Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 349 (2006) 197–211

VIROLOGY

www.elsevier.com/locate/yviro

Initiation of encapsidation as evidenced by deoxycholate-treated Nucleocapsid protein in the Chandipura virus life cycle

Raja Bhattacharya, Soumen Basak [1], D.J. Chattopadhyay *

Dr. B.C. Guha Centre for Genetic Engineering and Biotechnology, Department of Biochemistry, University College of Science, University of Calcutta, 35 Ballygunge Circular Road, Calcutta 700 019, India

Received 24 October 2005; returned to author for revision 11 November 2005; accepted 12 January 2006
Available online 17 February 2006

Abstract

Encapsidation of nascent genome RNA into an RNase-resistant form by nucleocapsid protein, N is a necessary step in the rhabdoviral life cycle. However, the precise mechanism for viral RNA specific yet processive encapsidation remains elusive. Using Chandipura virus as a model system, we examined RNA binding specificity of N protein and dissected the biochemical steps involved in the rhabdoviral encapsidation process. Our analysis suggested that N protein in its monomeric form specifically binds to the first half of the leader RNA in a 1:1 complex, whereas, oligomerization imparts a broad RNA binding specificity. We also observed that viral P protein and dissociating detergent deoxycholate, both were able to maintain N in a monomeric form and thus promote specific RNA recognition. Finally, use of a minigenome length RNA in an in vitro encapsidation assay revealed the monomeric N and not its oligomeric counterpart, to be the true encapsidating unit. Based on our observations, we propose a model to explain encapsidation that involves two discrete biochemically separable steps, initiation and elongation.

Keywords: Chandipura virus; Encapsidation; Leader RNA; Nucleocapsid protein; Initiation

Introduction

Rhabdoviruses in addition to being deadly human pathogens are also responsible for causing epidemics in cattle, fish and plant (Brown et al., 1979). Recent outbreaks of Chandipura virus (Arankalle et al., 2005; Rao et al., 2004), a member of the Rhabdoviridae family (Bhatt and Rodrigues, 1967), in parts of India necessitates a detailed understanding of the viral multiplication process, both for better therapeutic intervention as well as for providing a general model system to understand the rhabdovirus life cycle. Chandipura virus packages an 11,119 nucleotide long nonsegmented, negative sense genome RNA, enwrapped with viral nucleocapsid protein (N) into a ribonucleoprotein (RNP) particle (Marriott, 2005). The genome of the Chandipura virus encodes for 5 polypeptides in the order 3′-N-P-M-G-L-5′, flanked by two mutually complementary sequences of ~50 bases, 3′leader (l) and 5′trailer (t) (Banerjee, 1987). Viral RNA-dependant RNA polymerase (RdRp) is composed of large protein (L, 238.5 kDa) bearing the catalytic activity and the 32 kDa phosphoprotein (P), which acts as its cofactor (Banerjee and Barik, 1992). The encapsidated genome RNA is resistant to RNase action and acts as the template for RNA polymerization. Subsequent to viral infection, RdRp initiates transcription at the 3′end of the viral genome to sequentially synthesize leader RNA and 5 monocistronic mRNAs with progressive attenuation at each gene junction (Abraham and Banerjee, 1976; Iverson and Rose, 1981). During replication, the same polymerase reads through the stop signal at gene boundaries to produce a polycistronic complement of genome RNA, which acts as the template for subsequent synthesis of progeny viral genomes (Banerjee, 1987; Wertz and Levine, 1973). The molecular switch that leads to a change in polymerase function has remained an enigma for rhabdoviruses. Polymerase, with distinct compositions engaged in transcription or replication process has been proposed (Qanungo et al., 2004). Differential engagement of *cis*-acting elements in transcription or replication has also been postulated (Li and Pattnaik, 1997;

* Corresponding author. Fax: +91 33 2476 4419.
E-mail addresses: d_jc@sify.com, djcbcg@caluniv.ac.in (D.J. Chattopadhyay).
[1] Present address: UCSD.

0042-6822/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.01.017

Whelan and Wertz, 2002) with potential for internal entry of transcriptase complex at the promoter for N gene (Chuang and Perrault, 1997). Furthermore, previous work on VSV (Banerjee and Barik, 1992; Richardson and Peluso, 1996) or Chandipura system (Basak et al., 2003, 2004; Chattopadhyay et al., 1997) led to identification of phosphoprotein phosphorylation as a major determinant for polymerase function. While CKII-mediated phosphorylation at Ser62 of Chandipura virus P protein is indispensable for transcription (Chattopadhyay et al., 1997), a phosphorylation-defective mutant of P was shown to boost up viral replication presumably by its ability to bind to nascent leader RNA (Basak et al., 2003).

Nonetheless, a requirement for de-novo protein synthesis was reported during the viral replication process (Davis and Wertz, 1982; Patton et al., 1983; Wertz, 1983; Wertz and Levine, 1973). Interestingly, on-going synthesis of N protein was shown to satisfy this requirement in an in vitro replication assay (Patton et al., 1984). Accordingly, it was suggested that N-mediated nucleation and progressive encapsidation of nascent leader chain result in anti-termination at the gene boundaries that eventually leads to the onset of the replication process (Blumberg et al., 1981, 1983; Leppert et al., 1979). Although, this model does not exclude the possibility of synergistic involvement of other host factors or viral components, it advocates the need for encapsidation competent N during replication. Furthermore, a stoichiometric amount of N is necessary for encapsidation, to protect newly synthesized genome RNA from cellular RNase action. Thus, N-mediated encapsidation of nascent (+) sense genome that initiates at leader sequence signifies a critical event in viral multiplication process. However, N protein of vesiculoviruses had been consistently found to self-assemble into inactive aggregates when expressed in mammalian cell or in *E. coli* (Das and Banerjee, 1993; Howard and Wertz, 1989; Majumder et al., 2004; Patton et al., 1983). This aggregated form of N protein although capable of interacting with leader RNA lacks the ability to encapsidate 129nt long (+) sense VSV RNA into an RNase-resistant form (Moyer et al., 1991). Moreover, these aggregates were shown to interact with nonviral RNAs when expressed in the cell (Green et al., 2000; Iseni et al., 1998). Interestingly, phosphoprotein inhibits the concentration-dependent aggregation of N and it was believed that complex formation between N and P was one of the ways of maintaining a pool of replication competent N (Howard and Wertz, 1989). P protein was found to act as a specific chaperone that acts at the nucleation step to prevent N protein aggregation (Majumder et al., 2001, 2004). Accordingly, multiple N–P complexes of various molar ratios were observed within VSV-infected cells (Davis et al., 1986; Masters and Banerjee, 1988a; Peluso, 1988; Peluso and Moyer, 1988) or when coexpressed in *E. coli* (Green et al., 2000; Gupta and Banerjee, 1997). Furthermore, P was shown to suppress nonspecific RNA binding ability of N protein without interfering with leader RNA recognition by N (Masters and Banerjee; 1988b). Though P could not revert presynthesized N into a form that is suitable for encapsidation (Majumder et al., 2004; Masters and Banerjee, 1988a), prior complex formation with P was found to be a prerequisite for specific recognition of viral sequences. Thus, it was hypothesized that P protein channels N protein towards nascent viral leader sequence within the infected cell to ensure successful encapsidation of de novo synthesized viral genome. Nonetheless, aggregation prone characteristics so far prohibited detailed biochemical characterization for different modes of RNA binding by N protein in vitro. Although, when coexpressed with P in *E. coli*, a soluble form of vesicular stomatitis virus N protein was obtained as an N–P complex (Green et al., 2000), lack of a homogeneous N preparation hindered studies on properties of N protein complexes upon P binding.

