Significant differences in nucleocapsid morphology within the *Paramyxoviridae*

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Nucleocapsid (N) proteins from representative viruses of three genera within the *Paramyxoviridae* were expressed in insect cells using recombinant baculoviruses. RNA-containing structures, which appear morphologically identical to viral nucleocapsids, were isolated and subsequently imaged under a transmission electron microscope. Analysis of these images revealed marked differences in nucleocapsid morphology among the genera investigated, most notably between viruses of the *Paramyxovirinae* and the *Pneumovirinae* subfamilies. Helical pitch measurements were made, revealing that measles virus (MV, a *Morbillivirus* within the subfamily *Paramyxovirinae*) N protein produces helices that adopt multiple conformations with varying degrees of flexibility, while that of the *Rubulavirus* simian virus type 5 (SV5, subfamily *Paramyxovirinae*) produces more rigid structures with a less heterogeneous pitch distribution. Nucleocapsids produced by respiratory syncytial virus (RSV, subfamily *Pneumovirinae*) appear significantly narrower than those of MV and SV5 and have a longer pitch than the most extended form of MV. In addition to helical nucleocapsids, ring structures were also produced, image analysis of which has demonstrated that rings assembled from MV N protein consist of 13 subunits. This is consistent with previous reports that Sendai virus nucleocapsids have 13–07 subunits per turn. It was determined, however, that SV5 subnucleocapsid rings have 14 subunits, while rings derived from the radically different RSV nucleocapsid have been found to contain predominantly 10 subunits.

Introduction

The *Paramyxoviridae*, a family of viruses within the order *Mononegavirales*, contains important human and veterinary pathogens, including measles virus (MV), rinderpest virus, mumps virus, the parainfluenza viruses and respiratory syncytial virus (RSV). The *Paramyxoviridae* are divided into two subfamilies, *Paramyxovirinae* (containing the *Morbillivirus*, *Respirovirus* and *Rubulavirus* genera) and *Pneumovirinae* (containing the *Pneumovirus* and *Metapneumovirus* genera) (Pringle, 1997, 1999). Paramyxoviruses have a single-stranded non-segmented negative-sense RNA genome of between 15 and 19 kb in length (Lamb & Kolakofsky, 2001). This is encapsidated by multiple copies of the viral nucleocapsid (N) protein, forming a helical ribonucleoprotein complex. Together with the viral RNA polymerase, which is composed of the L protein and associated co-factors (P, and in the case of the *Pneumovirinae*, M2-1), this constitutes the functional nucleocapsid (or holonucleocapsid) (Collins et al., 1996; Curran, 1996; Horikami et al., 1992). The morphology of N-bound RNA (hereafter referred to as the nucleocapsid) is considered one of the defining features of the *Paramyxoviridae* and is described as having a ‘herringbone’ appearance when imaged under a transmission electron microscope (TEM).

The nucleocapsid, rather than naked genomic RNA, is the template for replication and transcription (reviewed by Lamb & Kolakofsky, 2001). How the polymerase accesses the RNA is not known but there is currently no evidence for disassembly of the nucleocapsid during replication or transcription. Thus, N plays a key role in mediating the interaction between the polymerase and the genome (Galinski, 1991). In virus-infected cells, only viral genomes and anti-genomes are encapsidated. It has been demonstrated in Sendai virus (SeV)-infected cells that, prior to nucleocapsid assembly, N associates with the phosphoprotein (P), which prevents non-specific binding to cellular RNAs (Curran et al., 1995). Co-expression of MV N and P has been shown to reduce non-specific encapsidation of RNA by N
(Spehner et al., 1997). These findings have led to the suggestion that the N–P interaction confers specificity on N, targeting it to genome leader and anti-genome trailer sequences. As well as that the N–P interaction confers specificity on N, targeting it (Spehner D. Bhella and others D. Bhella and others)

replication to transcription (Fearns demonstrated that higher levels of N do not affect the ratio of incorporation of the nucleocapsid into the virion (Coronel et al., 1999; Kato et al., 2001). As well as interacting with the viral polymerase, it has been demonstrated with human parainfluenza virus type 1 that a specific interaction between the matrix protein (M) and the C-terminal domain of N is required for incorporation of the nucleocapsid into the virion (Coronel et al., 2001).

