Paramyxovirus mRNA editing, the ‘rule of six’ and error catastrophe: a hypothesis

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The order Mononegavirales includes three virus families that replicate in the cytoplasm: the Paramyxoviridae, composed of two subfamilies, the Paramyxovirinae and Pneumovirinae, the Rhabdoviridae and the Filoviridae. These viruses, also called non-segmented negative-strand RNA viruses (NNV), contain five to ten tandemly linked genes, which are separated by conserved junctional sequences that act as mRNA start and poly(A)/stop sites. For the NNV, downstream mRNA synthesis depends on termination of the upstream mRNA, and all NNV RNA-dependent RNA polymerases reiteratively copy ('stutter' on) a short run of template uridylates during transcription to polyadenylate and terminate their mRNAs. The RNA-dependent RNA polymerase of a subset of the NNV, all members of the Paramyxovirinae, also stutter in a very controlled fashion to edit their phosphoprotein gene mRNA, and Ebola virus, a filovirus, carries out a related process on its glycoprotein mRNA. Remarkably, all viruses that edit their phosphoprotein mRNA are also governed by the ‘rule of six’, i.e. their genomes must be of polyhexameric length (6n + 0) to replicate efficiently. Why these two seemingly unrelated processes are so tightly linked in the Paramyxovirinae has been an enigma. This paper will review what is presently known about these two processes that are unique to viruses of this subfamily, and will discuss whether this enigmatic linkage could be due to the phenomenon of RNA virus error catastrophe.

The requirement for polyhexameric genome length

(i) The mostly proteinaceous non-segmented negative-strand RNA virus (NNV) nucleocapsid (NC) template

Helical NNV NCs are assemblies of nucleoprotein (N) and genomic (or antigenomic) RNA, associated along their entire length with N protein monomers packed side-by-side (Fig. 1). For Sendai virus (SeV), each N subunit is associated with precisely 6 nt (Egelman et al., 1989), and for vesicular stomatitis virus (VSV), a rhabdovirus, there are a mean of 9 nt associated with each N subunit (Thomas et al., 1985). These NCs are thus 97 and 85 % protein by weight, respectively. The RNA is tightly associated with N, as the genomic RNA of SeV and VSV NCs are resistant to micrococcal nuclease and RNase A digestion independent of salt concentration, and this structure resists the pressures of CsCl density-gradient centrifugation that would normally act to dissociate RNA from proteins (Lynch & Kolakofsky, 1978). It is necessary to at least partially denature the N protein to remove it from the RNA (e.g. by including 3 M guanidine HCl in CsCl gradients). It is this very stable N:RNA assembly, as opposed to free RNA, that is the template for viral RNA synthesis.

When NCs of the Paramyxovirinae are viewed in the electron microscope (EM), successive turns of the helical assembly packed closely together are observed, giving rise to the herringbone pattern that is characteristic of this subfamily (Fig. 1a, left). NCs of the Rhabdoviridae and Pneumovirinae do not show this pattern, and appear as more flexible coils, with less regular structure. Even the more regular measles (MeV) or SeV NCs are, in fact, very flexible and consist either of a continuum of helical states or of a distinct number of rapidly interchanging helical states with variable helical pitch and number of subunits per turn (Bhella et al., 2004). This variability may be necessary for structural transitions that occur during RNA synthesis. The NCs become less flexible after removal of the C-terminal domain of N by trypsin digestion (Mountcastle et al., 1974; Schoenh et al., 2004) (Fig. 1a, left). Several helical states of the digested NCs could be defined, and one was analysed structurally from cryo-electron micrographs to a resolution of 12 Å (1.2 nm) (Fig. 1a). Remarkably, in spite of the close

