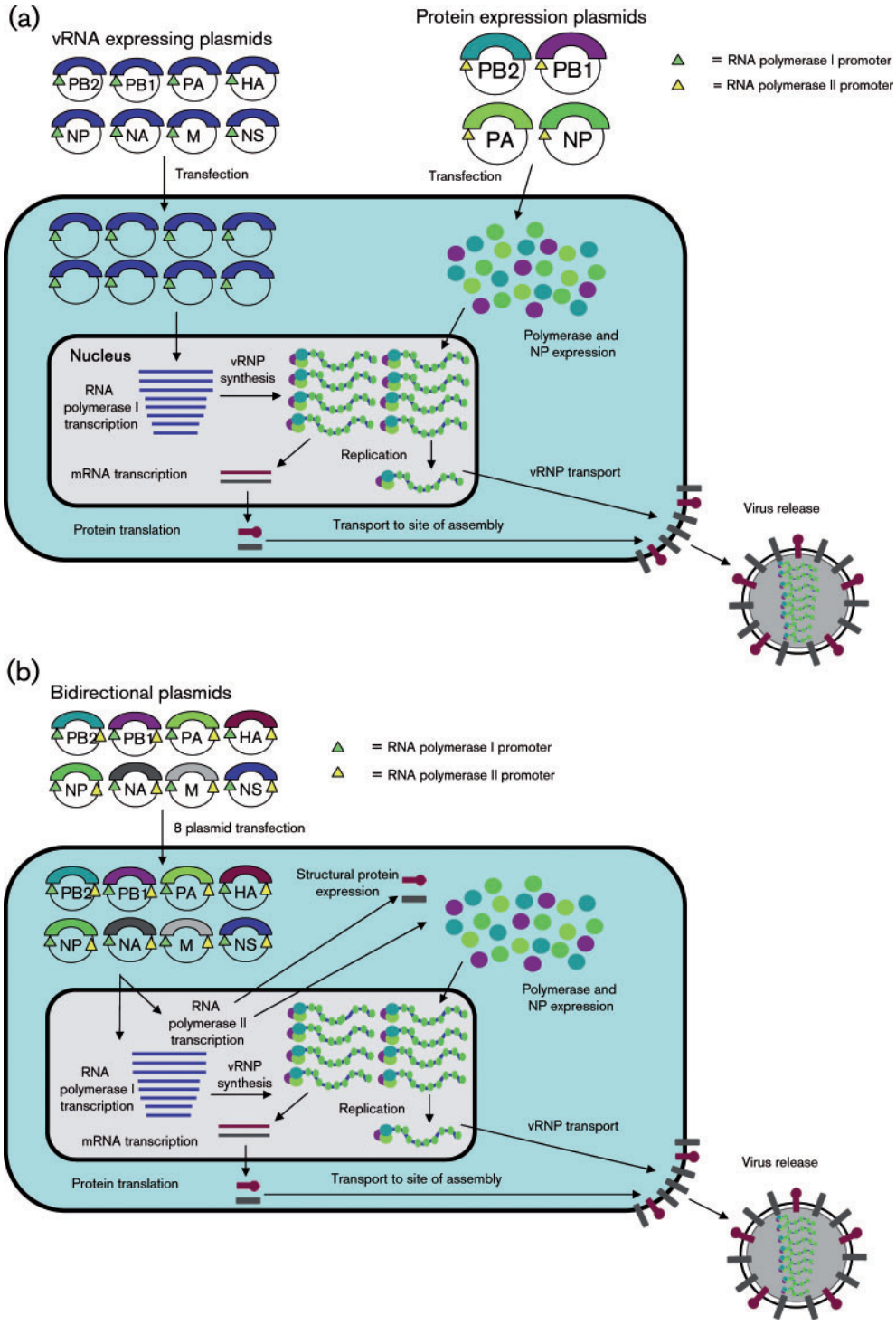

Fig. 1. Schematic diagrams of reverse genetics systems for recovery of influenza viruses from plasmid DNA. (a) 293T cells are co-transfected with 12 plasmids, eight encoding each of the eight vRNA gene segments and four expressing the viral polymerase complex and nucleoprotein (NP). After transcription 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).

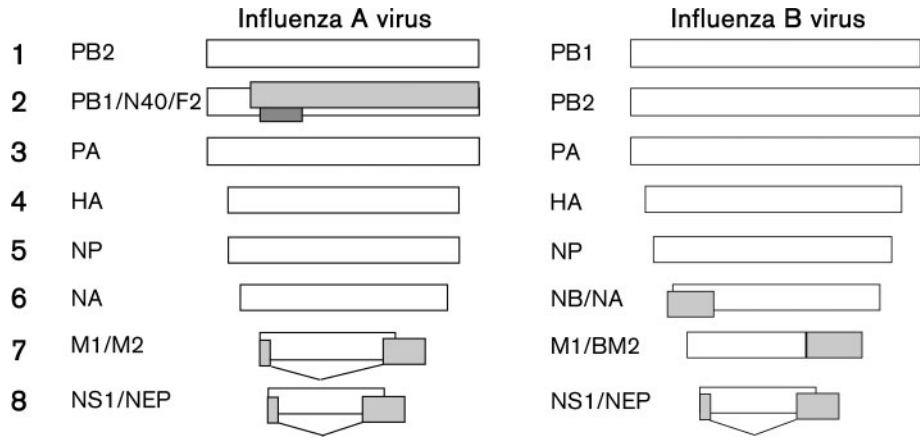

Fig. 2. Schematic diagram of the protein coding ORFs encoded on the eight segments of the influenza A and B virus genomes. Numbers in bold represent the vRNA segment number on which the proteins are encoded. The influenza A virus PB1-F2 and N40 proteins and the influenza B virus NB protein are encoded 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.

