



Investigations into the amino-terminal domain of the respiratory syncytial virus nucleocapsid protein reveal elements important for nucleocapsid formation and interaction with the phosphoprotein

Lindsay B. Murphy,^{a,1} Colin Loney,^a Jillian Murray,^a David Bhella,^a
Peter Ashton,^b and Robert P. Yeo^{a,*}

^a Medical Research Council Virology Unit, Church Street, Glasgow, G11 5JR UK

^b Department of Biology, University of York, P.O. Box 373, York, YO10 5YW, UK

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Abstract

Bacterially expressed nucleocapsid (N) protein, from respiratory syncytial virus (RSV), was used to investigate RNA binding in a modified North–Western blotting protocol. The recombinant protein demonstrated no sequence specificity in binding RNA representing either the antigenomic leader sequence or the nonspecific sequence derived from a plasmid vector. When recombinant N was purified on CsCl gradients, two types of structure, both with densities indicating that they contained RNA, could be visualised by negative-stain electron microscopy. Structures similar to nucleocapsids (NC) from RSV-infected cells were observed, as were ring structures. A small fragment of the N (amino acids 1–92) was all that was required for the production of NC-like structures. Another mutant with an internal deletion could form rings but not NC-like structures. This suggests that this domain (amino acids 121–160) may be important for maintaining helical stability. Further analysis has also identified a potential site in the amino-terminus that may be involved in an interaction with the phosphoprotein. A domain model of the RSV N protein is presented which, similar to that of other paramyxoviruses, supports the idea that the amino-terminus is important for NC assembly.

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Introduction

Respiratory syncytial virus (RSV), a *pneumovirus*, has a negative-sense RNA genome encapsidated by the nucleocapsid (N) protein forming a helical nucleocapsid (Collins et al., 2001). This ribonucleoprotein (RNP) structure, as is the case for all members of the *Mononegavirales*, is the template for replication and transcription of the genome; uncomplexed RNA cannot function as a template for the viral polymerase. The RNA is protected from ribonuclease attack and the nucleocapsid

may mask the genome from host cell surveillance via the interferon response. The N protein thus has a multitude of interactions for its functionality: with itself, the RNA, the polymerase (L), and associated cofactors (e.g., the P protein). In addition an interaction with the matrix (M) protein may be responsible for the uptake of the nucleocapsid into the virion particle, as has been demonstrated for human parainfluenza virus type 1 (hPIV1) and Sendai virus (SeV) (Coronel et al., 2001). Attempts to map domains on N proteins responsible for these interactions have been made on a number of *Mononegavirales* members, for example, rabies virus (RV) (Kouznetsoff et al., 1998), SeV (Myers et al., 1999), measles virus (MV) (Bankamp et al., 1996), and RSV (Garcia-Barreno et al., 1996; Khattar et al., 2000; Murray et al., 2001). Although differences exist between the viruses, the N proteins have

* Corresponding author. Fax +44-141-337-2236.

E-mail address: p.yeo@vir.gla.ac.uk (R.P. Yeo).

¹ Present address: University Medicine, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, U.K.

a similar modular compartmentation of the interacting regions. The amino-terminus appears to be responsible for the assembly of the nucleocapsid, while the carboxyl-terminus has elements that are necessary for the interaction with the polymerase and the M protein.

The assembly of nucleocapsids has been best studied on SeV, which appears to be an appropriate model for other members of the *Paramyxoviridae* (Lamb and Kolakofsky, 2001). The formation of the nucleocapsid is coupled with genome replication; the N being delivered possibly in the form of complex with P (N^oP). The N^oP complex maintains N in a soluble state and promotes specific binding to both viral genomic and antigenomic RNA (Curran, 1996; Curran et al., 1995). For a number of viruses within the *Paramyxoviridae*, sole expression of N results in assembly of nucleocapsid-like structures around cellular RNAs (Bhella et al., 2002); coexpression of P protein apparently inhibits this effect (Spehner et al., 1991; Curran et al., 1995).

Several groups have studied the interaction between the RSV N and P proteins. Deletion of the carboxyl-terminus of N, and monoclonal antibodies (Mabs) that bind to this region, have a deleterious effect on the interaction (Garcia-Barreno et al., 1996; Khattar et al., 2000; Murray et al., 2001). The amino-terminus of the RSV N has not been as well studied, although by analogy to other mononegaviruses, it is likely that the RNA-binding domain and the N–N interacting region are located within this region (Heggeness et al., 1980; Myers et al., 1997; Schoehn et al., 2001). Attempts to map functional domains to the amino-terminus of RSV N have not been overly successful, possibly due to altered conformations of expressed proteins. Khattar et al. (2000) attempted to determine the effect of deletion on the ability of N to encapsidate a minigenome RNA using protection from nuclease activity as an assay. Removal of the amino-terminal eight amino acids apparently ablated encapsidation, but surprisingly, such a deletion had a minimal effect on transcription and replication. It is likely, therefore, that the mutant N was still able to bind RNA and act as a component of the template, but not able to convert it into a nuclease resistant structure, a feature considered one of the defining attributes of *Mononegavirales* nucleocapsids. Buchholz et al. (1993) observed a similar phenomenon with the N of SeV.

In this article, we demonstrate that RSV N, when expressed and purified from bacteria, binds RNA in a non-sequence-specific manner and is able to form nucleocapsid-like structures. The binding to RNA apparently induces a conformational change in N. We show that the RNA, and possibly the N–N, interacting domains reside between amino acids 1 and 92 of N and that this region is sufficient to form a structure where the RNA is resistant to nuclease attack. We also show that the amino-terminus of N has a region that may be involved with P.

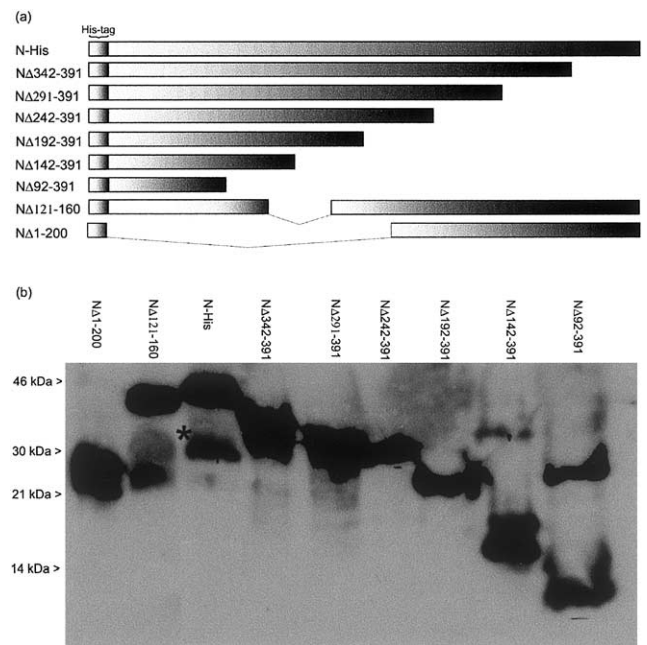


Fig. 1. (a) Graph of His-N and mutant proteins thereof. The deletion mutants were named according to the amino acids removed by the mutagenesis procedure. All the proteins were expressed and had an in-frame histidine tag. (b) Western blot analysis of crude cell extracts of bacteria expressing the proteins shown in (a). The lysates were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. The proteins were probed with an anti-histidine tag monoclonal conjugated to HRP; captured antibody was visualised using ECL. * indicates the amino-terminal breakdown product (N*) routinely observed in preparations of His-N and in some of the mutants (see text).

