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Review

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Genome packaging in influenza A virus

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The negative-sense RNA genome of influenza A virus is composed of eight segments, which encode 12 proteins between them. At the final stage of viral assembly, these genomic virion (v)RNAs are incorporated into the virion as it buds from the apical plasma membrane of the cell. Genome segmentation confers evolutionary advantages on the virus, but also poses a problem during virion assembly as at least one copy of each of the eight segments is required to produce a fully infectious virus particle. Historically, arguments have been presented in favour of a specific packaging mechanism that ensures incorporation of a full genome complement, as well as for an alternative model in which segments are chosen at random but packaged in sufficient numbers to ensure that a reasonable proportion of virions are viable. The question has seen a resurgence of interest in recent years leading to a consensus that the vast majority of virions contain no more than eight segments and that a specific mechanism does indeed function to select one copy of each vRNA. This review summarizes work leading to this conclusion. In addition, we describe recent progress in identifying the specific packaging signals and discuss likely mechanisms by which these RNA elements might operate.

Introduction

Influenza A virus is the prototype of the family *Orthomyxoviridae* and, like all members of this family, the negative-sense RNA that comprises its genome is divided into separate segments (Cheung & Poon, 2007; Neumann *et al.*, 2004). These virion (v)RNA segments share a common organization; a long central coding region (in antisense), sometimes encoding more than one polypeptide, flanked by relatively short untranslated regions (UTRs) and at the termini, sequences conserved between segments that show partial complementarity (Fig. 1a). The vRNA segments are separately encapsidated into ribonucleoprotein (RNP) structures by viral polypeptides: stoichiometric amounts of a single-strand RNA-binding nucleoprotein (NP; at one monomer per 24 nt) and a single copy of a heterotrimeric RNA-dependent RNA polymerase composed of the PB1, PB2 and PA polypeptides (Fig. 1b–d) (Neumann *et al.*, 2004; Portela & Digard, 2002). The RNPs act as independent units for the purposes of vRNA synthesis, which occurs in the nuclei of infected cells (Neumann *et al.*, 2004). Replicated vRNAs are exported (as RNPs) from the nucleus via the cellular CRM1 pathway (Boulo *et al.*, 2007; Neumann *et al.*, 2004),

and at the final stage of viral assembly, are incorporated into the virion as it buds from the apical plasma membrane of the cell (Fig. 2) (Cheung & Poon, 2007). The process of virion assembly is not well understood but is thought to involve a series of protein–protein interactions between the cytoplasmic tails of the viral integral membrane proteins, the matrix protein and the RNPs (Schmitt & Lamb, 2005).

Genome segmentation confers evolutionary advantages on influenza viruses, but also poses a problem in virion assembly. The eight segments encode 12 identified polypeptides, only two of which are non-essential outside of the laboratory setting (Chen *et al.*, 2001; Wise *et al.*, 2009). As these two non-essential proteins are encoded by segment 2, along with the essential PB1 polymerase subunit, it follows that at least one copy of each of the eight vRNAs must be packaged for a single virion to be able to initiate a productive infection. Until recently, the process by which this was achieved was poorly understood, but a clearer picture has begun to emerge of a mechanism for specifically packaging a full genome, mediated by *cis*-acting packaging signals in the vRNAs. This review aims to summarize the thought processes and experimental evidence leading up to the currently accepted model for influenza A genome packaging and to highlight the main questions remaining.

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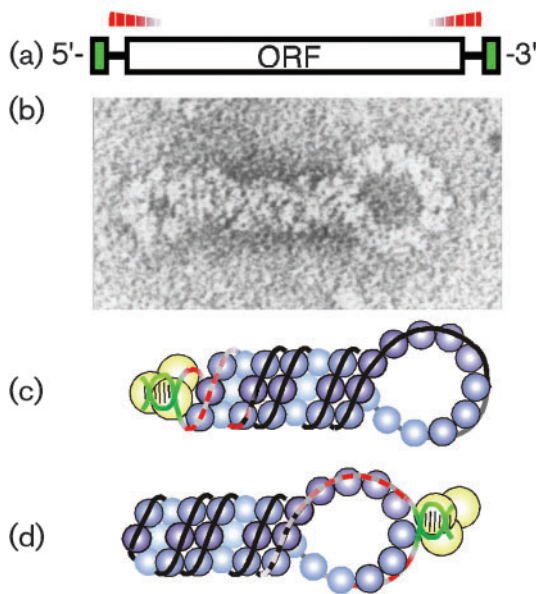


Fig. 1. Structure of influenza virus vRNA and RNPs. (a) Schematic diagram of a typical vRNA segment. A large ORF (open box) is flanked by short UTRs (lines) containing terminal promoter sequences (green boxes) that form the polymerase binding site and are essentially identical in all segments. Specific packaging signals (red wedges) overlap the UTRs and terminal coding regions and are apparently discontinuous. (b) EM image of an RNP negatively stained with uranyl acetate. Approximate magnification $\times 300\,000$. Adapted from the paper by Jennings *et al.* (1983) with permission from Elsevier. (c, d) Possible cartoon models of RNP structure. NP and the polymerase are represented as blue and yellow spheres respectively, while vRNA is represented as a line coloured according to (a). Adapted from the paper by Portela & Digard (2002) with permission of the Society for General Microbiology.

Genome segmentation: a mixed blessing

The majority of known virus families have monopartite genomes, and although segmentation has probably arisen independently on various occasions – suggesting that there is no substantial barrier to its evolution – it is much more common in RNA viruses (Ball, 2007). That segmentation evolved at all suggests that it can confer an evolutionary advantage, but it also results in problems for a virus, notably the increased complexity of genome packaging.

Genome segmentation could in theory provide various advantages. Chief among these for RNA viruses is probably the increased control it allows over gene expression (Belshaw *et al.*, 2008). However, other evolutionary consequences may follow. For influenza viruses, segmentation can be considered as providing a rudimentary sexual mechanism, taking a broad definition of sex as an exchange of genetic material (Chao *et al.*, 1997). A nascent virion assembles its genome from a pool of segments. Some of these will be miscopied, and if the cell has been infected by more than one virus, some may be derived from different

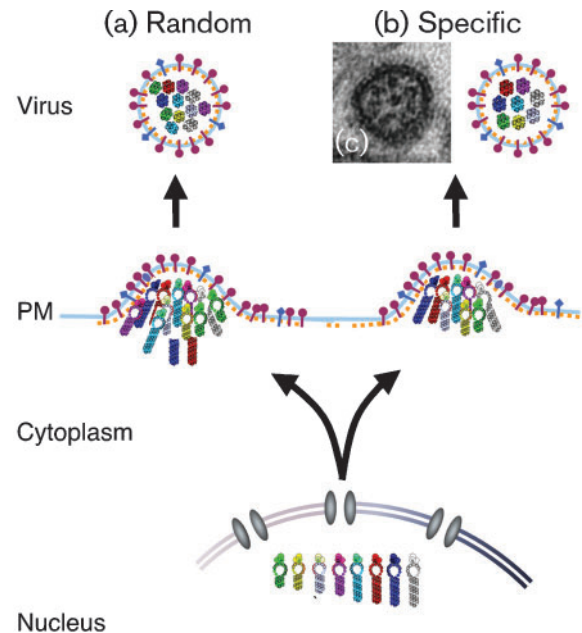


Fig. 2. Cartoon models of influenza virus particle assembly and genome packaging. The eight individual segments (differentiated by colour) are replicated independently in the nucleus before being exported to the cytoplasm and migrating to the apical plasma membrane (PM). There, they interact with other viral structural proteins and new virus particles form by budding. (a) The random model for genome packaging proposes that more than eight RNPs are incorporated in a segment non-specific manner such that a reasonable proportion of virions contain at least one copy of each segment. (b) The specific model proposes that unique segment-specific packaging signals operate to form a defined array of eight RNPs containing one copy of each segment. (c) A negatively stained EM section through an A/PuertoRico/8/34 (H1N1) influenza virion showing the distinctive 7+1 array of RNPs (appearing as dots in transverse section). Adapted, with permission from the American Society for Microbiology, from the paper by Hutchinson *et al.* (2008).