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

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)

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/cRNA 5′ non-coding regions	Nucleotide sequence of the vRNA 5′ non-coding regions
PB1	**AGCAGAAGC**GGAGCCUUUAAG	**AGUAGAAAC**ACGAGCCUUUUUUCAUUUUA AUCAUUUGUUUAUCGCAUGUAUUCAAU
PB2	**AGCAGAAGC**GGAGCG**UUUU**CAAG	**AGUAGAAAC**ACGAGCAUUUUUUCACUCAAUUG UGUUUAUUGAAUUAAUGGAUAAAUUUAUA
PA	**AGCAGAAGC**GGUGCGUUUGAUUUGCCAUA	**AGUAGAAAC**ACGUGCAUUUUUGAUUCUCGAU UCUUUUUAUUGGACGUUUAGAUACAUAAU GAACAAAAUAGUACCAAAUUGAGUACUAUGUUUUU
HA	**AGCAGAAGC**AGAGCAUUUUCUAAUAUCCACAAA	**AGUAGUAAC**AAGAGCAUUUUUCAAUAACGUU UUUUGUAAUGGUAACAAGCAAACAAGCACU ACAAUAAAGGAAAAUACAGGGCUUAAUUUUCC
NP	**AGCAGAAGC**A**C**AGCAUUUUCUUGUGAGC UUCGAGCACUAAUAAAACUGAAAAUCAAA	**AGUAGAAAC**AACAGCAUUUUUUACAUUUAUU UCAAUAAGAGUAAACACCCACAUCCC AAACGUACUGAAACAGUCACAGCCAUA GUGUCUAUUUUAUUGCU
NA	**AGCAGAAGC**AGAGCAUCUUCUCAAAACUG AAGCAAAUAGGCCAAAA	**AGUAGUAAC**AAGAGCAUUUUUUCAGAAACAAU UAAGUCCAGUAAGGACAAUUGUUCAAA CAAAAUAGGAACAAAGGGUUUAGAACAGA UUCAACCAUUCCUCCA
M	**AGCAGAAGC**ACGCACUUUCUUAAA	**AGUAGAAAC**AACGCACUUUUUCCAGUUUAUUUG CUGACAUUGAUUACAAUUUGC
NS	**AGCAGAAGC**AGAGGAUUUGUUUAGUCACUGG CAAACGAAAA	**AGUAGUAAC**AAGAGGAUUUUUAUUUUAAAU

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 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

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

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 antigens.

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).

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.

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

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 HXXXW 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

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 (PABPII). 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–

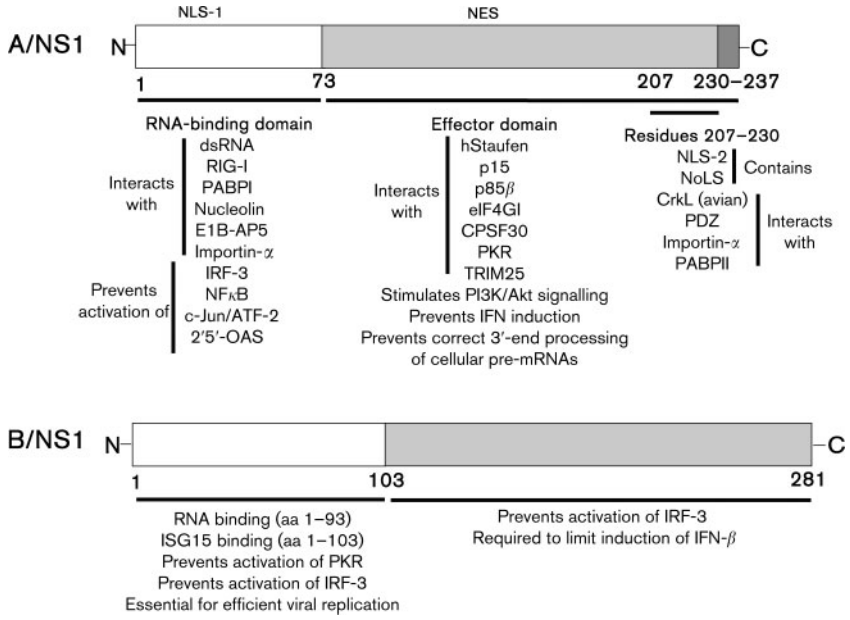

Fig. 3. Schematic diagram of the A/NS1 and B/NS1 proteins, highlighting the two distinct domains and the known protein interactions/functions of each. Numbers represent amino acid positions. NLS, Nuclear localization sequence; NES, nuclear export sequence.

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

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 polylactosaminoglycans (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.

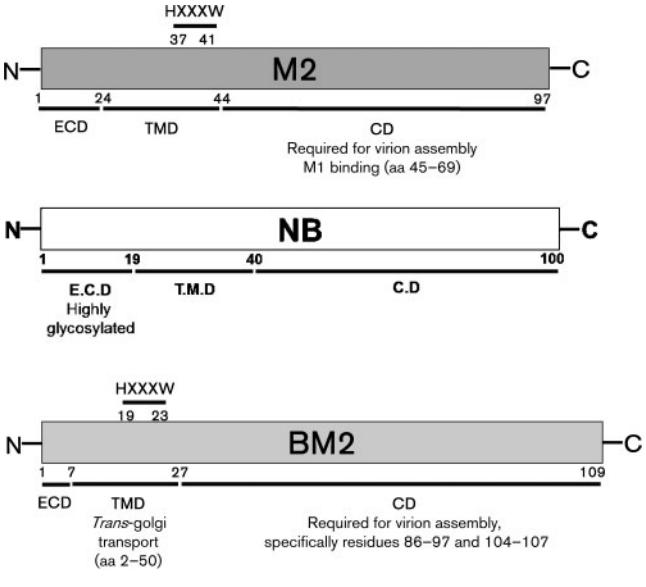

Fig. 4. Schematic diagram of the influenza A virus M2 protein and the influenza B virus NB and BM2 proteins. All three proteins are type III integral membrane proteins consisting of an N-terminal extracellular domain (ECD), a single transmembrane domain (TMD) and a C-terminal cytoplasmic domain (CD). 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).