Results and discussion

Generation of deletion mutants of the RSV N protein

The generation of full-length N protein with an amino-terminal histidine tag (His-N) has previously been described (Murray et al., 2001). A number of mutants (Fig. 1a), primarily C-terminal truncations, were subsequently prepared, using either PCR or the Promega Gene Editor kit. The C-terminal mutants were a series deleting the N by increments of 50 amino acids. Two mutants with deletions in the amino-terminus of N were also constructed; N Δ ₁₋₂₀₀ lacks the N-terminal 200 amino acids, while N Δ ₁₂₁₋₁₆₀ represents an internal deletion of 40 amino acids. Expression of the mutants and full-length His-N in BL21 (DE3) pLysS was readily detected (Fig. 1b). Subsequent work on the proteins demonstrated that a proportion of each was soluble (ca. 10–30% depending on the clone). The majority of the “insoluble” protein was subsequently determined to be oligomers of His-N with the morphological appearance of RSV nucleocapsids (see below), which were pelleted by the initial high speed centrifugation prior to isolation of protein using nickel affinity chromatography. Within the His-N, N Δ ₁₂₁₋₁₆₀, N Δ ₃₄₂₋₃₉₁, and N Δ ₂₉₂₋₃₉₁ preparations, we routinely observed a breakdown product of approximately 30

kDa (N*). This fragment could be detected with the His-tag antibody or with Mabs directed against the amino-terminus of N but not with Mabs against the carboxyl-terminus (not shown). Thus similar to other N proteins the C-terminus of the RSV N is susceptible to proteolytic removal (Heggeness et al., 1980, 1981; Mountcastle et al., 1974; Schoehn et al., 2001). We also observed that the smaller proteins (e.g., N_{Δ92-391}) had a propensity to form higher order oligomers that were not readily dissociated by boiling in SDS-PAGE loading buffer, suggesting that the first 92 amino acids may have a role in the N–N interaction.

Full-length recombinant N binds RNA in a non-sequence-specific manner

His-N was purified by nickel-affinity chromatography. Size exclusion chromatography showed that under the conditions used, the His-N preparations were largely monomeric protein with a small (<5%) amount of dimer. Various assays were used to test for RNA binding; however, when RNA of any nature was added, His-N aggregated rapidly and the majority of the complexes could not be adequately resolved by gel electrophoresis, although there was some indication of *in vitro* RNA binding (not shown). Addition of a molar excess of RNA, to promote the binding of fewer His-N molecules to each RNA molecule, did not alleviate the situation, which suggested that interaction of even a single His-N with an RNA molecule catalysed a cascade of binding events, resulting in aggregation. The binding of exogenously added RNA to His-N appeared inefficient possibly due to the presence of bacterial RNA already bound to the protein. This was confirmed by UV traces obtained during purification of His-N on an FPLC apparatus that indicated that nucleic acid was extracted in the same fractions as His-N (data not shown). As shown later (Fig. 4) His-N can form nucleocapsid-like structures containing bacterial RNA. However, no evidence was found for the presence of nucleocapsid-like structures after examining His-N preparations purified by nickel-affinity chromatography by electron microscopy (EM). Thus a significant proportion of the so-called soluble His-N must be bound to a small fragment of RNA in a nuclease-protected form.

To overcome the interference caused by the presence of bacterial RNA bound to His-N, we employed a modified North–Western blot assay. This included a protein denaturation and renaturation step so it was likely that incorporated bacterial RNA would be removed. The ability of His-N to bind RNA under these conditions was tested by the addition of either radiolabelled specific RNA (representing the plus leader strand) or a nonspecific RNA. As more RNA was added, the binding to His-N increased. However we did not observe any significant differences between the binding of the two RNAs (Fig. 2a, i and ii). No binding to a control protein (His-βGal) was observed.

To more fully explore this, a competition assay was developed. Fragments of nitrocellulose membrane, contain-

ing His-N, were excised and incubated in RNA-binding buffer containing a radiolabelled specific RNA probe and a cold competitor probe at varying molar ratios (Fig. 2b, i). The ability of the specific probe to compete with a radiolabelled nonspecific probe was also tested (Fig. 2b, ii). The relative intensities of captured probe, determined by densitometry, were plotted after normalisation of the protein levels bound to each fragment of membrane and of the specific activity of each of the probes (Fig. 2b, iv). We saw no significant differences between the two samples. A non-RNA-binding protein, β-galactosidase, was also tested (Fig. 2b, iii). At low competitor levels some degree of nonspecific RNA binding was apparent, but at higher levels, unlike His-N, no binding was detected. The pattern of binding indicates that there were two distinguishable modes of interaction between His-N and RNA in this assay. The major form is largely nonspecific, represented by a large decline in binding of the radiolabelled species in the presence of low amounts of cold competitor. We believe this to be a result of the RNA binding to His-N as a consequence of electrostatic attraction with basic amino acid residues; we assume that the His-N is present in both unfolded and folded forms, thus the residues may come from anywhere within the protein. The second form of binding appears to be of higher affinity, as large excesses of cold competitor failed to dislodge RNA from the N. We assume that this might represent RNA that has located to the true RNA-binding domain on native protein, and similar to the RNA bound to purified, monomeric N, this interaction would only be disrupted by protein denaturation. Our data could see no significant differences between the binding affinity of a specific and nonspecific RNA to His-N. We could not, therefore, assign a sequence specificity to the binding of RNA to RSV N, a feature that is apparently shared with other mononegaviruses when N proteins are expressed in the absence of other viral proteins (Bankamp et al., 1996; Buchholz et al., 1993; Iseni et al., 1998).

