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Review

Structural aspects of rabies virus replication

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Abstract. Rabies virus is a negative-strand RNA virus. Its RNA genome is condensed by the viral nucleoprotein (N), and it is this N-RNA complex that is the template for transcription and replication by the viral RNA-dependent RNA polymerase complex. Here we discuss structural and functional aspects of viral transcription and replication based on the atomic structure of a recombinant rabies virus N-RNA complex. We situate available biochemical data on N-RNA interactions with viral and cellular factors in the structural framework with regard to their implications for transcription and replication. Finally, we compare the structure of the rabies virus nucleoprotein with the structures of the nucleoproteins of vesicular stomatitis virus, Borna disease virus and influenza virus, highlighting potential similarities between these virus families.

Keywords. Nucleoprotein N, rabies virus, VSV, influenza virus, phosphoprotein P, nucleocapsid.

Introduction

Rabies virus infection causes a severe neurological disorder in humans. Its course of infection is always fatal when unrecognised [1]. Although rabies has been known since the Middle Ages, no effective treatment of the disease is available, resulting in more than 50,000 deaths worldwide per year [2]. Rabies is a member of the Rhabdoviridae, which comprise two types of rabies virus, bat and terrestrial virus. The first is found in a number of bat species (for example big brown bat (*Eptesicus fuscus*), silver-haired bat (*Lasionycteris noctivagans*) and eastern pipistrelle bat (*Pipistrellus subflavus*), and it is currently unknown whether it causes disease in bats. This virus is only very rarely transmitted to humans when they come in direct contact with bats [3] or possibly via aerosols in bat caves [4]. In Eurasia, many centuries ago, the bat virus entered the red fox population and established a replicative cycle in this new host through a series of mutations. This terrestrial rabies is a much bigger human health problem than bat rabies. Similar host changes have occurred in the USA, where terrestrial rabies mostly affects raccoons and skunks [5]. Infection is mainly transferred to dogs and can be introduced into humans by bites of infected dogs. Although the spread of rabies infection is kept under control by vaccinating domestic animals and the wild fox population in Europe, such vaccination programs are lacking in most developing countries, contributing to the spread of the disease [6]. Because of the various bat reservoirs, rabies is unlikely to be eradicated, and control of terrestrial rabies is only feasible via vaccination of terrestrial animal reservoirs through immense cost and effort. Knowledge of the structural details of the replication process of this virus may help in the design of anti-viral drugs to treat infection.

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Although rabies virus and vesicular stomatitis virus (VSV) belong to the same Rhabdoviridae family, their biology is different. Rabies virus is a neurotrophic virus and a serious human pathogen, while VSV is an arthropod-borne virus that primarily affects rodents, cattle, swine and horses, and can cause mild symptoms upon infection of humans and other species. It is thought that VSV is spread between hoofed animals and rodents via insect vectors [7]. Together with the families of the Paramyvoviridae [measles virus, Sendai virus, respiratory syncytial virus (RSV) and Nipah virus], the Filoviridae (Ebola and Marburg viruses) and the Bornaviridae (Borna disease virus) rhabdoviruses constitute the order Mononegavirales. The characteristics of these virus families are their single-stranded negative sense RNA genome that is encapsidated by a nucleoprotein (N) and the requirement of a viral L-P polymerase complex for transcription and replication; L (for large protein) is the enzymatically active RNA-dependent RNA polymerase, and P (for phosphoprotein) is the polymerase cofactor. Rabies virus nucleoprotein binds its viral genome with a stoichiometry of nine nucleotides per N-protomer similar to VSV [8, 9]. It is this N-RNA complex that is the template for the viral polymerase, as it cannot make messenger RNA (mRNA) from naked viral RNA [10]. L is bound to the N-RNA complex through P; P binds to L and to the N-RNA polymer and positions L onto its template (Fig. 1a). The complex of the nucleoprotein-encapsidated viral RNA plus the polymerase complex is called the nucleocapsid.

In contrast, segmented negative-strand RNA viruses, represented by families such as the Orthomyxoviridae (influenzaviruses A, B and C) and the Bunyaviridae (bunyaviruses, hantaviruses) also use a nucleoprotein to encapsidate their RNAs, but this is transcribed by the polymerase without additional viral cofactor(s).