Despite the importance of the Mononegavirales nucleocapsid in replication and morphogenesis, these structures have not been investigated extensively. This is due to the highly flexible nature of these assemblies that has, so far, prevented analysis by X-ray crystallography. Intermediate resolution studies, employing electron microscopy and three-dimensional image reconstruction, have been conducted on two systems, the nucleocapsid of SeV (Egelman et al., 1989) and N–RNA ring structures produced by heterologous expression of the N protein of rabies virus (RV, a Lyssavirus of the family Rhabdoviridae) (Iseni et al., 1998; Schohn et al., 2001). These investigations demonstrated distinct structural differences between the nucleocapsids of these two viruses. The helical SeV nucleocapsids were found to consist of 13–07 subunits per turn, while the RV subnucleocapsid rings were found to contain predominantly 10 subunits. It has been deduced, however, that similar quantities of RNA (approximately 80 bases) are contained in each turn of the SeV helix and the subnucleocapsid rings of RV (Egelman et al., 1989; Iseni et al., 1998).

Here, we report the production, purification and characterization of recombinant nucleocapsids from three distinct genera within the family Paramyxoviridae. In addition to the isolation of helical structures, we have found that large numbers of subnucleocapsid rings are produced, most likely by shedding of small fragments from the recombinant nucleocapsids during the purification process. Pitch and diameter measurements were taken from helical nucleocapsids and projection averages were calculated from top-views of subnucleocapsid rings. Our findings indicate that pneumovirus nucleocapsids are morphologically quite distinct from those produced by viruses of the subfamily Paramyxovirinae.

Methods

- **Cells and viruses.** SF21 cells were maintained at 28 °C in TC100 medium containing 10% foetal calf serum, either as a monolayer in T-180 flasks or in roller bottles as suspension cultures. Virus stocks of parental and recombinant baculoviruses were prepared by infecting SF21 cells at low multiplicity and allowing the infection to proceed for 8–10 days. Virus was harvested from the medium by centrifugation at 22000 × g in an SLA1500 rotor (Sorvall), re-suspended in TC100 medium and stored at −70 °C. Viruses were titrated before use. The baculovirus expressing MV N protein (Rbac09) was provided by the National Collection of Pathogenic Viruses (Centre for Applied Microbiology and Research, Porton Down, UK) and has been described previously (Fooks et al., 1993).

- **Construction of recombinant baculoviruses.** Baculoviruses expressing the N genes of RSV and SV5 (simian virus type 5) were constructed using PCR cloning methodologies. For RSV, RNA from infected CV-1 cells was prepared and reverse-transcribed using random primers in the AMV RT kit (Roche). cDNA was amplified by polymerase (Roche) using primers to amplify the N protein ORF. The primers (details not shown) had BamHI restriction sites that were used to clone the fragment into the BamHI site of the homologous recombination vector pAcACh-1. The vector and PAK-6 baculovirus DNA (Invitrogen), digested previously with BsaI, were co-transfected into SF21 cells using Lipofectamine (GibcoBRL). After 3 days, the supernatant was used to screen for recombinant baculoviruses. The SV5 baculovirus was constructed by PCR amplification of the N ORF from a cDNA clone (kindly provided by R. Randall, University of St Andrews, UK), again with primers that contained BamHI sites. The Bac-to-Bac system (Life Technologies) was used to produce SV5 N baculoviruses. Recombinant viruses were screened using monoclonal antibodies (mAbs) for either the RSV N protein (Murray et al., 2001) or the SV5 N protein (kindly provided by R. Randall).