Previously, we had reported the expression of recombinant Chandipura virus N protein in bacteria and its purification in a soluble form that is capable of RNA binding (Majumder et al., 2001). Here, we show that consistent with aggregation prone characteristics, affinity-purified N exists mostly in an oligomeric form. Treatment with dissociating detergent, such as deoxycholate, results in a pool of predominantly monomeric N protein, which, unlike oligomeric N, specifically recognizes viral leader RNA in vitro. The monomer N-leader RNA complex is shown to be biochemically distinct from a higher order N–RNA complex. Furthermore, competition gel shift assay and toe-printing analysis revealed that monomeric N binds within the first half (20 nucleotides) of leader RNA that is highly conserved among different vesiculoviruses. We found that P protein, independent of its phosphorylation status, maintains N protein in a monomeric form, thus mimicking deoxycholate action. Moreover, we show that this N–P complex and not N alone is a prerequisite for processive encapsidation of 597 nucleotides viral sequence into an RNase-resistant form in vitro. Accordingly, a mechanism elucidating the specific yet processive encapsidation of viral RNA by N protein has been discussed, in the context of the Chandipura virus life cycle.

Results

N protein can form two complexes with leader RNA in vitro

To understand the mechanism for specific viral RNA binding by Chandipura virus Nucleocapsid protein, N, we used a previously described soluble preparation of recombinant N in an RNA binding assay (Majumder et al., 2001). Accordingly, in vitro synthesized radiolabeled leader RNA was incubated with N protein and the RNA–protein complexes were resolved through native gel (Materials and methods). Fig. 1A shows formation of high molecular weight complex upon incubation of N protein with leader RNA (lane2), where the intensity of the shifted band progressively increased with an increasing gradient of N (lanes 2–7). Subsequently, we examined RNA binding ability of N protein by EMSA in the presence of a variety of salts or detergents to elucidate the biochemical nature of the RNA–protein interaction. As reported earlier (Iseni et al., 2000), we found the N–RNA complex to be modestly salt-sensitive due to its ionic nature (data not shown). Surprisingly, pretreatment of N protein with a mild ionic detergent, deoxycholate (DOC) at two different concentrations (0.5% and 1%) revealed an additional faster migrating complex

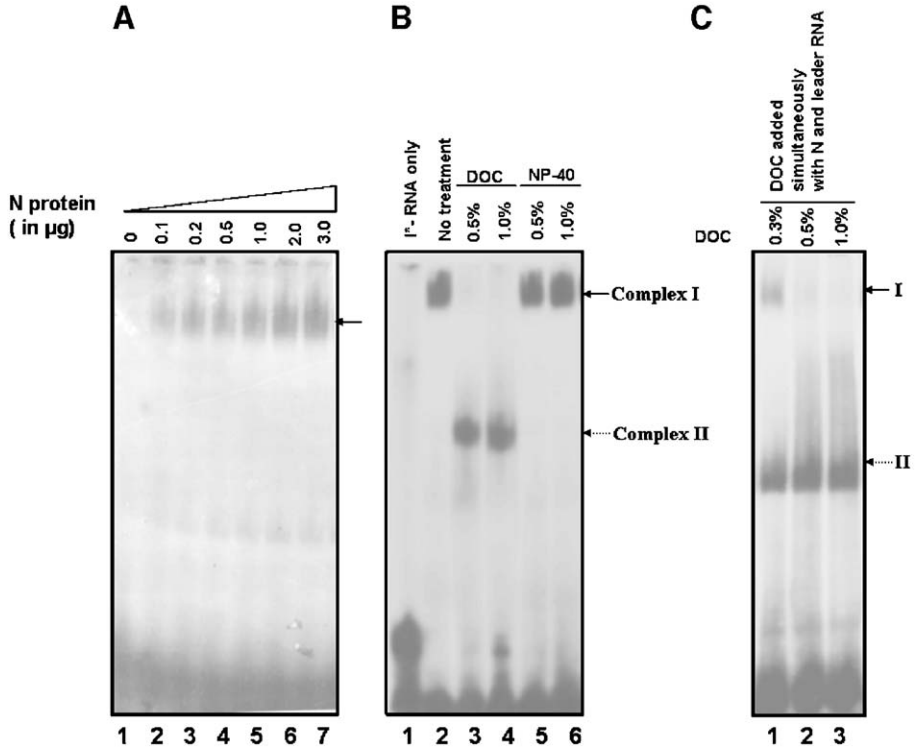

Fig. 1. N protein forms two different complexes with leader RNA in vitro. (A) The ability of purified recombinant N protein to encapsidate leader RNA in vitro was tested by electrophoresis mobility shift assay (EMSA). In vitro synthesized radiolabeled leader (l*) RNA was incubated in 1× binding buffer either alone (lane 1) or in the presence of increasing N protein concentrations as indicated (lanes 2 to 7), at 37 °C for 15 min. Complexes were resolved through a 4% native PAGE. (B) To test detergent sensitivity of RNA binding ability of N protein, l*-RNA probe was incubated with 3 µg of N protein as in panel A (lane 2) or after prior treatment with two different concentration (0.5% and 1%) of deoxycholate (DOC) (lanes 3 and 4) or NP-40 (lanes 5 and 6) for 10 min in binding buffer at 37 °C before loading on a native gel. (C) N protein was incubated simultaneously with l*RNA and 0.3%, 0.5% and 1% DOC (lanes 1, 2 and 3, respectively) for 15 min as usual and the complexes were resolved through a native gel.