parents. The evolutionary advantages of the resulting reassortment are twofold. Firstly, the ability to reconstitute an error-free genome from a selection of miscopied and correctly copied segments – by an assembly checkpoint or by multiplicity reactivation – may provide a way to escape from Muller's ratchet (the irreversible accumulation of deleterious mutations in an asexually reproducing genome; Muller, 1964; Belshaw *et al.*, 2008; Chao *et al.*, 1997; Pressing & Reaney, 1984). The pressure of deleterious mutations is likely to be acute in influenza viruses because their RNA genomes are reproduced with relatively low fidelity and are prone to damage (Nobusawa & Sato, 2006; Suarez *et al.*, 1992) while seasonal genetic bottlenecks periodically reduce the effective population size of the virus (Gog *et al.*, 2003; Rambaut *et al.*, 2008), increasing the importance of genetic drift (Moya *et al.*, 2004). The ability of segmentation to restore a functional influenza A genome

after damage or mutation can be observed experimentally by complementation (Sugiura *et al.*, 1972) and multiplicity reactivation (Barry, 1961). A second notable evolutionary advantage provided by a sexual mechanism such as segmentation is the acquisition of beneficial alleles. This is well-documented in influenza A virus through the phenomenon of antigenic shift, in which segments of human and animal virus genomes reassort to produce antigenically novel strains with the potential to cause pandemics and to establish themselves in new host populations (Webster *et al.*, 1992). Such reassortment events triggered the 1957 and 1968 pandemic outbreaks as well as playing a major role in the evolution of the current swine-derived H1N1 pandemic virus (Garten *et al.*, 2009; Smith *et al.*, 2009).

Genome segmentation does, however, come at the cost of increasing the complexity of virion assembly, by necessitating the transmission of every segment for an infection to succeed. For those segmented viruses that initiate infection at high multiplicities, for example through vertical infection or propagation by insect vectors, correct genome packaging is not critical. An infected cell is likely to receive multiple copies of each segment due to co-infection, and infection is even possible with a ‘multicompartment’ strategy where each segment is separately encapsidated (Pressing & Reaney, 1984). This is particularly true of plant viruses, in which the majority of segmented viruses employ multicompartment genome packaging (Lazarowitz, 2007). Conversely, the transmission of influenza virus by aerosol and/or fomite spread (or, for waterfowl, by contact with highly diluted virus in water) presumably means that many influenza infections are initiated at a low m.o.i. Consequently, it is necessary for the virus to adopt a ‘monocompartment’ strategy, with at least one copy of each individual segment in a single virion (Pressing & Reaney, 1984). Segmentation therefore poses a packaging problem – as influenza viruses cannot guarantee consistently high m.o.i. values, virus particles that fail to incorporate all eight genome segments will not replicate.

Genome packaging: random or segment-specific?

There are two models for the packaging of a segmented, monocompartment genome. In a random model, a mechanism exists to distinguish pieces of the viral genome from cellular RNA and non-genomic viral RNAs and incorporate them into virions, but has no way of distinguishing between different segments (Fig. 2a). Under this scheme, a fully infectious virion would acquire a complete genome purely through chance, with the probability of success being increased by packaging more segments than the minimum required for a complete genome (Compans *et al.*, 1970; Hirst, 1962). Infectious bursal disease virus, with a two segment double-stranded (ds)RNA genome, may provide an example of such a strategy, as distinct populations of virions with increasing numbers of segments and specific infectivities can be

isolated (Luque *et al.*, 2009). Conversely, in a specific packaging model, a mechanism ensures that one copy of each different segment is specifically selected during viral assembly (Fig. 2b) (Kingsbury, 1970). The dsRNA bacteriophage $\Phi 6$ is a good example of this strategy as a specific packaging mechanism has been demonstrated for its three segments and the particle : p.f.u. ratio of the virus is close to 1 (Mindich, 2004). These models represent the extreme cases of segment-specificity – absolutely no specificity in the former case and unfailingly rigorous selection in the latter. The extent to which packaging of the influenza A genome is in fact segment-specific has been debated, and evidence for varying degrees of segment-specificity has been presented.

Initial studies appeared to favour segment-specific packaging. Along with the realization that the influenza A genome was segmented, it was shown that genetic markers in influenza viruses fell into distinct complementation groups (Duesberg, 1968; Pons & Hirst, 1968). It was noted that although the genes of co-infecting viruses readily complemented each other, even when the parent viruses did not have plaque-forming ability in their own right (Barry, 1961; Hirst, 1973; Hirst & Pons, 1973), the progeny virions seldom carried more than one allele at a specific locus (Laver & Downie, 1976; Lubeck *et al.*, 1979; Nakajima & Sugiura, 1977; Varich *et al.*, 2008). Plausible instances of partial heterozygotes were rare (Laver & Downie, 1976; Nakajima & Sugiura, 1977); when one such virus was examined it was found to be unstable when passaged (Scholtissek *et al.*, 1978). It was inferred from this that influenza A virus normally has a haploid genome in which each segment is present only once (Laver & Downie, 1976; Nakajima & Sugiura, 1977). With only eight segments present, the probability of assembling a complete genome by random incorporation is very low; simple combinatorial calculations give a value of $8!/8^8$ or $1/416$ (Fig. 3) (Enami *et al.*, 1991; Nakajima & Sugiura, 1977).

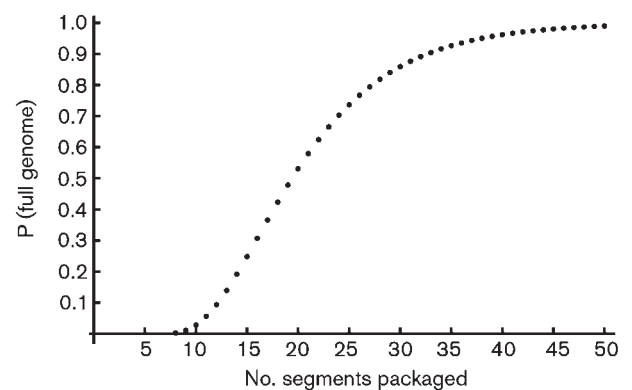


Fig. 3. A simple mathematical model of random genome packaging. The probabilities of obtaining one copy of each segment in a single virus particle are plotted for random selection with increasing numbers of RNPs per virion [using standard probability theory (Enami *et al.*, 1991)].

This value is incompatible with the approximately 1/10–1/100 proportion of infectious influenza virions estimated from particle : p.f.u. measurements (Donald & Isaacs, 1954; Hutchinson *et al.*, 2008; Kingsbury, 1970; Nakajima & Sugiura, 1977). Consequently, it was concluded that there must be some form of segment specificity in genome packaging (Laver & Downie, 1976; Nakajima & Sugiura, 1977). Further support was lent to the segment-specific packaging model by the observation that segments were present at equimolar levels in virions (Hatada *et al.*, 1989; McGeoch *et al.*, 1976) even when their ratios in infected cells differed (Bergmann & Muster, 1995; Smith & Hay, 1982), suggesting that some form of selection process was operating.