- **Purification of N protein complexes.** SF21 cells were infected with the appropriate baculovirus at an m.o.i. of 5. After 96 h, the cells were harvested by centrifuging at 3000 × g for 5 min and washing in NTE buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA). The cells were pelleted and re-suspended in 3-0 ml NTE buffer, to which was added NP-40 to a final concentration of 0.6% (v/v). This lysate was then treated with RNase A (200 µg/ml, Sigma) and DNase I (5 units/ml, Sigma) for 30 min at 37 °C and clarified by centrifuging at 14000 × g for 15 min at 4 °C. The supernatant was then layered onto a preformed 20–40% (w/v) CsCl gradient, prepared in NTE, and centrifuged at 25000 × g in a TST41.14 rotor (Sorvall) for 16 h at 16 °C. A band with a density of approximately 1.3 g/cm³ was extracted, dialysed against NTE and concentrated to a volume of less than 300 µl in a centrifugal microfiltration unit (Millipore). This was layered onto a 2 ml 10–40% (w/v) sucrose gradient prepared in NTE and centrifuged in a TLS 55 rotor (Beckman) at 215000 × g at 4 °C for 30 min for the SV5 and MV preparations or 60 min for the RSV preparation. The gradient was fractionated into 100 µl aliquots and analysed by negative-stain TEM (see below). Fractions containing high concentrations of subnucleocapsid rings or helices were dialysed against NTE and concentrated as necessary. For equilibrium analysis, N protein isolated previously on a linear CsCl gradient was diluted into 5 ml 30% (w/v) CsCl and the final density was verified by determining the refractive index. The solution was allowed to come to buoyant density equilibrium by centrifuging for 36 h at 155 000 × g in an AH650 rotor (Sorvall). The gradients were fractionated, analysed by Western blot using a mAb specific for the N proteins and the density of each fraction determined by refractometry.

- **Western blot analysis.** Fractions from density gradients were separated by SDS–PAGE and transferred to nitrocellulose membranes.

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Membranes were blocked with 5% (w/v) Marvel in PBS containing 0.1% Tween 20 (PBS–T). Primary mAbs in PBS–T were added, followed by addition of an anti-mouse antibody conjugated to horseradish peroxidase (Sigma). Protein was detected using Amersham ECL reagent on X-ray film. The developed films were digitized using a Bio-Rad FluorS CCD and densitometry performed using the Bio-Rad Quantity One software.

Electron microscopy. Nucleocapsids and subnucleocapsid rings were visualized by negative-stain TEM. A suspension of 5 µl of protein was loaded onto a freshly glow-discharged, carbon-coated, 400 mesh copper TEM grid for 30 s. This was then washed for 30 s in a 50 µl droplet of dH2O before staining for 10 s in a 20 µl droplet of 2% ammonium molybdate (pH 7.1). Grids were then drained and allowed to air dry before imaging under the TEM. Low-dose electron micrographs were recorded using a JEOL 1200 EXII TEM at 30000 or 50000 x magnification on SO163 film (Kodak) at an approximate defocus of 1 µm under focus.

Image analysis. Electron micrographs were digitized at a raster step-size corresponding to 3.5 Å per pixel in the specimen (calibrated from negative stain images of tobacco mosaic virus) on a Dunvegan Hi-Scan drum scanner and analysed on a dual-processor SGI Octane workstation.

To measure the helical pitch of nucleocapsids from each virus, sections of helix were manually selected and analysed using the SnuRAX image-processing package (Schroeter & Bretaudiere, 1996; Whittaker et al., 1995). Helices were first straightened using a β-spline-fitting algorithm applied to manually selected points along the helix. Their pitch was then determined by measuring the spacing of layer lines in their power spectra. Nucleocapsid diameters were manually measured directly from images of individual helices in the course of determining their pitch.

Projection averages were calculated from top-views of subnucleocapsid rings using the SnuRAX image-processing package (Frank et al., 1996). Rings were either manually selected (in the case of RSV rings) or automatically selected by cross-correlation of raw micrographs against ring models of the appropriate dimensions. These were then cut out and processed to remove low frequency variations in density. Data sets were compiled containing approximately 2000 particle images for each virus investigated. For RSV rings, images were first classified by cross-correlation against fuzzy ring models covering a range of radii from 5 to 20 nm. MV and SV5 data were found to be more homogeneous and were not sorted by radius. Each data set was then subject to rotational and translational reference-free alignment (Penczek et al., 1992), classified by multivariate statistical analysis (MSA) (van Heel & Frank, 1981) and projection averages calculated accordingly. Data sets were then sorted by cross-correlating the raw data against averages derived from the reference-free procedure, before final density averages were calculated from those data that best matched the group average. Finally, radial density profiles were calculated by rotational averaging of each projection.