(complex II) upon RNA binding, at the expense of the higher order N–RNA complex (complex I) (Fig. 1B, lanes 3–4). Furthermore, nonionic detergent NP-40 was unable to elicit such a change in the oligomeric status of N–RNA complexes (lanes 5–6), suggesting a specific mechanism underlying DOC-action. Pretreatment with as low as 0.3% DOC was sufficient for complete abrogation of higher order N–RNA complex and formation of complex II (data not shown). Furthermore, increasing concentration of DOC, added during formation of the N–RNA complex, results in formation of the faster migrating complex II exclusively (Fig. 1C). These results indicate that the formation of complex II might be directly dependent on the disruption of N–N interaction, solely attributed to the probable action of DOC to prevent N protein oligomerization without affecting its leader RNA recognition ability.

Deoxycholate induces a change in the oligomeric status of N protein

Previously, we described self-aggregation property of N protein, where a gradual increase in the hydrated diameter of N protein was observed in complete absence of detergents over a period of 24 h. Presence of mild detergent, such as Triton X-100, was shown to partly prevent this self-aggregation process (Majumder et al., 2001). As treatment with dissociating detergents, such as DOC, results in formation of a unique faster migrating N–RNA complex, we sought to examine any potential alterations in the oligomeric status of N protein itself, upon DOC-treatment. To this end, affinity-purified N protein either alone or after treatment with 1% DOC was subjected to gel filtration chromatography through a Sephacryl S-300 column (Fig. 2A). Accordingly, different fractions were collected, absorbance was measured at 280 nm and molecular weight was estimated assuming a globular solution behavior for N protein in its different oligomeric forms (Materials and methods). As depicted in Fig. 2A, majority of the N protein (closed circle) elutes in the 27th fraction, with an estimated molecular weight of approximately 250 kDa. DOC treatment results in a change in the elution profile with majority of N (open circle) eluting in fraction 52 as a monomer protein, with an estimated molecular weight of 45 kDa. This result indicates a change in the oligomeric status of N protein upon treatment with dissociating ionic detergent, which probably interferes with N–N interaction and thus maintains N protein in a monomeric form.

Additionally, we carried out sucrose density gradient centrifugation to examine sedimentation properties of N protein in the absence or presence of detergent. N protein, either untreated or treated with 1% DOC, was loaded on a 10%–60% preformed sucrose gradient and alternate fractions were subjected to SDS-PAGE followed by silver staining (Fig. 2B).

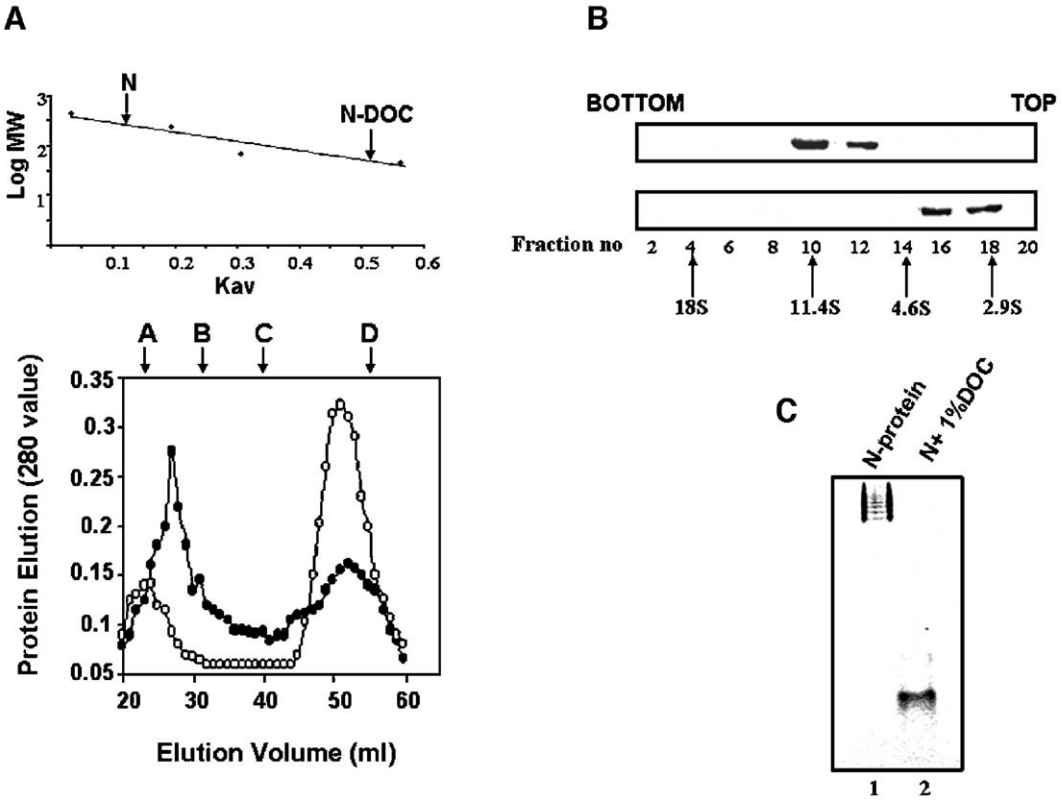

Fig. 2. Deoxycholate induces a change in the oligomeric status of N protein. (A) Size exclusion chromatography. Purified preparation of N protein in the presence (open circle) or absence (closed circle) of 1%DOC was analyzed by gel filtration chromatography on a Sephacryl S-300 column (1.5 × 45 cm) equilibrated in binding buffer at 4 °C. Elution profile of N and N-DOC is expressed as the relative absorbance at 280 nm. Arrows indicate the elution position of protein molecular weight standards with known molecular weights, used to calibrate the column-A: Ferritin (440 kDa), B: Catalase (232 kDa), C: BSA (67 kDa) and D: Ovalbumin (45 kDa). Above: Calibration graph of the column obtained from the elution profiles of protein standards. The estimated molecular weights of untreated (N) and DOC-treated (N-DOC) N protein were determined by comparing their Ve/Vo ratios with the calibration graph. (B) Silver-stained 10% SDS-PAGE profile of alternate fractions of purified N protein either alone or treated with 1% DOC after fractionation through a preformed 10–60% sucrose step gradient by centrifugation at 32,000 rpm for 16 h at 4 °C. Arrows below the gels indicate sedimentation positions of standard proteins with known sedimentation coefficients, run in parallel; Carbonic anhydrase (2.9 S), BSA (4.6 S), Catalase (11.4 S) and Ferritin (18 S). (C) Fractions from the previous sucrose gradient designating both untreated (fraction 10–12) and DOC-treated (fraction 16–18) N protein (lanes 1 and 2, respectively) were pooled separately, mixed with native dye and loaded onto a 6% native PAGE, run at 4 °C in native buffer. The gel was stained with Coomassie Brilliant Blue R-250.