The way in which the BM2 ORF is translated has recently been elucidated. The sequence upstream of the initiator

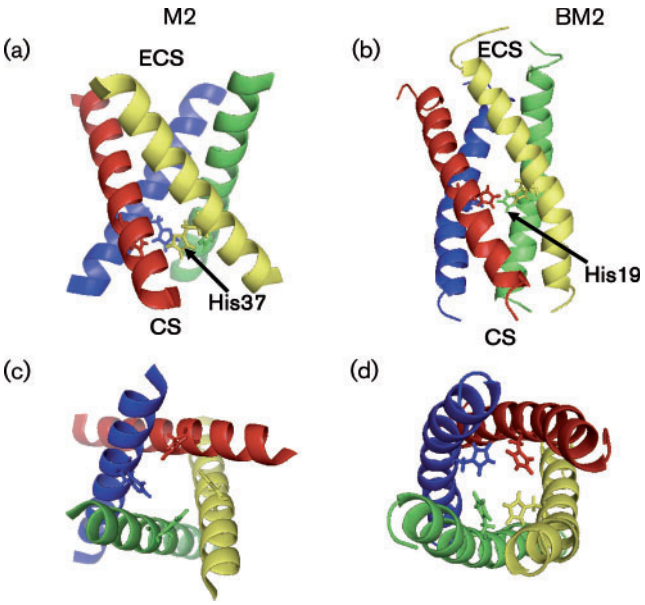

Fig. 5. Comparison of the (a) M2 and (b) BM2 transmembrane domain structures. Cartoon representation of the tetrameric ion channel pore formed by four individually coloured monomers. The critical His residue within the HXXXW ion channel motif for allowing the passage of protons across the channel is highlighted for each protein (His37 in M2; His19 in BM2). ECS, Extracellular surface; CS, cytoplasmic surface. The view from the extracellular surface looking down through the pore of (c) M2 and (d) BM2 highlights the similarities between the pore structures of the two proteins and demonstrates that His19 protrudes further into the BM2 pore compared to His37 of M2. Images were prepared by using MacPyMol and PDB files 2KAD (M2) and 2KIX (BM2).

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 reinforces 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 passaging 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 (McAuley *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.

Acknowledgements

Work performed with influenza B viruses by D. J. and R. A. E. was funded by BBSRC research studentships.

References

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Altschul, S. F., Wootton, J. C., Gertz, E. M., Agarwala, R., Morgulis, A., Schaffer, A. A. & Yu, Y. K. (2005). Protein database searches using compositionally adjusted substitution matrices. *FEBS J* **272**, 5101–5109.

Balannik, V., Lamb, R. A. & Pinto, L. H. (2008). The oligomeric state of the active BM2 ion channel protein of influenza B virus. *J Biol Chem* **283**, 4895–4904.

Barclay, W. S. & Palese, P. (1995). Influenza B viruses with site-specific mutations introduced into the HA gene. *J Virol* **69**, 1275–1279.

Baum, E. Z., Wagaman, P. C., Ly, L., Turchi, I., Le, J., Bucher, D. & Bush, K. (2003). A point mutation in influenza B neuraminidase confers resistance to peramivir and loss of slow binding. *Antiviral Res* **59**, 13–22.

Belshe, R. B. (2010). The need for quadrivalent vaccine against seasonal influenza. *Vaccine* **28** (Suppl 4), D45–D53.

Belshe, R. B., Coelingh, K., Ambrose, C. S., Woo, J. C. & Wu, X. (2010). Efficacy of live attenuated influenza vaccine in children against influenza B viruses by lineage and antigenic similarity. *Vaccine* **28**, 2149–2156.

Betakova, T., Nermut, M. V. & Hay, A. J. (1996). The NB protein is an integral component of the membrane of influenza B virus. *J Gen Virol* **77**, 2689–2694.

Brassard, D. L., Leser, G. P. & Lamb, R. A. (1996). Influenza B virus NB glycoprotein is a component of the virion. *Virology* **220**, 350–360.

Briedis, D. J. & Lamb, R. A. (1982). Influenza B virus genome: sequences and structural organization of RNA segment 8 and the mRNAs coding for the NS1 and NS2 proteins. *J Virol* **42**, 186–193.

Briedis, D. J., Lamb, R. A. & Choppin, P. W. (1982). Sequence of RNA segment 7 of the influenza B virus genome: partial amino acid homology between the membrane proteins (M1) of influenza A and B viruses and conservation of a second open reading frame. *Virology* **116**, 581–588.

Cady, S. D., Schmidt-Rohr, K., Wang, J., Soto, C. S., Degrado, W. F. & Hong, M. (2010). Structure of the amantadine binding site of influenza M2 proton channels in lipid bilayers. *Nature* **463**, 689–692.

Camilloni, B., Neri, M., Lepri, E. & Iorio, A. M. (2009). Cross-reactive antibodies in middle-aged and elderly volunteers after MF59-adjuvanted subunit trivalent influenza vaccine against B viruses of the B/Victoria or B/Yamagata lineages. *Vaccine* **27**, 4099–4103.

Carrat, F. & Flahault, A. (2007). Influenza vaccine: the challenge of antigenic drift. *Vaccine* **25**, 6852–6862.

Chen, Z. & Krug, R. M. (2000). Selective nuclear export of viral mRNAs in influenza-virus-infected cells. *Trends Microbiol* **8**, 376–383.

Chen, Z., Li, Y. & Krug, R. M. (1999). Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3′-end processing machinery. *EMBO J* **18**, 2273–2283.

Chen, W., Calvo, P. A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J. & other authors (2001). A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med* **7**, 1306–1312.

Chen, Z., Aspelund, A., Kemble, G. & Jin, H. (2006). Genetic mapping of the cold-adapted phenotype of B/Ann Arbor/1/66, the master donor virus for live attenuated influenza vaccines (FluMist). *Virology* **345**, 416–423.

Chen, Z., Aspelund, A. & Jin, H. (2008a). Stabilizing the glycosylation pattern of influenza B hemagglutinin following adaptation to growth in eggs. *Vaccine* **26**, 361–371.

Chen, Z., Aspelund, A., Kemble, G. & Jin, H. (2008b). Molecular studies of temperature-sensitive replication of the cold-adapted B/Ann Arbor/1/66, the master donor virus for live attenuated influenza FluMist vaccines. *Virology* **380**, 354–362.