Results

Expression of Paramyxoviridae N proteins leads to the production of structures that are similar to viral nucleocapsids

Sf21 cells were infected with recombinant baculoviruses containing the ORF for RSV N. The intracellular proteins were separated on linear 20–40% (w/w) CsCl gradients and the distribution of N within the gradient was analysed by Western blot (0–5 ml fractions). The resultant autoradiograms were digitized, analysed by densitometry software and the relative levels of protein plotted (Fig. 1a). Four distinct bands of N were detected and TEM of these fractions showed that bands 1–3 contained no structures of interest, while band 4 had structures that were morphologically identical to RSV nucleocapsids. The density of each fraction was determined by refractometry.

We decided to extend the investigation to include two other members of the Paramyxoviridae, MV (a Morbillivirus) and SV5 (a Rubulavirus). A recombinant baculovirus expressing SV5 N was constructed and a baculovirus expressing the MV N (Rbac69) was obtained (Fooks et al., 1993). Nucleocapsid-like structures were isolated on density gradients following expression of MV and SV5 N proteins, as described for RSV N. Buoyant density equilibrium centrifugation showed that these structures banded at a density similar to that of RSV-derived material (ca. 1.3 g/cm3). RNA was found subsequently to be associated with RSV and SV5 N proteins isolated from density gradients (R. P. Yeo, unpublished data). The ability of MV N to bind RNA in a heterologous system has been demonstrated already (Spehner et al., 1997).

Fig. 1. Distribution of RSV N protein in CsCl density gradients. (a) RSV N protein expressed by recombinant baculovirus was separated on a preformed linear 20–40% (w/w) CsCl. The gradient was fractionated and the distribution of RSV N determined by Western blotting. The resultant autoradiograms were quantified by densitometry and plotted as a function of relative protein units for each fraction. (b) N derived from band 4 was analysed further on a CsCl buoyant density equilibrium gradient and compared to genuine RSV nucleocapsids. The density of each fraction was determined by refractometry.
Electron micrographs of nucleocapsids and subnucleocapsid rings produced by heterologous expression of (a) RSV, (b) MV and (c) SV5 N proteins isolated on CsCl gradients. Inset in (a) is a micrograph of a viral nucleocapsid isolated from RSV-infected cells. Bar, 50 nm.

**Characterization of helical nucleocapsids**

Electron micrographs of nucleocapsids and subnucleocapsid rings isolated for each N protein investigated are presented in Fig. 2. Qualitative observations and measurements taken from these images revealed distinct morphological differences between the nucleocapsids produced by expression of N proteins from the *Paramyxovirinae* (MV and SV5) and the *Pneumovirinae* (RSV). We also observed subtle differences in both pitch (the axial rise per turn of the helix) and flexibility between the recombinant nucleocapsids produced by expression of SV5 N and MV N.

Electron microscopy of nucleocapsids isolated after the expression of RSV N revealed a population of helices that were highly flexible and appeared to be less well ordered than those of the other N proteins under investigation. Pitch measurements were therefore somewhat harder to take from these data. In those nucleocapsids that gave reasonable power spectra, we have determined that pitches range from 68 to 74 Å. These nucleocapsids are also narrower than those of the *Paramyxovirinae*, measuring between 14 and 16 nm in diameter.

Nucleocapsids derived from the expression of MV N were found to exhibit considerable heterogeneity of pitch. Our measurements indicate that recombinant MV N is capable of producing helices with pitches ranging from 47 to 64 Å (Fig. 3).
The shortest pitch helices (< 50 Å) appear rigid, while the longer pitch helices are more flexible and have a pronounced chevron pattern. We have observed that the distribution of pitches found varies for different preparations, isolated under ostensibly the same conditions. Fig. 3(d) presents a histogram showing the pitch distributions for three different preparations. It has been suggested previously that changes in pitch may be mediated by salt conditions (Heggeness et al., 1980). Without the fixation step employed by Heggeness et al. (1980), pitch measurements from negative-stain data will invariably reflect those found in high-salt conditions. We have observed wide variation in pitch and flexibility under these conditions, while preliminary cryomicroscopy data suggest that MV nucleocapsids are found in rigid conformations at salt concentrations as low as 10 mM (D. Bhella and R. P. Yeo, unpublished data). These findings suggest that salt concentration is not the sole factor to influence pitch in these structures. Diameter measurements do not deviate significantly from the value of 20 nm reported previously (Egelman et al., 1989).