N protein was almost exclusively detected in fractions 10–12, from the bottom of the gradient. However, detergent treatment results in a population of slower sedimenting N, which could be found in fractions 16–18. Elution of a higher order N assembly in an identical position to 232 kDa protein Catalase as an 11.5 S particle in sucrose gradient (Fig. 2B, upper panel), along with the previous gel filtration results further suggests that N may form a pentamer in solution. Importantly, DOC-treated N elutes as a 3 S particle along with ovalbumin (45 kDa), thus confirming existence of monomeric N upon detergent treatment (Fig. 2B, lower panel). Finally, we separately pooled either N protein eluted in fraction 10–12 or detergent-treated N protein eluted in fraction 16–18, concentrated them and loaded the two fractions on a 6% native PAGE. The untreated N protein migrated near the top of the gel as a conglomeration of closely spaced bands as earlier reported for VSV (Masters and Banerjee, 1988b) while DOC-treated proteins entered into the gel as a single faster migrating band (Fig. 2C). These results in conjunction with our previous analysis suggest that DOC promotes formation of monomeric N protein by disrupting oligomeric assemblies. While oligomeric N protein showed certain heterogeneity on native gel, it apparently revealed characteristics of defined complex on gel filtration analysis. Therefore, it seems likely that subtle conformational heterogeneity could exist within oligomeric N population that is otherwise identical in terms of subunit composition.

Stoichiometry of different N–RNA complexes

Deoxycholate prevents N protein oligomerization and keeps it in the monomeric form. Accordingly, RNA binding analysis revealed presence of a faster migrating N-leader RNA complex in the presence of detergent. These results suggest a possible change in the stoichiometry of N–RNA complex upon DOC treatment. To investigate this possibility, we examined the number of N molecules bound per leader RNA utilizing gel filtration and sucrose density gradient centrifugation assays. Radiolabeled leader RNA was incubated with either N protein alone or N pretreated with DOC as described (Materials and methods). Subsequently, the complexes were resolved through a

S-300 gel filtration column pre-equilibrated with binding buffer. RNA elution profile was monitored by Cerenkov counting of each fraction as a measure for complex elution. Consistently, we observed two distinct peaks for the DOC-treated (open circles) and untreated (closed circles) sets indicating the presence of two stable RNA–protein complexes, separable with respect to their molecular weights (Fig. 3A). Estimated molecular weight for complex I was found to be ~300 kDa suggesting that five molecules of N protein bind to RNA to form the higher order complex. In contrast, the radioactivity peak was observed to coelute with BSA in the presence of DOC, indicating a molecular weight of 67 kDa. This implies that one N binds to leader RNA to form the intermediate complex II. Leader-RNA alone eluted at a different position sufficiently separated from that of either complexes (not shown).

Next, we examined the N protein–leader RNA complexes by sucrose density gradient centrifugation. While some free N protein unassociated with RNA eluted as protein oligomer in 10–12 fraction, as observed before, the higher order complex I was exclusively found in the 6th fraction from the bottom of the gradient as evident from the coelution of N protein and labeled RNA (Fig. 3B, panel i). This gives a sedimentation value of ~18 S for the higher order complex that corroborates with previous findings by Blumberg and Kolakofsky (1981). Nonetheless, separate elution profile for oligomeric N and RNA-bound oligomer also confirms our N preparation, per se, to be free from any endogenous bacterial RNA. Complex II formed in the presence of DOC, however, sediments predominantly in ~14th fraction with an *S* value of ~4.6, suggesting the presence of a molecular species of 67 kDa, composed of one N molecule and an RNA (Fig. 3B, panel ii). These results along with gel filtration analysis, thus confirm a change in the stoichiometry of N–RNA complex upon DOC treatment, concomitant with a DOC-induced disruption of protein oligomer.

Complex II is different from the leader RNA encapsidation complex

The higher order RNA–protein complex consisting of oligomeric N formed an encapsidation complex that was irreversible and resistant to RNase (Basak et al., 2003; Majumder et al., 2001). We asked if an RNA–protein complex consisting of DOC-treated N retained such characteristics. To test RNase sensitivity, leader RNA was incubated with N protein in the absence or presence of deoxycholate. Subsequently, the preformed complexes were treated with RNaseA prior to loading on a native gel. Unlike complex I, monomeric N–RNA complex was found to be RNase sensitive with complete loss of shifted band upon RNase treatment (Fig. 4A, compare lanes 3 and 4). When preformed N-labeled leader RNA was challenged with excess unlabeled leader RNA, only a minor decrease in the shifted band intensity was observed implying that the higher order N–RNA complex formation was irreversible (Fig. 4B, lanes 1–4). However, complex II formed in the presence of DOC was found to be reversible in nature. Addition of 2–10-fold molar excess of unlabeled leader RNA to preformed complex II results in proportional decrease in the shifted band intensity within 10 min of cold RNA addition