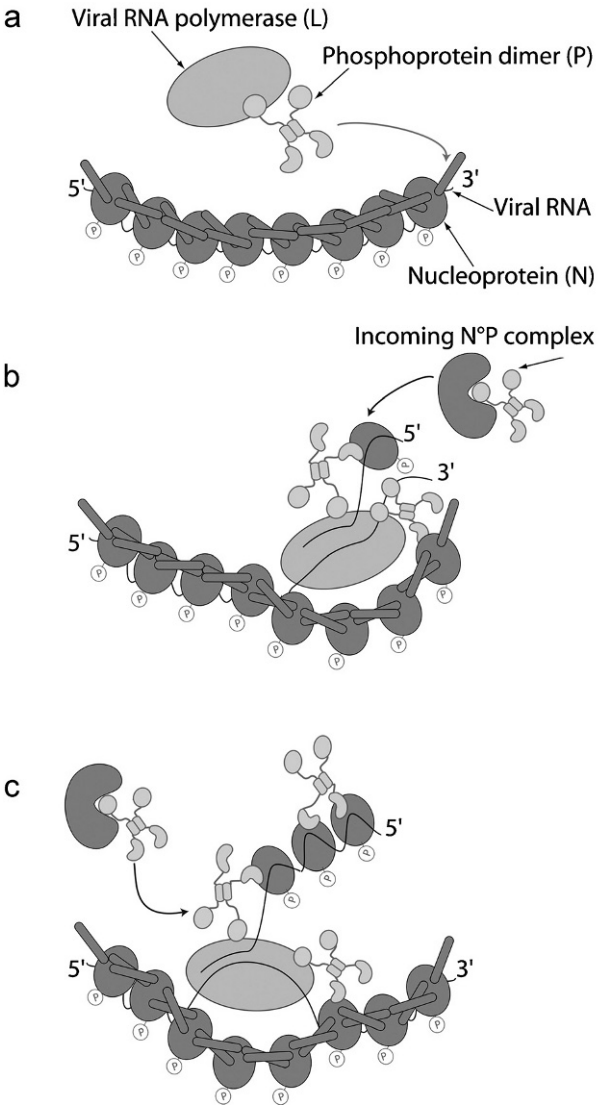

Figure 1. Schematic model of the function of the rabies virus nucleoprotein (N), the phosphoprotein (P) and the polymerase (L) in transcription/replication on an N-RNA polymer template. (*a*) Replication initiation. We suppose that N subunits associated with RNA are phosphorylated (marked by P), and that the catalytic polymerase subunit (L) is associated with one phosphoprotein dimer (P). The L-P complex recognizes the polymerase entry site at the 3′ end of the RNA. (*b*) Genome replication. L-P binding to the N-RNA template triggers RNA release and allows access of the polymerase to the RNA. Newly produced viral RNA is immediately encapsidated by N freshly released from the N°-P complex. Note that when the polymerase is in the transcription mode, the produced mRNA is not encapsidated by N. (*c*) Elongation of the replication reaction results in the production of a new N-RNA complex.

Rhabdovirus transcription and replication

After receptor binding, rabies virus enters its host cells through the endosomal transport pathway [11] via a low-pH-induced membrane fusion process catalyzed by the glycoprotein G, a major determinant for RV neuropathogenicity [1, 12]. All transcription and replication events take place in the cytoplasm inside a specialized 'virus factory', the Negri body [13]. These inclusion bodies are typical for a rabies infection of the brain and have been used as definite histological proof of such infection.

Much of the understanding of rabies virus transcription and replication comes from studies on VSV (prototype of the Rhabdoviridae family). The first step of the replication cycle is transcription of the viral genome. The L-P polymerase complex enters the nucleocapsid at the 3′ end and starts transcription with the production of a short RNA molecule, the leader RNA, that is neither capped nor polyadenylated. After the transcription stop, the polymerase restarts with the transcription of nucleoprotein mRNA, which

is capped and polyadenylated by the viral polymerase complex as shown for VSV [14, 15]. Subsequently, mRNAs are produced for N, P, the matrix protein, the glycoprotein and L. There is a gradient of transcription of these messengers, because after every transcription stop, a percentage of the polymerases do not restart [14, 16, 17]. Later in infection, the activity of L switches to replication in order to produce full-length positive-strand RNA copies without caps or poly (A) tails. These complementary or cRNAs are also encapsidated by N and bind the L-P complex. They are used as templates to make new negative-strand RNA genomes for encapsidation by N in order to form new nucleocapsids. The molecular trigger that induces the switch from transcription to replication is not known, but it can only take place if enough newly produced nucleoprotein is available for encapsidation of the replicated genome [18, 19].

In the virus-infected cell, newly produced nucleoprotein binds only to viral RNA or cRNA (but not mRNA). This association is strictly regulated by a second function of the phosphoprotein. Newly made nucleoprotein binds to the phosphoprotein to form an N°-P complex, in which P plays the role of a viral chaperone that prevents it from binding to cellular RNA and from polymerisation (Fig. 1b,c) [20–22]. Rabies virus phosphoprotein is a dimer in solution [23] and in complex with N° (N°-P) [24] (see Fig. 1b). P binds to N in complex with RNA through its C-terminal domain [25], while the N-terminal 40 amino acids of P are required for interaction with N° (without RNA) [26]. This is consistent with the proposal that P has two N binding sites exerting two different functions (Fig. 1b,c) [27]. Although the mechanism by which N° is released from N°-P to allow N-RNA interaction and polymerisation is not known, it is likely that interaction of N°-P with the replicating viral polymerase complex releases P from N° and induces binding of N° to newly produced viral RNA (Fig. 1b,c). Binding of N° to viral RNA is concomitant with phosphorylation of N at Ser 389 [28] which, in itself, is necessary for optimal binding of P to N in the N-RNA complex.

Structure of recombinant nucleoprotein-RNA complexes

In the absence of viral infection, when recombinant nucleoprotein of rabies virus is expressed in bacteria or in insect cells, it polymerises onto cellular RNA and forms helical structures that are indistinguishable from viral nucleocapsids (Fig. 2) [8, 29, 30]. The length of the bound cellular RNA determines the observed structures that include long helical coils and closed circular complexes (Fig. 2a–c). Similar helical N-RNA structures have been observed for most members of the Mononegavirales upon recombinant expression of nucleoproteins in the absence of other viral proteins (measles virus [31–33]; Marburg virus [34]), with the exception of bornavirus nucleoprotein expression, which spontaneously forms homotetramers without RNA binding and higher-order polymerisation [35] (see below).

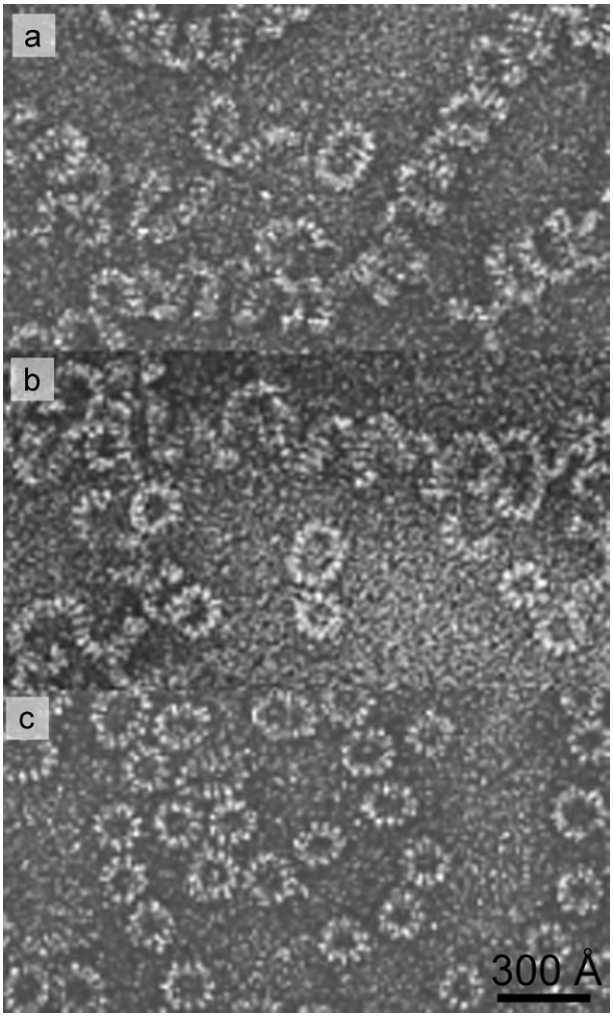

Figure 2. Negative staining of viral and recombinant rabies virus N-RNA complexes. Panel *a* shows N-RNA purified from virus particles. Panels *b* and *c* show recombinant N-RNA produced in insect cells in which N is bound to long or short cellular RNAs, respectively. The scale bar indicates 30 nm.