Cox, N. J., Kitame, F., Kendal, A. P., Maassab, H. F. & Naeve, C. (1988). Identification of sequence changes in the cold-adapted, live attenuated influenza vaccine strain, A/Ann Arbor/6/60 (H2N2). *Virology* **167**, 554–567.

Crescenzo-Chaigne, B., Naffakh, N. & van der Werf, S. (1999). Comparative analysis of the ability of the polymerase complexes of influenza viruses type A, B and C to assemble into functional RNPs that allow expression and replication of heterotypic model RNA templates in vivo. *Virology* **265**, 342–353.

Dauber, B., Heins, G. & Wolff, T. (2004). The influenza B virus nonstructural NS1 protein is essential for efficient viral growth and antagonizes beta interferon induction. *J Virol* **78**, 1865–1872.

Dauber, B., Schneider, J. & Wolff, T. (2006). Double-stranded RNA binding of influenza B virus nonstructural NS1 protein inhibits protein kinase R but is not essential to antagonize production of alpha/beta interferon. *J Virol* **80**, 11667–11677.

Dauber, B., Martinez-Sobrido, L., Schneider, J., Hai, R., Waibler, Z., Kalinke, U., Garcia-Sastre, A. & Wolff, T. (2009). Influenza B virus ribonucleoprotein is a potent activator of the antiviral kinase PKR. *PLoS Pathog* **5**, e1000473.

Davies, W. L., Grunert, R. R., Haff, R. F., McGahen, J. W., Neumayer, E. M., Paulshock, M., Watts, J. C., Wood, T. R., Hermann, E. C. & other authors (1964). Antiviral activity of 1-Adamantanamine (amantadine). *Science* **144**, 862–863.

DeBorde, D. C., Donabedian, A. M., Herlocher, M. L., Naeve, C. W. & Maassab, H. F. (1988). Sequence comparison of wild-type and cold-adapted B/Ann Arbor/1/66 influenza virus genes. *Virology* **163**, 429–443.

Desselberger, U. & Palese, P. (1978). Molecular weights of RNA segments of influenza A and B viruses. *Virology* **88**, 394–399.

Desselberger, U., Racaniello, V. R., Zazra, J. J. & Palese, P. (1980). The 3′ and 5′-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. *Gene* **8**, 315–328.

Donelan, N. R., Dauber, B., Wang, X., Basler, C. F., Wolff, T. & Garcia-Sastre, A. (2004). The N- and C-terminal domains of the NS1 protein of influenza B virus can independently inhibit IRF-3 and beta interferon promoter activation. *J Virol* **78**, 11574–11582.

Ehrhardt, C., Wolff, T. & Ludwig, S. (2007a). Activation of phosphatidylinositol 3-kinase signaling by the nonstructural NS1 protein is not conserved among type A and B influenza viruses. *J Virol* **81**, 12097–12100.

Ehrhardt, C., Wolff, T., Pleschka, S., Planz, O., Beermann, W., Bode, J. G., Schmolke, M. & Ludwig, S. (2007b). Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. *J Virol* **81**, 3058–3067.

Fischer, W. B., Pitkeathly, M. & Sansom, M. S. (2001). Amantadine blocks channel activity of the transmembrane segment of the NB protein from influenza B. *Eur Biophys J* **30**, 416–420.

Flandorfer, A., Garcia-Sastre, A., Basler, C. F. & Palese, P. (2003). Chimeric influenza A viruses with a functional influenza B virus neuraminidase or hemagglutinin. *J Virol* **77**, 9116–9123.

Garcia-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D. E., Durbin, J. E., Palese, P. & Muster, T. (1998). Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* **252**, 324–330.

Garten, R. J., Davis, C. T., Russell, C. A., Shu, B., Lindstrom, S., Balish, A., Sessions, W. M., Xu, X., Skepner, E. & other authors (2009). Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* **325**, 197–201.

Ghenkina, D. B. & Ghendon, Y. Z. (1979). Recombination and complementation between orthomyxoviruses under conditions of abortive infection. *Acta Virol* **23**, 97–106.

Gog, J. R., Afonso Edos, S., Dalton, R. M., Leclercq, I., Tiley, L., Elton, D., von Kirchbach, J. C., Naffakh, N., Escriou, N. & other authors (2007). Codon conservation in the influenza A virus genome defines RNA packaging signals. *Nucleic Acids Res* **35**, 1897–1907.

Goodeve, A. C., Jennings, R. & Potter, C. W. (1985). Reassortants of influenza B viruses for use in vaccines: an evaluation. *Arch Virol* **83**, 169–179.

Gotlieb, T. & Hirst, G. K. (1954). The experimental production of combination forms of virus. III. The formation of doubly antigenic particles from influenza A and B virus and a study of the ability of individual particles of X virus to yield two separate strains. *J Exp Med* **99**, 307–320.

Gubareva, L. V., Kaiser, L., Matrosovich, M. N., Soo-Hoo, Y. & Hayden, F. G. (2001a). Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. *J Infect Dis* **183**, 523–531.

Gubareva, L. V., Webster, R. G. & Hayden, F. G. (2001b). Comparison of the activities of zanamivir, oseltamivir, and RWJ-270201 against clinical isolates of influenza virus and neuraminidase inhibitor-resistant variants. *Antimicrob Agents Chemother* **45**, 3403–3408.

Hai, R., Martinez-Sobrido, L., Fraser, K. A., Ayllon, J., Garcia-Sastre, A. & Palese, P. (2008). Influenza B virus NS1-truncated mutants: live-attenuated vaccine approach. *J Virol* **82**, 10580–10590.

Hale, B. G., Jackson, D., Chen, Y. H., Lamb, R. A. & Randall, R. E. (2006). Influenza A virus NS1 protein binds p85β and activates phosphatidylinositol-3-kinase signaling. *Proc Natl Acad Sci U S A* **103**, 14194–14199.