Opposition to the segment-specific packaging model developed with the ability to introduce artificial segments into influenza viruses through reverse genetics (Luytjes *et al.*, 1989). Synthetic vRNAs were produced in which viral open reading frames (ORFs) were replaced by reporter genes, retaining only the terminal UTRs of the original segment. As discussed above (Fig. 1a), the UTRs of each segment consist of 12–13 nt of highly conserved sequence, forming a panhandle structure required for polymerase binding (Neumann *et al.*, 2004), as well as a variable amount (typically around 25 nt) of segment-specific sequence. Studies showed that the UTRs of segments 4, 5 or 8 were sufficient, in the presence of a helper virus, to direct replication of the segment, to package it into virions and, in some experiments, to maintain it for several passages, indicating that the UTRs contained the minimal determinants of segment packaging (Luytjes *et al.*, 1989; Neumann *et al.*, 2004; Neumann & Hobom, 1995; Tchatalbachev *et al.*, 2001). This approach was then used to produce a virus with nine distinct segments (Enami *et al.*, 1991). Segment 8 was duplicated such that one copy contained only the NS1 gene, while the other encoded both NS2/NEP and a temperature-sensitive NS1 protein. When introduced into a virus, the short segment 9 complemented the temperature-sensitive NS1 allele in the full-length segment 8. At the non-permissive temperature, it was shown that nine-segment viruses, including both the full-length mutant segment 8 and the NS1-only construct, could be propagated clonally with single hit infectivity kinetics. The artificial nine-segment virus showed reduced infectivity at the non-permissive temperature and this was discussed with respect to segment packaging. Using a random packaging model based on standard probability methods, the observed proportion of infectious particles was consistent with the incorporation of around 10–11 segments per particle (Enami *et al.*, 1991). However, maintenance of this nine-segment virus required strong selection, without which the synthetic segment was rapidly lost (Enami *et al.*, 1991), consistent with the unstable nature of a naturally occurring partial heterozygote (Scholtissek *et al.*, 1978). More recently, it was shown that the UTRs of segments 5 (encoding NP), 6 (NA) and 8 were also able to promote the packaging of reporter genes, and

that, consistent with the random packaging model, constructs containing only the UTRs did not compete for packaging in a segment-specific fashion (Bancroft & Parslow, 2002). By using two-colour reporter genes flanked by UTRs from the same segment, it was estimated that 3–5% of virions were functionally diploid. This was proposed to be consistent with the packaging of 9–11 segments per virion (Bancroft & Parslow, 2002).

Thus, the conclusions from the first applications of molecular biology to influenza genetics conflicted with the interpretation of classical viral genetics about the mechanism of genome packaging. However, further evidence in favour of specific packaging was already available, from the characterization of defective-interfering (DI) RNAs. These are truncated forms of genome segments which retain their ability to replicate and be packaged and which, due to their smaller size, out-compete full-length segments. Viruses containing DI RNAs arise in influenza A during high-multiplicity passage (von Magnus, 1954), particularly in certain genetic backgrounds (Nakajima *et al.*, 1979; Odagiri & Tobita, 1990; Ueda *et al.*, 1980). Sequencing of DI RNAs showed that they were derived from genomic segments by internal deletion, and retained the terminal regions of the segment (Davis *et al.*, 1980; Davis & Nayak, 1979; Duhaut & Dimmock, 1998, 2000; Duhaut & McCauley, 1996; Hughes *et al.*, 2000; Jennings *et al.*, 1983; Moss & Brownlee, 1981; Nakajima *et al.*, 1979; Nayak & Sivasubramanian, 1983; Nayak *et al.*, 1982; Noble & Dimmock, 1995). As it became possible to determine the segment from which a DI RNA was derived, it became apparent that in many cases, their presence correlated with reduced amounts of the parent segment in virus particles (Akkinia *et al.*, 1984; Nakajima *et al.*, 1979; Odagiri & Tobita, 1990; Ueda *et al.*, 1980). Furthermore, this interference was shown to act at the level of packaging (rather than solely at the point of synthesis in cells) and also to affect the incorporation of the homologous segment in mixed infections of DI-containing and non-defective wild-type virus stocks (Duhaut & Dimmock, 2002; Duhaut & McCauley, 1996; Odagiri & Tashiro, 1997; Odagiri *et al.*, 1994). This segment-specific competition implied that packaging involved selection for distinctive features shared by the segment and its DI RNA. The terminal regions retained in DI RNAs included portions of the coding region as well as the UTRs (Davis *et al.*, 1980; Duhaut & Dimmock, 1998, 2000; Duhaut & McCauley, 1996; Hughes *et al.*, 2000; Jennings *et al.*, 1983; Liu & Air, 1993; Nayak & Sivasubramanian, 1983; Nayak *et al.*, 1982; Noble & Dimmock, 1995) (see also Fig. 4). Although this could be explained by constraints on the minimum length of a DI RNA or the mechanism by which they were generated, it is plausible that the coding regions were part of a specific packaging signal. Consistent with this, when reverse genetics was used to systematically vary the length of segment 1-derived DI RNAs, it was found that portions of the 5' coding region increased DI stability during passage (Duhaut & Dimmock, 2000) and were necessary for