Structure of rabies virus N-RNA

The rabies virus nucleoprotein expressed in insect cells polymerises into cellular RNA containing ring-like structures. The N-RNA rings were purified by native gel electrophoresis, and their N content (from 9 to 15 N-protomers per ring) was determined by

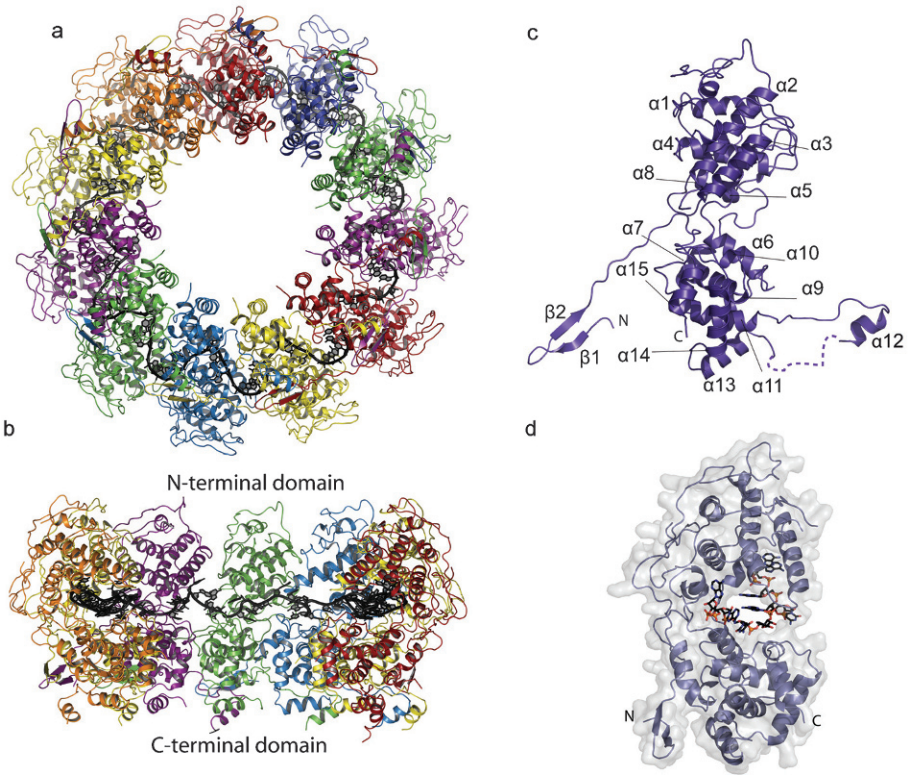

Figure 3. Crystal structure of the rabies virus N-RNA polymer. (*a*) Ribbon diagram of the 11 N-protomers containing RNA, forming a ring structure as viewed from the top (similar to the rings shown in Figure 2*c*). (*b*) Side view of the ring showing the interior of 6 protomers. The RNA is bound to a central cavity formed between the two domains and wraps around the centre of the ring, twisted clockwise in a left-handed helical manner. (*c*) Ribbon diagram of the N-protomer reveals two main domains and two smaller extra domains extending from the N- terminal and C-terminal domains. Secondary structure elements are labelled. (*d*) Ribbon diagram within a space-filling model (light blue) of the monomer showing the complete enclosure of the bound RNA (all-atom model).

electron microscopy [36]. Several of the classified N-RNA rings were tested for crystallisation, and the rings with 11 protomers gave the best-diffracting crystals and were used for structure determination [37]. Independently, the N-RNA structure of the VSV N-RNA rings produced by co-expression of VSV N and P proteins in *Escherichia coli* was solved by X-ray crystallography [38]. The rabies structure reveals the RNA occluded in the centre, twisted clockwise in a left-handed helical manner along the ring composed of 11 N protomers (Fig. 3a, b). The RNA wraps around the N-terminal domain in a way that permits exploitation of the surface for maximal nucleotide protein contacts. A further characteristic of this binding mode is the complete occlusion of the RNA between the N- and C-terminal domains, which appear to clamp down onto the bound RNA (Fig. 3d), consistent with its resistance to dissociation under CsCl conditions. Each N protomer contacts 9 nucleotides (Fig. 4a, b) as determined biochemically [8]. Since the RNA has been taken up from the host cell RNA pool, the nucleoprotein RNA recognition is nucleotide-unspecific; each nucleotide is thus coordinated mainly by polar interactions between phosphate groups and basic residues, highlighted by the overall basic charge of the RNA binding cleft (Fig. 4c, d). Such an interaction is a common sequence independent nucleotide-protein interaction mode. A close-up view of the RNA binding site shows that bases 1–5 (yellow in Fig. 4b) point away from the back of the cleft towards the solvent and stack onto each other. Then the RNA molecule makes three turns. The first turn places base 6 (in green) towards the back of the cleft, the next turn points base 7 (in red) away from the cleft and the final turn positions bases 8 and 9 (in green) towards the back of the cleft while stacking onto base 6 and each other (Fig. 4b). Since the RNA is completely enclosed by the protein, the structure presents most likely the storage form of the nucleocapsid as it is packaged in virus particles.