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Fig. 1. Paramyxovirinae. (a) NC structure. Electron micrographs of MeV NCs containing intact N proteins, and those with tail-less N protein (trypsin digested), are shown on the left. Note the herringbone pattern characteristic of NCs of the Paramyxovirinae. A high-resolution [11.8 Å (1-18 nm)] model of the tail-less (trypsin-treated) MeV NC (Schoehn et al., 2004) is shown at the top right. The outside and inside surfaces of the left-handed coil are shown separately for clarity and the dotted line in the inside (cut-away) view shows the proposed path of the RNA. A blow-up of 4 N subunits in which their lateral N:N contacts are clear, is shown on the bottom right. Note the solvent-accessible space between the two N:N contacts. Cisplatin staining of the RNA within these NCs suggests that the RNA (dotted line, each dot represents 1 nt) is located near the inside surface of the coil [107 Å (10-7 nm) from the helix axis], near this space, as indicated. In a helix with 13 N subunits/turn containing 13 × 6 nt, the mean distance between nucleotides in the RNA genome would be 4.3 Å (0-43 nm), i.e. the RNA would be fairly extended. In this blown up part of the figure, black dots represent nucleotide positions one and six, whereas white dots represent positions two to five. The tight association of the RNA bases with the N subunits presumably contributes to the structural regularity of these NCs. (b) Model of genomic promoters. A lower-resolution model of slightly more than one turn of the SeV N subunit assembly, based on the original image reconstruction of Egelman et al. (1989), with 13-1 subunits/turn and a pitch (distance between turns) of 5-3 nm. For easy reading, the nucleotides are shown on the outside of the helix. The 3′ end of the genome is denoted by a hydroxyl group, and the N subunits and the 6 nt associated with each subunit are numbered from this end. Only the bases of the 3′ end core promoter (PrE-I plus PrE-II) are shown. The downstream element (PrE-II) of the bipartite promoter for respiroviruses and morbilliviruses is 5′ [nnnnnC]3 in subunits 14, 15 and 16, and 5′ [CGnnnn]3 in subunits 13, 14 and 15 for rubulaviruses. G/Pr, Genomic promoter. For further details, see text.
packing of the coil, all N:N subunit contacts in the MeV assembly are in fact lateral. There are no direct contacts between N subunits on adjacent turns of the helix, and this presumably facilitates the structural transitions of the N:RNA that may be required for RNA synthesis (Schoehn et al., 2004). Naturally occurring rhabdovirus and pneumovirus NCs are too flexible and irregular in the EM for detailed structural analysis. However, rings of rabies N:RNA s that contain N plus short cellular RNAs, such as tRNAs, have been produced in closed form by recombinant means and are highly regular, presumably because of the absence of free ends (Iseni et al., 1998; Schoehn et al., 2001, 2004). A VSV NC ring structure has recently been reconstructed from electron micrographs to a resolution of 18 Å (1.8 nm) (Chen et al., 2002) and the VSV N-monomer shows overall similarity to N in the MeV NC structure.

The 3’ termini of NNV genomes and antigenomes contain cis-acting sequences (promoters) that allow the viral RNA-dependent RNA polymerase (vRdRp) to initiate RNA synthesis. For phosphodiester bond formation to begin at the 3’ end of the genome, the large (L) subunit of vRdRp must interact with the N:RNA such that the initiating NTP (a 1 nt primer) is paired with the 3’ base of the template and the next NTP is paired with the penultimate 3’ base. Promoter recognition and the initiation of RNA synthesis is not a trivial problem for any RNA polymerase. In eukaryotes, for example, a finger domain of general transcription factor IIB is inserted into the RNA polymerase-II active centre during initiation, where it is thought to help position the initiating NTPs (Bushnell et al., 2004). Closer to home, RdRp of plus-strand RNA viruses (hepatitis C virus, HCV) and dsRNA viruses (phi6 and reovirus) contain a C-terminal domain (referred to as an ‘initiation platform’ or ‘priming loop’) that is present in the active site of the molecule (Butcher et al., 2001; Hong et al., 2001; Laurila et al., 2002; Tao et al., 2002). These C-terminal domains are thought to stabilize the initiation complex by interacting with the initiation nucleotides, and they also serve as a physical barrier to prevent the 3’ end of the template from slipping through the active site, thus ensuring terminal initiation of replication (reviewed by van Dijk et al., 2004). The problem of just where to start RNA synthesis is especially acute for NNV, where the template is composed mostly of the N protein. Moreover, if chemical reactivity of the RNA bases within NCs is a guide to the initial vRdRp interaction with its promoter, most of the SeV promoter sequences are initially inaccessible to SeV RdRp (Iseni et al., 2000). For VSV, the bases of the cis-acting sequences in resting VSV NCs are more accessible to the solvent (Iseni et al., 2002). VSV RdRp can then initially contact a more extensive region of the cis-acting promoter sequence that is not occluded by the N subunits.