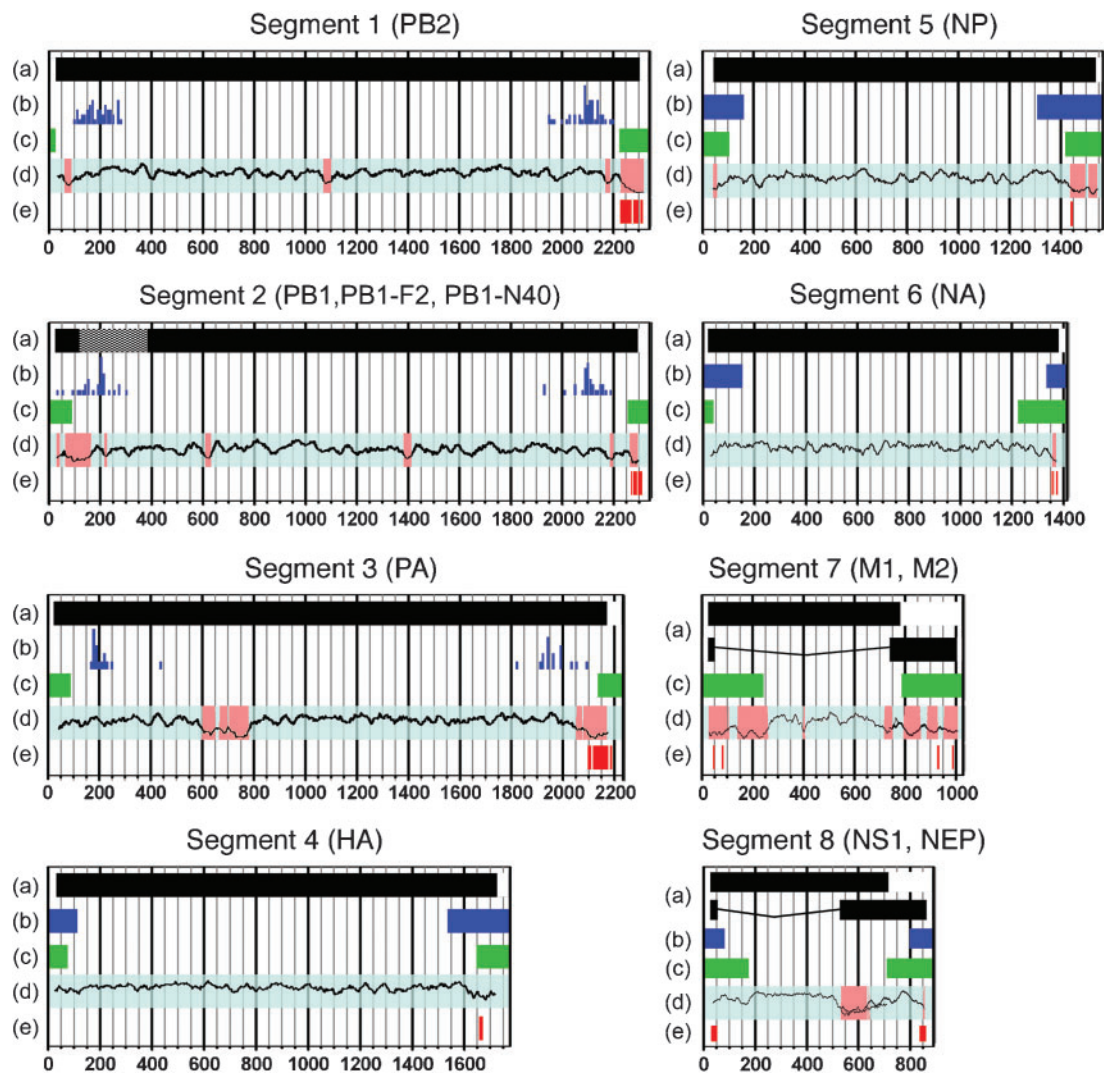


Fig. 4. Location of specific packaging signals in the influenza A virus genome. The eight segments are shown to approximate scale, with nucleotide coordinates (cRNA sense) shown beneath. (a) ORFs are shown in black (or for the overlapping PB1-F2 gene, as cross hatching), with untranslated regions in white. Introns in spliced genes are indicated by angled lines. (b) DI RNAs. For segments 1–3, blue histograms (with a bin size of 10 nt) show the boundary positions of the major internal deletions of a number of sequenced DI RNAs (Duhaut & Dimmock, 1998, 2000; Duhaut & McCauley, 1996; Jennings *et al.*, 1983; Nayak & Sivasubramanian, 1983; Nayak *et al.*, 1982; Noble & Dimmock, 1995). For segments 4–8, where fewer sequences are available, blue bars show the shortest known terminal sequences retained by DI RNAs (Hughes *et al.*, 2000; Jennings *et al.*, 1983). No sequence information is available for segment 7. (c) Packaging of reporter genes. The shortest sequences shown to cause efficient packaging of reporter genes are indicated by green bars (Fujii *et al.*, 2003; Fujii *et al.*, 2005; Liang *et al.*, 2005; Marsh *et al.*, 2007; Ozawa *et al.*, 2007, 2009). The region indicated for segment 7 promotes packaging relatively poorly, and may not contain the complete packaging signal (Muramoto *et al.*, 2006; Ozawa *et al.*, 2009). (d) Sequence conservation. A measure of normalized codon variation averaged over a window of 11 positions is shown as a black line, with lower values indicating greater conservation (Gog *et al.*, 2007). Pink shading indicates areas where the probability of the averaged value of codon variation arising through chance (given the observed codon distributions per site in the segment) is less than 5%. (e) Point mutagenesis. Red lines indicate regions where clusters of mutations or small deletions have substantially reduced segment packaging (Fujii *et al.*, 2005; Gog *et al.*, 2007; Hutchinson *et al.*, 2008, 2009; Liang *et al.*, 2008; Marsh *et al.*, 2007, 2008).

interference with a wild-type virus (Duhaut & Dimmock, 2002). Although not widely accepted at the time, these studies can be viewed as the first to demonstrate a *cis*-acting RNA packaging signal in influenza A virus.

A further study that used reverse genetics to follow up classical virology provided the evidence that has led to widespread acceptance of the specific-packaging model (Fujii *et al.*, 2003). Work in the 1990s showed that NA-

deficient influenza viruses could be selected for in tissue culture using exogenously supplied bacterial neuraminidase and antibodies against the viral NA (Liu & Air, 1993; Yang *et al.*, 1997). These viruses all retained internally deleted versions of segment 6, structurally akin to DI RNAs, strongly suggesting a positive selection pressure to retain the terminal regions of the vRNA (Yang *et al.*, 1997). At the time, it could not be determined whether this reflected a need for the truncated vRNAs and/or the short NA peptides they encoded. However, Fujii *et al.* (2003) utilized reverse genetics to examine this question and found that efficient virus particle formation required the presence of eight different vRNA segments, independent of the presence or absence of their encoded peptide(s). Furthermore, inclusion of the terminal coding regions of segment 6 in addition to the UTRs promoted packaging of a reporter gene far more efficiently than the UTRs alone (Fujii *et al.*, 2003). Similar studies have now been reported for all eight segments (summarized in Fig. 4c), and in every case, the terminal coding regions, comprising sequences unique for each segment, have been found to promote more efficient packaging than the UTRs alone (Dos Santos Afonso *et al.*, 2005; Fujii *et al.*, 2003; Fujii *et al.*, 2005; Gog *et al.*, 2007; Liang *et al.*, 2005, 2008; Marsh *et al.*, 2007; Muramoto *et al.*, 2006; Ozawa *et al.*, 2007, 2009; Watanabe *et al.*, 2003). The observation that virus formation is inefficient in the absence of the terminal regions of all eight vRNAs has also been corroborated and extended by examining the effects of removing other segments (de Wit *et al.*, 2006; Fujii *et al.*, 2009; Gao *et al.*, 2008; Gao & Palese, 2009; Marsh *et al.*, 2007).

Taken as a whole, data gained from analysing reassortant viruses, DI RNAs, and most tellingly, the fact that reverse genetics can be used to map unique sequences in all eight segments which promote incorporation of the vRNA into virus particles, provides compelling evidence in favour of a segment-specific packaging mechanism and against a purely random-packaging model. Further supporting evidence comes from electron microscopy (EM) of virus particles. The random-packaging model requires incorporation of more than eight segments per virion to be compatible with measured values for particle:p.f.u. ratios. Most versions of this model have suggested fairly conservative levels of overpackaging: typically 10–12 segments, the minimum required to produce between 10 and 20% of particles with a complete genome (Fig. 3) (Bancroft & Parslow, 2002; Enami *et al.*, 1991; Lamb & Choppin, 1983; Luo *et al.*, 1992). Although this is minimally compatible with estimates for influenza A virus particle:p.f.u. ratios, these conservative models thereby attribute the high proportion of apparently non-infectious influenza virions solely to defective packaging of the genome. Non-segmented negative-sense viruses, such as Newcastle disease virus and mumps virus, have similar particle:infectivity ratios to influenza viruses (Isaacs & Donald, 1955; Kingsbury, 1970), so these proposals require every other stage of influenza virus assembly and infection

to be disproportionately robust in comparison. To take just one example arguing against this, it has been estimated that in one particular system, around two-thirds of influenza virions were intrinsically defective for membrane fusion (Lakadamyali *et al.*, 2003). Variability in the cell population is also likely to contribute to lowering the apparent infectivity of a virus preparation (Snijder *et al.*, 2009). In theory, the virus could package even higher numbers of segments, thereby increasing the chance of obtaining a full genome and allowing a greater proportion of non-infectious particles to be attributed to other defects. However, the mathematics of random incorporation of eight segments dictate that to obtain around 50% viable genomes, 20 segments must be packaged, and that as many as 38 are required to produce a 95% success rate (Fig. 3). Not only are such levels of overpackaging incompatible with observed viral genetics, as discussed above, but also routine polyploidy to even a limited degree is inconsistent with EM imaging of virus particles. Early studies that examined negatively stained virus sections revealed electron-dense interior structures, sometimes with apparent organization suggesting bundles of aligned RNPs (Apostolov & Flewett, 1969; Bachi *et al.*, 1969; Birch-Andersen & Paucker, 1959; Compans & Dimmock, 1969; Morgan *et al.*, 1956) that in cross section could be seen to be organized into a '7+1' array of seven particles surrounding a central member (Oxford & Hockley, 1987). A recent analysis confirmed that the interior density of virions did indeed represent RNPs and moreover, showed that many particles from a variety of influenza virus strains contained eight RNPs organized into this characteristic 7+1 array (Noda *et al.*, 2006). An example of this virion morphology is shown in Fig. 2(c) and a representation is shown in Fig. 5. Application of more specialized EM imaging techniques to influenza viruses has provided data that are, at least in part, consistent with this arrangement; although not all particles were seen to contain organized bundles of RNPs, no evidence for routine incorporation of more than eight RNPs has been presented (Harris *et al.*, 2006; Yamaguchi *et al.*, 2008).

Thus, overall, a large body of data contradict the purely random-packaging model, leaving the specific model as the option that better fits observation. The favoured current model is therefore that the predominant route of viral assembly involves the packaging of eight RNPs including one copy of each of the segments.