Pitch measurements of helices produced by expression of SV5 N indicated a population of nucleocapsids of which the vast majority were in a rigid conformation, with pitches ranging from 47 to 52 Å. These preparations were more consistent in this respect, with very little preparation-to-preparation variation in the distribution of pitch length. As with the nucleocapsids produced with MV N, the SV5 N helices had an average diameter of 20 nm.

**Projection averaging of subnucleocapsid rings**

We have found that our method of expression and purification produces a large number of ring structures. These are most likely shed from the helices as they pass through the CsCl gradient, as we do not see them in crude cell extracts of SV5 N. It was noted that upon further attempts to purify the rings from helical structures, using sucrose gradients, they were found throughout the gradient, suggesting that they are shed continuously from helices passing through the gradient (data not shown). Furthermore, we frequently see ‘fraying’ from both ends of helices in our images, suggesting that the majority of ring structures are most likely representative of a single turn of the helix (Fig. 4).

We have calculated projection averages from top-views of the ring structures using a reference-free alignment procedure (Penczek et al., 1992) (Fig. 5). MV and SV5 N ring averages appear to have a broadly similar structure to a cross-section through the SeV nucleocapsid reconstruction (Egelman et al., 1989), consisting of a continuous central ring with an internal radius of approximately 3 nm and an external radius of approximately 7 nm. Spokes approximately 3 nm long protrude from this central core and terminate in a second globular domain. Reference-free alignments of the MV N ring data set consistently yielded averages consisting of 13 subunits per
ring (Fig. 5c); a similar value was determined for the number of subunits per turn of the SeV helix (Egelman et al., 1989). When applied to the SV5 N rings, this method produced averages containing exclusively 14 subunits per ring (Fig. 5d). As the reference-free alignment procedure is likely to align the data to the consensus structure (an average of 13 subunits in MV and 14 subunits in SV5), we considered it possible that small populations may be present in both data sets that contained a different number of subunits. In preliminary analyses of our RSV data, we found this to be the case (see below). Although the RSV N rings were separated readily by the MSA classification procedure, we found that classifying the RSV N data by radius, prior to alignment, gave a less ambiguous result. MV and SV5 N ring data sets were far less heterogeneous in radius and so could not be sorted meaningfully by cross-correlation with ring models. To test whether there were minority populations in the MV and SV5 N data sets, they were sorted by cross-correlation, in a projection matching procedure, comparing the raw data to the MV N ring average (13 subunits per ring) and concurrently to the SV5 N ring average (14 subunits per ring). The majority of rings produced by MV N (approximately 75%) correlated to the 13 subunit (MV) average, while 25% were found to be a better match with the average of 14 subunits (SV5). In the SV5 data, some 30% of the data gave higher cross-correlation coefficients with the MV average (i.e. 13 subunits per ring). However, reference-free alignment of these minor subsets gave ambiguous averages that were not reproducibly consistent with the model used to sort them (data not shown). These averages were defined poorly and may indicate the presence of a subpopulation of lock-washer structures consisting of a non-integer number of subunits per turn. Indeed, inspection of the averages produced by both MV and SV5 rings reveals less well-defined regions, suggesting that these may be derived from top-views of lock-washer structures rather than rings. This variation in definition may also be attributed to variation in staining; however, in all cases, we have observed that averages will contain a region of lighter staining where the features of the ring are less well defined. It does seem likely, however, that at least a proportion of our data are derived from top-views of short helices rather than rings in the strictest sense, as we occasionally observe particles with very heavy staining, suggesting that these structures are longer in the view axis.