Even though the conformation of the bound RNA is identical in both the rabies virus and the VSV N-RNA structures, the residues implicated in RNA coordination vary slightly [37,38]. Green et al. report two conserved basic VSV N residues (R214 and R312) along the RNA binding site which are not involved in RNA coordination but could contact RNA with different side-chain rotamers. In contrast, homologous residues contact the RNA in the rabies virus N-RNA complex (VSV R214 corresponds to rabies R225; VSV R312 corresponds to rabies virus R323; Fig. 4d). Vice versa, two conserved basic residues (K152 and K297) of rabies virus could also contact the RNA with alternative side-chain rotamer conformations; however, both coordinate the RNA in VSV N (K155 and K286). This observed variability in RNA coordination suggests that some residues could act as alternative RNA anchors, and the differential binding

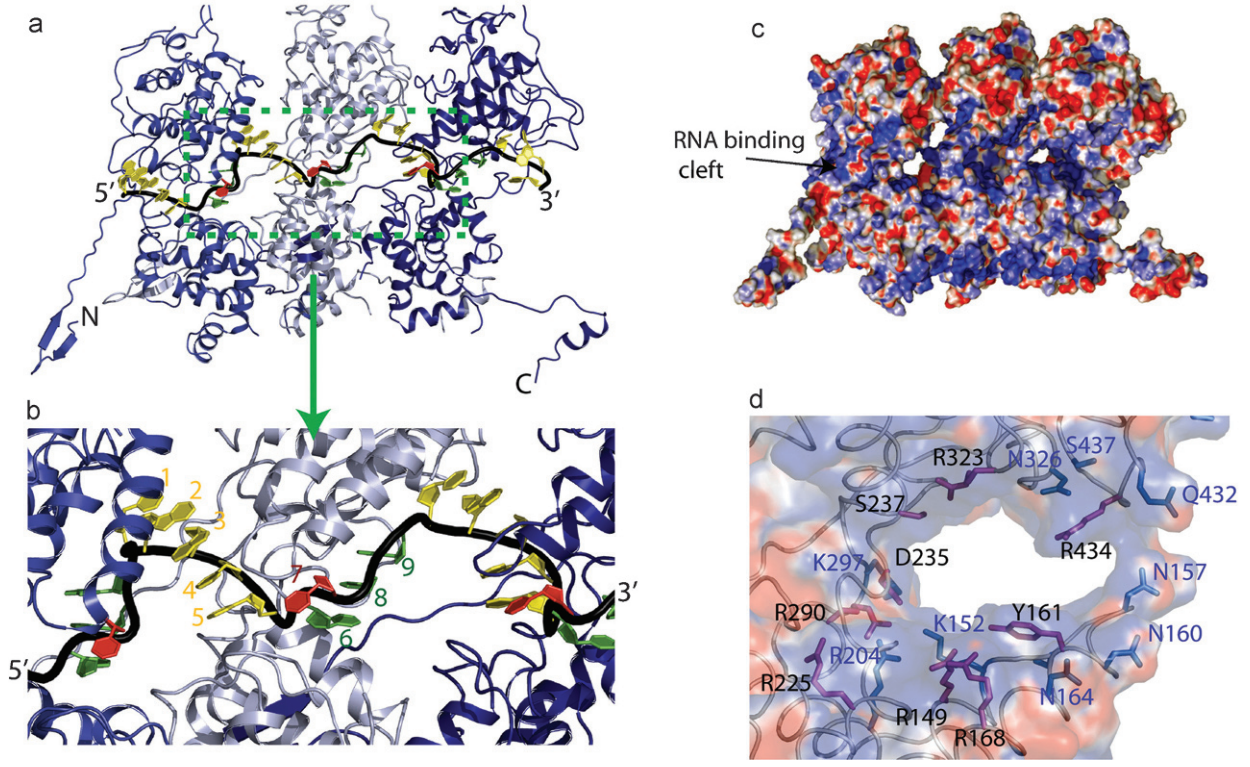

Figure 4. Close-up of the RNA binding site. (*a*) Ribbon diagram of 3 N-protomers with bound RNA; the ribose phosphate backbone of the RNA is shown as a black coil, and the bases are coloured according to their stacking orientation. (*b*) Close-up of the bound RNA: bases oriented away from N are drawn in yellow and red, and those oriented towards the protein are shown in green. (*c*) Electrostatic potential map of 3 N protomers reveals a mostly basically charged surface of the cleft between the N- and C-terminal domains, which constitute the RNA binding cleft. (*d*) Close-up and surface representation of the RNA binding cleft; basic residues implicated in RNA coordination are shown in purple (black label) as all-atom models; residues shown in blue (blue label) are either in a position to contact RNA with a different side-chain rotamer and/or are alternatively used for RNA binding by either rabies or VSV.

might play a role during specific steps of replication and/or transcription such as transcription start and stop, accommodate newly synthesized RNA or simply act as transient anchors during polymerase activity.

The closed form of the N-RNA may protect the viral genome from recognition by cellular Toll-like receptors (TLR) such as RIG-1, which recognises the 5′ PPP [39–41], or TLR3, which recognises double-stranded RNA [42] that could result from transcription and replication reactions. However, this structure may also be one of the Achilles' heels of the virus. Drugs that mimic the factor that opens up the nucleoprotein would expose the viral RNA to innate immunity receptors, and drugs that stabilise the closed form of N would inhibit polymerase processivity.

Nucleoprotein polymerisation and nucleocapsid formation

Polymerisation is achieved mainly by domain exchange between protomers. Two small extra domains extend each from the N- and the C-terminal domains and reach over to the neighbouring molecules in the N polymer, while contacting each other (Fig. 3c, 5a). Although the 11-nucleoprotein ring structure can be viewed as an artificial mini nucleocapsid, it is of note that there are extensive contacts between the C-terminal domains (buried surface 2700 Å^2), but there are no significant interactions between the N-terminal domains, which provide most of the RNA contacts (8 out of 11). This implies indirectly that the N-terminal domain requires some freedom of movement for function. A similar feature is observed in case of the VSV nucleoprotein, although the ring structure reveals a tighter packing of the N protomers [38]. Despite the appearance that the domain exchanges provide a tight interconnection of the N-protomers, each individual N-N interaction appears to be flexible, underlined by relatively high temperature factors for the extending domains.

N must preferentially encapsidate its own negative-sense genome. This might be spatially regulated together with specific affinities for genomic RNA. Some studies suggest that the 5′ leader RNA plays a pivotal role in conferring RNA encapsidation specif-

icity, since rabies virus N binds preferentially to leader RNA [43, 44]. This process is further modulated by P [45, 46] and by phosphorylation of N [28]. In a linear and viral nucleocapsid, the extended 'arm' emanating from the N-terminal domain of N at the beginning of the nucleocapsid would not be involved in domain exchange in the absence of a neighbouring N protomer; it is thus reasonable to speculate that this small domain might be employed to confer leader RNA specificity for N interaction with genomic RNA. Alternatively, or in addition, the extra basic residues within the RNA binding cleft might confer higher affinity to the leader RNA than to any other RNA.