The RNA binding domain is located in the amino-terminus of the RSV N protein

Attempts were made to use the deletion mutants in the modified North–Western assay to determine the approximate locale of the RNA-binding domain. However, variation in expression levels and purity made the assay unmanageable. An alternative protocol employed the use of CsCl density gradients and Trizol extraction of RNA. The gradients were fractionated and the presence of protein determined by Western blotting using an anti-His tag Mab (not shown). The densities of fractions that contained protein were determined by refractometry and by reference to published tables and are listed in Table 1. We noted that there was variation in the densities, but generally the mutants had lower densities than that obtained for His-N or for N prepared from RSV-infected CV-1 cells. However, all the densities were significantly higher than that of BSA (1.19

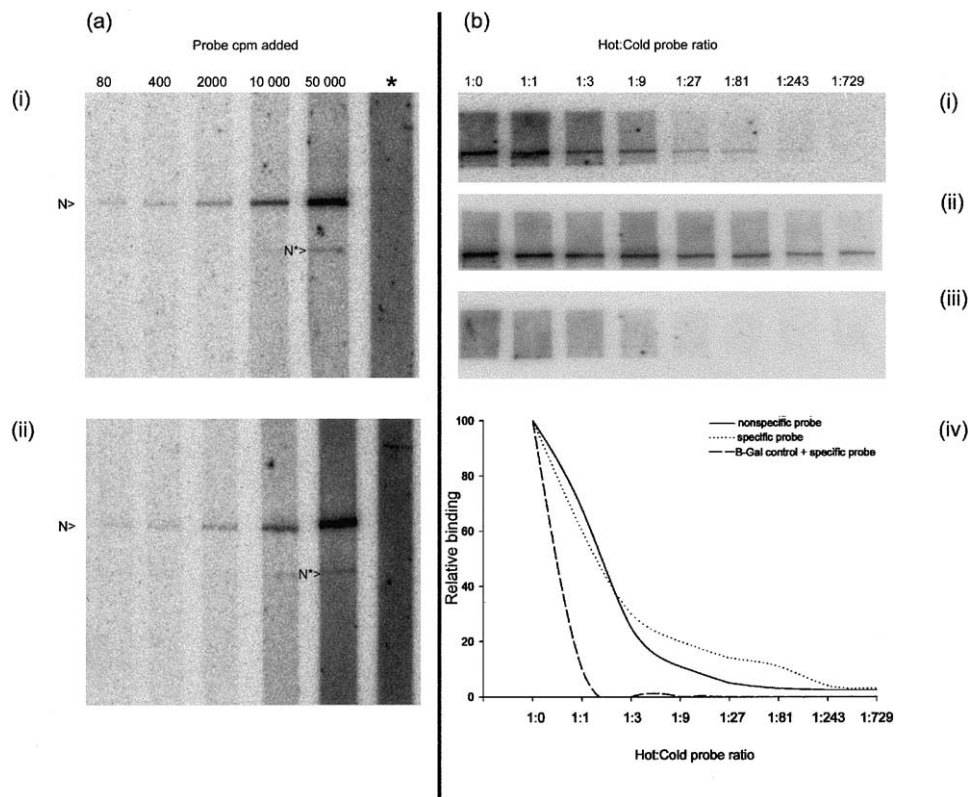


Fig. 2. RNA binding by His-N shows no sequence specificity. (a) 50 μ g of His-N, isolated by Ni-affinity chromatography, was separated by SDS-PAGE in an 8-cm well and Western blotted. After transference, the nitrocellulose was cut into 1-cm strips and subjected to North-Western blotting after renaturation of the protein. Increasing amounts of radiolabelled RNA was added to each strip (indicated above) and allowed to bind. After washing, the strips were exposed to a phosphorImager screen and scanned using a Bio-Rad phosphorImager with Quantity One software. Two different RNA probes were employed: (i) a specific probe representing the RSV plus-strand Leader RNA and (ii) a nonspecific RNA probe. The last strip (*) was his- β -Gal probed with 50,000 cpm of the appropriate probe. The smaller band noticeable in the strip exposed to 50,000 cpm of probe is N* (see Fig. 1). In all cases yeast tRNA was included to inhibit nonspecific interactions. (b) Competitive North-Western using His-N. Small fragments of nitrocellulose containing renatured His-N were exposed to a radiolabelled probe, either (i) the specific leader probe or (ii) the nonspecific probe. The binding of each probe was performed in competition with increasing molar ratios of cold competitor. After binding, the strips were washed and exposed to a phosphorImager screen and scanned. After taking into account protein levels (determined by Western blot of the fragments) and probe activities, the competition profiles were plotted (iv). As a control His- β -Gal was probed with hot specific probe and cold nonspecific competitor (iii).

g/cm^3). This suggested that all the mutants bound RNA, although the ratio of RNA to protein was probably variable, which may explain the variation in densities.

Table 1
Densities obtained by caesium chloride gradients for various N protein mutants

Protein	Density (g/cm^3)
RSV N	1.31 ± 0.02
N-His	1.30 ± 0.02
N Δ 342–391	1.29 ± 0.02
N Δ 292–391	1.29 ± 0.02
N Δ 242–391	1.28 ± 0.02
N Δ 192–391	1.28 ± 0.02
N Δ 142–391	1.29 ± 0.02
N Δ 92–391	1.28 ± 0.02
N Δ 121–160	1.30 ± 0.02
N Δ 1–200	1.26 ± 0.03
BSA ^a	1.19 ± 0.05

^a Bovine Serum Albumin

To test the assumption that RNA has been encapsidated by His-N, or mutants thereof, the peak fractions were dialysed into RIPA buffer and precipitated with an anti-His tag Mab and recovery was confirmed by Western blot (not shown). Nucleic acid within the potential RNP complexes was recovered by Trizol extraction and end-labelled with ^{32}P . TCA precipitable material was recovered from all of the C-terminal mutants and N Δ ₁₂₁₋₁₆₀ (Fig. 3). However, N Δ 1-200, despite having a density higher than that of BSA, had no detectable nucleic acid. When micrococcal nuclease was added, after Trizol extraction, the radioactivity was converted to an acid-soluble form, confirming that the recovered nucleic acid was RNA. This implies that the RNA-binding domain of the RSV N protein, or at least significant elements of it, is located within amino acids 1-92. Thus it appears that the elements necessary for nucleocapsid formation, similar to other members of the *Paramyxoviridae*, are found within the amino-terminus of N (Bankamp et al., 1996; Buchholz et al., 1993; Myers et al., 1997). The be-

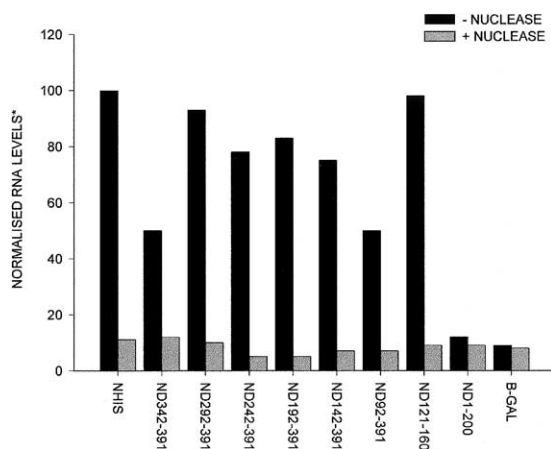


Fig. 3. Radiolabelling of RNA extracted from potential RNP structures. After expression and isolation on CsCl density gradients the N mutants were immunoprecipitated by an anti-histidine tag Mab. The complexes were treated with Trizol reagent, dephosphorylated, and end-labelled with ^{32}P . The samples were either left untreated or digested with micrococcal nuclease and precipitated with TCA and insoluble complexed captured onto glass filters. The level of radioactivity from each sample was normalised to protein levels that had been determined by Western blotting of a portion of the sample prior to Trizol extraction.

haviour of $N_{\Delta 1-200}$ raises the possibility that the RNA-binding domain might be bipartite. The protein banded at a density higher than BSA suggests the presence of bound RNA, but no RNA could be extracted. $N_{\Delta 1-200}$ may bind RNA nonspecifically; we note that our previous explanation of RNA binding suggested that the majority observed in vitro was due to nonspecific electrostatic interactions. Prior to purification on CsCl gradients, the crude lysates were subjected to RNase treatment to overcome such a problem. However, it is apparent that the majority of the RNA-binding ability of N is a feature of residues 1-92.