(ii) Two groups of NNV

Despite their overall similarity as single-subunit (N) assemblies in which the genome RNA is tightly associated with N, VSV and SeV NCs differ in other respects. NNV can be broadly divided into two groups; these are typified by the Paramyxovirinae on the one hand, and rhabdo- and pneumoviruses on the other. Ebola virus (EboV), a filovirus, is an exception and is treated below.

Genome length. The precise length of rhabdo/pneumovirus genomes does not appear to be important; they are not subject to a hexamer (or any integer) rule (Pattnaik et al., 1995; Samal & Collins, 1996). For the Paramyxovirinae, only genomes of integer hexamer length replicate efficiently and are found naturally (Calain & Roux, 1993; Kolakofsky et al., 1998).

mRNA editing. Phosphoprotein (P) mRNAs of the Paramyxovirinae are co-transcriptionally edited in response to a cis-acting sequence, by pseudo-templated transcription (Jacques & Kolakofsky, 1991; Thomas et al., 1988). Rhabdo/pneumovirus RdRp, like all NNV, stutter to form poly(A) tails, but they do not edit their mRNAs.

Replication promoters. For the Paramyxovirinae, the genomic and antigenomic promoters (G/Pr and AG/Pr) are bipartite in nature (Murphy et al., 1998; Pelet et al., 1996; Tapparel et al., 1998) (Fig. 1b). There is both an end element (PrE-I or CR-I) comprising approximately the first 30 nt of the leader/trailer regions (in which the first 12 nt are conserved), and a downstream element within the 3’ UTR of the N gene or the 5’ UTR of the L gene (PrE-II or CR-II) (Fig. 1b). The downstream element is a simple but phased sequence repeat 3’ [C[n2n3n4n5n6], for respiro- and morbilliviruses (Hoffman & Banerjee, 2000; Tapparel et al., 1998) and 3’ [n2n3n4n5n6G2C2], for rubulaviruses (Murphy & Parks, 1999), that always includes the fourteenth and fifteenth hexamers that are adjacent to the first two N subunits in helical NCs with 13 subunits per turn. G/Pr is thus spread across two helical turns of the NC. This contiguous surface of the template (N subunits 1, 2, 14 and 15) may serve as a recognition site for vRdRp initiation at the template 3’ end, as both the hexamer phase of PrE-II and its spacing from PrE-I are critical under normal conditions (Murphy et al., 1998; Tapparel et al., 1997).

In contrast, rhabdo/pneumovirus replication promoters are more compact, and the essential sequences are contained entirely within the leader/trailer regions (Collins et al., 1991; Fears et al., 2000; Pattnaik et al., 1995).

Location of G/Pr and AG/Pr. Only rhabdo/pneumo G/Pr or AG/Pr that are at (or near) the 3’ end of the genome are active in reverse genetic systems (Collins et al., 1991; Pattnaik et al., 1992). The 3’ end of the genome itself appears to be an essential feature of these replication promoters. For the Paramyxovirinae, vRdRp can initiate at an internal AG/Pr hundreds of nucleotides away from the 3’ end of the genome, but only if AG/Pr is in the bona fide hexamer phase. The 3’ end of the genome itself is not an
essential part of this replication promoter, but its hexamer phase is critical (Vulliemoz & Roux, 2001).

**RNA base reactivity.** The Watson–Crick (W-C) positions of the bases in VSV NCs are, in general, almost as accessible to chemical attack as they are in deproteinized RNA. There is some variation of reactivity for specific nucleotides, but this variation does not fall into any phase pattern (Iseni et al., 2000). The W-C positions of the bases in SeV NCs are, in general, protected against chemical attack. However, cytidines in hexamer positions one and six do react strongly with dimethyl sulphate (DMS), which is a highly reactive reagent that specifically modifies the N3 position of cytidine and N1 of adenosine, whereas cytidines at other hexamer positions are much less reactive (Iseni et al., 2002). Adenosines, in contrast, are very poorly reactive with DMS regardless of their hexamer position, whereas most adenosines in resting VSV NCs are highly reactive. Inspection of the MeV NC structure shows a space between the two regions of lateral N:N contact (Fig. 1a). Since the RNA is thought to be located in this area, this space may allow access of the cytidines in N-subunit positions one and six to the solvent. Note also that the conserved cytidines in the PrE-II elements are in a solvent accessible position.