Comparison of the N-RNA ring structure with the structure of free viral nucleocapsids and the nucleocapsid coil inside virus particles

The relation of the N-RNA rings with isolated, intact viral nucleocapsids and with nucleocapsid incorporated into virus particles has been described in Schoehn et al. [29]. In order to make a closed ring structure with a diameter of 160 Å, the 11 N-protomers are packed with an angle between two monomers of ~147°. Isolated viral nucleocapsids, released from viral particles, have a diameter of ~240 Å and contain ~15 protomers per helical turn. There the angle between the protomers is ~156° (schematically represented in Fig. 5b). In virus particles, the diameter of the tightly packaged nucleocapsid coil is about 750 Å, with ~53 N protomers per helical turn and an angle between two N protomers of ~173°. This tight packaging of the N-RNA inside the virus particle is mediated by the binding of the viral matrix protein to the N-RNA, which must induce stress in the N-RNA structure to extend its diameter [47, 48]. The condensation of the N-RNA coil takes place at the inside of the cell membrane, just before the budding process in which the membrane studded with glycoprotein spikes is acquired [49]. The released, loose N-RNA coils are probably the real target for the polymerase complex, thus constituting the transcription and replication competent nucleocapsid rather than the tightly packaged nucleocapsid inside the virus particle. Based on the flexible linkage of N-protomers and the available electron microscopy (EM) models of rabies virus N-RNA complexes, a model of a nucleocapsid in the virus particle was constructed containing 53 N per helical turn (Fig. 5c).

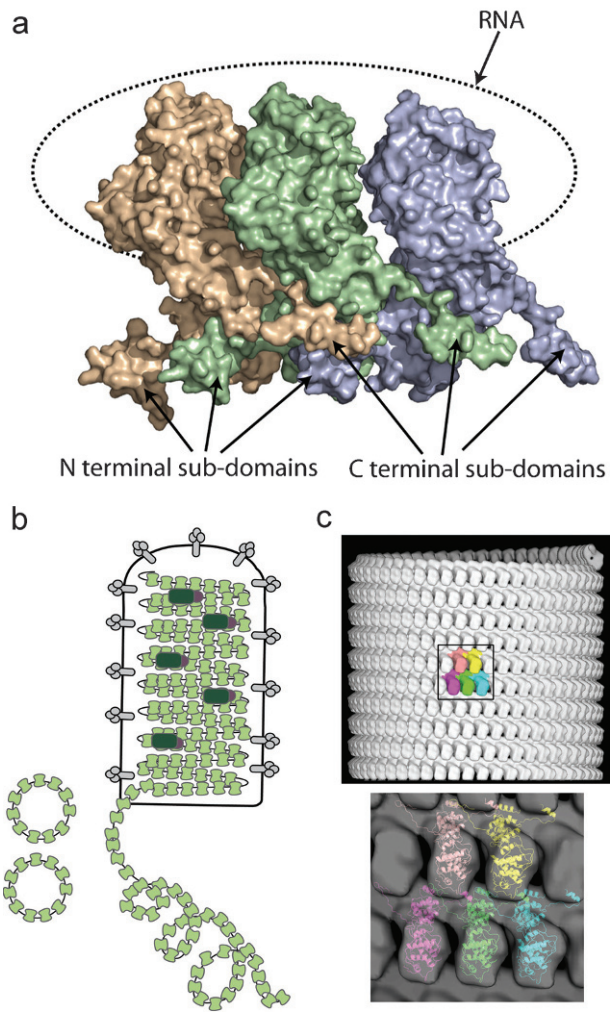

Figure 5. Domain exchange between N-protomers stabilizes the N-RNA polymer and allows nucleocapsid formation. (*a*) Surface representation of 3 protomers showing the intimate interconnection of the N- and C-terminal extra domains on the back of the neighbouring C-terminal domain. Both subdomains contact each their neighbouring N partner and each other. (*b*) Schematic model of the nucleocapsid present in infectious virions showing a 'tight' coil structure within the virion, 'loose' coils when released from the virus and artificial rings when N is produced in insect cells. (*c*) A nucleocapsid model from rabies virus was generated based on the electron microscopy model of N-RNA ring structures [29] containing 53 N-protomers per helical turn. The close-up shows the docking of the crystal structure into the electron microscopy density.

Implications of the N-RNA structure for transcription and replication

The most obvious implication of the N-RNA structure in the resting state is the postulation of a mechanism that can catalyse opening or at least allow local access to the bound RNA by the polymerase complex. Because the C-terminal domains of N are tightly bound to their neighbouring domains, it is most likely that the N-terminal domains will move to open up the

cleft. For Sendai virus, it has been shown that apart from the phosphoprotein bound to the polymerase in the P-L complex, an excess amount of P is needed for efficient polymerase activity [50]. It is thus possible that an excess of P is needed to pry open the closed rabies virus N and prepare it for access to the polymerase complex. Another possibility is that phosphorylation of N may be implicated in this process, although its precise role is still elusive, and it should be noted that VSV N does not acquire phosphorylation for its function [51].

The connections between N-neighbours via the C-terminal domain and the exchanged domains are most likely essential for transcription and replication in order to maintain the N-polymer during potential release of the RNA while the polymerase complex moves ahead (Fig. 1b,c). After the passage of the polymerase complex, the template RNA will need to bind again tightly to the nucleoprotein polymer, thus efficiently preventing the formation of double-stranded RNA (Fig. 1c). Because the polymerase complex is bound both to the nucleoprotein and to the RNA during transcription and replication, a break in the protein polymer would most likely halt the polymerisation process.