Formation of nucleocapsid-like structures as visualised by electron microscopy

The observation that the carboxyl-terminal mutants, and $N_{\Delta 121-160}$, bound RNA such that it was protected from nuclease digestion suggested that the N proteins may form nucleocapsid-like structures. The ability of *Mononegavirales* N proteins to form such structures in the absence of other viral protein has been documented previously (Bhella et al., 2002; Fooks et al., 1993; Green et al., 2000; Iseni et al., 1998; Mavrikis et al., 2002; Meric et al., 1994; Myers et al., 1999; Spehner et al., 1991). The N mutants, and full-length His-N, were separated on CsCl gradients, stained with 1% (w/v) PTA, and visualised by transmission electron microscopy (TEM). When visualised by TEM, His-N formed two types of structure (Fig. 4). Nucleocapsid-like structures similar to that formed in RSV-infected cells were observed, as was a population of subnucleocapsid rings (arrows). Our previous analysis of the N-RNA rings formed when RSV N is expressed from recombinant baculoviruses

(Bhella et al., 2002) demonstrated that they were composed of 10 subunits, similar to structures obtained by heterologous expression of VSV and RV N proteins (Green et al., 2000; Iseni et al., 1998).

The C-terminal mutants also formed ordered structures with similarities to RSV nucleocapsids and the majority formed distinguishable ring structures. The largest deletion mutants, $N_{\Delta 142-391}$ and $N_{\Delta 92-391}$, formed nucleocapsid-like structures (Fig. 4a), suggesting that both the RNA binding and the N self-interacting domains were found within amino acids 1-92. The $N_{\Delta 121-160}$ mutant was particularly interesting. Whereas most of the N proteins, not including $N_{\Delta 1-200}$, produced nucleocapsid-like and ring structures, $N_{\Delta 121-160}$ produced only ring structures. This would suggest that helical stability relies on more than lateral (i.e., side-by-side) contacts and may involve interaction between N molecules on adjacent turns of the helix and thus there may be up to six contacts for any one subunit within a helix (see Fig. 4b); thus a nucleocapsid could be considered a curved sheet with N packed in a hexagonal arrangement. The $N_{\Delta 121-160}$ mutation may disrupt an interaction between protein on adjacent turns, either by loss of one of the contacts or by adopting a conformation that prevents an interaction occurring. The result could be the inability of $N_{\Delta 121-160}$ to form more than one helical turn or that the stability of helices is decreased to such an extent that purification disrupts any that may have existed. We have tried to visualise whether $N_{\Delta 121-160}$ forms helices, within bacteria, but have not been able to identify any such structures.

$N_{\Delta 1-200}$ formed neither nucleocapsid-like nor ring structures, but we observed aggregates of what we assume to be $N_{\Delta 1-200}$. It could be that there may be an additional N-N interaction site within amino acids 201-391 that allowed the aggregation to occur. The possible bipartite nature of the N-N interacting domain may also exist in MV N (Bankamp et al., 1996).

Identification of sites of interaction for the P protein

We previously demonstrated, using Mabs and peptide blocking studies, that there was a P-binding site in the N protein carboxyl-terminus (Murray et al., 2001). Further peptide studies were undertaken to determine whether the amino-terminus was involved in any way with the P protein. Peptides representing the amino-terminus from amino acids 1 to 252 (the region not covered previously) were used in preliminary studies either to bind P to a solid support or to block the binding of N to P (data not shown). N4 (amino acids 46-65) demonstrated reasonable binding to P (Fig. 5a), but in blocking assays had a dramatic effect on the binding of N to P (Fig. 5b). Peptide N17 (amino acid 241-260) bound P strongly but was unable to block the N-P interaction. The binding was specific as P did not bind significantly to other peptides nor did N4 and N17 bind control proteins (not shown). The sequence of N4 and N17 are presented in Fig. 5d.