Defining segment-specific packaging signals

Both the random and segment-specific packaging models require that segments possess packaging signals. In the case of the random model, all that is required is a means of distinguishing viral genomic RNA from non-genomic and cellular RNA, whereas the specific model requires this plus a means of distinguishing individual vRNA segments. For influenza A virus, it appears that the two processes are achieved through adjacent but largely separate *cis*-acting

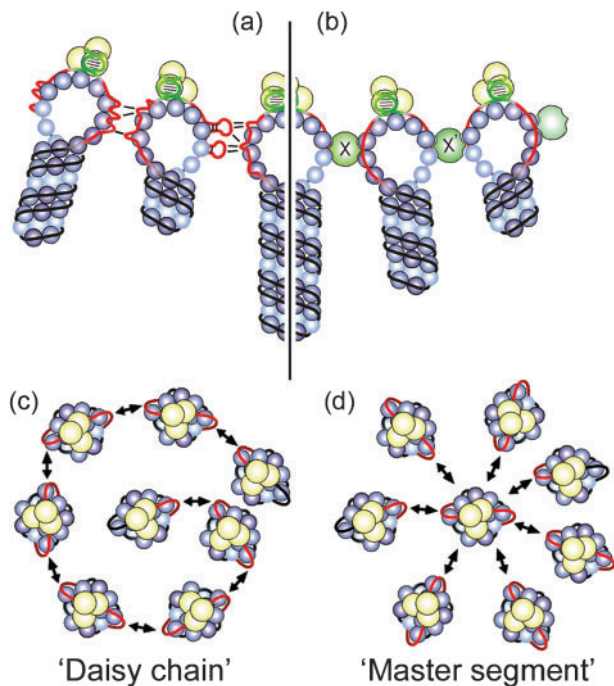


Fig. 5. Models of possible selective packaging methods for influenza A virus. It is proposed that the selective packaging of the segments is brought about by the assembly of a non-covalently linked higher-order genome complex, containing each of the eight vRNAs. The formation of this complex is mediated by specific interactions between the packaging signals of the segments, either (a) by direct RNA–RNA interactions between the packaging signals, possibly with the involvement of short secondary structures or (b) by as-yet-unidentified protein factors (X, X' etc). (c, d) Possible organizations of the 7 + 1 genome complex utilizing the minimum possible number of between-segment interactions (arrows). A greater number of nearest-neighbour interactions between segments is possible. RNPs are drawn as in Fig. 1(d).

RNA elements present in all vRNAs. Genomic RNA is distinguished from non-genomic by the presence of the conserved terminal promoter sequences located at the 5' and 3' end of all segments (Fig. 1, depicted in green). These sequences are partially base-paired in RNPs to form a panhandle or corkscrew structure (Fig. 1c, d) although the 5'-arm contains the primary sequence-specific motif recognized by the viral RNA polymerase (Neumann *et al.*, 2004). Without a functionally intact terminal panhandle structure, an RNA will not be packaged into an RNP and thus will not be a substrate for packaging into virions, irrespective of the presence or absence of a specific packaging signal (Luytjes *et al.*, 1989; Neumann & Hobom, 1995). The partial complementarity of the 5'- and 3'-termini means that a similar but not identical panhandle structure is present in the plus-sense cRNA replicative intermediates from which progeny vRNAs are transcribed (Neumann *et al.*, 2004). In the context of a synthetic vRNA lacking an influenza gene and therefore a

specific packaging signal, the unpaired A10 residue in the 5'-arm of vRNA is apparently crucial for differentiating vRNA from cRNA, operating to prevent packaging of the latter by not supporting its nuclear export (Tchatalbachev *et al.*, 2001).

As discussed above, segment-specific packaging signals are found in unique regions adjacent to the panhandle of each segment, including the UTRs and coding regions. Sequences contributing to these specific packaging signals have been identified by various means: (i) the structure of DI RNAs, (ii) the flanking sequences required to efficiently package reporter genes, (iii) sequence conservation and (iv) the effect of point mutations on packaging.

Naturally occurring DI RNAs are predominantly derived from the three largest segments (Davis *et al.*, 1980; Davis & Nayak, 1979; Duhaut & Dimmock, 1998; Jennings *et al.*, 1983). Examples from every segment have been identified, but as yet, no sequence data have been reported for a segment 7 DI RNA (Davis *et al.*, 1980; Davis & Nayak, 1979; Duhaut & Dimmock, 1998, 2000; Duhaut & McCauley, 1996; Hughes *et al.*, 2000; Jennings *et al.*, 1983; Liu & Air, 1993; Nayak & Sivasubramanian, 1983; Nayak *et al.*, 1982; Noble & Dimmock, 1995). The majority of DI RNAs studied have a single major internal deletion (Jennings *et al.*, 1983; Nayak *et al.*, 1982; Winter *et al.*, 1981) and retain the terminal UTRs of their parent segment along with a variable amount of coding sequence (Davis *et al.*, 1980; Davis & Nayak, 1979; Moss & Brownlee, 1981; Nakajima *et al.*, 1979). The mechanism of DI RNA generation is not known, but it is likely that the size and location of deletions is determined by several factors, including the juxtaposition of non-contiguous sequences brought about by the coiled path of the vRNA in the RNP (Jennings *et al.*, 1983). In addition, for a DI RNA to be selected for and reach detectable levels it must out-compete other segments (most importantly its parent segment) during replication and at the point of genome packaging. It can therefore be assumed that a DI RNA will contain signals sufficient for both processes, while being sufficiently smaller than the parent segment to have a significant replicative advantage. In general, this seems to equate to retaining between 100 and 300 nt of sequence from each end of the segment, but wide variation is evident (Duhaut & Dimmock, 1998, 2000; Jennings *et al.*, 1983; Nayak & Sivasubramanian, 1983; Nayak *et al.*, 1982; Noble & Dimmock, 1995). Noting the regions incorporated in DI RNAs thus provides a first approximation of the regions required for segment packaging (Fig. 4b).

Further evidence for the location of specific packaging signals comes from applying reverse genetics to the DI principle. Artificial segments, usually carrying an easily assayable reporter gene replacing the majority of the influenza ORF, can readily be expressed in cells and potentially packaged into a superinfecting influenza A virus (Luytjes *et al.*, 1989; Neumann *et al.*, 1994). Infection of a second set of cells then allows a quantitative measure of the

efficiency with which the segment is acquired by virions. By varying the amount of flanking sequence, regions of segments promoting efficient packaging have been mapped (Dos Santos Afonso *et al.*, 2005; Duhaut & Dimmock, 2000; Enami *et al.*, 1991; Fujii *et al.*, 2003; Fujii *et al.*, 2005; Liang *et al.*, 2005; Luytjes *et al.*, 1989; Marsh *et al.*, 2007; Muramoto *et al.*, 2006; Ozawa *et al.*, 2007, 2009; Watanabe *et al.*, 2003). There are certain caveats for this approach. Depending on the experimental setup, detection of reporter gene expression in the second set of cells may depend on the ability of the infecting virus to replicate its genome. This presupposes the presence of the four segments encoding the viral polymerase and NP (Bancroft & Parslow, 2002) and, as discussed below, disruption of the packaging of one segment can affect the incorporation of others. It is also important to control for adventitious effects of the mutations on synthesis and/or stability of the mutant vRNA molecule or its mRNA. Although the core promoter sequences for vRNA synthesis and encapsidation reside in the conserved terminal regions of the segments (Neumann *et al.*, 2004), instances have been noted where alteration of more distal sequences within the unique regions of a segment have affected its accumulation and/or transcription (Bergmann & Muster, 1996; Hutchinson *et al.*, 2008; Zheng *et al.*, 1996). Also, depending on the experimental design, the reporter construct may or may not be competing for packaging with the genomic segment from which it was derived, while requirements for packaging may differ for single-cycle and multi-cycle growth. Factors such as these (as well as the use of different virus strains) may explain differences in outcomes when different laboratories have attempted to map signals on the same segment (e.g. Liang *et al.*, 2005; Marsh *et al.*, 2007; Muramoto *et al.*, 2006; Watanabe *et al.*, 2003). It is also not known to what extent the length of a packagable vRNA is limited by the maximum total size of the segment, or by the stoichiometry of nucleotides to NP monomers in the RNP, though neither has been shown yet to have an obvious effect on packaging (Dos Santos Afonso *et al.*, 2005; Marsh *et al.*, 2007).