The structures of a number of viral RNA-dependent RNA polymerases and reverse transcriptases have been determined which reveal central pores where the RNA template has to pass through during the polymerisation process [52]. It is thus reasonable to assume that rhabdoviral polymerases will have a similar architecture, which would support a model where the viral RNA would have to come completely free from the nucleoprotein. Furthermore, the newly produced RNA molecule has to form a hybrid double-stranded RNA helix with the resident RNA so that the polymerase can stay in register with the template. Without such a hybrid structure, the polymerase could slip back on the substrate and add non-coded nucleotides [53]. Therefore, the RNA must come off the nucleoprotein and there must be enough distance between the nucleoprotein skeleton and the liberated RNA to make space for the polymerase complex. Because of the helical path that the RNA takes on the nucleoprotein, the distance taken up by the 9 nucleotides that bind to a single N-protomer is only 22 Å. The maximum distance that could be stretched by 9 nucleotides is 45 Å. This suggests that dissociating the RNA from 1, 2 or even 3 subsequent N-protomers could indeed generate enough space for the polymerase complex in action, as suggested previously [37].

Comparison of the structure of N in the N-RNA complex with that of N in the N°-P complex

In addition to positioning the polymerase on the N-RNA complex and its putative role in the local release of RNA from N, the phosphoprotein P plays an important chaperone role to prevent newly synthesized nucleoprotein from polymerisation on free RNA. Biochemical analyses have shown that N forms a 1:2 complex with P, and limited proteolysis indicated that the N-terminus of P comprising its first 40 residues is important for interaction [26]. Protease digestions also removed N amino acids 1–24, 120–129 and 356–400, consistent with the parts which had been determined to be flexible within the nucleoprotein-RNA structure (Fig. 6a). From this comparison it is obvious that there is very good correspondence between the two highlighted characteristics. Residues 1–24 correspond to the N-terminal domain involved in domain swapping, residues 120–129 are close to a flexible loop at the tip of the N-terminal domain of N, and residues 356–400 correspond to helix 12 plus the disordered part of the C-terminal domain swapped part of N. This close equivalence of flexible regions strongly suggests that the nucleoprotein in the N°-P complex has the same structure as N in the N-RNA rings. It thus reinforces the suggestion made by Mavrakis et al. [26] that the acidic, N-terminal peptide of P occupies the space that is normally occupied by the RNA in the N-RNA complex. An interesting detail that differs in the proteolytic behaviour of N in complex with RNA and in complex with P is that extensive digestion by trypsin leads to removal of the entire C-terminal part of N (376–450; see also below) in the N-RNA complex, whereas the very C-terminal residues 400–450 remain associated with the rest of the protein in the N°-P complex (Fig. 6a,b) [26,29] [M. Mavrakis et al., unpublished results]. Although there are several contacts to the RNA (R434, R323, R290), it does not seem to suffice to keep the ensemble of helices 13 to 15 (residues 396 to 450) attached to the core of the C-terminal domain. In contrast, because residues 400–450 are retained in the trypsin-digested N°-P complex (Fig. 6a), it is likely that these helices contribute to P interaction.

The phosphoprotein N-RNA interaction

The polymerase binds to the N-RNA complex in the form of a P-L complex, in which P tethers L to the N-RNA complex (Fig. 1). The details of the N-P-binding site in the N-RNA complex are not known, although phosphorylation of Ser 389 (Fig. 6c) is necessary for efficient binding of P [28]. Cryo-EM single-particle

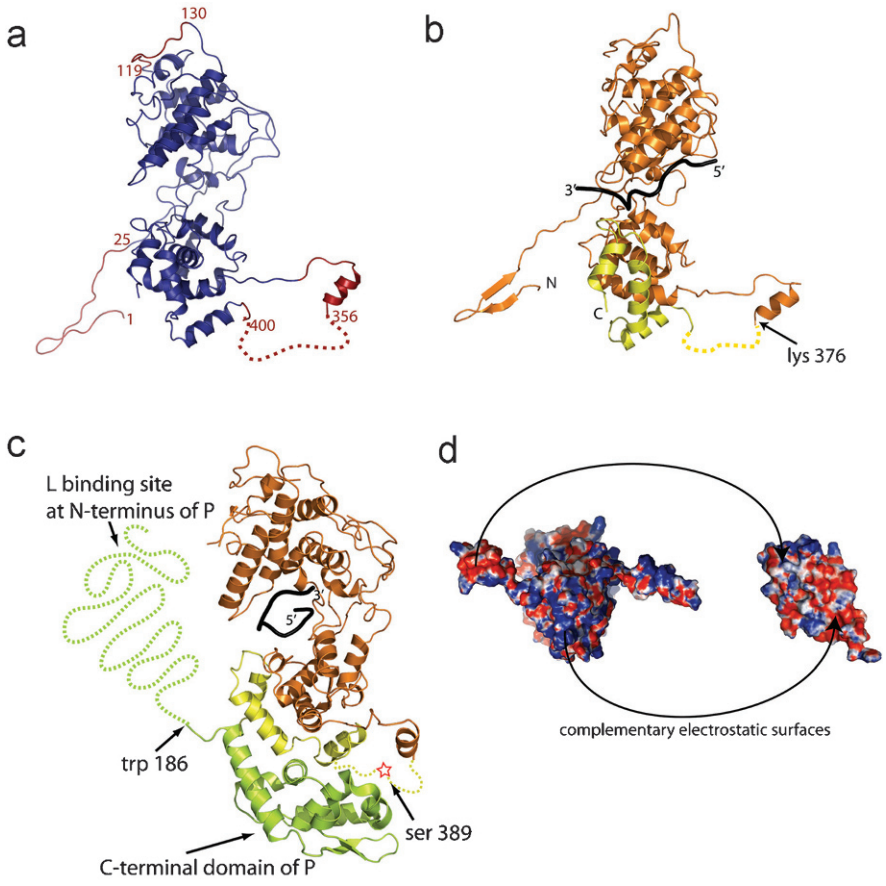

Figure 6. Model of the N-P interaction based on the proteolytic digestion of N-RNA and N-P complexes and the partial crystal structure of P. (*a*) Ribbon diagram of N showing the regions removed upon N°-P trypsin and subtilisin double digestion in red. (*b*) Ribbon diagram of the N-RNA monomer showing the parts removed by trypsin digestion in yellow. The RNA is shown as a coil in black. (*c*) Ribbon diagram of N docked manually to P (green). The helices removed by trypsin as presented in panel b are shown in yellow. The N-terminal extension of P (including the oligomerisation domain) points into the correct direction to place L close to the bound RNA. (*d*) Electrostatic potential map of a N-protomer as viewed from the bottom of the C-terminal domain (left panel) and that of the structure of the C-terminal domain of P (right panel), which was shown to bind to N-RNA complexes.

reconstruction of native N-RNA rings containing 10 N-protomers and of rings in which residues 376–450 had been removed by trypsin digestion revealed that a bulky part of the bottom of the rings was removed by the protease [29]. As already mentioned above, the trypsin treatment removes helices 13–15 plus the disordered part of the C-terminal-exchanged domain up to helix 12 (Fig. 6b). The position of these helices at the inside and bottom of the rings corresponds well with the absent density observed in the cryo-EM reconstructions of trypsinized N-RNA ring structure [29]. Independently, biochemical experiments showed that P could no longer bind to the trypsinized N-RNA rings. Therefore, N-residues 376–450 are involved in binding to P. Thus, it is conceivable that P interacts with the C-terminal exchange domain. Such an interaction could affect the N-terminal-exchanged domain in close proximity (Fig. 3c, 5a), which might transfer a signal to the N-terminal domain and trigger the release of the RNA.