Projection averages of subnucleocapsid rings formed by RSV N were found to be radically different from those produced by MV and SV5 N proteins. The majority of RSV N subnucleocapsid rings (approximately 70%) were classified into groups, averages of which had 10 subunits per ring (Fig. 5a), suggesting that this was the preferred structure and most likely the true number of subunits per turn of the helix. A second average with 11 subunits per ring was also identified (Fig. 5b), as well as a group of smaller rings that contained too few images to produce a meaningful average. Variation in subunits per ring is a phenomenon shared with ring structures produced by the expression of RV N protein, whereby rings of 9, 10 and 11 subunits were identified (Iseni et al., 1998). The distribution of density in RSV rings appears to be markedly different from MV and SV5, with smaller spokes than are seen in the Paramyxovirinae, although measurements of the central continuous ring indicated similar dimensions, giving a 3 nm internal radius and ~ 6.5 nm external radius for the 10 subunit ring. The external radius of the whole ring is approximately 8 nm. Radial density profiles were calculated for each average showing that these rings have similar internal radii (Fig. 5e). The similar outer radii for the continuous central region of these rings is highlighted by the pronounced shoulder in the rotational averages for SV5 and MV, which coincides with a slight shoulder in the 11 subunit RSV ring and the fall-off in radial density for the 10 subunit RSV ring.

Discussion

We have described the production of recombinant paramyxovirus nucleocapsids by expression of the N proteins from three genera within this family. As has been reported previously for viruses within the Mononegavirales, these structures are formed around cellular RNAs, protecting these molecules from nuclease digestion (Fooks et al., 1993; Iseni et al., 1998; Meric et al., 1994; Myers et al., 1999; Spehner et al., 1991). We have demonstrated that structures derived from RSV N are morphologically indistinguishable from viral nucleocapsids and that they band on CsCl gradients at similar densities. Ring structures were also isolated that we suggest represent single turns of the helical nucleocapsid. Further characterization of these structures by negative-stain TEM has led us to conclude that the pneumovirus nucleocapsid is quite different from those of the Paramyxovirinae. We have also uncovered some differences between the structures produced by heterologous expression of SV5 N protein and that of MV. Our findings have implications in our understanding of transcription and replication in these viruses and widen the already well-defined gap between the subfamilies of the Paramyxoviridae.

Multiple conformations in nucleocapsids specified by the Paramyxovirinae

We have observed that, in our expression system, MV N protein assembles into helices with pitches ranging from 47 to 64 Å. It is unclear from our data whether these represent discrete helical conformations or a continuously variable range of pitches. Viral nucleocapsids from SeV have been found to adopt discrete conformations of 53 and 68 Å pitch (Egelman et al., 1989). It is possible that this may also be the case in MV-infected cells and that nucleocapsid conformation is influenced by some as yet undetermined factor. SV5 N protein assembles into rigid nucleocapsids in our system and these are more prone to shearing than those produced by MV and RSV N.
proteins. This is apparent from the presence of short, straight nucleocapsids and many more subnucleocapsid rings in these preparations. We have, however, observed greater flexibility and a more pronounced chevron pattern in micrographs of SV5 nucleocapsids published by other workers (Mountcastle et al., 1970). It is possible that the tendency to form helices in this conformation may be due to our expression system. Proteolytic treatment of SV5 nucleocapsids has been shown to remove a significant fragment (ca. 15 kDa) from the C terminus of the SV5 N protein, resulting in the generation of rigid nucleocapsids similar to those observed in this study (Mountcastle et al., 1970, 1974). Western blot analysis of our preparations gives a single band of approximately 60 kDa molecular mass, indicating that no such loss of protein has occurred. It is unclear, at this stage, what role in the virus replication strategy variation in helical pitch plays, although it seems likely that nucleocapsid flexibility would be important during morphogenesis so that nucleocapsids may be packaged in a spherical virion. It is also possible that destabilization of the helix may be required to allow the polymerase complex to access the RNA genome. A further possibility is that these conformational changes may regulate the switch between transcription and replication, as the spatial orientation of promoter elements has been found to be critical for virus replication (see below). Although we have observed variation in helical pitch in our SV5 data, we have found a distinct lack of flexibility in these nucleocapsids. Further work is therefore necessary to establish the validity of this model system for functional studies. The increased rigidity and propensity to form rings, however, does make this an attractive system for further structural analysis.