Hale, B. G., Randall, R. E., Ortin, J. & Jackson, D. (2008). The multifunctional NS1 protein of influenza A viruses. *J Gen Virol* **89**, 2359–2376.

Hatakeyama, S., Sugaya, N., Ito, M., Yamazaki, M., Ichikawa, M., Kimura, K., Kiso, M., Shimizu, H., Kawakami, C. & other authors (2007). Emergence of influenza B viruses with reduced sensitivity to neuraminidase inhibitors. *JAMA* **297**, 1435–1442.

Hatta, M. & Kawaoka, Y. (2003). The NB protein of influenza B virus is not necessary for virus replication in vitro. *J Virol* **77**, 6050–6054.

Hatta, M., Goto, H. & Kawaoka, Y. (2004). Influenza B virus requires BM2 protein for replication. *J Virol* **78**, 5576–5583.

Hatta, M., Kohlmeier, C. K., Hatta, Y., Ozawa, M. & Kawaoka, Y. (2009). Region required for protein expression from the stop-start pentanucleotide in the M gene of influenza B virus. *J Virol* **83**, 5939–5942.

Hay, A. J. (1992). The action of adamantanamines against influenza A viruses: inhibition of the M2 ion channel protein. *Semin Virol* **3**, 21–30.

Hayden, F. G., Treanor, J. J., Fritz, R. S., Lobo, M., Betts, R. F., Miller, M., Kinnersley, N., Mills, R. G., Ward, P. & other authors (1999). Use of the oral neuraminidase inhibitor oseltamivir in experimental human influenza: randomized controlled trials for prevention and treatment. *JAMA* **282**, 1240–1246.

Hayden, F. G., Gubareva, L. V., Monto, A. S., Klein, T. C., Elliot, M. J., Hammond, J. M., Sharp, S. J. & Ossi, M. J. (2000). Inhaled zanamivir for the prevention of influenza in families. Zanamivir Family Study Group. *N Engl J Med* **343**, 1282–1289.

Hiebert, S. W., Williams, M. A. & Lamb, R. A. (1986). Nucleotide sequence of RNA segment 7 of influenza B/Singapore/222/79: maintenance of a second large open reading frame. *Virology* **155**, 747–751.

Hoffmann, E., Mahmood, K., Yang, C. F., Webster, R. G., Greenberg, H. B. & Kemble, G. (2002). Rescue of influenza B virus from eight plasmids. *Proc Natl Acad Sci U S A* **99**, 11411–11416.

Hoffmann, E., Mahmood, K., Chen, Z., Yang, C. F., Spaete, J., Greenberg, H. B., Herlocher, M. L., Jin, H. & Kemble, G. (2005). Multiple gene segments control the temperature sensitivity and attenuation phenotypes of *ca* B/Ann Arbor/1/66. *J Virol* **79**, 11014–11021.

Horimoto, T., Takada, A., Iwatsuki-Horimoto, K., Hatta, M., Goto, H. & Kawaoka, Y. (2003). Generation of influenza A viruses with chimeric (type A/B) hemagglutinins. *J Virol* **77**, 8031–8038.

Horimoto, T., Iwatsuki-Horimoto, K., Hatta, M. & Kawaoka, Y. (2004a). Influenza A viruses possessing type B hemagglutinin and neuraminidase: potential as vaccine components. *Microbes Infect* **6**, 579–583.

Horimoto, T., Takada, A., Iwatsuki-Horimoto, K. & Kawaoka, Y. (2004b). A protective immune response in mice to viral components other than hemagglutinin in a live influenza A virus vaccine model. *Vaccine* **22**, 2244–2247.

Horvath, C. M., Williams, M. A. & Lamb, R. A. (1990). Eukaryotic coupled translation of tandem cistrons: identification of the influenza B virus BM2 polypeptide. *EMBO J* **9**, 2639–2647.

Hsu, M. T., Parvin, J. D., Gupta, S., Krystal, M. & Palese, P. (1987). Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. *Proc Natl Acad Sci U S A* **84**, 8140–8144.

Huber, V. C., Kleimeyer, L. H. & McCullers, J. A. (2008). Live, attenuated influenza virus (LAIV) vehicles are strong inducers of immunity toward influenza B virus. *Vaccine* **26**, 5381–5388.

Hutchinson, E. C., von Kirchbach, J. C., Gog, J. R. & Digard, P. (2010). Genome packaging in influenza A virus. *J Gen Virol* **91**, 313–328.

Imai, M., Watanabe, S., Ninomiya, A., Obuchi, M. & Odagiri, T. (2004). Influenza B virus BM2 protein is a crucial component for incorporation of viral ribonucleoprotein complex into virions during virus assembly. *J Virol* **78**, 11007–11015.

Imai, M., Kawasaki, K. & Odagiri, T. (2008). Cytoplasmic domain of influenza B virus BM2 protein plays critical roles in production of infectious virus. *J Virol* **82**, 728–739.

Isaacs, A. & Baron, S. (1960). Antiviral action of interferon in embryonic cells. *Lancet* **2**, 946–947.

Iwatsuki-Horimoto, K., Horimoto, T., Noda, T., Kiso, M., Maeda, J., Watanabe, S., Muramoto, Y., Fujii, K. & Kawaoka, Y. (2006). The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly. *J Virol* **80**, 5233–5240.

Iwatsuki-Horimoto, K., Hatta, Y., Hatta, M., Muramoto, Y., Chen, H., Kawaoka, Y. & Horimoto, T. (2008). Limited compatibility between the RNA polymerase components of influenza virus type A and B. *Virus Res* **135**, 161–165.

Jackson, D., Cadman, A., Zurcher, T. & Barclay, W. S. (2002). A reverse genetics approach for recovery of recombinant influenza B viruses entirely from cDNA. *J Virol* **76**, 11744–11747.

Jackson, D., Zurcher, T. & Barclay, W. (2004). Reduced incorporation of the influenza B virus BM2 protein in virus particles decreases infectivity. *Virology* **322**, 276–285.

Jackson, D., Barclay, W. & Zurcher, T. (2005). Characterization of recombinant influenza B viruses with key neuraminidase inhibitor resistance mutations. *J Antimicrob Chemother* **55**, 162–169.

