Chapter 6
Nucleocapsid Structure and Function

S. Longhi

Contents

Abstract Measles virus belongs to the *Paramyxoviridae* family within the Mononegavirales order. Its nonsegmented, single-stranded, negative-sense RNA genome is encapsidated by the nucleoprotein (N) to form a helical nucleocapsid. This ribonucleoproteic complex is the substrate for both transcription and replication. The RNA-dependent RNA polymerase binds to the nucleocapsid template via its co-factor, the phosphoprotein (P). This chapter describes the main structural information available on the nucleoprotein, showing that it consists of a structured core (N_{CORE}) and an intrinsically disordered C-terminal domain (N_{TAIL}). We propose a model where the dynamic breaking and reforming of the interaction between N_{TAIL} and P would allow the polymerase complex (L–P) to cartwheel on the nucleocapsid template. We also propose a model where the flexibility of the disordered N and P domains allows the formation of a tripartite complex (N°–P–L) during replication, followed by the delivery of N monomers to the newly synthesized genomic RNA chain. Finally, the functional implications of structural disorder are also discussed in light of the ability of disordered regions to establish interactions with multiple partners, thus leading to multiple biological effects.

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The Replicative Complex of Measles Virus

The genome of measles virus (MV) is encapsidated by the nucleoprotein (N) within a helical nucleocapsid. The viral RNA is tightly bound within the nucleocapsid and does not dissociate during RNA synthesis, as shown by its resistance to silencing by siRNA (Bitko and Barik 2001). Hence, this ribonucleoproteic (RNP) complex, rather than naked RNA, is the template for both transcription and replication. These latter activities are carried out by the RNA-dependent RNA polymerase (RdRp), which is composed of the large (L) protein and of the phosphoprotein (P). The P protein is an essential polymerase co-factor in that it tethers the L protein onto the nucleocapsid template. This ribonucleoproteic complex made of RNA, N, P, and L constitutes the replicative unit (Fig. 1A).

MV nucleocapsids, as visualized by negative stain transmission electron microscopy, have a typical herringbone-like appearance (Fig. 1B). The nucleocapsid of all *Paramyxoviridae* has a considerable conformational flexibility and can adopt different helical pitches (the axial rise per turn) and twists (the number of subunits per turn), resulting in conformations differing in their extent of compactness (Bhella et al. 2002, 2004; Egelman et al. 1989; Heggeness et al. 1980, 1981; Schoehn et al. 2004).

Once the viral RNPs are released in the cytoplasm of infected cells, transcription of viral genes occurs using endogenous NTPs as substrates. Following primary transcription, the polymerase switches to a processive mode and ignores the gene junctions to synthesize a full, complementary strand of genome length. This positive-stranded RNA (antigenome) does not serve as a template for transcription and its unique role is to provide an intermediate in replication. The intracellular concentration of the N protein is the main element controlling the relative rates of transcription and replication. When N is limiting, the polymerase is preferentially engaged in transcription, thus leading to an increase in the intracellular concentration of viral proteins, including N. When N levels are high enough to allow encapsidation of the nascent RNA chain, the polymerase switches to a replication mode (Plumet et al. 2005) (see also the chapter by B. Rima and W.P. Durprex, this volume and Albertini et al. 2005; Lamb and Kolakofsky 2001; Longhi and Canard 1999; Roux 2005 for reviews on transcription and replication).

The nucleoprotein is the most abundant structural protein. Its primary function is to encapsidate the viral genome. However, as we will see throughout this chapter, N is not a simple structural component, serving merely to package viral RNA. Rather, it plays several functions. Within *Mononegavirales*, each N monomer interacts with a precise number of nucleotides. The number of nucleotides varies among *Mononegavirales*, being specific to each family: six nucleotides for *Paramyxovirinae* (Egelman et al. 1989) and *Pneumovirinae* (Tran et al. 2007), nine for *Rhabdoviridae* (Albertini et al. 2006; Flamand et al. 1993; Green et al. 2006), and 12–15 for *Filoviridae* (Mavrakis et al. 2002). The fact that in *Paramyxovirinae* N wraps exactly six nucleotides imposes the so-called rule of six to these viruses, i.e., their genome must be of polyhexameric length (6n + 0) to efficiently replicate (see Kolakofsky et al. 2005; Roux 2005; Vulliemoz and Roux 2001 for reviews).

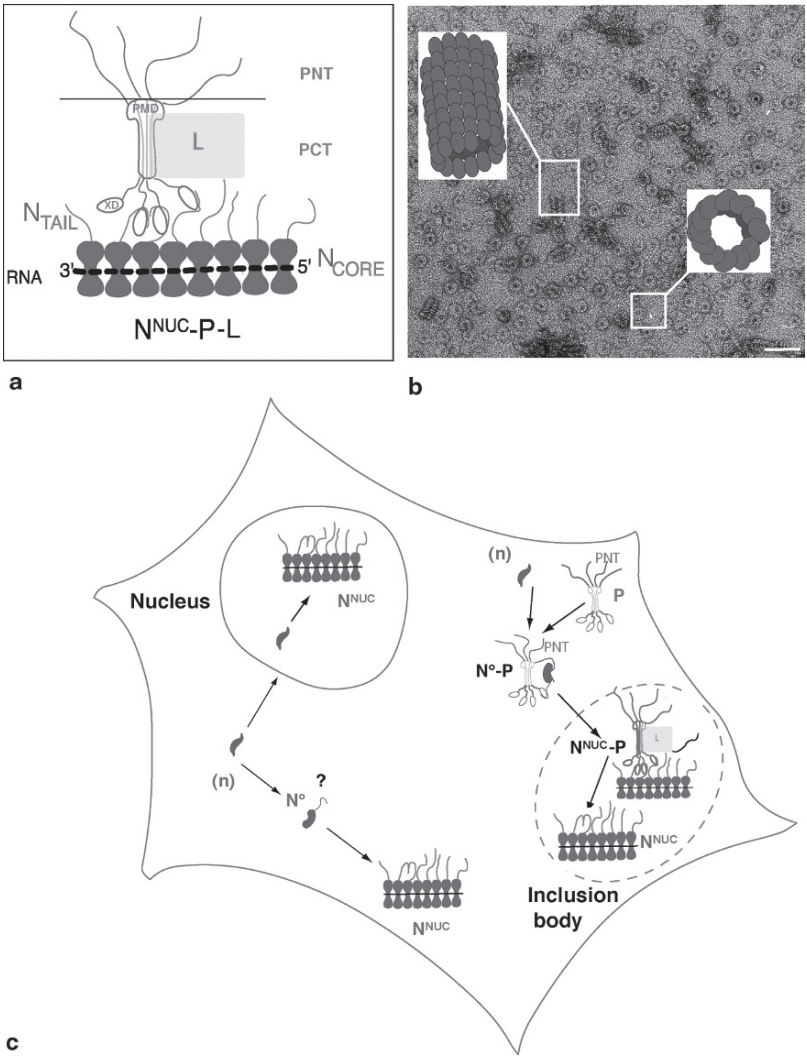

Fig. 1 **A** Schematic representation of the N^{NUC}–P–L complex of measles virus. The disordered N_{TAIL} (aa 401–525) and PNT (aa 1–230) regions are represented by lines. The encapsidated RNA is shown as a dotted line embedded in the middle of N by analogy with *Rhabdoviridae* N-RNA complexes (Albertini et al. 2006; Green et al. 2006). The multimerization domain of P (aa 304–375, PMD) is represented with a dumbbell shape according to Tarbouriech et al. (2000). The tetrameric P (Rahaman et al. 2004) is shown bound to N^{NUC} through three of its four C-terminal XD (aa 459–507) arms, as in the model of Curran and Kolakofsky (1999). The segment connecting PMD and XD is represented as disordered according to Longhi et al. (2003) and Karlin et al. (2003).The L protein is shown as a rectangle contacting P through PMD by analogy with SeV (Smallwood et al. 1994). **B** Negative-staining electron micrographs of bacterially expressed MV N. Nucleocapsid-like herringbone structures are shown on the left, while rings, corresponding to short nucleocapsids seen perpendicularly to their axis, are shown on the right. The bar represents 50 nm. Data shown in **B** were taken from Karlin et al. 2002a. **C** Maturation of MV N according to Gombart et al. (1993). Interior of an infected cell, with the nucleus (*top left*) and a cytoplasmic