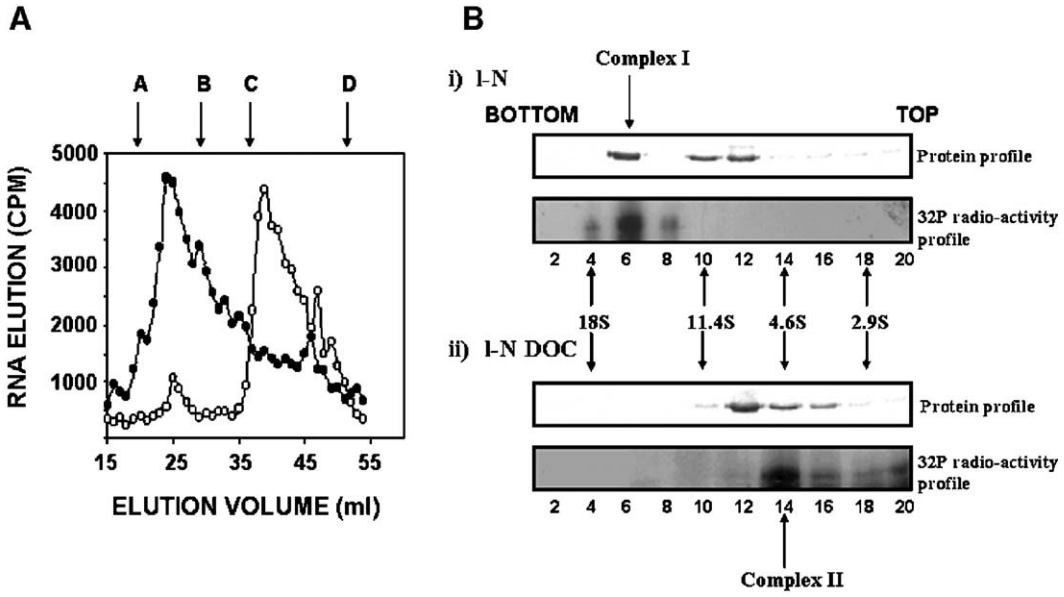

Fig. 3. (A) N protein either untreated (closed circle) or treated with 1% DOC for 30 min (open circle) was mixed with α^{32}P-UTP-labeled in vitro synthesized leader RNA and incubated in binding buffer for 30 min. The resulting complexes were loaded on a Sephacryl S-300 column equilibrated in the same buffer at 4 °C. RNA elution profile was monitored by Cerenkov counting and expressed as counts per minute for each 1 ml fraction. Arrows represent the elution of protein molecular weight standards—A: Ferritin (440 kDa), B: Catalase (232 kDa), C: BSA (67 kDa) and D: Ovalbumin (45 kDa). (B) Sedimentation analysis of the N protein–leader RNA complexes. The I*RNA–N protein complexes, both untreated and treated with DOC (i and ii, respectively), were formed essentially as in gel filtration and sedimented through 10–60% sucrose gradients in binding buffer by ultracentrifugation as mentioned earlier. Alternate fractions, collected from the bottom of the tubes, were analyzed through 10% SDS-PAGE for the presence of RNA–protein complexes. Both autoradiographic impressions as well as silver staining of the same gels are shown to give an estimate of the elution profile of α^{32}P-UTP-labeled RNA and protein in the complexes. Sedimentation of protein standards is marked by arrows: Carbonic anhydrase (2.9 S), BSA (4.6 S), Catalase (11.4 S) and Ferritin (18 S).

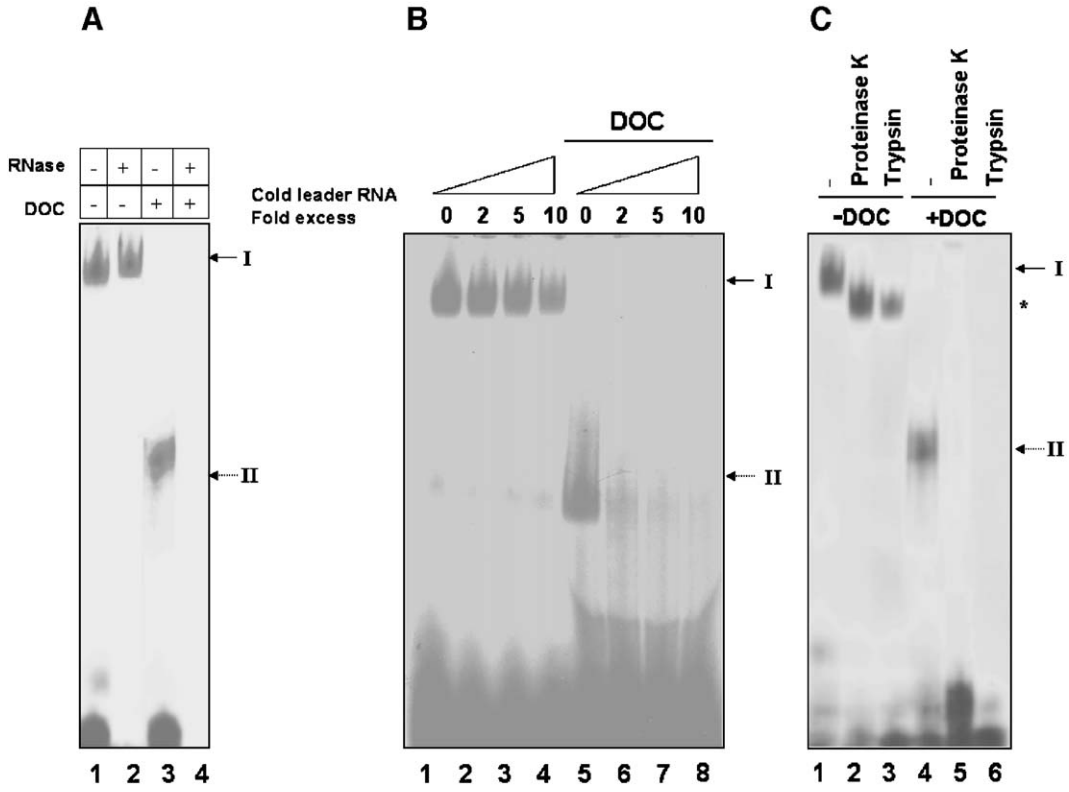

Fig. 4. Complex II is different from the leader RNA encapsidation complex. (A) To test the RNase sensitivity of two complexes, N protein, either untreated (lane 1) or pretreated for 10 min with 1% DOC (lane 3), was incubated with l*RNA probe. The two complexes thus formed were additionally incubated for 10 min with RNaseA (final concentration 60 μg/ml) (lanes 2 and 4, respectively). (B) To check for the differential mode of interaction, complexes I and II were formed essentially as mentioned earlier (lanes 1 and 5). The preformed complexes were challenged with 2-fold (lanes 2 and 6), 5-fold (lanes 3 and 7) or 10-fold (lanes 4 and 8) molar excess of cold leader RNA for another 10 min before loading onto a 4% native gel. (C) For the protease sensitivity experiment, complexes I (lane 1) and II (lane 4) were treated with either Proteinase-K (lanes 2 and 5) or Trypsin (lanes 3 and 6) for 10 min and resolved through gels. *Indicates the altered position of complex I upon protease treatment.