Jackson, D., Killip, M. J., Galloway, C. S., Russell, R. J. & Randall, R. E. (2010). Loss of function of the influenza A virus NS1 protein promotes apoptosis but this is not due to a failure to activate phosphatidylinositol 3-kinase (PI3K). *Virology* **396**, 94–105.

Kawai, N., Ikematsu, H., Iwaki, N., Maeda, T., Satoh, I., Hirotsu, N. & Kashiwagi, S. (2006). A comparison of the effectiveness of oseltamivir for the treatment of influenza A and influenza B: a Japanese multicenter study of the 2003–2004 and 2004–2005 influenza seasons. *Clin Infect Dis* **43**, 439–444.

Kilbourne, E. D. (1969). Future influenza vaccines and the use of genetic recombinants. *Bull World Health Organ* **41**, 643–645.

Kim, C. U., Lew, W., Williams, M. A., Liu, H., Zhang, L., Swaminathan, S., Bischofberger, N., Chen, M. S., Mendel, D. B. & other authors (1997). Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. *J Am Chem Soc* **119**, 681–690.

Koudstaal, W., Hartgroves, L., Havenga, M., Legastelois, I., Ophorst, C., Sieuwerts, M., Zuijdgeest, D., Vogels, R., Custers, J. & other authors (2009). Suitability of PER.C6 cells to generate epidemic and pandemic influenza vaccine strains by reverse genetics. *Vaccine* **27**, 2588–2593.

Lackenby, A., Thompson, C. I. & Democratis, J. (2008). The potential impact of neuraminidase inhibitor resistant influenza. *Curr Opin Infect Dis* **21**, 626–638.

Lamb, R. A. & Krug, R. M. (2001). *Orthomyxoviridae*: The Viruses and Their Replication. In *Fields Virology*, 4th edn, pp. 1487–1531. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Lee, Y. S. & Seong, B. L. (1996). Mutational analysis of influenza B virus RNA transcription in vitro. *J Virol* **70**, 1232–1236.

Lee, Y. S. & Seong, B. L. (1998). Nucleotides in the panhandle structure of the influenza B virus virion RNA are involved in the specificity between influenza A and B viruses. *J Gen Virol* **79**, 673–681.

Lenschow, D. J., Lai, C., Frias-Staheli, N., Giannakopoulos, N. V., Lutz, A., Wolff, T., Osiak, A., Levine, B., Schmidt, R. E. & other authors (2007). IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proc Natl Acad Sci U S A* **104**, 1371–1376.

Lin, Y. P., Gregory, V., Bennett, M. & Hay, A. (2004). Recent changes among human influenza viruses. *Virus Res* **103**, 47–52.

Lugovtsev, V. Y., Vodeiko, G. M., Strupczewski, C. M., Ye, Z. & Levandowski, R. A. (2007). Generation of the influenza B viruses with improved growth phenotype by substitution of specific amino acids of hemagglutinin. *Virology* **365**, 315–323.

Lugovtsev, V. Y., Smith, D. F. & Weir, J. P. (2009). Changes of the receptor-binding properties of influenza B virus B/Victoria/504/2000 during adaptation in chicken eggs. *Virology* **394**, 218–226.

Luo, C., Nobusawa, E. & Nakajima, K. (1999). An analysis of the role of neuraminidase in the receptor-binding activity of influenza B virus: the inhibitory effect of Zanamivir on haemadsorption. *J Gen Virol* **80**, 2969–2976.

Luytjes, W., Krystal, M., Enami, M., Parvin, J. D. & Palese, P. (1989). Amplification, expression, and packaging of foreign gene by influenza virus. *Cell* **59**, 1107–1113.

Ma, C., Soto, C. S., Ohigashi, Y., Taylor, A., Bournas, V., Glawe, B., Udo, M. K., Degrado, W. F., Lamb, R. A. & other authors (2008). Identification of the pore-lining residues of the BM2 ion channel protein of influenza B virus. *J Biol Chem* **283**, 15921–15931.

Maassab, H. F. (1967). Adaptation and growth characteristics of influenza virus at 25 degrees c. *Nature* **213**, 612–614.

Maassab, H. F. & DeBorde, D. C. (1985). Development and characterization of cold-adapted viruses for use as live virus vaccines. *Vaccine* **3**, 355–369.

Mazur, I., Anhlan, D., Mitzner, D., Wixler, L., Schubert, U. & Ludwig, S. (2008). The proapoptotic influenza A virus protein PB1–F2 regulates viral polymerase activity by interaction with the PB1 protein. *Cell Microbiol* **10**, 1140–1152.

McAuley, J. L., Hornung, F., Boyd, K. L., Smith, A. M., McKeon, R., Bennink, J., Yewdell, J. W. & McCullers, J. A. (2007). Expression of the 1918 influenza A virus PB1–F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. *Cell Host Microbe* **2**, 240–249.

McCown, M. F. & Pekosz, A. (2005). The influenza A virus M2 cytoplasmic tail is required for infectious virus production and efficient genome packaging. *J Virol* **79**, 3595–3605.

McCown, M. F. & Pekosz, A. (2006). Distinct domains of the influenza a virus M2 protein cytoplasmic tail mediate binding to the M1 protein and facilitate infectious virus production. *J Virol* **80**, 8178–8189.

McCullers, J. A., Wang, G. C., He, S. & Webster, R. G. (1999). Reassortment and insertion-deletion are strategies for the evolution of influenza B viruses in nature. *J Virol* **73**, 7343–7348.

McCullers, J. A., Saito, T. & Iverson, A. R. (2004). Multiple genotypes of influenza B virus circulated between 1979 and 2003. *J Virol* **78**, 12817–12828.

McCullers, J. A., Hoffmann, E., Huber, V. C. & Nickerson, A. D. (2005). A single amino acid change in the C-terminal domain of the matrix protein M1 of influenza B virus confers mouse adaptation and virulence. *Virology* **336**, 318–326.