**Nucleocapsid structure and the ‘rule of six’**

It has been observed in viruses from two genera within the Paramyxovirinae (the Respirovirinae and the Morbillivirinae) that virus replication has a strict requirement for the genome to consist of a number of bases that is a multiple of six (Calain & Roux, 1993; Radecke et al., 1995). For SeV, it has been inferred that each N binds six nucleotides (Egelman et al., 1989) but it has been suggested that the rule of six is more than a simple reflection of this stoichiometry (Tapparel et al., 1998; Vulliemoz & Rouz, 2001). The SeV genome promoter, which drives both replication and transcription, is bipartite, consisting of a region at the extreme 3′ end of the genome and a second element consisting of a triplet repeat of hexamers, located between bases 79 and 96. The position of this second element has been found to be crucial for efficient virus replication (Tapparel et al., 1998). In the context of the three-dimensional reconstruction of the SeV nucleocapsid (Egelman et al., 1989), it has been suggested that this may be due to the spatial arrangement of the first and second elements of the promoter. In a helix containing approximately 80 bases per turn, the two elements of the promoter would be found adjacent to each other on successive turns, potentially constituting a landing platform for the viral polymerase (Lamb & Kolakofsky, 2001; Tapparel et al., 1998). Encapsidation proceeds from the 5′ end of the genome or anti-genome as they are synthesized. Changes in the length of the RNA that disrupt the rule of six would therefore result in a phase change in the hexameric motifs relative to the N protein. This region would therefore be incorrectly presented to the polymerase complex for subsequent rounds of replication. Our findings indicate the presence of conformations consisting of 14 subunits per turn in SV5, and possibly in MV. Although SV5 has a less stringent requirement for a genome that contains a multiple of six bases (Murphy & Parks, 1997), the positioning of the bipartite promoter is the same as in SeV and MV. Clearly, in a Paramyxovirinae helix with a number of subunits per turn other than 13, the positioning of the two promoter elements would be profoundly affected. However, the potential for such radical conformational changes suggests a possible mechanism for regulating the switch between transcription and replication. As our SV5 rings are derived from helices predominantly in the rigid < 50 Å pitch conformations, we cannot discount the possibility that virus-derived nucleocapsids may be found in a conformation similar to that which has been described for SeV, i.e. 53 Å pitch with 13±7 subunits per turn. Further structural characterization of the different conformations observed for these assemblies would therefore seem essential to establish whether changes in pitch are accompanied by a change in helical twist and, hence, the number of subunits per turn.

**Differences in nucleocapsid morphology, implications for the study and classification of the Paramyxoviridae**

The division of the Paramyxoviridae into two subfamilies is based on a number of biological differences, including the numbers and sizes of genes and radical differences in their attachment proteins (Collins et al., 2001). Pneumoviruses, unlike the Paramyxovirinae, do not follow the rule of six as a requirement for replication (Samal & Collins, 1996). This may reflect the organization of the viral promoter, which, unlike those of members of the Paramyxovirinae, appears to be contained within a single functional unit (Fearns et al., 2000). Differences observed previously in nucleocapsid morphology were, however, attributed to the influence of the negative-staining procedure rather than any significant structural difference (Lamb & Kolakofsky, 2001). Our results demonstrate that there are striking differences in the organization of helical nucleocapsids of the Paramyxovirinae and the Pneumovirinae. These structural differences may have a bearing on the replication strategies employed by each virus subfamily. Radial density profiles and measurements made from projection averages indicate that, despite the different numbers of subunits found in the subnucleocapsid rings, the continuous cores of these rings have similar dimensions. Its seems possible that, like SeV and RV, the SV5, MV and RSV rings may contain 80–90 bases of RNA. Further work is therefore needed to
establish the number of RNA bases associated with each turn of the helix and thereby each N subunit. We have observed that subnucleocapsid rings composed of RSV N protein are closer in size and morphology to those of RV than to other viruses within the Paramyxoviridae. We would wish, however, to avoid making assumptions, based on limited structural information, about possible similarities in nucleocapsid structure and function between RSV and RV. We anticipate that further structural analysis of nucleocapsids will shed light on the evolutionary, and mechanistic, relationships between viruses within the Mononegavirales.

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