owing to an exchange between N-bound-labeled RNA with unlabeled leader (Fig. 4B, lanes 5–8). Furthermore, we checked protease sensitivity of preformed complexes I and II by partial digestion of RNA–protein complexes with Proteinase K or Trypsin. Complex I showed resistance towards both the proteases as evident from the formation of slightly faster migrating encapsidation complex on a native gel (Fig. 4C, lanes 1–3). This could be due to digestion of a protease-sensitive region of N protein in higher order RNA–protein complex leaving a protease-resistant core of the protein still bound to leader RNA. This result is complementary to the finding of Iseni et al. (1998) who showed that Trypsin cleaved a 17 kDa fragment of rabies virus N protein without altering the overall nucleocapsid structure. Complex II, on the contrary, was susceptible to both Proteinase K and Trypsin treatment (lanes 4–6). Therefore, this observation indicates that the nucleocapsid structure confers protease resistance to N protein, while in the absence of any protein–protein interaction (i.e. in presence of DOC), N protein is sensitive to protease action, even in RNA-bound form.

N monomer recognizes a specific sequence element in the leader RNA

Our present analysis shows that N protein can interact with leader RNA both as an oligomer or monomer, albeit differently, forming distinct complexes. Previous studies from our group in the Chandipura virus system and also by other researchers have consistently shown that N protein possesses a nonspecific RNA binding activity (Basak et al., 2003; Masters and Banerjee, 1988b). In this context, we asked if nonspecific RNA binding ability of N protein is intrinsic to individual N polypeptide or an acquired trait arising from protein multimerization. To this end, N protein without additional treatment with DOC was incubated with an increasing gradient of unlabeled leader RNA (lanes 2–4, Fig. 5A), leader RNA (1–21) (lanes 5–7, Fig. 5A), unrelated tRNA (lanes 8–10) or unrelated RNA derived from pGEM3z vector (lanes 11–13). Subsequently, radiolabeled leader RNA was added to the reaction mixture prior to resolving the complexes in a native gel (Materials and methods). Our competition gel shift analysis revealed that leader RNA binding could be efficiently competed by unlabeled RNA of viral or nonviral origin as evident from complete absence of shifted band intensity, demonstrating the nonspecific RNA binding ability of oligomeric N (Fig. 5A). This result suggests that oligomeric N utilizes a single site with broad RNA recognition specificity for complex I formation thus allowing competition by nonviral RNA. Interestingly, DOC-treated N protein was able to form the complex II even in the presence of 2–5-fold molar excess of cold unrelated tRNA (Fig. 5B, lanes 8–10) or nonviral RNAs similar in size to leader, using either pGEM3z

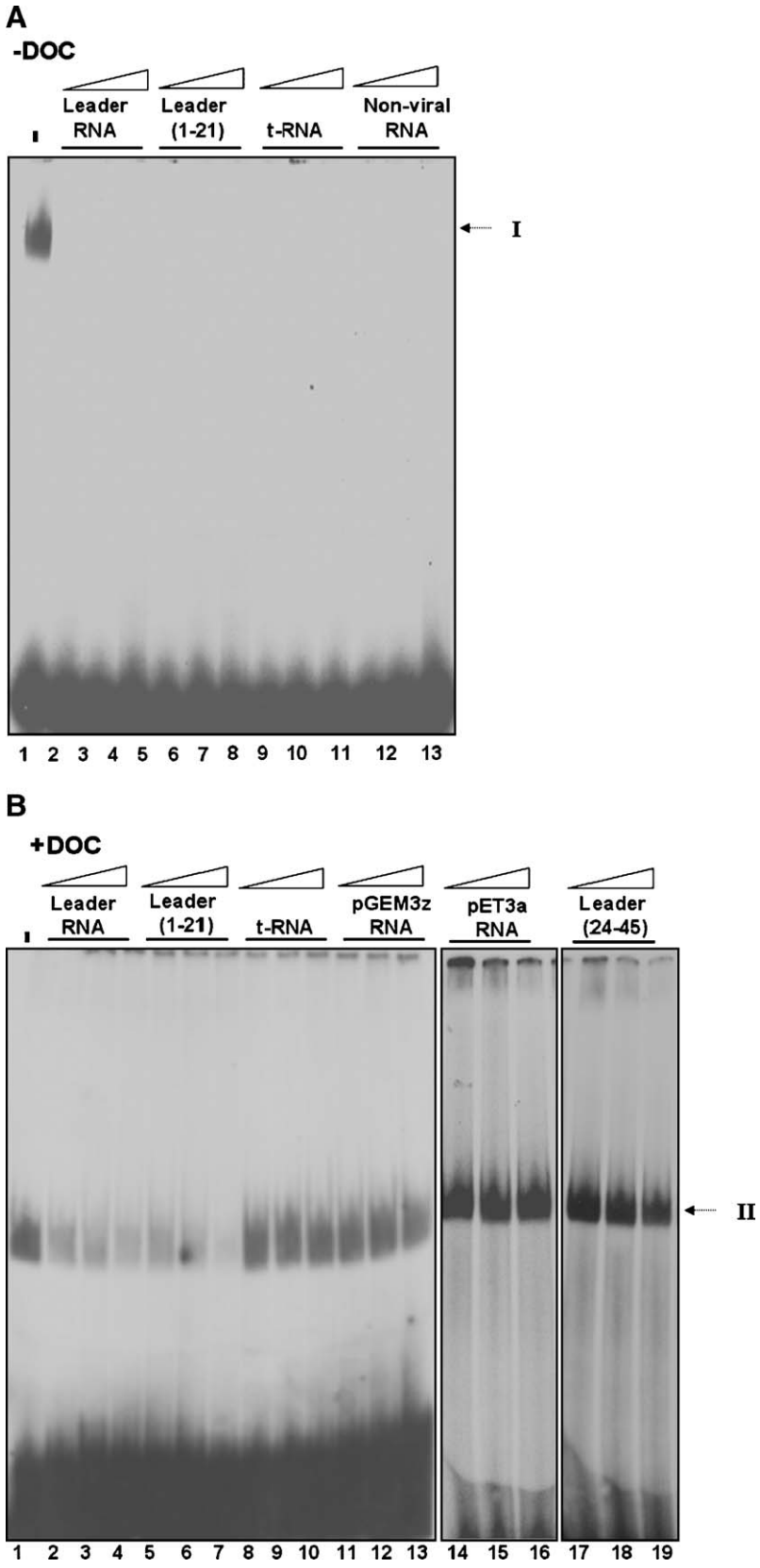

Fig. 5. Monomer N-leader RNA interaction is specific in nature. (A and B) N protein either alone (A) or after treatment with 1% DOC (B) was incubated in buffer alone (lane 1) or in the presence of equal (lanes 2, 5, 8 and 11), 2-fold (lanes 3, 6, 9 and 12) and 5-fold (lanes 4, 7, 10 and 13) molar excess of cold leader, first half of leader l (1–21), tRNA and a 3z vector derived nonviral RNA, respectively, in binding buffer at 37 °C for 15 min. The specificity of complex II formation was also tested in the presence of similar molar excesses of either pET3a-derived nonviral RNA of comparable base composition to leader (B, lanes 14–16) or the second half of leader l (24–45) (B, lanes 17–19). The reaction mixtures were chased with l*RNA probe for 10 min and resolved through a 4% native PAGE.