The specific encapsidation signal is thought to lay within the 5′ *Leader* and *Trailer* extremities of the antigenome and genome RNA strands, respectively, as demonstrated for another *Mononegavirales* member (Blumberg et al. 1983). However, in the absence of viral RNA and of other viral proteins, *Mononegavirales* nucleoproteins are able to self-assemble onto cellular RNA to form nucleocapsid-like particles. Therefore, a regulatory mechanism is necessary to prevent the illegitimate self-assembly of N in the absence of ongoing genomic RNA synthesis. Indeed, in infected cells N is found in various forms: a soluble, monomeric form (referred to as N°) and an assembled form (referred to as N^{NUC}). Two forms of soluble N are likely to occur in the cytosol: a neosynthesized, transient form (herein referred to as n) and a more mature form, N° (Fig. 1C) (Gombart et al. 1993). The assembled form of N is localized in both the cytosol and the nucleus (Fig. 1C) (Gombart et al. 1993; Horikami and Moyer 1995). Sato and co-workers have recently identified the determinants of the intracellular trafficking within the nucleoprotein sequence of MV and canine distemper virus (CDV), a closely related *Morbillivirus*. They both possess a novel nuclear localization signal (NLS) at positions 70–77 and a nuclear export signal (NES). The NLS has a novel leucine/isoleucine-rich motif (TGILISIL), whereas the NES is composed of a leucine-rich motif (LLRSLTLF). While in CDV the NES occurs at positions 4–11, in MV it is located in the C-terminus (Sato et al. 2006). In both viruses, the nuclear export of N is CRM1-independent.

Once synthesized, the monomeric form of N requires the presence of a chaperone. This role is played by the P protein, whose association with N prevents illegitimate self-assembly of N and also retains the soluble form of N in the cytoplasm (Huber et al. 1991; Spehner et al. 1997). This soluble N°–P complex is used as a substrate for the encapsidation of nascent genomic RNA chain during replication. The assembled form of N also forms complexes with P, either isolated (N^{NUC}–P) or bound to L (N^{NUC}–P–L), which are essential to RNA synthesis by the viral polymerase (Buchholz et al. 1994; Ryan and Portner 1990) (Fig. 1C).

As the nucleoprotein, the P protein provides several functions in transcription and replication. Beyond serving as a chaperone for N, P binds to the nucleocapsid, thus tethering the polymerase onto the nucleocapsid template. P is a modular

Fig. 1 (Continued) inclusion body (*bottom right*). N is found under several conformations. The neosynthesized form (n) and a more mature form (N°) are both cytosolic, whereas the assembled form (N^{NUC}) is probably bound to the cytoskeleton (not shown). The polymerase complex is formed by L and by a tetramer of P. In the absence of P (*left*), n can self-assemble illegitimately on cellular RNA (*bottom*) and migrates to the nucleus, where it forms nucleocapsid-like particles. It is not known whether it undergoes a conformational change directly from n to N^{NUC} or whether N° is an intermediate conformation in the process. P forms a complex with N° (*right*) thereby preventing illegitimate self-assembly of N. The N°–P complex has been represented with a 1:4 stoichiometry by analogy to SeV (J. Curran, personal communication). Within the N°–P complex, the N_{CORE} region has been represented with a shape slightly differing from that of the N^{NUC}–P complex according to Gombart et al. (1993). The N°–P is used by the polymerase to encapsidate neosynthesized RNA during replication (which takes place in the cytoplasmic inclusion bodies). Reprinted with permission from Nova Publishers Inc.

protein, consisting of at least two domains: an N-terminal disordered domain (aa 1–230, PNT) (Karlin et al. 2002b) and a C-terminal domain (aa 231–507, PCT) (for a more detailed description of the modular organization of P see Bourhis et al. 2005a, 2006). Transcription requires only the PCT domain, whereas genome replication also requires PNT.

The viral polymerase, which is responsible for both transcription and replication, is poorly characterized. It is thought to carry out most (if not all) enzymatic activities required for transcription and replication, including nucleotide polymerization, mRNA capping, and polyadenylation. However, no *Paramyxoviridae* polymerase has been purified so far, implying that most of our present knowledge arises from bioinformatics studies. Notably, using bioinformatics approaches, a ribose-2'-O-methyltransferase domain involved in capping of viral mRNAs was identified within the C-terminal region of *Mononegavirales* polymerases (with the exception of *Bornaviridae* and *Nucleorhabdoviruses*) (Ferron et al. 2002). The methyltransferase activity of the C-terminal region of Sendai virus (SeV) polymerase (aa 1756–2228) has been recently demonstrated biochemically (Ogino et al. 2005). Interestingly, the polymerase of vesicular stomatitis virus (VSV, a *Rhabdoviridae* member) has been recently shown to possess both ribose-2'-O and guanine-N-7-methyltransferase activities (Li et al. 2006).

In all *Mononegavirales* members, the viral genomic RNA is always encapsidated by the N protein, and genomic replication does not occur in the absence of N°. Therefore, during RNA synthesis, the viral polymerase has to interact with the N:RNA complex and use the N°–P complex as the substrate of encapsidation. Hence, the components of the viral replication machinery – namely P, N, and L – engage in a complex macromolecular ballet. Although the understanding of the roles of N, P, and L within the replicative complex of MV has benefited from significant breakthroughs in recent years (see Bourhis et al. 2006 for a review), rather limited three-dimensional information on the replicative machinery is available. The scarcity of high-resolution structural data stems from several facts: (a) the problems in obtaining homogenous polymers of N suitable for X-ray analysis (Karlin et al. 2002a; Schoehn et al. 2001), (b) the low abundance of L in virions and its very large size, which renders its heterologous expression difficult, and (c) the structural flexibility of N and P (see below) (Bourhis et al. 2004, 2005b, 2006; Karlin et al. 2002b, 2003; Longhi et al. 2003).

Structural Disorder Within the N and P Proteins

In the course of the structural and functional characterization of MV replicative complex proteins, my group discovered that the N and P proteins contain long (up to 230 residues) disordered regions possessing sequence features that typify intrinsically disordered proteins (IDPs) (Bourhis et al. 2004, 2005a, 2005b, 2006; Karlin et al. 2003; Karlin et al. 2002b; Longhi et al. 2003). By using bioinformatics approaches (see Ferron et al. 2006), structural disorder was shown to be a conserved

and widespread property within *Mononegavirales* N and P proteins, thus implying functional relevance (Karlin et al. 2003).

IDPs, also referred to as natively unfolded proteins, are functional proteins that fulfill essential biological functions while lacking highly populated constant secondary and tertiary structure under physiological conditions (Dunker et al. 2001; Dyson and Wright 2005; Fink 2005; Tompa 2003, 2005; Uversky 2002a; Uversky et al. 2005). Although there are IUPs that carry out their function while remaining disordered all the time (e.g., entropic chains) (Dunker et al. 2001), many of them undergo a disorder-to-order transition upon binding to their physiological partner(s), a process termed induced folding (Dyson and Wright 2002; Fuxreiter et al. 2004; Uversky 2002b).