**(ii) Possible N protein sliding on the RNA genome**

The N subunits of VSV N:RNA do not protect the RNA bases from chemical attack, but they do protect the ribose-Po backbone from free radical attack (Iseni et al., 2000). The N subunits of VSV (and probably all NNV) NCs thus interact tightly with the RNA backbone. However, if all N:N subunit contacts in NNV NCs are in fact lateral, this not only facilitates the structural transitions of the N:RNA required for RNA synthesis, it also removes a major obstacle to lateral N movement on the RNA. For the *Paramyxovirinae*, we expect that N sliding along the ribose-Po backbone would still be prevented by the close interactions of N and the RNA bases. However, when N subunits interact less tightly with the RNA bases (like in VSV), there may be little to prevent N subunit sliding along the RNA backbone.

The reason for this speculation is as follows: the N subunits of VSV and MeV NCs are highly asymmetric, elongated proteins that are aligned side-to-side, perpendicular to the helix axis (Fig. 1a, right) (Chen et al., 2004; Sakaguchi et al., 1996; Schoenh et al., 2004). The location of their RNAs will soon be known with certainty from X-ray studies, but in both image reconstructions there is good evidence that the RNA is located near the inside of the coil (Fig. 1a, right). Given the asymmetry of MeV N and its association with precisely 6 nt, and the inaccessibility of most of the RNA bases in SeV NCs to the solvent, it is hard to see how the initial interaction of MeV RdRp with its promoter would not include the irregular features of the N surface as recognition determinants. The available evidence suggests that VSV N subunits are also associated with an integral number of nucleotides, namely nine (Chen et al., 2004; Thomas et al., 1985). It is not unlikely that other NNV like VSV also use features of the N subunit surface for promoter recognition by RdRp. As these other NNV genomes do not require any particular integer length to replicate efficiently, their N subunits may need to slide along the RNA genome so as to place the 3’ end promoter sequences in an N-subunit context that is optimal for initiation.

**NNV RdRp pseudo-templated transcription or ‘stuttering’**

(i) **Bona fide mRNA editing**

All DNA-dependent RNA polymerases (DdRp) respond to pause sites that are crucial elements of the mechanisms that regulate DdRp processivity (e.g. HIV TAT and anti-TAR) or that lead to chain termination (Palangat et al., 1998). DdRp pausing is thought to occur by a two-step mechanism. Prior to pausing, DdRp transcribes rapidly, oscillating in each round of nucleotide addition between the post-translocated conformation in which NTP is bound efficiently and the pre-translocated conformation that forms upon nucleotide addition. In the first pause step, DdRp isomerizes to an inactivated state that binds NTP less efficiently. This sets up the second step, in which DdRp backtracks ~1 bp along the hybrid, displacing the 3’ end of the RNA from the active site (Gnatt et al., 2001; Palangat & Landick, 2001). For bacterial and eukaryotic DdRp, reversible backtracking has been proposed to account for the pause.

The structures of the three plus-strand RNA vRdRp and the two dsRNA vRdRp determined to date show that they all have a basic right hand–like structure (Ollis et al., 1985), with fingers, palm and thumb subdomains, similar to other polymerases (Ollis et al., 1985; van Dijk et al., 2004). Several motifs conserved in all RdRp have been identified (Hansen et al., 1997; Koonin, 1991; Poch et al., 1989) and the most prominent (A, B and C) are in the palm domain, where the active site, defined by two Mg2+ ions, is located. The two metal ions are coordinated by two conserved aspartates, one in motif A and one in C. Based on structural similarities and the presence of conserved motifs, all polymerases are thought to use the same two metal ion mechanism for catalysis (Joyce & Steitz, 1995).