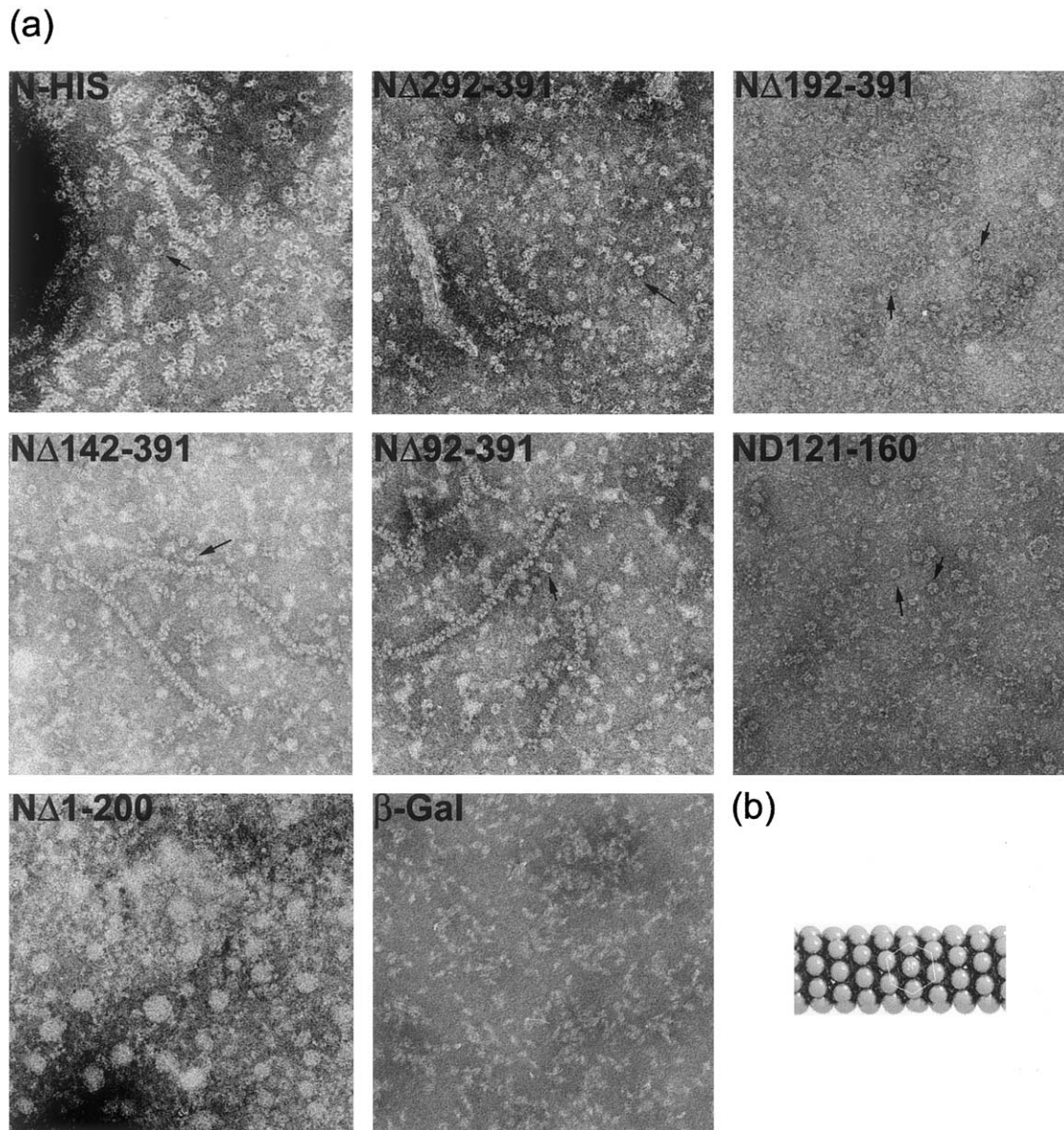


Fig. 4. (a) Formation of nucleocapsid-like structures by His-N and the mutant proteins. The various proteins were extracted from bacteria by separating cell lysates, previously treated with RNase and DNase, on a CsCl gradient. The appropriate bands were dialysed into NTE and dropped onto a carbon-coated grid and stained with 1% PTA. The grids were then observed in a Phillips electron microscope at a nominal magnification of $\times 32,000$. Mutants N Δ 342-391 and N Δ 242-391 are not included as we were not able to obtain preparations that were suitable for EM due to bacterial contamination. (b) A diagram of the RSV nucleocapsid. The hexagonal order of the N within the helix is discernible, the N within the “core” of a hexagon may contact up to six other N molecules.

Expression of mutants containing these regions showed that whereas the carboxy domain, as represented by N Δ 1-200, could bind P, the amino-terminus, as represented by N Δ 192-392, was unable to do so (Fig. 5c). It is possible that the N4 sequence at the amino-terminus, in the context of the full-length protein, required the presence of the carboxyl-terminus for functionality: use of the peptide in binding/blocking experiments overcomes the requirement *in vitro*. We have preliminary evidence, using tryptic digests and mass spectroscopy analysis, that the sequence represented by N4 on N is exposed to the solvent and thus would be available for some form of interaction (L.B. Murphy and P. Ashton,

unpublished observations). Since the sequence is within the proposed nucleocapsid assembly domain and RNA-binding domain, the interaction of P with this site could prevent unsolicited assembly of N onto cellular RNAs. Similarly, it has been reported for MV and HPIV-3 that there are at least two regions involved in P binding, one in the amino-terminus and another near the carboxyl-terminus (Bankamp et al., 1996; Zhao and Banerjee, 1995). Caution must be applied to such a comparison since the N proteins of the *Pneumovirinae* are considerably smaller than their *Paramyxovirinae* counterparts (ca. 43 kDa compared to 55 kDa and more). However the results do indicate that separate elements make