The protein flexibility inherent in structural disorder is of functional relevance. In particular, an increased plasticity would (a) enable binding of numerous structurally distinct targets, (b) provide the ability to overcome steric restrictions, enabling larger interaction surfaces in protein–protein and protein–ligand complexes than those obtained with rigid partners, and (c) allow protein interactions to occur with both high specificity and low affinity (Dunker et al. 1998, 2001, 2002; Dunker and Obradovic 2001; Dyson and Wright 2005; Fink 2005; Gunasekaran et al. 2003; Uversky et al. 2002; Wright and Dyson 1999).

Regions lacking specific 3D structure have been so far associated with approximately 30 distinct functions, including nucleic acid and protein binding, display of phosphorylation sites and proteolysis sites, prevention of interactions by means of excluded volume effects, and molecular assembly. Most proteins containing disordered regions are involved in signaling and regulation events that generally imply multiple partner interactions (see Chen et al. 2006b; Dunker et al. 2002, 2005; Iakoucheva et al. 2002; Tompa 2003 for reviews). The percentage of the genome encoding protein disorder increases from bacteria to eukaryotes and, more generally, it increases with increasing organism complexity. The increased prevalence of disorder in higher organisms is related to an increased need for cell regulation and signaling. In addition, a recent study published by Dunker and Uversky's group shows that viruses and Eukaryota have ten times more conserved disorder (roughly 1%) than archaea and bacteria (0.1%) (Chen et al. 2006a). The abundance of disorder within viruses likely reflects the need for genetic compaction, where a single disordered protein can establish multiple interactions and hence exert multiple concomitant biological effects.

Structural Organization of the Nucleoprotein

Deletion analyses and electron microscopy studies have shown that *Paramyxoviridae* nucleoproteins are divided into two regions: a structured N-terminal moiety, N_{CORE} (aa 1–400 in MV), which contains all the regions necessary for self-assembly and RNA binding (Bankamp et al. 1996; Buchholz et al. 1993; Curran et al. 1993; Karlin et al. 2002a; Kingston et al. 2004b; Liston et al. 1997; Myers et al. 1997;

Myers et al. 1999), and a C-terminal domain, N_{TAIL} (aa 401–525 in MV), which is intrinsically disordered (Longhi et al. 2003) (Fig. 2A). N_{TAIL} protrudes from the globular body of N_{CORE} and is exposed at the surface of the viral nucleocapsid (Heggeness et al. 1980, 1981; Karlin et al. 2002a). N_{TAIL} contains the regions responsible for binding to P in both N°–P and N^{NUC}–P complexes (Bankamp et al. 1996; Kingston et al. 2004b; Liston et al. 1997; Longhi et al. 2003).

The Structured N_{CORE} Domain

N_{CORE} contains all the regions necessary for self-assembly and RNA binding, since nucleoproteins composed only of the core region can encapsidate neosynthesized RNA into nucleocapsid-like particles. Within N_{CORE}, deletion studies have failed to identify independent, modular domains, but have identified regions involved in the N–N interaction. The region spanning aa 258–357, called the central conserved region (CCR), is well conserved in sequence, and mainly hydrophobic (Fig. 2A). Several studies have shown that it is one of the regions involved in self-association and in RNA binding (Karlin et al. 2002a; Liston et al. 1997). The location of the RNA-binding site(s) within N_{CORE} is unknown. When denatured, such as in Northwestern blots, N does not bind RNA, indicating that the RNA-binding site is probably formed by maturation of N during encapsidation (Lamb and Kolakofsky 2001). In agreement, all mutants in which self-association is impaired do not package RNA (Karlin et al. 2002a; Myers et al. 1999). In the closely related SeV (a *Respirovirus* member), more subtle mutations in the 360–375 region were found that disrupted neither RNA binding nor the morphology of N, but rendered N inactive in replication (Myers et al. 1999). The author suggested that particular residues in this region might be involved in binding the leader sequence of SeV RNA, as opposed to nonspecific RNA. However, it is more likely that mutations within this region could affect the ability of N to interact with PNT, thereby preventing either formation or proper conformation of the N°–P complex.

Beyond the CCR, another region of N–N interaction was found within the 189–239 residues of MV N (see Fig. 2A), as deletion of this region led to a nucleoprotein variant form that can still bind P but has lost its ability to self-assemble (Bankamp et al. 1996). In further support of a role of this additional region in N assembly, two point substitutions thereof (namely, S228Q and L229D) impair self-association of N and RNA binding without affecting either the overall secondary structure content or the gross domain organization and the ability to bind P (Karlin et al. 2002a).

Indeed, a major hurdle to X-ray crystallography techniques is the strong self-assembly of N to form large nucleocapsids with a broad size distribution when expressed in heterologous systems such as mammalian cells (Spehner et al. 1991), bacteria (Warnes et al. 1995) and insect cells (Bhella et al. 2002). Because of this property, *Paramyxoviridae* nucleoproteins have resisted high-resolution structure determination so far. Because of variable helical parameters, the recombinant or

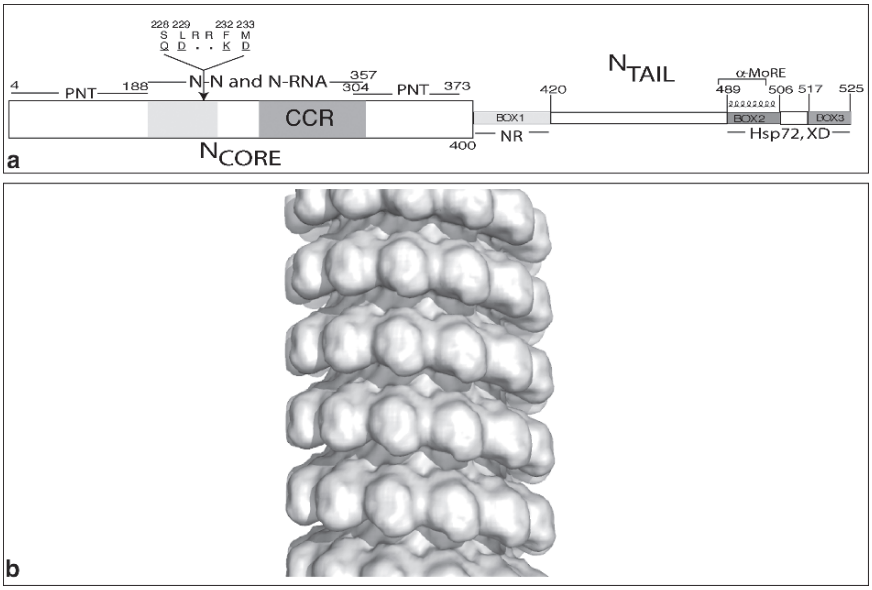

Fig. 2 **A** Organization of MV N. The location of N-N, N-P, and RNA binding sites is indicated. The central conserved region (CCR, aa 258–357) (*dark gray*) and the additional region involved in oligomerization (aa 189–239; *light gray*) are also shown. The positions targeted for mutagenesis (see text) are shown by an *arrow*. Wild-type residues are shown in the *top position*, while mutated residues are shown *below*. The location of the N_{CORE}-PNT sites (aa 4–188 and 304–373), as reported by Bankamp et al. (1996), is also shown. **B** Reconstruction of MV nucleocapsid as obtained from cryoelectron microscopy. Data were kindly provided by David Bhella (MRC, Glasgow, UK). The picture was drawn using Pymol (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA. http://www.pymol.org). (Reprinted with permission from Nova Publishers Inc.)

viral nucleocapsids are also difficult to analyze using electron microscopy coupled to image analysis. Despite these technical drawbacks, elegant electron microscopy studies by two independent groups led to real-space helical reconstruction of MV nucleocapsids (Bhella et al. 2004; Schoehn et al. 2004) (Fig. 2B). These studies pointed out a considerable conformational flexibility, with the most extended conformation having a helical pitch of 66 Å, while twist varies from 13.04 to 13.44 (Bhella et al. 2004). Notably, these studies also highlighted cross-talk between N_{CORE} and N_{TAIL}, based on the observation that removal of the disordered N_{TAIL} domain leads to increased nucleocapsid rigidity, with significant changes in both pitch and twist (see Bhella et al. 2004; Longhi et al. 2003; Schoehn et al. 2004).