McGeoch, D., Fellner, P. & Newton, C. (1976). Influenza virus genome consists of eight distinct RNA species. *Proc Natl Acad Sci U S A* **73**, 3045–3049.

McKimm-Breschkin, J. L. (2000). Resistance of influenza viruses to neuraminidase inhibitors – a review. *Antiviral Res* **47**, 1–17.

Meindl, P. & Tuppy, H. (1969). 2-Deoxy-2,3-dehydrosialic acids. II. Competitive inhibition of Vibrio cholerae neuraminidase by 2-deoxy-2,3-dehydro-N-acylneuraminic acids. *Hoppe Seylers Z Physiol Chem* **350**, 1088–1092.

Monto, A. S., Pichichero, M. E., Blanckenberg, S. J., Ruuskanen, O., Cooper, C., Fleming, D. M. & Kerr, C. (2002). Zanamivir prophylaxis: an effective strategy for the prevention of influenza types A and B within households. *J Infect Dis* **186**, 1582–1588.

Mould, J. A., Paterson, R. G., Takeda, M., Ohigashi, Y., Venkataraman, P., Lamb, R. A. & Pinto, L. H. (2003). Influenza B virus BM2 protein has ion channel activity that conducts protons across membranes. *Dev Cell* **5**, 175–184.

Nemeroff, M. E., Barabino, S. M., Li, Y., Keller, W. & Krug, R. M. (1998). Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3′ end formation of cellular pre-mRNAs. *Mol Cell* **1**, 991–1000.

Neumann, G. & Kawaoka, Y. (2001). Reverse genetics of influenza virus. *Virology* **287**, 243–250.

Odagiri, T., Hong, J. & Ohara, Y. (1999). The BM2 protein of influenza B virus is synthesized in the late phase of infection and incorporated into virions as a subviral component. *J Gen Virol* **80**, 2573–2581.

Ohigashi, Y., Ma, C., Jing, X., Balannick, V., Pinto, L. H. & Lamb, R. A. (2009). An amantadine-sensitive chimeric BM2 ion channel of influenza B virus has implications for the mechanism of drug inhibition. *Proc Natl Acad Sci U S A* **106**, 18775–18779.

Osterhaus, A. D., Rimmelzwaan, G. F., Martina, B. E., Bestebroer, T. M. & Fouchier, R. A. (2000). Influenza B virus in seals. *Science* **288**, 1051–1053.

Palese, P. & Shaw, M. L. (2007). *Orthomyxoviridae*: The viruses and their replication. In *Fields Virology*, 5th edn, pp. 1647–1689. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Paterson, R. G., Takeda, M., Ohigashi, Y., Pinto, L. H. & Lamb, R. A. (2003). Influenza B virus BM2 protein is an oligomeric integral membrane protein expressed at the cell surface. *Virology* **306**, 7–17.

Pau, M. G., Ophorst, C., Koldijk, M. H., Schouten, G., Mehtali, M. & Uytdehaag, F. (2001). The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines. *Vaccine* **19**, 2716–2721.

Powell, M. L., Napthine, S., Jackson, R. J., Brierley, I. & Brown, T. D. (2008). Characterization of the termination-reinitiation strategy employed in the expression of influenza B virus BM2 protein. *RNA* **14**, 2394–2406.

Qiu, Y. & Krug, R. M. (1994). The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). *J Virol* **68**, 2425–2432.

Robertson, J. S. (1979). 5′ and 3′ terminal nucleotide sequences of the RNA genome segments of influenza virus. *Nucleic Acids Res* **6**, 3745–3757.

Robertson, J. S., Naeve, C. W., Webster, R. G., Bootman, J. S., Newman, R. & Schild, G. C. (1985). Alterations in the hemagglutinin associated with adaptation of influenza B virus to growth in eggs. *Virology* **143**, 166–174.

Rota, P. A., Wallis, T. R., Harmon, M. W., Rota, J. S., Kendal, A. P. & Nerome, K. (1990). Cocirculation of two distinct evolutionary lineages of influenza type B virus since 1983. *Virology* **175**, 59–68.

Sato, Y., Yoshioka, K., Suzuki, C., Awashima, S., Hosaka, Y., Yewdell, J. & Kuroda, K. (2003). Localization of influenza virus proteins to nuclear dot 10 structures in influenza virus-infected cells. *Virology* **310**, 29–40.

Schneider, J., Dauber, B., Melen, K., Julkunen, I. & Wolff, T. (2009). Analysis of influenza B virus NS1 protein trafficking reveals a novel interaction with nuclear speckle domains. *J Virol* **83**, 701–711.

Schnell, J. R. & Chou, J. J. (2008). Structure and mechanism of the M2 proton channel of influenza A virus. *Nature* **451**, 591–595.

Shaw, M. W., Choppin, P. W. & Lamb, R. A. (1983). A previously unrecognized influenza B virus glycoprotein from a bicistronic mRNA that also encodes the viral neuraminidase. *Proc Natl Acad Sci U S A* **80**, 4879–4883.

Sheu, T. G., Deyde, V. M., Okomo-Adhiambo, M., Garten, R. J., Xu, X., Bright, R. A., Butler, E. N., Wallis, T. R., Klimov, A. I. & other authors (2008). Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. *Antimicrob Agents Chemother* **52**, 3284–3292.

Skehel, J. J. & Hay, A. J. (1978). Nucleotide sequences at the 5′ termini of influenza virus RNAs and their transcripts. *Nucleic Acids Res* **5**, 1207–1219.

Smee, D. F., Sidwell, R. W., Morrison, A. C., Bailey, K. W., Baum, E. Z., Ly, L. & Wagaman, P. C. (2001). Characterization of an influenza A (H3N2) virus resistant to the cyclopentane neuraminidase inhibitor RWJ-270201. *Antiviral Res* **52**, 251–259.

Snyder, M. H., Betts, R. F., DeBorde, D., Tierney, E. L., Clements, M. L., Herrington, D., Sears, S. D., Dolin, R., Maassab, H. F. & other authors (1988). Four viral genes independently contribute to attenuation of live influenza A/Ann Arbor/6/60 (H2N2) cold-adapted reassortant virus vaccines. *J Virol* **62**, 488–495.