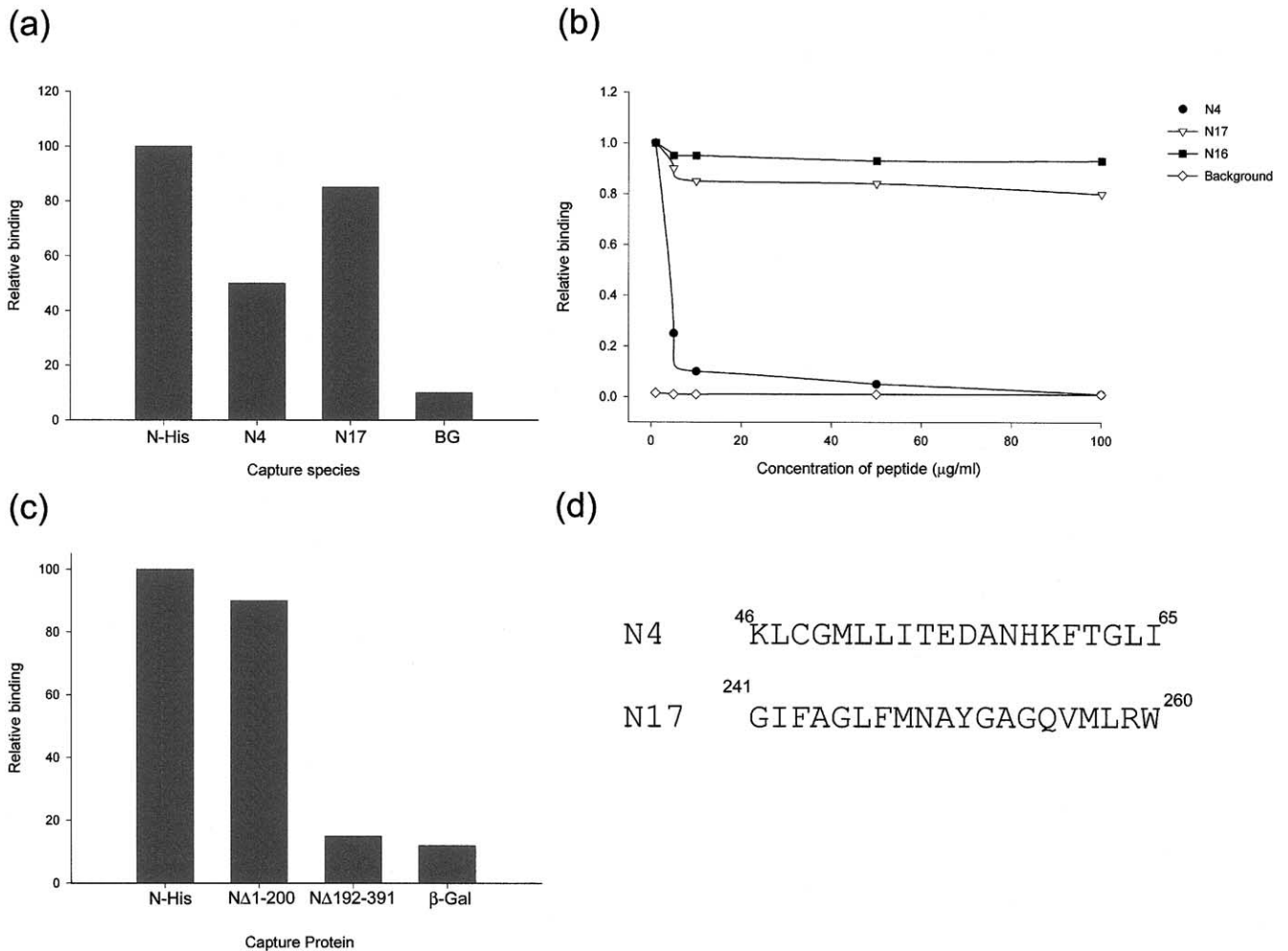


Fig. 5. There is a possible P-interacting domain in the N protein's amino-terminus. (a) Peptides N4 and N17 can bind P protein in vitro. The wells of a microtitre plate (Immulon 2, Thermo Labsystems) were coated with saturating amounts of His-N, peptide N4, peptide N17, or a nonspecific peptide (BG, supplied by H. Marsden, MRC Virology Unit, U.K.). His-P was added and allowed to bind. The plates were extensively washed and bound His-P was detected using an anti-P Mab. (b) Peptide N4, but not N17, can block the N–P interaction. Microtitre plate wells were coated with His-P protein. The peptides were added at varying concentrations before His-N was added. Bound His-N was detected using an anti-N Mab. 100% binding was taken as the level that the two proteins interacted in the absence of peptide. Background was determined by omitting His-N. Peptide N16 (amino acids 226–245) was used as a negative control, as in preliminary experiments it neither bound to His-P nor blocked the interaction. (c) The amino-terminus cannot bind P when expressed alone. Two of the mutant constructs were tested for their ability to bind P. NΔ1–200, which represents the carboxy region, and NΔ192–391, which represents the amino-terminus of N, were tested. As in (a) plates were coated with the appropriate protein and His-P added, the binding of which was determined using an anti-P Mab. His-β-Gal was used as a negative control. (d) The sequences of peptides N4 and N17 are given with their amino acid location. The numbers refer to the wt N sequence without any tags.

up P-binding sites for both subfamilies. Whether these constitute components of a single binding site, which is formed by folding of N, or represent distinct functional sites is not clear.

The domain structure of the RSV N protein

Figure 6 shows a proposed domain structure for the hRSV N protein based on the above data and from previous publications (Garcia-Barreno et al., 1996; Khattar et al., 2000; Murray et al., 2001).

Khattar et al. (2000) investigated the N of bovine RSV

(bRSV), which shares approximately 93% homology at the amino acid level with the human strain of RSV N protein. Two domains were identified in the bRSV N protein carboxyl-terminus that were involved in an interaction with P, represented by amino acids 244–290 and 338–364. Peptide N17 lies within the 244–290 region and we had placed the second P interacting domain at amino acids 305–335 (Murray et al., 2001), thus there is good agreement on the arrangement of the P interacting domain(s), at least in the carboxyl-terminus of N. Garcia-Barreno et al. (1996) had also indicated that P bound hRSV N near amino acid 352. We now show that there is a third potential region, between

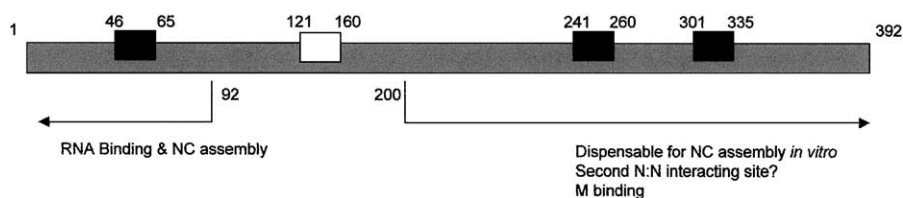


Fig. 6. A model for the domain organisation of the RSV N protein based on our observations. The potential P-binding sites are represented by filled boxes, while the region that may have a role in helical stability is represented by an open box.

amino acids 45 and 65, on N that may be involved in some interaction with P, possibly as suggested above to prevent N assembling on nonviral RNA.

Khattar et al. (2000) showed that deletions of only 28 amino acids at the carboxyl-terminus affect encapsidation of viral RNA by the bRSV N. Our data differ in that N missing 75% of its backbone (amino acids 1–92) was able to form structures that encapsidated RNA and formed nucleocapsid-like structures, indicating that the minimal requirements for assembly are contained within this small domain. The remainder of the N probably plays a supporting role in structural integrity, interactions with other viral and cellular components, and functionality. The differences between the data can be explained by the fact that Khattar et al. (2000) used a biological system dependent on replication, and specifically, looked for the incorporation of a minigenome RNA. It is possible that cellular RNAs were nonspecifically incorporated but not detected. However, the observations of Khattar et al. (2000) and the study presented here support the concept of N having at least two domains, one for nucleocapsid assembly/structure and the other essential for the interaction with the polymerase and/or other factors.

The features on N shown in Fig. 6 represent only some of the potential interactions that N must be involved in. The complexity of *Mononegavirales* nucleocapsid assembly, and indeed virion assembly, suggest that the N protein has other interactions, for example, with the M protein for packaging the nucleocapsid into the virion during the budding process. Preliminary data implicates the C-terminal region in this role (R.P. Yeo, unpublished results). Using the data above, and reagents developed previously (Murray et al., 2001), we aim to model the RSV nucleocapsid and map the N protein domains into three-dimensional space, rather than the linear model presented in Fig. 6, in an attempt to determine the mechanisms of the nucleocapsid functionality during RNA synthesis.

Materials and methods

Materials

*Bam*HI and T4 DNA ligase were purchased from Gibco-BRL. The plasmid pET 16b was obtained from Novagen as

were the bacterial strains DH5 α and BL12 (DE3) pLysS. Primers for PCR were purchased from MWG Biotech; the sequences are available on request. Reagents for RNA extraction from RSV-infected CV-1 cells and reverse transcription using random primers were obtained from Ambion and Boehringer Mannheim, respectively. The proofreading *Pwo* polymerase was used for PCR (Boehringer Mannheim). The Mabs to N and P have been described before (Murray et al., 2001); the anti-mouse HRP conjugate and the Mabs against the histidine tag were obtained from Sigma.

Plasmid construction

The production of plasmids expressing histidine-tagged versions of the RSV N (pETN) and P (pETP) proteins has been previously described (Murray et al., 2001). pETN was used as a template for the production of deletion mutants, either by PCR or by using the Gene Editor mutagenesis kit from Promega. Initially all constructs were transformed into DH5 α and sequences at their amino- and carboxyl-termini were confirmed. Again, primer design was such that cloning was in-frame with the pET 16b histidine tag. Plasmid DNA was used to transform BL21 (DE3) pLysS; a number of clones of each mutant were tested for expression before selecting one for analysis.