Conversely, high-resolution structural data are available for two *Rhabdoviridae* members, namely the rabies virus and the VSV (Albertini et al. 2006; Green et al. 2006; Luo et al. 2007). The nucleoprotein of these viruses consists of two lobes and possesses an extended terminal arm that makes contacts with a neighboring N monomer. The RNA is tightly packed in a cavity between the two N lobes. N establishes contacts with the sugar and phosphate moiety of nucleotides via basic

residues, in agreement with previous studies showing that the phosphate moieties of encapsidated RNA are not accessible to the solvent (Iseni et al. 2000). In both nucleoproteins, the RNA is not accessible to the solvent. Thus, it has to partially dissociate from N to become accessible to the polymerase.

Functional and structural similarities between the nucleoproteins of *Rhabdoviridae* and *Paramyxoviridae* are well established. In particular, they share the same organization in two well-defined regions, N_{CORE} and N_{TAIL}, and in both families the CCR is involved in RNA binding and self-assembly of N (Kouznetzoff et al. 1999). Moreover, incubation of the rabies virus nucleocapsid with trypsin results in the removal of the C-terminal region (aa 377–450) (Kouznetzoff et al. 1998). This N_{TAIL}-free nucleocapsid is no longer able to bind to P, thus suggesting that in *Rhabdoviridae*, N_{TAIL} plays a role in the recruitment of P, like in *Paramyxoviridae* (Schoehn et al. 2001). However, contrary to MV, the rabies virus N_{TAIL} domain is structured (Albertini et al. 2006). Presently, it is not known whether *Rhabdoviridae* and *Paramyxoviridae* nucleoproteins share the same bilobal morphology. In MV N, bioinformatics analyses predict that N_{CORE} is organized into two subdomains (aa 1–130 and aa 145–400) (see Bourhis et al. 2007; Ferron et al. 2006) separated by a hypervariable, antigenic loop (aa 131–149) (Giraudon et al. 1988), that would probably fold cooperatively into a bilobal morphology.

Although within the MV N^{NUC}–P complex, the N region responsible for binding to P is located within N_{TAIL}, the N°–P complex involves an additional interaction between N_{CORE} and the disordered N-terminal domain of P (PNT) (Fig. 1C). Within the N°–P complex, P-to-N binding is mediated by the dual PNT–N_{CORE} and PCT-N_{TAIL} interaction (Chen et al. 2003) (Fig. 1C). Studies on SeV suggested that an N°–P complex is absolutely necessary for the polymerase to initiate encapsidation (Baker and Moyer 1988). Therefore, formation of the N°–P complex would have at least two separate functions: (a) preventing illegitimate self-assembly of N and (b) allowing the polymerase to deliver N to the nascent RNA to initiate replication.

The regions within N_{CORE} responsible for binding PNT within the N°–P complex have been mapped to residues 4–188 and 304–373, with the latter region being not strictly required for binding but rather favoring it (Bankamp et al. 1996) (Fig. 2A). However, precise mapping of such regions is hard because N_{CORE} does not have a modular structure, and consequently it is difficult to distinguish between gross structural defects and specific effects of deletions.

The Intrinsically Unstructured N_{TAIL} Domain

In *Morbilliviruses*, N_{TAIL} is responsible for binding to P in both N°–P and N^{NUC}–P complexes (Bankamp et al. 1996; Kingston et al. 2004; Liston et al. 1997; Longhi et al. 2003). Within the N^{NUC}–P complex, N_{TAIL} is also responsible for the interaction with the polymerase (L–P) complex (Bankamp et al. 1996; Kingston et al. 2004; Liston et al. 1997; Longhi et al. 2003). Several features distinguish N_{TAIL} from N_{CORE}. Indeed, N_{TAIL} possesses features that are hallmarks of intrinsic disorder:

(a) it is hypersensitive to proteolysis (Karlin et al. 2002a), (b) it is not visualizable in cryoelectron microscopy reconstructions of nucleocapsids (Bhella et al. 2004), and (c) it has an amino acid sequence that is hypervariable among *Morbillivirus* members.

In agreement with these features suggesting disorder, the sequence properties of N_{TAIL} conform to those of IDPs (Dunker et al. 2001). Indeed, while the amino acid composition of N_{CORE} does not deviate from the average composition of proteins found in the Protein Data Bank (PDB), the N_{TAIL} region is depleted in order-promoting residues (W, C, F, Y, I, L) and enriched in disorder-promoting residues (R, Q, S, and E) (Longhi et al. 2003). Moreover, N_{TAIL} is predicted to be mainly (if not fully) disordered (data not shown; Bourhis et al. 2004; Longhi et al. 2003) by the secondary structure and disorder predictors implemented within the MeDor metaserver (Lieutaud et al., in press).

The disordered state of N_{TAIL} was experimentally confirmed by several spectroscopic and hydrodynamic approaches (see Receveur-Bréchot et al. 2006 for a review on methods to assess structural disorder and induced folding). Altogether, these studies pointed out that N_{TAIL} is a premolten globule (Bourhis et al. 2004; Longhi et al. 2003), i.e., it has a conformational state intermediate between a random coil and a molten globule (Dunker et al. 2001; Uversky 2002a). In solution, premolten globules possess a certain degree of residual compactness due to the presence of residual and fluctuating secondary and tertiary structures. As for the functional implications, it has been proposed that the residual intramolecular interactions that typify the premolten globule state may enable a more efficient start of the folding process induced by a partner (Fuxreiter et al. 2004; Lacy et al. 2004; Tompa 2002).

That N_{TAIL} does indeed undergo induced folding was documented by CD studies, where N_{TAIL} was shown to undergo an α-helical transition in the presence of PCT (Longhi et al. 2003). Using computational approaches, an α-helical *M*olecular *R*ecognition *E*lement (α-MoRE, aa 488–499 of N) has been identified within N_{TAIL} (see Fig. 2A). MoREs are regions within IDPs that have a certain propensity to bind to a partner and thereby undergo induced folding (Garner et al. 1999; Mohan et al. 2006; Oldfield et al. 2005; Vacic et al. 2007). The role of the α-MoRE in binding to P and in the α-helical induced folding has further been confirmed by spectroscopic and biochemical experiments carried out on a truncated N_{TAIL} form devoid of the 489–525 region (Bourhis et al. 2004).

The P region responsible for the interaction with N_{TAIL} and its induced folding has been mapped to the C-terminal module (XD, aa 459–507; see Fig. 1A) of P (Johansson et al. 2003). The crystal structure of XD has been solved and consists of a triple α-helical bundle. A model of the interaction between XD and the α-MoRE of N_{TAIL} was then built in which N_{TAIL} is embedded in a large XD hydrophobic cleft delimited by helices α2 and α3 of XD. According to this model, burying of hydrophobic residues of the α-MoRE would provide the driving force to induce its folding, thus leading to a pseudo-four-helix arrangement occurring frequently in nature (Johansson et al. 2003). This model was thereafter validated by Kingston and co-workers who solved the crystal structure of a chimeric form mimicking this complex (Fig. 3) (Kingston et al. 2004).