Snyder, M. H., London, W. T., Maassab, H. F. & Murphy, B. R. (1989). Attenuation and phenotypic stability of influenza B/Texas/1/84 cold-adapted reassortant virus: studies in hamsters and chimpanzees. *J Infect Dis* **160**, 604–610.

Sridharan, H., Zhao, C. & Krug, R. M. (2010). Species specificity of the NS1 protein of influenza B virus: NS1 binds only human and non-human primate ubiquitin-like ISG15 proteins. *J Biol Chem* **285**, 7852–7856.

Stevens, M. P. & Barclay, W. S. (1998). The N-terminal extension of the influenza B virus nucleoprotein is not required for nuclear accumulation or the expression and replication of a model RNA. *J Virol* **72**, 5307–5312.

Stoeckle, M. Y., Shaw, M. W. & Choppin, P. W. (1987). Segment-specific and common nucleotide sequences in the noncoding regions of influenza B virus genome RNAs. *Proc Natl Acad Sci U S A* **84**, 2703–2707.

Stouffer, A. L., Acharya, R., Salom, D., Levine, A. S., Di Costanzo, L., Soto, C. S., Tereshko, V., Nanda, V., Stayrook, S. & other authors (2008). Structural basis for the function and inhibition of an influenza virus proton channel. *Nature* **451**, 596–599.

Sugaya, N., Mitamura, K., Yamazaki, M., Tamura, D., Ichikawa, M., Kimura, K., Kawakami, C., Kiso, M., Ito, M. & other authors (2007). Lower clinical effectiveness of oseltamivir against influenza B contrasted with influenza A infection in children. *Clin Infect Dis* **44**, 197–202.

Sunstrom, N. A., Premkumar, L. S., Premkumar, A., Ewart, G., Cox, G. B. & Gage, P. W. (1996). Ion channels formed by NB, an influenza B virus protein. *J Membr Biol* **150**, 127–132.

Sweet, T. M., Maassab, H. F. & Herlocher, M. L. (2004). Reverse genetics studies of attenuation of the ca A/AA/6/60 influenza virus: the role of the matrix gene. *Biomed Pharmacother* **58**, 509–515.

Varghese, J. N., Laver, W. G. & Colman, P. M. (1983). Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. *Nature* **303**, 35–40.

Versteeg, G. A., Hale, B. G., van Boheemen, S., Wolff, T., Lenschow, D. J. & Garcia-Sastre, A. (2010). Species-specific antagonism of host ISGylation by the influenza B virus NS1 protein. *J Virol* **84**, 5423–5430.

von Itzstein, M., Wu, W. Y., Kok, G. B., Pegg, M. S., Dyason, J. C., Jin, B., Van Phan, T., Smythe, M. L., White, H. F. & other authors (1993). Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* **363**, 418–423.

Wang, Z. & Duke, G. M. (2007). Cloning of the canine RNA polymerase I promoter and establishment of reverse genetics for influenza A and B in MDCK cells. *Virol J* **4**, 102.

Wang, W. & Krug, R. M. (1996). The RNA-binding and effector domains of the viral NS1 protein are conserved to different extents among influenza A and B viruses. *Virology* **223**, 41–50.

Watanabe, S., Imai, M., Ohara, Y. & Odagiri, T. (2003). Influenza B virus BM2 protein is transported through the *trans*-Golgi network as an integral membrane protein. *J Virol* **77**, 10630–10637.

Williams, M. A. & Lamb, R. A. (1986). Determination of the orientation of an integral membrane protein and sites of glycosylation by oligonucleotide-directed mutagenesis: influenza B virus NB glycoprotein lacks a cleavable signal sequence and has an extracellular NH2-terminal region. *Mol Cell Biol* **6**, 4317–4328.

Williams, M. A. & Lamb, R. A. (1988). Polylactosaminoglycan modification of a small integral membrane glycoprotein, influenza B virus NB. *Mol Cell Biol* **8**, 1186–1196.

Williams, M. A. & Lamb, R. A. (1989). Effect of mutations and deletions in a bicistronic mRNA on the synthesis of influenza B virus NB and NA glycoproteins. *J Virol* **63**, 28–35.

Wise, H. M., Foeglein, A., Sun, J., Dalton, R. M., Patel, S., Howard, W., Anderson, E. C., Barclay, W. S. & Digard, P. (2009). A complicated message: identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. *J Virol* **83**, 8021–8031.

Wressnigg, N., Shurygina, A. P., Wolff, T., Redlberger-Fritz, M., Popow-Kraupp, T., Muster, T., Egorov, A. & Kittel, C. (2009a). Influenza B mutant viruses with truncated NS1 proteins grow efficiently in Vero cells and are immunogenic in mice. *J Gen Virol* **90**, 366–374.

Wressnigg, N., Voss, D., Wolff, T., Romanova, J., Ruthsatz, T., Mayerhofer, I., Reiter, M., Nakowitsch, S., Humer, J. & other authors (2009b). Development of a live-attenuated influenza B ΔNS1 intranasal vaccine candidate. *Vaccine* **27**, 2851–2857.

Yuan, W. & Krug, R. M. (2001). Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. *EMBO J* **20**, 362–371.

Yuan, W., Aramini, J. M., Montelione, G. T. & Krug, R. M. (2002). Structural basis for ubiquitin-like ISG 15 protein binding to the NS1 protein of influenza B virus: a protein-protein interaction function that is not shared by the corresponding N-terminal domain of the NS1 protein of influenza A virus. *Virology* **304**, 291–301.

Zambon, M. & Hayden, F. G. (2001). Position statement: global neuraminidase inhibitor susceptibility network. *Antiviral Res* **49**, 147–156.

Zebedee, S. L. & Lamb, R. A. (1988). Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *J Virol* **62**, 2762–2772.

Zhirnov, O. P. (1992). Isolation of matrix protein M1 from influenza viruses by acid-dependent extraction with nonionic detergent. *Virology* **186**, 324–330.