Given these similarities between RdRp and DdRp, the DdRp pausing mechanism has been extrapolated to explain NNV RdRp pseudo-templated transcription (stuttering), with one notable qualification. RdRp stuttering is thought to occur when the polymerase active site backtracks on the template at a ‘slippery’ sequence and the hybrid containing the 3’ end of the mRNA is realigned on the template as well (Fig. 2, black to red arrow). This hybrid realignment makes use of non-destabilizing G:U base pairs, and leads to pseudo-templated insertions upon chain elongation (Lamb & Kolakofsky, 2001; Vidal et al., 1990). Different paramyxoviruses insert one, two or a broader distribution of pseudo-templated Gs to the mRNA at the editing site, which is matched to the genetic organization of each P gene (Fig. 3,
bottom). Studies of SeV mRNA editing using varying NTP concentrations, and nucleoside analogues that induce RNA polymerase pausing, are consistent with the stuttering mechanism (Vidal et al., 1990). Moreover, altering the stability of the hybrid between the 3’ end of the mRNA and the template (Fig. 2, black arrows) affects editing as expected (editing frequency is inversely proportional to hybrid stability (Hausmann et al., 1999). The stability of the hybrid in the SeV transcription elongation complex thus appears to act similarly to some pause sites for cellular DdRp, where weakness of the hybrid by itself induces DdRp pausing (Chan & Landick, 1993).

The various paramyxovirus P gene editing signals are shown in Fig. 2 as minus-strand RNA, aligned according to their pyrimidine tracts (at least 8 nt in length), and the upstream purine that defines its start. The stutter site for SeV, i.e. the presumed position of the RdRp active site during stuttering, appears to be a unique template cytidine (Hausmann et al., 1999) (Fig. 2, boxed), and is the eighth pyrimidine. Most of the pyrimidine tract is proposed to be hybridized to the nascent mRNA during stuttering, but the hybrid can only be realigned by 1 or 2 nt, respectively. In the case of PIV3, this cycle repeats itself one to six times. Paramyxoviruses listed are: hPIV/bPIV, human/bovine parainfluenza type 3 virus; SeV, Sendai virus; MeV, measles virus; PDV, Porcine distemper virus; RPV, Rinderpest virus; CDV, Canine distemper virus; DMV, dolphin morbillivirus; HeV, hendra virus; NiV, nipah virus; SalV, saliviruses; TPMV, tupaia paramyxovirus; NDV, Newcastle disease virus; SV5, Simian virus 5; MuV, Mumps virus; PI4, parainfluenza virus type 4; LPMV, La Piedad-Michoacan-Mexico virus; PI2, parainfluenza virus type 2; and FDLV, Fer de Lance virus.
appears to be modulated by the sequence directly upstream of the pyrimidine tract, and the hexamer phase as well (Iseni et al., 2002). Whereas bona fide G/Pr (at least PrE-II) hexamer phase is critical for promoter activity, the SeV editing signal is active in all phases, and phase exerts only modulatory effects on editing patterns. Nevertheless, the fact that hexamer phase has any effect at all on mRNA editing implies that the N subunits of the template remain in sufficiently close contact with vRdRp during transcription elongation to affect the process. Whether this implies that the N subunits remain in close association with the phosphodiester backbone during transcription is unclear. There is no information as yet about NC structure within an elongation complex, and we have only the structure of the inactive or resting NC as a guide.