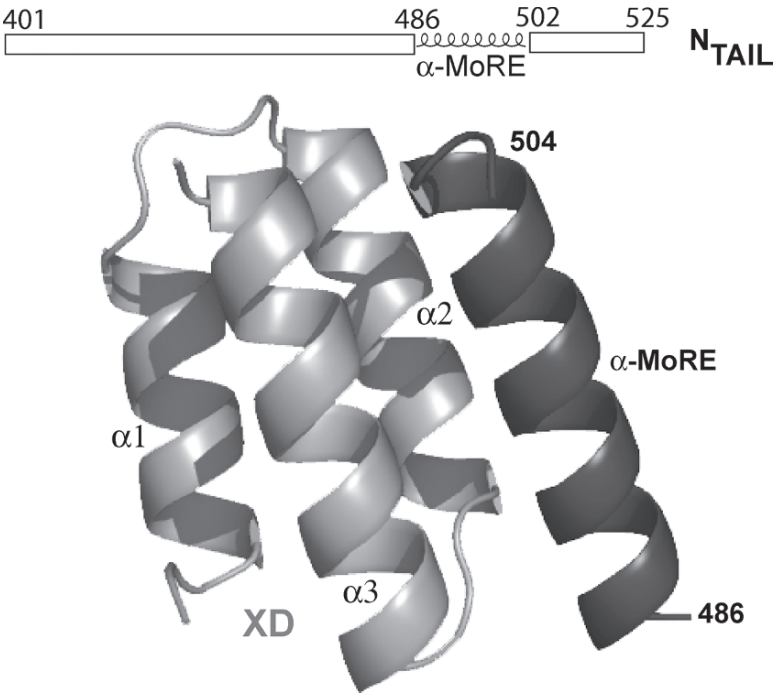

Fig. 3 Ribbon representation of the crystal structure of the complex between XD and the 486–504 region of N_{TAIL} (pdb code: 1T60) (Kingston et al. 2004). The picture was drawn using Pymol (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA, http://www.pymol.org). The schematic representation of N_{TAIL} with the α-MoRE highlighted is shown on the top

Small-angle X-ray scattering studies provided a low resolution model of the N_{TAIL}–XD complex, which showed that most of N_{TAIL} (aa 401–488) remains disordered within the complex and supported a role for the C-terminus in binding to XD (Bourhis et al. 2005). The involvement of the C-terminus of N_{TAIL} in binding to XD was indeed confirmed by spectroscopic and surface plasmon resonance (BIAcore) studies, where removal of either Box3 alone or Box2 plus Box3 (see Fig. 2A) results in a strong increase (three orders of magnitude, 10μM K_D) in the equilibrium dissociation constant (Bourhis et al. 2005). When synthetic peptides mimicking Box1, Box2, and Box3 were used, Box2 peptide was found to display an affinity toward XD (20nM K_D) similar to that of N_{TAIL} (80nM K_D), consistent with the role of Box2 as the primary binding site (S. Longhi and M.J. Oglesbee, unpublished data). Interestingly however, Box3 peptide exhibits an insignificant affinity for XD (approximately 1mM K_D) (S. Longhi and M.J. Oglesbee, unpublished data). The discrepancy between the data obtained with N_{TAIL} truncated proteins and with peptides can be accounted for by assuming that Box3 would act only in the context of N_{TAIL} and not in isolation. Thus, according to this model, Box3 and Box2 would be functionally coupled in the binding of N_{TAIL} to XD. One can speculate that burying

the hydrophobic side of the α-MoRE in the hydrophobic cleft formed by helices α2 and α3 of XD would provide the primary driving force in the N_{TAIL}-XD interaction and that Box3 would act by stabilizing the bound conformation.

Heteronuclear NMR (HN-NMR) studies using ^{15}N-labeled N_{TAIL} or a truncated form devoid of Box3 and unlabeled XD, revealed that while Box2 undergoes α-helical folding upon binding to XD, Box3 does not acquire any regular secondary structure element (Bourhis et al. 2005).

The molecular mechanism of the XD-induced folding of N_{TAIL} has been also investigated by using site-directed spin-labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy. The basic strategy of SDSL involves the introduction of a paramagnetic nitroxide side chain at a selected protein site. This is usually accomplished by cysteine-substitution mutagenesis, followed by covalent modification of the unique sulphydryl group with a selective nitroxide reagent, such as the methanethiosulphonate derivative (see Biswas et al. 2001; Feix and Klug 1998; Hubbell et al. 1998 for reviews). From the EPR spectral shape of a spin-labeled protein, one can extract information in terms of radical mobility, which reflects the local mobility of residues in the proximity of the radical. Variations in the radical mobility can therefore be monitored in the presence of partners, ligands, or organic solvents.

Fourteen single-site N_{TAIL} cysteine mutants were designed, purified, and labeled, thus enabling grafting of a nitroxide paramagnetic probe on 12 sites scattered in the 488–525 region and on two sites located outside the reported region of interaction with XD (Morin et al. 2006; V. Belle et al., in press). EPR spectra were then recorded in the presence of either the secondary structure stabilizer 2,2,2-trifluoroethanol (TFE) or XD.

Different regions of N_{TAIL} were shown to contribute to a different extent to the binding to XD: while the mobility of the spin labels grafted at positions 407 and 460 was unaffected upon addition of XD, that of the spin labels grafted within the 488–502 and the 505–522 regions was severely and moderately reduced, respectively. Furthermore, EPR experiments in the presence of 30% sucrose (i.e., under conditions in which the intrinsic motion of the protein becomes negligible with respect to the intrinsic motion of the spin label), allowed precise mapping of the N_{TAIL} region undergoing α-helical folding to residues 488–502. The drop in the mobility of the 505–522 region upon binding to XD was shown to be comparable to that observed in the presence of TFE, thus suggesting that the restrained mobility that this region experiences upon binding to XD is not due to a direct interaction with XD.

The mobility of the 488–502 region was found to be restrained even in the absence of the partner, a behavior that could be accounted for by the existence of a transiently populated folded state. This may reflect the predominance of an α-helical conformation among the highly fluctuating conformations sampled by unbound N_{TAIL}, in agreement with previous biochemical and spectroscopic data that mapped the region involved in the α-helical-induced folding to residues 489–506 (Bourhis et al. 2004). That the conformational space of MoREs (Oldfield et al. 2005) in the unbound state is restricted by their inherent conformational propensities, thereby reducing the entropic cost of binding, has already been proposed (Fuxreiter et al. 2004; Lacy et al. 2004; Sivakolundu et al. 2005; Tompa 2002).

Finally, equilibrium displacement experiments showed that the XD-induced folding of N_{TAIL} is a reversible phenomenon (Morin et al. 2006). These results represent the first experimental evidence indicating that N_{TAIL} adopts its original premolten globule conformation after dissociation from its partner. This latter point is particularly relevant taking into consideration that the contact between XD and N_{TAIL} within the replicative complex has to be dynamically made and broken to allow the polymerase to progress along the nucleocapsid template during both transcription and replication. Hence, the complex cannot be excessively stable for this transition to occur efficiently at a high rate.

In conclusion, using a panel of various physicochemical approaches, the interaction between N_{TAIL} and XD was shown to imply the stabilization of the helical conformation of the α-MoRE, which is otherwise only transiently populated in the unbound form. The occurrence of a transiently populated α-helix, even in the absence of the partner, suggests that the molecular mechanism governing the folding of N_{TAIL} induced by XD may rely on conformer selection (i.e., selection by the partner of a preexisting conformation) (Tsai et al. 2001a, 2001b) rather than on a fly-casting mechanism (Shoemaker et al. 2000), contrary to what has been reported for the pKID–KIX couple (Sugase et al. 2007).

Stabilization of the helical conformation of the α-MoRE is also accompanied by a reduction in the mobility of the downstream region. The lower flexibility of the region downstream Box2 is not caused by a gain of α-helicity, nor can it be ascribed to a restrained motion brought by a direct interaction with XD. Rather, it likely arises from a gain of rigidity brought by α-helical folding of the neighboring Box2 region.