The C-terminal domain of P (P-CTD, residues 186–297) folds into a small helical domain that is required for N-RNA interaction [25]. P-CTD has the shape of a half-pear, the pear being cut lengthwise. The flat face of the half-pear is strongly charged with a basic patch at its C-terminal end and an acidic patch at its N-terminal end. Based on the shape and the charge complementarity of N and P-CTD (Fig. 6d) as well as on mutagenesis studies [54], a hypothetical model of the N-P interaction was constructed. In this model P-CTD covers helices 14–15 of N, and the basic patch of P-CTD would be in close proximity to the proposed Ser 389 phosphorylation site required for P binding (Fig. 6c). This model also places the N-terminal end required for L interaction [55] at a position where L could contact the RNA. This model predicts further that modification of the C-terminal end of N would inhibit transcription and replication, which is consistent with the failed rescue of recombinant virus upon green fluorescent protein (GFP) fusion to the C-terminus of N. However, coexpression of native N and N-GFP permitted incorporation of N-GFP into the viral nucleocapsid, and the recombinant virus grew with similar kinetics as wild-type rabies virus. In these nucleocapsids about 30 % of N was modified; the rest was wild-type N [56]. This suggests that the functional interaction of the P-L complex with the N-RNA is rugged and that it is apparently not necessary that P bind to every N in the N-RNA for a productive interaction between L and the RNA. The C-terminus

of P points away from the proposed N-P interface (Fig. 6c). In accordance with this, addition of GFP to the C-terminus of P did not have much effect on transcription but impaired a later step in virus production [57].

All known viral P proteins form oligomers and require multimerization for proper L-P-N-RNA positioning. VSV P contains a central domain with a mixed alpha-beta structure that forms parallel homodimers [58]. The other known P oligomerization domain structure is that of Sendai virus, which folds into a parallel tetrameric coiled coil [59]. Although the central function of each negative-strand RNA virus P protein, namely forming the polymerase complex together with L, is the same, it seems that these related viruses have developed different structural solutions to solve the transcription and replication processes using a N-RNA template. This is also highlighted by the different structural module used by measles virus and Sendai virus P to bind to their nucleoproteins [60–63] as compared to the rabies virus P-N attachment domain [25].

Phosphoprotein interactions with cellular factors

Rabies virus P was shown to interact with the dynein light chain 8 (LC8), which could link P-N-RNA complexes to the dynein motor complex and thus to the cellular transport system [64, 65]. This is consistent with VSV nucleocapsid transport towards the cell periphery via a microtubule-mediated process [66]. However, deletion of the putative dynein binding site of P did not change rabies virus entry into the central nervous system but attenuated both viral transcription and replication primarily in neurons [67]. Another clearly defined function of P is to modulate the innate immune response in infected cells. P interacts with PML (promyelocytic leukemia) protein, an interferon-induced protein, and reorganizes PML nuclear bodies in the nucleus of the infected cell [68]. P also interacts with activated STAT1 and STAT2, preventing their transport to the nucleus and thus acts as an interferon antagonist [69, 70]. The function of rabies P is further regulated by phosphorylation via protein kinase C plus a rabies virus-specific kinase [71], while VSV P seems to be phosphorylated by casein kinase-II [72, 73]. Although the role of phosphorylation is still unclear, evidence suggest that it might influence P multimerization [72] and/or P-L complex formation [74].

Comparison of the structure of rabies virus nucleoprotein with those of three other negative-strand RNA viruses, VSV, Borna disease virus and influenza virus

Besides the nucleoprotein structures of rabies (Fig. 7a) and VSV (Fig. 7b), which are basically identical, two other nucleoprotein structures are known: the Borna disease virus (BDV) nucleoprotein, a member of the Mononegavirales and that of the influenza virus nucleoprotein. Both structures have been solved in the unliganded form. BDV N forms a tetramer in solution and in the crystal structure (Fig. 7c) and does not spontaneously associate with cellular RNA [35]. However, the BDV monomer contains two helical domains with short N- and C-terminal extensions which are involved in domain exchange and stabilize the tetramer (Fig. 7c). Furthermore, the cleft formed between the two main domains carries a mostly basic charge and could accommodate RNA similar to the binding mode observed in rhabdovirus N-RNA complexes (Fig. 7a). Even though at first sight the structures of the rabies virus and BDV nucleoproteins seem to be similar – they are both helical arrangements – it was not possible to structurally align the two proteins in a significant manner, indicating that it is unclear whether they evolved from a common ancestor or by convergent evolution.

Influenza virus, a member of the Orthomyxoviridae, has a segmented negative-strand RNA genome that is encapsidated by the nucleoprotein, whose structure has been determined in the absence of RNA [75]. Influenza virus N forms trimers in solution and in the crystal asymmetric unit; its structure contains two mainly helical domains. They are arranged in a way that a cleft is formed between the two domains, which carries an overall basic charge and might thus constitute the putative RNA binding site (Fig. 7d). Although the overall shape and architecture is again similar to that of rhabdovirus N and BDV N, the sequence assignment of both helical domains of influenza virus N is non-linear; no significant structural homology can be postulated to the other known nucleoprotein structures. In addition, influenza virus N contains only one extra domain that might be implicated in N polymerisation; in fact, the tail loop emanating from the C-terminal domain is implicated in trimerisation and was suggested to function in NP polymerisation [75] (Fig. 7d).