(ii) Polyhexameric genome length correction via ‘illegitimate’ editing

Unlike mRNA polyadenylation, mRNA editing is associated with continued elongation. Should this process occur during replication, it will lead to antigenomes with insertions at the editing site, which in turn are copied into genomes (‘illegitimate’ editing). These non-polyhexameric antigenomes will replicate poorly, and will thus be under-represented in the population. The frequency with which ‘illegitimate’ editing actually occurs during replication is thus unclear. However, work with SeV has suggested that this frequency may not be trivial. Using a transfected cell system, Hausmann et al. (1996) examined the effect of varying the total length of SeV mini-replicons that contained a mRNA editing site, on the frequency and nature of G insertions at this site during replication. When non-polyhexameric constructs were used, insertions (or deletions) at the editing site occurred during antigenome synthesis that strikingly restored polyhexameric length. When 6n+1 and 6n+5 constructs were amplified, fully 50% of the antigenomes had deleted a single nucleotide at the editing site in the former case or had added a single nucleotide at the editing site in the latter case. The remaining 50%, in each case, were unaltered at the editing site, but may have altered other sites to recover polyhexameric length (see below). Under these conditions, the mRNA editing site is a natural hotspot for RdRp slippage during replication, accounting for at least 50% of the length corrections. This SeV study stands in contrast to that of Skiadopoulos et al. (2003) who engineered full-length HPIV2 cDNAs that deviated from the rule of six by 1 to 5 nt. Infectious virus with wild-type growth properties was recovered in all cases, and all the constructs yielded viruses that contained small nucleotide deletions or insertions that regenerated polyhexameric genomes. Virus recovery here appeared to depend on the occurrence of spontaneous deletions and insertions that were all consistent with RdRp slippage. However, in contrast to the SeV mini-replicon study, none of the HPIV2 genome length corrections were at the editing site.

In the context of a complete infectious virus, editing site insertions are presumably not tolerated, as this strongly alters expression of the various P gene products whose relative proportions are critical (Fig. 3). However, in a mini-genome context where these alterations have no consequence on genome replication (because viral proteins are provided from plasmids), their frequency more likely reflects the true rate. This number is still hard to pin down, as we do not know how many rounds of replication have occurred in the SeV study, nor the relative replication disadvantage of non-polyhexameric genomes. However, even if we assume that illegitimate editing during replication occurs 100 times less frequently than the bona fide event during transcription (ca. 50%), this is still approximately 100 times as frequent as mis-incorporations (9 \times 10^{-5} for MeV; Schrag et al., 1999). More importantly, unlike mis-incorporations that may have a range of effects on viral fitness, illegitimate editing is inevitably highly deleterious for virus viability in most cases (EboV is an exception, see below).

**Negative effects of illegitimate editing and error catastrophe**

High mutation rates due to RdRp errors and quasi-species dynamics confer great adaptability to RNA viruses.
(Domingo, 2000; Drake & Holland, 1999). However, when the error rate during copying of the genetic material exceeds a threshold value, the integrity of the genetic information cannot be maintained (Eigen, 2002). Because the ability to respond to different environmental conditions is so important for virus survival, RNA viruses are thought to exist close to the edge of the error threshold.

The remarkable success of ribavirin in combating HCV infection has renewed interest in how this nucleoside analogue acts. Recent studies of ribavirin action have come to the equally remarkable conclusion that in some viral systems the drug acts not by inhibiting virus replication per se, but that it increases the error frequency of vRDrp (Snell, 2001), leading to error catastrophe and virus extinction (Graci & Cameron, 2002). In this light, the exceptional limitation on genome variation imposed by the rule of six may finally make some sense. NNV like VSV, respiratory syncytial virus and EboV are not subject to any integer rule, and except for EboV, do not edit their mRNAs. This exception is instructive. The EboV GP gene contains a run of seven adenosines and directly encodes a soluble, truncated sGP. This run is expanded to eight in 30% of the GP mRNAs, which then encodes full-length GP (Sanchez et al., 1996; Volchkov et al., 1995). This same insertion must also occur during antigenome synthesis at a significant rate, as EboV tissue culture stocks contain two variants, one with a run of seven, and the other with eight adenosines in the GP gene. It was in fact the latter eight adenosines virus that was first selected for analysis, as it grows rapidly and forms clear plaques (Philippe Calain and Tony Sanchez, personal communication). Clearly, illegitimate editing during EboV replication does not generate debilitated virus. This process may even play a role in EboV biology. Thus, NNV that are not subject to any integer rule either do not contain a dedicated mRNA editing site (rhabdo/pneumoviruses), or if they do (EboV) the operation of this process during replication does not require a mechanism to stringently remove genomes with inserted nucleotides from the virus population. We propose that the situation is very different if they do (EboV) the operation of this process during antigenome synthesis at a significant rate, as beyond this, however, the mechanism(s) by which pre-extinction FMDV RNA suppresses infectious virus production remains unclear. If Paramyxovirinae genomes with illegitimately edited P genes have this negative property of pre-extinction FMDV RNA, this might be one reason that they must be stringently removed from the virus population.

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References


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