In agreement with these data and with the BIAcore data obtained using a synthetic Box3 peptide, preliminary titration studies using heteronuclear NMR suggest that XD does not establish direct interactions with Box3 (H. Darbon and S. Longhi, unpublished data). A tentative model can be proposed, where binding to XD might take place through a sequential mechanism that could involve binding and α-helical folding of Box2, followed by a conformational change of Box3, whose overall mobility is consequently reduced, probably through tertiary contacts with the neighboring Box2 region.

Functional Role of Structural Disorder of N_{TAIL} for Transcription and Replication

The K_D value between N_{TAIL} and XD is in the 100nM range (Bourhis et al. 2005). This affinity is considerably higher than that derived from isothermal titration calorimetry studies which pointed out a KD of 13μM (Kingston et al. 2004). A weak binding affinity, implying fast association and dissociation rates, would ideally fulfill the requirements of a polymerase complex that has to cartwheel on the nucleocapsid template during both transcription and replication. However, a K_D in the micromolar range would not seem to be physiologically relevant considering the low

intracellular concentrations of P in the early phases of infection and the relatively long half-life of active P–L transcriptase complex tethered on the NC template, which has been determined to be well over 6 h (Plumet et al. 2005). Moreover, such a weak affinity is not consistent with the ability to readily purify nucleocapsid–P complexes using rather stringent techniques such as CsCl isopycnic density centrifugation (Oglesbee et al. 1989; Robbins and Bussell 1979; Robbins et al. 1980; Stallcup et al. 1979). A more stable XD–N_{TAIL} complex would be predicted to hinder the processive movement of P along the nucleocapsid template. In agreement with this model, the elongation rate of MV polymerase was found to be rather slow (three nucleotides/s) (Plumet et al. 2005). In addition, the C-terminus of N_{TAIL} has been shown to have an inhibitory role upon transcription and replication, as indicated by minireplicon experiments, where deletion of the C-terminus of N enhanced basal reporter gene expression (Zhang et al. 2002). Deletion of the C-terminus of N also reduces the affinity of XD for N_{TAIL}, providing further support for modulation of XD/N_{TAIL} binding affinity as a basis for polymerase processivity. Thus, Box3 would dynamically control the strength of the N_{TAIL}-XD interaction, by stabilizing the Box2–XD interaction. Removal of Box3 or interaction of Box3 with other partners (see the next paragraph) would reduce the affinity of N_{TAIL} for XD, thus stimulating transcription. Modulation of XD/N_{TAIL} binding affinity could be dictated by interactions between N_{TAIL} and cellular and/or viral co-factors. Indeed, the requirement for cellular or viral co-factors in both transcription and replication has been already documented in MV (Vincent et al. 2002) and other *Mononegavirales* members (Fearns and Collins 1999; Hartlieb et al. 2003). Furthermore, in both CDV and MV, viral transcription and replication are enhanced by the major heat shock protein (hsp72), and this stimulation relies on interaction with N_{TAIL} (Zhang et al. 2002, 2005). These co-factors may serve as processivity or transcription elongation factors and could act by modulating the strength of the interaction between the polymerase complex and the nucleocapsid template (see the next paragraph).

N_{TAIL} also influences the physical properties of the nucleocapsid helix that is formed by N_{CORE} (Longhi et al. 2003; Schoehn et al. 2004). Electron microscopic analysis of nucleocapsids formed by either N or N_{CORE} indicates that the presence of N_{TAIL} was associated with a greater degree of fragility, evidenced by the tendency of helices to break into individual ring structures (Fig. 4A). This fragility is associated with evidence of increased nucleocapsid flexibility, with helices formed by N_{CORE} alone forming rods (Fig. 4A) (see also Schoehn et al. 2004). It is therefore conceivable that the induced folding of N_{TAIL} resulting from the interaction with P (and/or other physiological partners) could also affect the nucleocapsid conformation in such a way as to affect the structure of the replication promoter (Fig. 4B). Indeed, the replication promoter, located at the 3′ end of the viral genome, is composed of two discontinuous elements building up a functional unit because of their juxtaposition on two successive helical turns (Tapparel et al. 1998) (Fig. 4B). The switch between transcription and replication could be dictated by variations in the helical conformation of the nucleocapsid, which would result in a modification in the number of N monomers (and thus of nucleotides) per turn, thereby disrupting the replication promoter in favor of the transcription promoter (or vice-versa).

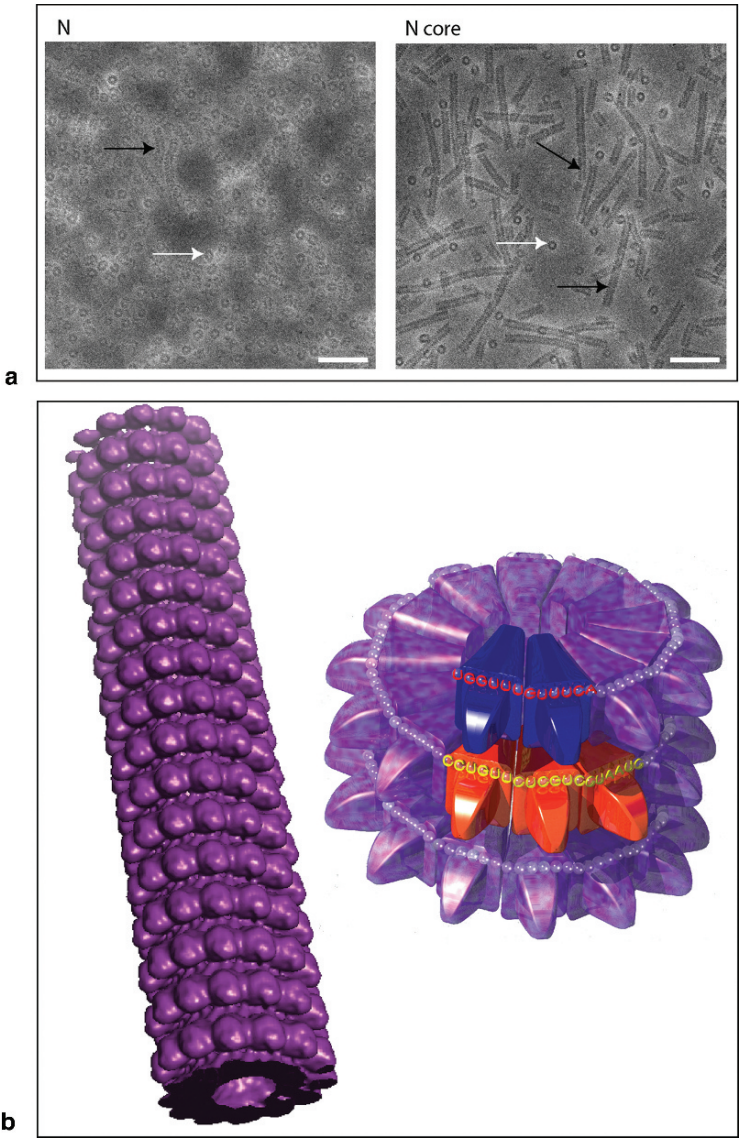

Fig. 4 **A** Negative stain electron micrographs of N and N_{CORE}. The bar corresponds to 100 nm. Rings and herringbone structures are indicated by *white and black arrows*, respectively. **B** Cryoelectron microscopy reconstructions of MV nucleocapsid (*left*) and schematic representation of the nucleocapsid (*right*), highlighting the structure of the replication promoter composed of two discontinuous units juxtaposed on successive helical turns (see regions wrapped by the *red* and *blue* N monomers). (Courtesy of D. Bhella, MRC, Glasgow, UK). Data in **A** were taken from Longhi et al. (2003). Reprinted with permission from Nova Publishers Inc.