Caveats notwithstanding, this approach has been used successfully to map packaging elements in all eight segments (Dos Santos Afonso *et al.*, 2005; Fujii *et al.*, 2003; Fujii *et al.*, 2005; Liang *et al.*, 2005; Marsh *et al.*, 2007; Muramoto *et al.*, 2006; Ozawa *et al.*, 2007, 2009; Watanabe *et al.*, 2003). In all cases, segments were found to have a bipartite specific packaging signal that involved extended sequences at both ends of the vRNA (Fig. 4c), in broad agreement with the inferences drawn from the study of DI RNA structure. For most segments, the regions defined as containing the minimal specific packaging signals by deliberate deletion mutagenesis tend to be slightly smaller than those contained in the smallest reported DI RNA (Fig. 4b, c). To some extent, this apparent difference may be more artificial than real. In most cases, incremental deletion of flanking sequences reduced the packaging efficiency of the reporter vRNA gradually rather than

revealing a sharp cut-off point between functional and non-functional. Thus, as well as making the definition of a minimal 'efficient' packaging signal somewhat arbitrary, this also suggests that complete packaging signals are composed of numerous contributing parts rather than a single discrete sequence element. Although both ends of the segment contribute to the packaging signal in all segments, the 5' end was shown to be more important for segments 1–3 (Liang *et al.*, 2005; Muramoto *et al.*, 2006), whereas the 3' end contributed more for segments 6 and 8 (Fujii *et al.*, 2003; Fujii *et al.*, 2005).

Examination of sequence conservation has been further used to define the location of specific packaging signals. As *cis*-acting RNA sequences, they will be subject to functional selection and should therefore be conserved. Since packaging signals overlap with coding regions, it is difficult to tease out whether conservation of a particular nucleotide sequence arose from selection for protein and/or for RNA function. The large numbers of influenza sequences available makes it possible to address this by considering the levels of synonymous variation within the coding regions (Gog *et al.*, 2007; Liang *et al.*, 2008; Marsh *et al.*, 2008). These analyses identified codons with very low levels of synonymous variation at positions where variation should have been possible, consistent with functional conservation of the primary RNA sequence beyond that required for its coding capacity. A large-scale analysis that considered all eight segments of the genome (Gog *et al.*, 2007) found that these conserved codons tended to cluster in the terminal regions of each segment, in the same areas implicated by experimental or DI RNA structure as containing packaging signals (Fig. 4d, pink shading). Although the conserved codons formed clusters, these were not necessarily totally contiguous and the degree of conservation tended to decrease with increasing distance from the termini (Gog *et al.*, 2007), consistent with the 'fuzzy' boundaries of packaging elements defined by deletion mapping. Other statistically significant clusters of low variability codons in regions outside of the terminal packaging signals were also identified, some attributable to features such as overlapping ORFs or splice sites, others in areas with no known constraint (Fig. 4d; Gog *et al.*, 2007).

Further information on the identity of the packaging signals comes from point mutagenesis of regions thought to be part of the signal. A major advantage of this approach is that it can be applied to otherwise wild-type virus (Fujii *et al.*, 2005; Hutchinson *et al.*, 2008, 2009; Liang *et al.*, 2008; Marsh *et al.*, 2007, 2008; Ozawa *et al.*, 2009) as well as to constructs containing reporter genes (Fujii *et al.*, 2005; Fujii *et al.*, 2009; Gog *et al.*, 2007; Liang *et al.*, 2008). For all segments, point mutagenesis has identified nucleotides, individually or in small clusters, whose alteration causes significant reductions in packaging of the altered segment as well as (where examined) reductions in the titre of replicated virus (Fujii *et al.*, 2005; Fujii *et al.*, 2009; Gog *et al.*, 2007; Hutchinson *et al.*, 2008, 2009; Liang *et al.*, 2008; Marsh *et al.*, 2007, 2008) (Fig. 4e). The finding that

alteration of a small number of nucleotides (even as few as one) can have a profound effect on segment incorporation is perhaps surprising, given the apparently large size of the signals. However, in many cases, other mutations within areas predicted to be part of the packaging signal had no apparent effect on its function (Fujii *et al.*, 2005; Gog *et al.*, 2007; Hutchinson *et al.*, 2008, 2009; Liang *et al.*, 2008; Marsh *et al.*, 2007, 2008). This may, in part, reflect the discontinuous nature of the signals, as suggested by analysis of sequence conservation (Gog *et al.*, 2007). However, in some instances, mutation of conserved nucleotides had no phenotypic effect and indeed, studies have found that a variety of randomly selected sequences within the 3'-end of the segment 8 or the 5'-end of the segment 7 packaging signals supported substantial levels of segment packaging despite having little homology to the wild-type sequence other than perhaps a tendency to being U-rich (Fujii *et al.*, 2009; Ozawa *et al.*, 2009). Large or small deletions with the region of the signal, however, do not seem to be well tolerated, leading to the suggestion that length is as important as sequence (Liang *et al.*, 2008; Ozawa *et al.*, 2009). This apparent functional plasticity of the packaging signals is puzzling and awaits explanation.

Thus, taking all the available threads of evidence together, the conclusion is that all segments contain a generic bipartite packaging signal contained within their 5'- and 3'-terminal 12–13 nt that (through its operation as the promoter for the viral RNA polymerase) serves to identify them as vRNAs and substrates for encapsidation into RNPs (Fig. 1a, green boxes). In addition, each of the eight segments also contains specific packaging signals adjacent to these conserved promoter sequences (at 5'- and 3'-ends) that span the UTRs and extend some distance into the coding regions. Within these sequences, the functionally significant elements are probably discontinuous and, rather than ending at a discrete boundary, tend to wane in importance with increasing distance from the termini (Fig. 1a, red dashes). This organization of packaging signals bears comparison with the dsRNA cystoviruses such as bacteriophage $\Phi 6$, where the three segments contain a conserved 18 nt element at their 5'-ends followed by unique *pac* signals of around 200 nt each (Mindich, 2004). In contrast with influenza A virus, the conserved and unique signals of $\Phi 6$ are separated by 50 nt, are located only at the 5'-end of the segment and the *pac* sequences are contained entirely within the segment UTRs. The discontinuity of the influenza packaging signals can perhaps be explained by encapsidation of the vRNAs into RNPs, even though the vRNAs are still partially accessible to RNases and other modifying agents. A variety of approaches estimate a stoichiometry of around 24 nt per unit of NP protein in the RNP (Portela & Digard, 2002), which, given the physical sizes of the components, implies a degree of looping of the RNA (Jennings *et al.*, 1983). Taken together with the existence of a likely RNA-binding cleft in the NP structure (Ng *et al.*, 2008; Ye *et al.*, 2006), it is therefore unlikely that all regions of the encapsidated vRNA

molecule will be equally accessible. It is also worthwhile considering the 'tertiary' structure of the RNP. EM imaging studies show that the rod-like structure of the RNP is in fact helical, often opening out (when examined in physiological salt conditions) into a terminal loop (Fig. 1b). Cartoon models of this often place the viral polymerase at the opposite end of the RNP (Fig. 1c). However, this organization is uncertain as negative stain techniques have not permitted unambiguous visualization of the viral polymerase on authentic RNPs. Although an immuno-gold labelling study showed that the polymerase was located at one end of the RNP, terminal loops in the backbone of the RNP were not seen in this case (Murti *et al.*, 1988). Indeed, one study presenting a detailed form of the RNP model considered it more likely that the polymerase was located at the open end (Fig. 1d), based on a possible mechanism for the generation of DI RNAs (Jennings *et al.*, 1983). In light of the extended and perhaps flexible nature of the packaging signals, this latter organization is plausible. Thus, although the position of the packaging signals in the primary nucleotide sequence is increasingly clearly delineated, their position in the 3D structure of the RNP remains ambiguous. Ongoing work characterizing the structure of intact RNPs (e.g. Coloma *et al.*, 2009) may well rectify this gap in the future.