vector-derived RNA (lanes 11–13) or pET3a vector-derived RNA of similar base composition (lanes 14–16). Viral leader sequence and more importantly leader (1–21) efficiently compete with the labeled probe for the complex II formation (lanes 2–7). These results indicate the presence of a distinct virus-specific RNA recognition motif in monomeric N, which could not be saturated by even 5-fold molar excess of nonviral RNA. However, saturation of this specific RNA binding motif by unlabeled leader RNA (1–21) indicates that a specific *cis*-acting sequence present in the first half of the leader could be responsible for complex formation between monomeric N and leader RNA. Interestingly, the first 24 nucleotides of the leader RNA contain high sequence homology among different rhabdoviruses (Marriott, 2005) and were shown to be necessary for efficient encapsidation and genome replication (Li and Pattnaik, 1997). Moreover, the second half of leader RNA (24–45) could not chase out the complex II (Fig. 5B, lanes 17–19), further indicating that the first half of the leader is responsible for N monomer binding. From these data, it also appears that oligomerization of N protein compromises its RNA binding specificity, presumably by masking specific RNA recognition motif and creating additional altered surface of broader specificity at the expense of specific RNA binding domain.

Subsequently, we performed toe-printing analysis on leader RNA bound to N protein monomer to define specific target sequence of monomeric N. For this purpose, we utilized an in vitro synthesized 70 nt long leader RNA with a fusion of additional 21 residues at the 3′end of 49 nt leader sequence (Materials and methods). We also designed a primer that anneals to the extended 3′end sequence of leader RNA and produces a 70 nt long cDNA product in reverse transcription reaction (lane 1, Fig. 6A). Subsequently, leader RNA, bound to N protein in the absence or presence of DOC, was annealed with the primer and reverse transcribed. Binding of N oligomer to leader interfered with primer annealing and thus no cDNA product was obtained (lane 2). However, RNA present in the complex II could be partially copied to form a cDNA product, suggesting the presence of a block at the 14th residue, mediated by the specific binding of N monomer on the leader RNA (lane 3) that inhibits cDNA elongation. Furthermore, intensity of the inhibited cDNA product increased in a manner that is dependent on the concentration of deoxycholate treated N (lanes 3–5).

The trailer region positioned at the extreme 5′end of the genome RNA shows a high degree of complementarity to the leader sequence. The encapsidation process is initiated on the (+) sense leader sequence during formation of the viral replication intermediate, the positive strand genome. During the synthesis of progeny negative strand genome RNAs from the replication intermediate, RNA synthesis and consequently encapsidation begin from the trailer sequence. We tested, if (−) trailer is also a substrate for specific binding by monomeric N protein. To this end, trailer sequence cloned under T7 promoter

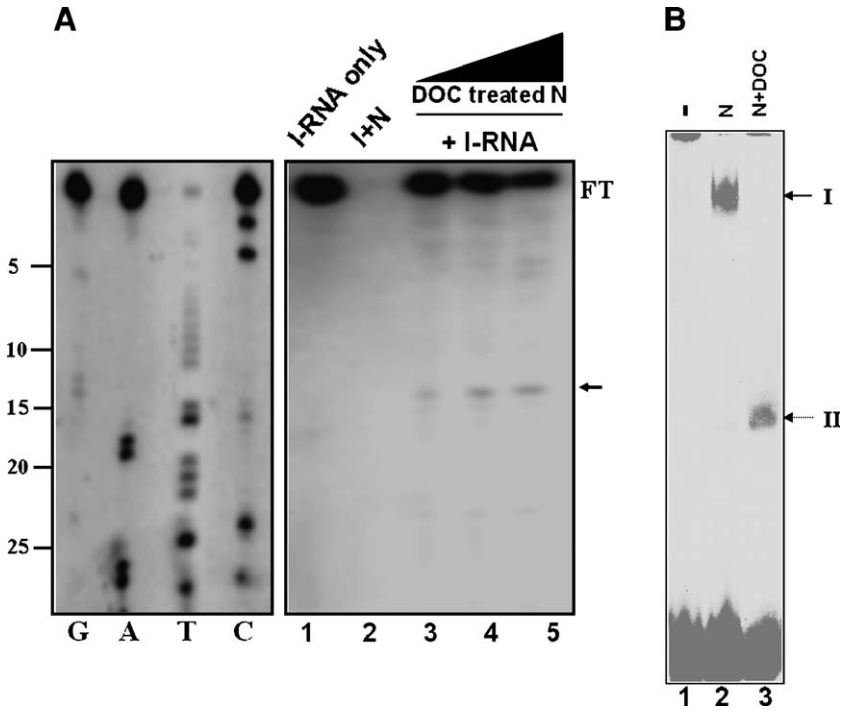

Fig. 6. N monomer recognizes a specific sequence element in the leader RNA. (A) Toeprint analysis of the formation of initiation complex for encapsidation. Autoradiogram of a 8% sequencing gel showing inhibition of extension reaction from a γ-^{32}P end-labeled primer PE1 on in vitro synthesized CHP virus leader RNA (Chpl-T7) (lane 1) or after incubation with N protein that is untreated (lane 2) or treated with DOC (lane 3). An increasing concentration gradient of 1%DOC treated N was used in binding reaction (lanes 3 to 5). FT is the signal corresponding to the full length reverse transcript, arrow indicates the position of the major toeprint signal, 14 nt less than FT. Lanes G, A, T and C correspond to the individual sequencing reactions with respective ddNTPs, performed on free Chpl-T7 RNA with the same primer to determine the nucleotide bases of interaction. (B) To test possible interaction between trailer sequence and N protein, in vitro synthesized radiolabeled RNA encompassing trailer sequence (lane 1) was incubated with N, untreated or DOC-treated (lanes 2 and 3, respectively). Subsequently, complexes were resolved on 4% native PAGE.

in pGEM3z vector was utilized to synthesize trailer RNA (Materials and methods). Gel shift analysis revealed that N protein both as oligomer or monomer was able to form distinct complexes with trailer RNA, thus, resembling leader RNA binding (Fig. 6B). Our competition gel shift experiments revealed that unlabeled trailer RNA efficiently competes with leader as well as trailer probe for virus-specific RNA binding region of monomeric N (data not shown).