Morphological analyses, showing the occurrence of a large conformational flexibility within *Paramyxoviridae* nucleocapsids (Bhella et al. 2002, 2004; Oglesbee et al. 1989, 1990), tend to corroborate this hypothesis.

Finally, preliminary data indicate that incubation of MV NCs in the presence of XD triggers unwinding of the NC, thus possibly enhancing the accessibility of genomic RNA to the polymerase complex (D. Bhella and S. Longhi, unpublished data). Hence, it is tempting to propose that the XD-induced α-helical folding of N_{TAIL} could trigger the opening of the two lobes of N_{CORE}, thus rendering the genomic RNA accessible to the solvent.

Unstructured regions are considerably more extended in solution than globular regions. For instance, MV PNT has a Stokes radius of 4 nm (Karlin et al. 2002). However, the Stokes radius only reflects a mean dimension. Indeed, the maximal extension of PNT, as measured by SAXS (S. Longhi et al., unpublished data) is considerably larger (>40 nm). In comparison, one turn of the nucleocapsid is 18 nm in diameter and 6 nm high (Bhella et al. 2002). Thus PNT could easily stretch over several turns of the nucleocapsid, and since P is multimeric, N°–P might have a considerable extension (Fig. 5). In the same vein, it is striking that SeV and MV PCT, which interacts with the intrinsically disordered N_{TAIL} domain, comprises a flexible linker (Bernado et al. 2005; Blanchard et al. 2004; Longhi et al. 2003; Marion et al. 2001). This certainly suggests the need for a great structural flexibility. This flexibility could be necessary for the tetrameric P to bind several turns of the helical nucleocapsid. Indeed, the promoter signals for the polymerase are located on the first and the second turn of the SeV nucleocapsid (Tapparel et al. 1998).

Likewise, the maximal extension of N_{TAIL} in solution is of 13 nm (Longhi et al. 2003). The very long reach of disordered regions could enable them to act as linkers and to tether partners on large macromolecular assemblies. Accordingly, one role of the tentacular N_{TAIL} projections in actively replicating nucleocapsids could be to put into contact several proteins within the replicative complex, such as the N°–P and the P–L complexes (see Fig. 5).

In *Respiroviruses* and *Morbilliviruses*, PNT, which is also disordered (Karlin et al. 2003), contains binding sites for N° (Curran et al. 1994; Harty and Palese 1995; Sweetman et al. 2001) and for L (Curran et al. 1995; Curran and Kolakofsky 1991; Sweetman et al. 2001). This pattern of interactions among N°, P, and L, mediated by unstructured regions of either P or N, suggests that N°, P, and L might interact simultaneously at some point during replication. Notably, the existence of a N–P–L tripartite complex has been proved by co-immunoprecipitation studies in the case of VSV, where this tripartite complex constitutes the replicase complex, as opposed to the L–P binary transcriptase complex (Gupta et al. 2003).

A model can be proposed where during replication, the extended conformation of PNT and N_{TAIL} would be key to allowing contact between the assembly substrate (N°–P) and the polymerase complex (L–P), thus leading to a tripartite N°–P–L complex (Fig. 5). This model emphasizes the plasticity of intrinsically disordered regions, which might give a considerable reach to the elements of the replicative machinery.

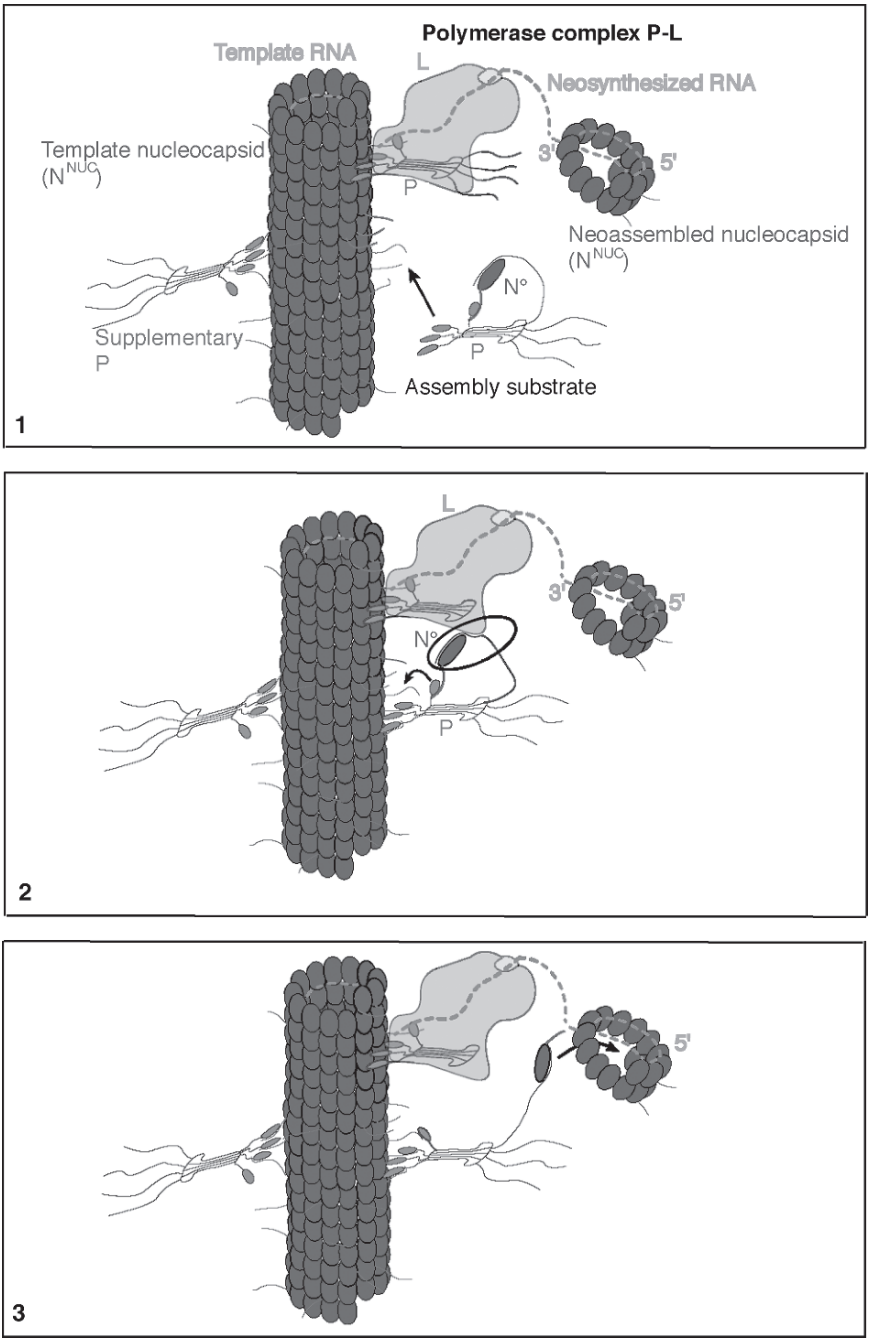

Fig. 5 Model of the polymerase complex actively replicating genomic RNA. Disordered regions are represented by *lines*. The location of the viral RNA (*dotted line*) within the N^{NUC}–P complex is schematically represented at the interior of the nucleocapsid by analogy with *Rhabdoviridae* N-RNA complexes (Albertini et al., 2006; Green et al., 2006). Within the N^{NUC}–P

Interestingly, there is a striking parallel between the N_{TAIL}–XD interaction and the PNT-N_{CORE} interaction (Fig. 5). Both interactions are not stable by themselves and must be strengthened by the combination of other interactions. This might ensure easy breaking and reforming of interactions. The relative weak affinity that typifies interacting disordered regions, together with their ability to establish contacts with other partners serving as potential regulators, would ensure dynamic breaking and reforming of interactions. This would result in transient, easily modulated interactions. One can speculate that the gain of structure of N_{TAIL} upon binding to XD could result in stabilization of the N–P complex. At the same time, N_{TAIL} folding would result in a modification in the pattern of solvent-accessible regions, resulting in the shielding of specific regions of interaction. As a result, N_{TAIL} would no longer be available for binding to its other partners. Although induced folding likely enhances the affinity between interacting proteins, the dynamic nature of these interactions could rely on (a) the intervention of viral and/or cellular cofactors modulating the strength of such interactions and (b) the ability of the IDP to establish weak affinity interactions through residual disordered regions.