Mechanisms of segment selection

Although there is now compelling evidence for segment-specific packaging in influenza A virus, the mechanism of this process remains unclear. The best characterized mechanism of specific packaging in a segmented virus is that of the bacteriophage $\Phi 6$. Here, three segments of plus-strand RNA (later converted into dsRNA) are sequentially recruited into a preformed capsid through a series of conformational changes driven by interactions with the segment packaging signals that expose and then mask sequence/structure-specific RNA binding sites while simultaneously expanding the capsid to allow incorporation of more RNA (Huiskonen *et al.*, 2006; Mindich, 2004). This model seems unlikely to be applicable to influenza virus for several reasons. During influenza infections, preformed capsids are not seen in the cytoplasm, with the virus instead assembling at the point of budding through the plasma membrane (Cheung & Poon, 2007). In addition, the pleiomorphy of influenza virus particles seems intrinsically ill-suited to a mechanism founded on ordered conformational changes of the capsid that regulate specific RNA-binding sites in the virion interior. Indeed, it has even been proposed that the viral glycoproteins alone can drive particle assembly and that not all influenza virions even contain a matrix protein layer (Chen *et al.*, 2007; Harris *et al.*, 2006). Instead, as EM analyses suggest that budding influenza A virions incorporate a parallel 7+1 array of eight RNPs (Harris *et al.*, 2006; Noda *et al.*, 2006; Oxford & Hockley, 1987; Yamaguchi *et al.*, 2008), it has been plausibly hypothesized that this represents a specific multi-segmental 'genome complex' containing one copy of

each of the eight vRNAs whose assembly confers specificity on packaging (Heggeness *et al.*, 1982; Noda *et al.*, 2006).

The way in which this putative viral genome complex assembles remains a matter of speculation (as does the existence of the complex itself) but it presumably would involve recognition of the various specific packaging signals present on the eight vRNAs. Theoretically, this might involve the action of sequence-specific RNA-binding proteins (Fig. 5b), of either viral or cellular origin. However, the formation of a specific complex of eight RNPs requires a minimum of seven inter-RNP interactions (Fig. 5c, d) and no evidence has been found so far for even a single protein able to recognize an influenza virus specific packaging signal, let alone the 14 or more separate RNA elements required to bring together eight RNPs. This is not to say that such a mechanism is impossible; although not strictly analogous, the structures of ribosomal subunits illustrate how megadalton ribonucleoprotein assemblies can be constructed through multiple individual proteins binding to non-contiguous RNA elements that are primarily recognized as structures rather than as nucleotide sequences (Brodersen *et al.*, 2002; Klein *et al.*, 2004). Some cellular proteins have been shown to be incorporated into released influenza virus particles (Shaw *et al.*, 2008); further low abundance components (perhaps only present at one copy per virion) could still remain to be discovered. A more parsimonious (but not necessarily exclusive) hypothesis is that the RNA signals themselves function as the recognition surfaces via direct RNA–RNA interactions (Fig. 5a) (Fujii *et al.*, 2003; Kingsbury & Webster, 1969). This hypothesis has viral precedents in the formation and packaging of homodimers of the retroviral genome (Greatorex, 2004), as well as (in a heterodimeric context) during RNA synthesis in red clover necrotic mosaic virus (Sit *et al.*, 1998). It is also generally consistent with the structural diversity of RNA, even when in the form of an RNP (Brodersen *et al.*, 2002; Holbrook, 2005; Klein *et al.*, 2004). However, attempts to identify plausible interacting motifs by *in silico* analysis of vRNA sequences have so far been unsuccessful, as might be expected given the complex way in which vRNA coils around NP to form the RNP, the discontinuous nature of the signals and that the hypothesized RNA–RNA interactions need not be limited to Watson–Crick base-pairs. Thus at present, our understanding of the mechanism by which specific genome packaging in influenza A virus is achieved is at the stage of testing plausible hypotheses. Nevertheless, some prior evidence, albeit equivocal, has a bearing on these proposals.

A key component of the currently favoured hypothesis is the formation of a specific genome complex of the eight RNPs. Although EM imaging indicates that some virions contain a 7+1 array of RNPs, this is not proof of a complex with a specific composition or even that the RNPs are actually associated; the apparent order could potentially result from the geometry of packing eight rods into a spherical particle and/or the mechanism of the budding process itself. Images showing the 7+1 array appear to be

less common when released particles are examined, in comparison to sections across budding virions (Booy *et al.*, 1985; Harris *et al.*, 2006; Noda *et al.*, 2006; Oxford & Hockley, 1987; Yamaguchi *et al.*, 2008; Yazaki *et al.*, 1984). This may well be because released particles are free to rotate, reducing the chance of seeing RNPs in cross section, whereas cell-associated virions maintain the directionality of budding. However, it could also indicate that the RNPs are not bound together in the particle and redistribute once freed from an initial constraint imposed by the budding process; a hypothesis supported by a tomographic study in which ordered arrays of RNPs were found more frequently in elongated virions than in larger spherical particles (Harris *et al.*, 2006). As a counter-argument against general disorder in virion contents, magnetic birefringence analysis of released virions indicated that the interior components were probably ordered to some degree (Torbet, 1983). Similarly, EM studies have also provided striking images of long helical structures released from partially disrupted virions, proposed to be RNP (Almeida & Brand, 1975; Murti *et al.*, 1980). These helical structures were often too long to represent individual RNPs and the authors speculated they might represent non-covalently linked complexes formed as part of the genomic packaging process. However, again in counter-argument, a subsequent study disputed this, proposing instead that the structure were helices of M1 (Ruigrok *et al.*, 1989). Furthermore, sedimentation analysis of material released from purified virus particles has so far failed to provide evidence for the existence of a packaging complex, with RNPs instead migrating at the approximate positions expected for their individual molecular masses (Compans *et al.*, 1972; Duesberg, 1969; Krug, 1971; Pons *et al.*, 1969). It should be noted, however, that these early experiments were all performed in buffer conditions that would not necessarily maintain structured RNA–RNA interactions. Therefore, overall, microscopic and biochemical evidence for the existence of a specific influenza A genome complex is ambivalent; further work in this area is required.

A further key prediction of the current hypothesis for how specific packaging operates is that there is a specific suite of inter-segment interactions. Within a 7+1 array of rod-like structures all containing their packaging signals at one end, not every RNP is likely to interact with all others; instead, various specific linkage schemes can be envisaged. In a ‘daisy chain’ network, most segments would interact with two others while the end ones only interact with one (Fig. 5c). Alternatively, a ‘master’ segment (perhaps in the centre of the array) that interacts with many or all other segments can be envisaged (Fig. 5d). More complex ‘hybrid’ schemes with additional interactions between adjacent RNPs are also plausible, but in any case, the model predicts that deleterious mutations in the packaging signal of a given segment have the possibility of affecting the incorporation of adjacent, interacting segments. Initial evidence consistent with this hypothesis came from studying the incorporation of reporter vRNAs with