There are several differences between the nucleoproteins of the rhabdoviruses and influenza virus. First, rhabdoviruses bind 9 nucleotides per N-protomer, which are completely shielded off from the environment, resistant against high salt treatments and

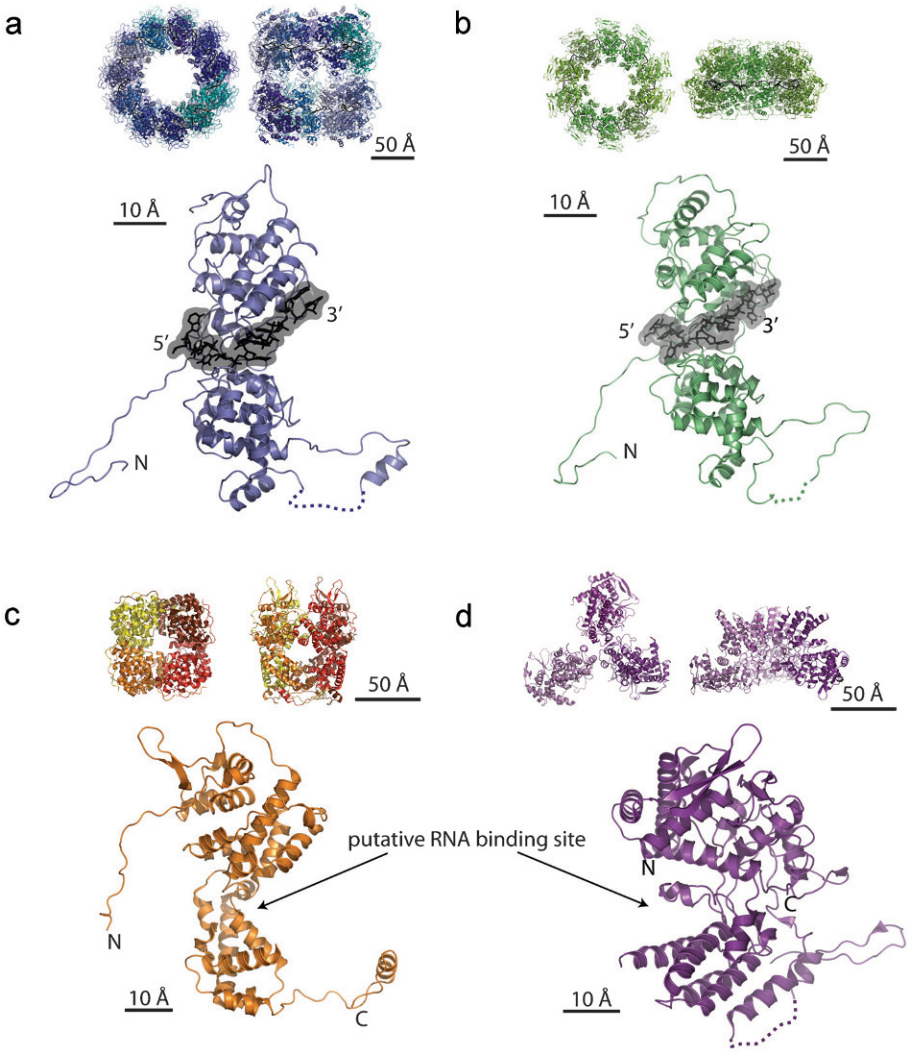

Figure 7. Comparison of known viral nucleoprotein structures in polymeric and monomeric states. (*a*) Ribbon diagram of the rabies virus N-RNA complex; upper panel shows the two 11-mer N-RNA ring complexes contained in the crystal asymmetric unit; lower panel shows the monomer in complex with RNA. (*b*) Ribbon diagram of the VSV N-RNA complex; upper panel shows the recombinant 10 mer N-RNA ring complex; lower panel shows the monomer in complex with RNA. (*c*). Ribbon diagram of the BDV nucleoprotein in its unliganded form. The upper panel shows the tetrameric form found in solution and in the crystal; lower panel shows the monomer composed of two main domains and two small extra domains emanating from the N- and C-terminal domains and involved in domain exchange in the tetramer structure. The putative RNA binding site in the monomer is indicated by an arrow. (*d*) Ribbon diagram of the influenza virus nucleoprotein in its RNA-free conformation. The upper panel shows the trimer found in solution and in the crystal. The lower panel shows the monomer composed of two helical domains whose arrangement generates a cleft, which was proposed to constitute the RNA binding site (arrow). A flexible tail loop extends from the C-terminal domain is implicated in trimerisation and polymerisation.

protected from RNase degradation. In contrast, influenza virus binds 24 nucleotides per N-protomer [76]; the RNA is easily released by high salt treatment [77] and sensitive to RNase treatment within the RNPs [78]. Furthermore, in influenza virus nucleocapsids the nucleotide bases are more exposed to the solvent than in the nucleocapsids of the rhabdo- and paramyxoviruses, as shown by chemical modification experiments [77, 79, 80]. Nothing is yet known about the RNA bound within BDV nucleocapsids. Given the size of the structure of the putative RNA cleft, the number of nucleotides bound to each protomer may vary further, consistent with the fact that other families of the Mononegavirales such as measles and Sendai virus (Paramyxoviridae) bind only 6 nucleotides per monomer [81].

Both influenza virus N and BDV N do not efficiently interact with cellular RNA and do not polymerise upon binding in the absence of other viral factors. Such RNA binding-driven polymerisation is, however, observed for all other members of the Mononegavirales, such as filoviruses and paramyxoviruses (see third paragraph). A major difference in the life cycle of these viruses is that rhabdoviruses, filoviruses and paramyxoviruses replicate in the cytoplasma, while BDV as well as influenza virus replicate in the nucleus. The latter two thus do not need to employ a strategy which prevents constant perfect nucleocapsid protection, since it will be safe from cellular attacks once it is in the nucleus. Furthermore, the higher concentration of cellular RNA in the nucleus might require a more efficient way to switch the nucleoprotein from the RNA free form to the one specifically encapsidating genomic RNAs.

In summary, the limited structural information on nucleoproteins suggests that they all employ common motifs and strategies to bind to RNA and polymerise. However, the major structural differences prevent predicting a common ancestor for these nucleoproteins. The current information sug-

gests rather that they might be the result of convergent evolution.

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