Purification of his-tagged proteins

The expressed proteins were purified using Ni-affinity chromatography. An overnight culture of each clone was used to inoculate 100 ml YT broth and allowed to grow to OD₆₀₀ of 0.5 at 37°C before the addition of 1 mM IPTG. After 3 h induction the bacteria were pelleted, resuspended in phosphate buffer (20 mM sodium phosphate, 500 mM NaCl pH 7.4), and lysed by three rounds of freeze/thaw in a dry-ice/methanol bath. The lysate was treated with DNase I and RNase A to reduce viscosity and clarified by centrifugation at 85,000 *g* for 20 min. The his-tagged proteins were isolated on a Hi-Trap chelating column (Pharmacia), previously loaded with nickel, and eluted using increasing concentrations of imidazole as per the manufacturer's instructions. The eluted proteins were dialysed against PBS

and stored at 4°C before use. As a control his-tagged β -galactosidase (Novagen) was used.

Western blot analysis

Proteins, denatured by boiling in SDS-sample loading buffer, were separated by SDS-PAGE. The proteins were transferred to nitrocellulose membrane (Hybond-C, Amersham) blocked with 5% (w/v) Marvel in PBS + 0.1% (v/v) Tween 20 and the proteins probed with an anti-his-tag monoclonal (Sigma) which was conjugated to horseradish peroxidase (HRP). Bound antigen-antibody was visualised by ECL. All washes were carried out with PBS/Tween.

Production of radiolabelled RNA transcripts

Two ^{32}P -labelled transcripts were prepared. One, termed the “specific target,” represented the leader region of the RSV antigenome, supposedly the site responsible for encapsidation. This was generated by annealing two complementary oligonucleotide primers that contained a T7 polymerase promoter and the RSV leader sequence (Collins et al., 1991). The nonspecific target was produced by linearising a pBluescript KS plasmid with *Sst*I. Runoff transcripts were prepared using the Mega-Shortscript kit (Ambion). To produce radiolabelled RNAs, the cold CTP was replaced with [^{32}P]CTP. The RNA transcripts were purified on a 15% polyacrylamide gel containing 6 M urea, eluted into TE buffer with 0.1% (w/v) SDS, and precipitated using ethanol. The RNAs were pelleted before use and resuspended in RNase-free water.

North-Western analysis

The RNA-binding specificity of full-length His-N protein was analysed by North-Western blot protocol, modified from that described by Blackwell and Brinton (1995). Fifty micrograms of His-N was denatured and loaded into a single 5-cm-wide lane on a 12% polyacrylamide gel, electrophoresed, and transferred to nitrocellulose as above. The membrane was blocked in 5% Marvel in PBS containing 1 mM DTT. The membrane was washed in HBB (25 mM HEPES pH 7.5, 25 mM NaCl, 5 mM MgCl_2 , and 1 mM DTT) to allow protein refolding to take place at 4°C for 16 h. The portion of the membrane containing the His-N protein was cut up into small (5 × 3 mm) fragments and inserted into 1.5-ml microtubes and covered with HYB100 buffer (20 mM HEPES-KOH, 100 mM KCl, 2.5 mM MgCl_2 , 0.1 mM EDTA, 0.05% (v/v) Nonidet P-40, and 1 mM DTT) with 20,000 counts per minute (cpm) of the appropriate RNA probe together with 1 μg yeast tRNA (Sigma) to reduce nonspecific binding and, when required, a cold competitor RNA at various concentrations. RNA was quantified by spectrophotometry. Binding was allowed to take place overnight before washing the membranes extensively in HYB100 buffer and exposing the blot to a phos-

phorImager screen (Bio-Rad). Band intensities were determined by densitometry using Bio-Rad Quantity One software. Each band was boxed and the background, determined for each individual nitrocellulose fragment, was determined. The level of binding was calculated by normalising to protein levels on each fragment (by Western blot) and taking into account the specific activities of the probes used.

Isolation of recombinant nucleocapsid-like structures

The various N proteins were expressed as above. After pelleting the bacteria, they were resuspended in NTE buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 2 mM EDTA). A 1/10 volume of 6% (v/v) NP-40 in NTE was added and the cells were subjected to three rounds of freeze/thaw. The lysate was clarified by centrifugation at 12,000 g for 20 min. The clarified lysate was layered on top of a linear 25–40% CsCl gradient, prepared in NTE, and centrifuged in a TST41 rotor (Sorvall) at 200,000 g for 18 h at 16°C. Bands were visualised by light scattering and isolated by side puncture. The bands were dialysed against NTE and used for EM analysis (see below). The RNP-like structures were also precipitated by an anti-histidine tag Mab before RNA extraction methodologies were employed.

Density gradient analysis

An alternative to linear gradients was to isolate RNP using buoyant density equilibrium centrifugation to determine the density of the nucleocapsids. The constructs were expressed as above and the clarified lysate layered on top of a CsCl step gradient (2 ml 40%, 2 ml 30%, 2 ml 25%, topped with a 2.5 ml 5% sucrose solution) and centrifuged at 200,000 g for 2 h in a TST41 rotor. The 30% band was isolated and the volume increased to 5 ml with 30% CsCl; the density of the solution was checked by refractometry. The solution was rebanded to equilibrium by centrifugation in a AH650 rotor at 150,000 g for 36 h at 16°C. The gradients were fractionated from the top and each fraction was subjected to Western blot analysis to detect the his-tagged proteins; the density of each fraction was determined by refractometry.

Detection of RNA from recombinant nucleocapsid-like structures

Prior to isolating the RNA, to ensure that only N-structures were the source, the RNPs from above were precipitated by an anti-histidine tag antibody (Sigma). RNA was recovered by extraction using Trizol LS (Invitrogen) as per the manufacturer's instructions. Recovered RNA was treated with calf intestinal alkaline phosphatase to remove 5' phosphate groups and then subsequently end-labelled with ^{32}P using T4 polynucleotide kinase. Each sample was divided into two; one was left untreated, and the other was digested with micrococcal nuclease in the presence of cal-

cium. After treatment, 10 volumes of ice-cold 10% (w/v) trichloroacetic acid was added and the samples were captured on glass filters (Sigma) which were washed with 5 ml of 10% (w/v) trichloroacetic acid. Captured radioactivity was measured by scintillation counting.

Electron microscopy

The bands obtained from the linear gradients were mounted on carbon-coated copper grids and stained using a 1% (w/v) phosphotungstic acid (PTA) stain. The grids were analysed in a Phillips 100 V electron microscope at a nominal magnification of $\times 32,000$.

Protein binding and competition assay

Microtitre plates were coated with peptides (Murray et al., 2001) or appropriate protein. His-P protein was added in saturating quantities, determined empirically, and allowed to bind. After washing, the His-P was detected using a P Mab and an anti-mouse HRP conjugate. ABTS (1 mg/ml; Sigma) color reagent in 50 mM citrate buffer (pH 4.0) with hydrogen peroxide was added to each well and the color reaction allowed to proceed for up to 30 min at room temperature. All binding and wash steps were performed in PBS containing 0.1% Tween 20. The plates were analysed on a Dynex microtitre plate reader at a wavelength of 405 nm. The blocking assays were carried out in a similar manner except that His-P was used to coat the plate. Peptides were added, before addition of His-N, at varying concentrations from 1–100 $\mu\text{g/ml}$ in PBS or water. After allowing the peptides to bind, His-N was added in saturating quantities and allowed time to interact. Captured His-N was detected using a N Mab followed by an anti-mouse HRP conjugate.