flanking sequences from the three largest segments. In a systematic examination of all eight segments, the presence of the coding regions from segment 1 (and in particular the 5'-end) was found to be especially important not only for packaging of the recombinant vRNA but also to the efficiency with which infectious virus-like particles (VLPs) were formed (Muramoto *et al.*, 2006). Detection of the VLPs relied on their incorporating either segment 4 (HA) or 5 (NP) so this effect could have arisen from knock-on effects on the incorporation of other segments and/or the actual budding process itself. Subsequent work has confirmed that both mechanisms potentially play a part. Several studies have since examined the phenotype of packaging mutants in the context of viable virus (rather than via artificial reporter constructs) and directly examined the relative amounts of segments incorporated per virion. Consistent with the previous VLP study by Muramoto *et al.* (2006), mutations to segment 1 were found to reduce packaging of not only itself but also all other segments (Marsh *et al.*, 2008). Similarly, mutations in the segment 7 packaging signals reduced incorporation of each of the eight segments by an approximately equal extent, such that the majority of virions contained an incomplete genome (Hutchinson *et al.*, 2008). Lesions in segment 7 also reduced virus budding (Hutchinson *et al.*, 2008), consistent with the prior data inferred from examining VLPs and viruses with less than eight segments (de Wit *et al.*, 2006; Fujii *et al.*, 2005; Fujii *et al.*, 2009; Gao *et al.*, 2008; Marsh *et al.*, 2007; Ozawa *et al.*, 2009). More specific secondary effects on segment incorporation after mutation of a single packaging signal have also been seen. Mutations to segment 4 that reduced its packaging also reduced incorporation of other segments: notably 2, but also (to lesser extents) 3, 5 and 6 (Marsh *et al.*, 2007). Similarly, mutations in the segment 3 packaging signal affected incorporation of segments 1 and 5, in some instances to the point where they showed a greater packaging deficit than the mutated segment 3 (Marsh *et al.*, 2008). Conversely, a packaging signal mutation in segment 5 reduced incorporation of segment 3 (Hutchinson *et al.*, 2009).

Thus, taken as a whole, evidence for *trans*-acting effects of packaging mutations on other segments are consistent with an important prediction of the 'genome complex' hypothesis; that packaging of individual segments is not independent but instead is interlinked. It is tempting to try and deduce likely patterns of inter-segment interaction from the available data. For instance, comparison of experiments suggests a reciprocal packaging interaction between segments 3 and 5 (Hutchinson *et al.*, 2009; Marsh *et al.*, 2008). However, other data suggest complexities arguing against such straightforward interpretations. For instance, a single nucleotide mutation to codon 745 of PB2 in the 5'-end of segment 1 vRNA reduced the relative packaging of the segment by nearly tenfold and also decreased incorporation of segments 2 and 5 by over fivefold. However, mutation of the adjacent codon 744

only significantly affected packaging of segment 1 itself (Marsh *et al.*, 2008), suggesting complex position-dependent effects of packaging signal mutations that may confound simple interpretation. Thus, while further experimentation of this sort may elucidate a proposed web of inter-segment interactions, other approaches are needed. At present, it is probably safe to conclude that the data do not support the hypothesis of a simple 'daisy chain' to assemble the genome complex (Fig. 5c), but instead suggest more complex interaction patterns. Segments 1 and 7 seem to be potential candidates for 'master segments' (Fig. 5d), based on the pleiotropic effects mutation of their packaging signals have on virus budding and incorporation of other vRNAs (Hutchinson *et al.*, 2008; Marsh *et al.*, 2008; Muramoto *et al.*, 2006; Ozawa *et al.*, 2009).

Packaging signals and influenza virus evolution

Genome segmentation clearly plays a major role in influenza virus evolution, both within a single host species and in facilitating jumps in host range (Dugan *et al.*, 2008; Garten *et al.*, 2009; Hatchette *et al.*, 2004; Kuiken *et al.*, 2006; Nelson & Holmes, 2007; Smith *et al.*, 2009). As discussed above, although the strategy of a divided genomic structure confers fitness benefits, it comes at the direct cost of increasing the complexity of virus assembly. It is clear that the virus has evolved a solution to this problem, although the exact mechanism by which specificity in packaging is achieved is still uncertain. Does the nature of the solution impose constraints on virus evolution? In one sense the answer is an unequivocal 'yes'; the footprints of conservation left by the specific packaging signals on the viral terminal coding regions are clear to see (Gog *et al.*, 2007; Liang *et al.*, 2008; Marsh *et al.*, 2008). This may be exploitable for intervention strategies. For instance, it has been suggested that packaging signals are good targets for oligonucleotide-based inhibition (Giannecchini *et al.*, 2009). In a similar vein, the M2 ectodomain has been proposed as a candidate immunogen for a 'universal' influenza vaccine, for which antigenic escape mutants will be less likely to develop because the M2e coding region is either congruent with or overlaps that of M1 (Saelens, 2008). We speculate that the fact that the first nine codons of the M2e coding region also overlap the segment 7 packaging signal (Gog *et al.*, 2007; Hutchinson *et al.*, 2008; Ozawa *et al.*, 2009) will further constrain the development of escape mutants. Better understanding of influenza virus genome packaging has also facilitated attempts to use the virus as a gene delivery vector (Gao *et al.*, 2008; Shinya *et al.*, 2004) as well as providing an ingenious approach to improving engineered virus biosafety through reducing the probability of successful reassortment with 'wild' viruses (Gao & Palese, 2009).

We also hypothesize that the evolutionary solution the virus has found to the packaging problem has broader implications for its biology. The fact that the same stretches

of RNA perform multiple functions in addition to acting as packaging signals [coding for proteins, as well as *cis*-acting functions such as promoter or splice signals (Gog *et al.*, 2007; Hutchinson *et al.*, 2008)] creates potentially conflicting requirements that reduce the chances of finding an optimal solution to the problems of genome packaging. Although the evidence for a specific packaging method is overwhelming, the mechanism is clearly not perfect. The existence of nine segment viruses (Enami *et al.*, 1991; Laver & Downie, 1976; Nakajima & Sugiura, 1977; Scholtissek *et al.*, 1978) and the low levels of 'background' packaging of reporter vRNAs lacking specific packaging signals seen by several laboratories and for all segments (Bancroft & Parslow, 2002; Dos Santos Afonso *et al.*, 2005; Fujii *et al.*, 2003; Luytjes *et al.*, 1989; Muramoto *et al.*, 2006; Neumann *et al.*, 1994; Tchatalbachev *et al.*, 2001) suggest that there is a measurable degree of imprecision in the mechanism. We are also presented with the apparent paradox, that in the laboratory, small mutations (even a single nucleotide) can significantly disrupt packaging of a particular segment to the point where the virus replicates noticeably more poorly (e.g. Gog *et al.*, 2007; Hutchinson *et al.*, 2008; Marsh *et al.*, 2008) and yet in other circumstances, selection of efficient packaging signals from a pool of randomized sequences reveals no clear consensus sequence (Fujii *et al.*, 2009; Ozawa *et al.*, 2009). Similarly, reassortment in nature seems readily capable of bringing together segments from diverged genetic backgrounds that could, on occasion, include changes to packaging signals (Dugan *et al.*, 2008; Ghedin *et al.*, 2009; Hatchette *et al.*, 2004). This discrepancy lacks a molecular explanation at present, but it provides further evidence that the virus tolerates a degree of imprecision in its packaging mechanism.

Why then, has packaging in influenza A virus not evolved a higher degree of precision? As already discussed, the overlap of packaging signals with open reading frames and *cis*-acting RNA functions may hinder the evolution of a 'perfect' genome packaging solution of the sort achieved by the bacteriophage $\Phi 6$ (Mindich, 2004), as indeed may constraints from other aspects of virus biology such as virion morphology or the mechanism of budding. However (to end on a speculative note), we wonder if an imperfect packing strategy is actually beneficial to virus fitness, by providing flexibility for reassortment. Studies of natural isolates suggest that widespread and continuous reassortment events drive influenza virus evolution (Dugan *et al.*, 2008; Ghedin *et al.*, 2009; Hatchette *et al.*, 2004), and in this respect the ability to package evolutionarily diverged segments would carry benefits. As well as increasing the selective advantage that the rudimentary sexual process of reassortment provides to the virus, this can perhaps be viewed in terms of selection acting on individual segments. Participation of a 'selfish segment' in a selective packaging mechanism would be balanced between the need to be correctly packaged in the context of its current genome and retaining sufficient flexibility to be able to occasionally 'jump ship' through facile reassortment with an evolutionarily

diverged genome. At the level of both the virus and its segments, a mechanism of selective genome packaging that is neither entirely random nor unfailingly rigorous may have proven to be the most successful evolutionary strategy for influenza A virus.

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