Finally, since binding of N_{TAIL} to XD allows tethering of the L protein on the NC template, the N_{TAIL}–XD interaction is crucial for both viral transcription and replication. Moreover, as neither N_{TAIL} nor XD have cellular homologs, this interaction is an ideal target for antiviral inhibitors. In silico screening of small compounds for their ability to bind to the hydrophobic cleft of XD is in progress. A few candidate molecules are being tested for their ability to bind to XD and to prevent interaction with N_{TAIL} using HN-NMR (X. Morelli and S. Longhi, unpublished data).

N_{TAIL} and Molecular Partnership

The disordered nature of N_{TAIL} confers to this N domain the ability to adapt to various partners and to form complexes that are critical for both transcription and replication. Indeed, given its exposure at the surface of the viral nucleocapsid, N_{TAIL}

Fig. 5 (Continued) complex, P is represented as bound to N^{NUC} through three of its four terminal XD arms according to the model of Curran and Kolakofsky (1999). Only a few N_{TAIL} regions are drawn. PNT regions within the L–P complex have not been represented in panels 2 and 3. The P molecule delivering N° has been represented as distinct from that within the L–P complex in agreement with the results of Tuckis et al. (2002). The numbering of the different panels indicates the chronology of events. (1) L is bound to a P tetramer. A supplementary P molecule, not bound to L, is also shown (*right*). The newly synthesized RNA is shown as already partially encapsidated. (2) The encapsidation complex, N°–P, binds to the nucleocapsid template through three of its four XD arms. The extended conformation of N_{TAIL} and PNT would allow the formation of a tripartite complex between N°, P, and the polymerase (*circled*). It is tempting to imagine that the proximity of the polymerase (or an unknown signal from this) may promote the release of N° by XD, thus leading to N° incorporation within the assembling nucleocapsid. The N° release would also lead to cartwheeling of the L–P complex through binding of the free XD arm onto the nucleocapsid template (*arrow*) as in the model of Curran and Kolakofsky (1999). (3) PNT delivers N° to the newly assembled nucleocapsid (*arrow*). Reprinted with permission from Nova Publishers Inc.

establishes numerous interactions with various viral partners, including P, the P–L complex, and possibly the matrix protein (Coronel et al. 2001). Beyond viral partners, N_{TAIL} also interacts with several cellular proteins, including the heat-shock protein hsp72 (Zhang et al. 2002, 2005), the cell protein responsible for the nuclear export of N (Sato et al. 2006) and possibly components of the cell cytoskeleton (De and Banerjee 1999; Moyer et al. 1990). Moreover, N_{TAIL} within viral nucleocapsids released from infected cells also binds to the yet unidentified nucleoprotein receptor (NR) expressed at the surface of human thymic epithelial cells (Laine et al. 2003).

The interaction between N_{TAIL} and hsp72 stimulates both transcription and genome replication. Two binding sites for hsp72 have been identified (Zhang et al. 2002, 2005). High-affinity binding is supported by the α-MORE, and hsp72 can competitively inhibit binding of XD to N_{TAIL} (Zhang et al. 2005). A second low-affinity binding site is present in the C-terminus of N_{TAIL} (Zhang et al. 2002). Variability in sequence of the N protein C-terminus gives rise to hsp72 binding and nonbinding variants. Analysis of infectious virus containing a nonbinding motif shows loss of hsp72-dependent stimulation of transcription but not genome replication (Zhang et al. 2005). These findings suggest two mechanisms by which hsp72 could enhance transcription and genome replication, and both involve reducing the stability of P/N_{TAIL} complexes, thereby promoting successive cycles of binding and release that are essential to polymerase processivity (Bourhis et al. 2005; Zhang et al. 2005). The first mechanism is competition between hsp72 and XD for α-MORE binding, and this would occur at low hsp72 concentrations. In the second mechanism, hsp72 would neutralize the contribution of the C-terminus of N_{TAIL} to the formation of a stable $P\text{-}N_{TAIL}$ complex, and this would occur in the context of elevated cellular levels of hsp72 and only for MV strains that support hsp72 binding in this region (Zhang et al. 2005). The basis for the separable effects of hsp72 on genome replication versus transcription remains to be shown, with template changes unique to a replicase versus transcriptase being a primary candidate. The latter could involve unique nucleocapsid ultrastructural morphologies, with hsp72-dependent morphologies being well documented for CDV (see Oglesbee et al. 1989, 1990).

As for the functional role of hsp72 in the context of MV infection, it has been proposed that the elevation in the hsp72 levels in response to the infection could contribute to virus clearance (Carsillo et al. 2004; Oglesbee et al. 2002). Indeed, the stimulation of viral transcription and replication by hsp72 is also associated with cytopathic effects, leading to apoptosis and release of viral proteins in the extracellular compartment (Oglesbee et al. 1993; Vasconcelos et al. 1998a, 1998b). These would stimulate the adaptive immune response, thereby leading to virus clearance.

Finally, after apoptosis of infected cells, the viral nucleocapsid is released in the extracellular compartment, where it becomes available to cell surface receptors. While N_{CORE} specifically interacts with FcγRII (Laine et al. 2005), N_{TAIL} interacts with the yet uncharacterized NR, which is expressed at the surface of dendritic cells of lymphoid origin (both normal and tumoral) (Laine et al. 2003), and of T and B lymphocytes (Laine et al. 2005). The N_{TAIL}-NR interaction triggers an arrest in the G_0/G_1 phase of cell cycle, whereas the N_{CORE}–FcγRII interaction triggers apoptosis

(Laine et al. 2005). Both mechanisms have the potential to contribute to immunosuppression, which is a hallmark of MV infections (Laine et al. 2005).

Flow cytofluorimetry studies conducted on truncated forms of N_{TAIL} identified the N_{TAIL} region responsible for the interaction with NR (Box1, aa 401–420) (Laine et al. 2005). SDSL EPR studies in the presence of TFE pointed out a structural propensity within Box1 (Morin et al. 2006), the biological relevance of which may reside in a gain of structure possibly arising upon binding to NR. It is tempting to speculate that Box1 could undergo induced folding upon binding to NR. However, definitive answers on gain of regular secondary structure elements by Box1 upon binding to NR awaits the isolation of this receptor and the molecular characterization of its interaction with N_{TAIL}.

Conclusions

As thoroughly discussed in this chapter, N is a pleiotropic protein exerting multiple biological functions. The molecular bases for this pleiotropy reside in the ability of the C-terminal domain to establish interactions with various cellular and viral partners. Since many, if not all, of these interactions are critical for transcription and replication, they provide excellent targets for antiviral agents. In this context, the discovery that the N domain supporting these multiple protein interactions is intrinsically disordered is particularly relevant: indeed, protein–protein interactions mediated by disordered regions provide interesting drug discovery targets with the potential to increase the discovery rate for new compounds significantly (Cheng et al. 2006). These could eventually be used against MV and/or other *Mononegavirales* pathogens.

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