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References

- Bankamp, B., Horikami, S.M., Thompson, P.D., Huber, M., Billeter, M., Moyer, S.A., 1996. Domains of the measles virus N protein required for binding to P protein and self-assembly. *Virology* 216, 272–277.
- Bhella, D., Ralph, A., Murphy, L.B., Yeo, R.P., 2002. Significant differences in nucleocapsid morphology within the Paramyxoviridae. *J. Gen. Virol.* 83, 1831–1839.
- Blackwell, J.L., Brinton, M.A., 1995. BHK cell proteins that bind to the 3' stem-loop structure of the West Nile virus genome RNA. *J. Virol.* 69, 5650–5658.
- Blumberg, B.M., Giorgi, C., Kolakofsky, D., 1983. N protein of vesicular stomatitis virus selectively encapsidates leader RNA in vitro. *Cell* 32, 559–567.
- Buchholz, C.J., Spehner, D., Drillien, R., Neubert, W.J., Homann, H.E., 1993. The conserved N-terminal region of Sendai virus nucleocapsid protein NP is required for nucleocapsid assembly. *J. Virol.* 67, 5803–5812.
- Collins, P.L., Chanock, R.M., Murphy, B.R., 2001. Respiratory syncytial virus, in: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.), *Fields Virology*, Lippincott-Raven, Philadelphia, pp. 1443–1485.
- Collins, P.L., Mink, M.A., Stec, D.S., 1991. Rescue of synthetic analogs of respiratory syncytial virus genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. *Proc. Natl. Acad. Sci. USA* 88, 9663–9667.
- Coronel, E.C., Takimoto, T., Murti, K.G., Varich, N., Portner, A., 2001. Nucleocapsid incorporation into parainfluenza virus is regulated by specific interaction with matrix protein. *J. Virol.* 75, 1117–1123.
- Curran, J., 1996. Reexamination of the Sendai virus P protein domains required for RNA synthesis: a possible supplemental role for the P protein. *Virology* 221, 130–140.
- Curran, J., Marq, J.B., Kolakofsky, D., 1995. An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. *J. Virol.* 69, 849–855.
- Fooks, A.R., Stephenson, J.R., Warnes, A., Dowsett, A.B., Rima, B.K., Wilkinson, G.W., 1993. Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures. *J. Gen. Virol.* 74, 1439–1444.
- Garcia-Barreno, B., Delgado, T., Melero, J.A., 1996. Identification of protein regions involved in the interaction of human respiratory syncytial virus phosphoprotein and nucleoprotein: significance for nucleocapsid assembly and formation of cytoplasmic inclusions. *J. Virol.* 70, 801–808.
- Green, T.J., Macpherson, S., Qiu, S., Lebowitz, J., Wertz, G.W., Luo, M., 2000. Study of the assembly of vesicular stomatitis virus N protein: role of the P protein. *J. Virol.* 74, 9515–9524.
- Heggeness, M.H., Scheid, A., Choppin, P.W., 1980. Conformation of the helical nucleocapsids of paramyxoviruses and vesicular stomatitis virus: reversible coiling and uncoiling induced by changes in salt concentration. *Proc. Natl. Acad. Sci. USA* 77, 2631–2635.
- Heggeness, M.H., Scheid, A., Choppin, P.W., 1981. The relationship of conformational changes in the Sendai virus nucleocapsid to proteolytic cleavage of the NP polypeptide. *Virology* 114, 555–562.
- Izeni, F., Barge, A., Baudin, F., Blondel, D., Ruigrok, R.W., 1998. Characterization of rabies virus nucleocapsids and recombinant nucleocapsid-like structures. *J. Gen. Virol.* 79, 2909–2919.
- Khattar, S.K., Yunus, A.S., Collins, P.L., Samal, S.K., 2000. Mutational analysis of the bovine respiratory syncytial virus nucleocapsid protein using a minigenome system: mutations that affect encapsidation, RNA synthesis, and interaction with the phosphoprotein. *Virology* 270, 215–228.
- Kouznetzoff, A., Buckle, M., Tordo, N., 1998. Identification of a region of the rabies virus N protein involved in direct binding to the viral RNA. *J. Gen. Virol.* 79, 1005–1013.
- Krishnamurthy, S., Samal, S.K., 1998. Identification of regions of bovine respiratory syncytial virus N protein required for binding to P protein and self-assembly. *J. Gen. Virol.* 79, 1399–1403.
- Lamb, R.A., Kolakofsky, D., 2001. Paramyxoviridae: the viruses and their replication, in: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.), *Fields Virology*, Vol. 2, Lippincott-Raven, Philadelphia, pp. 1305–1340.
- Mavrakis, M., Kolesnikova, L., Schoehn, G., Becker, S., Ruigrok, R.W., 2002. Morphology of Marburg virus NP-RNA. *Virology* 296, 300–307.
- Meric, C., Spehner, D., Mazarin, V., 1994. Respiratory syncytial virus nucleocapsid protein (N) expressed in insect cells forms nucleocapsid-like structures. *Virus Res.* 31, 187–201.
- Mountcastle, W.E., Compans, R.W., Lackland, H., Choppin, P.W., 1974. Proteolytic cleavage of subunits of the nucleocapsid of the paramyxovirus simian virus 5. *J. Virol.* 14, 1253–1261.

- Murray, J., Loney, C., Murphy, L.B., Graham, S., Yeo, R.P., 2001. Characterization of monoclonal antibodies raised against recombinant respiratory syncytial virus nucleocapsid (N) protein: identification of a region in the carboxy terminus of N involved in the interaction with P protein. *Virology* 289, 252–261.
- Myers, T.M., Pieters, A., Moyer, S.A., 1997. A highly conserved region of the Sendai virus nucleocapsid protein contributes to the NP-NP binding domain. *Virology* 229, 322–335.
- Myers, T.M., Smallwood, S., Moyer, S.A., 1999. Identification of nucleocapsid protein residues required for Sendai virus nucleocapsid formation and genome replication. *J. Gen. Virol.* 80, 1383–1391.
- Schoehn, G., Iseni, F., Mavrikis, M., Blondel, D., Ruigrok, R.W., 2001. Structure of recombinant rabies virus nucleoprotein-RNA complex and identification of the phosphoprotein binding site. *J. Virol.* 75, 490–498.
- Spehner, D., Kirn, A., Drillien, R., 1991. Assembly of nucleocapsidlike structures in animal cells infected with a vaccinia virus recombinant encoding the measles virus nucleoprotein. *J. Virol.* 65, 6296–6300.
- Zhao, H., Banerjee, A.K., 1995. Interaction between the nucleocapsid protein and the phosphoprotein of human parainfluenza virus 3. Mapping of the interacting domains using a two-hybrid system. *J. Biol. Chem.* 270, 12485–12490.