P protein maintains majority of N in the monomer form in vitro

The DOC-mediated disruption of oligomeric form of N protein demonstrated the monomeric status to be an important prerequisite for the specific interaction of N with l-RNA. So we questioned whether the P protein of CHP virus could substitute the in vitro action of DOC in keeping N in a predominantly monomeric form. In our experimental condition, we were unable to disrupt oligomeric or higher aggregates of N proteins, once formed, into monomer by incubation with P (data not shown). Therefore, we used detergent-dissociated N monomer, dialyzed against a deoxycholate free buffer in the absence or presence of P protein, to examine the ability of P to keep N as a monomer. After dialysis, the protein samples were loaded on sucrose density gradient to test any possible change in the oligomeric status of the N protein. Upon DOC removal in absence of P, N protein eluted in fraction 10–12 similar to that of untreated oligomer, thus implying detergent dissociation of N-oligomer to be a reversible process. However, an equimolar mixture of N and P protein formed upon DOC dialysis revealed most of the N protein to be present as a slower sedimenting particle coeluting with P protein as a complex (Fig. 7, bottom panel). The elution profile of the N–P complex in sucrose gradient with peak elution in fraction 14–16 closely matches that of DOC-treated monomeric N, thus implying maintenance of N in monomer form by P protein in solution, possibly in a 1:1 complex. We also noticed that a minor amount of N oligomer coelutes with P in fraction 4 as a faster sedimenting higher order

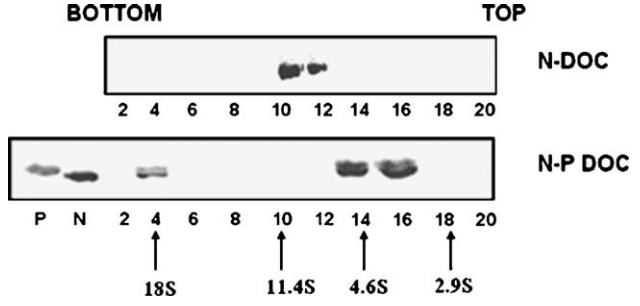

Fig. 7. P protein maintains majority of N in the monomeric form. Purified N protein was treated with 1% DOC for 30 min as mentioned earlier. Subsequently, DOC was dialyzed out over night against TET/150 mM NaCl, either in the absence or presence of equimolar amount of P protein. The DOC dialyzed N or N–P complexes (N-DOC and N–P DOC, respectively) were layered on top of a preformed 10–60% sucrose gradient in the same buffer and sedimented by ultracentrifugation as described. Fractions collected from the bottom were analyzed through silver staining of a (30:0.4) 10% SDS-PAGE. Purified N and P proteins were run in parallel to provide an estimate of their differences in migration. The protein standards are marked by arrows.

complex (bottom panel). In addition, we used FITC-labeled N protein and incubated with P in presence of DOC. Subsequently, detergent was removed by dialysis, the reaction mixture was resolved by gel filtration and the elution profile of N was monitored by measuring the emission at 520 nm. Chromatographic separation methods revealed similar results, viz, a predominant pool of N was maintained in a lower molecular weight form in the presence of P protein (data not shown).

N protein maintained in a monomer form by P can encapsidate viral sequences in a specific yet processive manner

P protein was shown to form complexes with nascent N synthesized in reticulocyte lysate in vitro to prevent the binding of vesicular stomatitis virus N protein with nonviral RNA (Masters and Banerjee, 1988b). However, a higher order N–P complex formed by coexpressing N and P protein in *E. coli* was shown to be associated with ~90 nt long bacterial RNA (Green et al., 2000). In this context, we sought to examine RNA binding specificity of N oligomer or monomer N–P complexes purified through sucrose gradient. For this purpose, radiolabeled ~65 nt long nonviral RNA derived from pGEM3z vector was incubated with N oligomer or N–P complexes (Fig. 8A) in the presence of increasing amount of unlabeled leader RNA (lanes 3–4 and 6–7) and the reaction mixtures were resolved through a native gel. As reported earlier, oligomeric N was able to bind to nonviral RNA forming a higher order complex (lane 2), while N–P complex did not recognize pGEM3z derived RNA sequences (lane 5). Furthermore, excess unlabeled leader RNA could be shown to compete for this nonspecific RNA binding by N oligomer (lanes 3–4), which further indicates that a single site on N-oligomer is utilized for both leader RNA binding and interaction with nonviral RNA (discussed later). Nonetheless, we confirm that Chandipura virus N protein in a 1:1 complex with P is identical to DOC-disrupted monomeric N in that it does not recognize nonviral sequences.

Although, aggregated N protein was shown to form RNase-resistant complexes with leader RNA (Blumberg et al., 1983), it was unable to encapsidate longer VSV RNA in vitro (Moyer et al., 1991). Based on our observations, it seems likely that oligomeric N bound to short leader RNA sterically hinders the nuclease action. In contrast, the longer transcripts remain RNase sensitive possibly due to inability of the oligomer to properly enwrap the RNA. However, it may also be possible that N oligomer does form a true encapsidation complex on leader sequence but a viral or host factor plays an essential role in continuation of the encapsidation process after a finite length. Preparation of N protein in a 1:1 complex with P, that preserves N as a monomer, provided an opportunity to directly address the encapsidation competency of this monomeric form. To this end, stoichiometric amount of leader RNA was incubated with oligomeric N (fraction 10–12) or purified N–P complex (fraction 14–16), from the sucrose gradients. Subsequently, complexes were treated with RNase, protected RNA was phenol extracted, ethanol precipitated and examined on a 10% denaturing urea PAGE. As depicted in Fig. 8B, N protein in a monomeric form complexed with P was equally proficient in

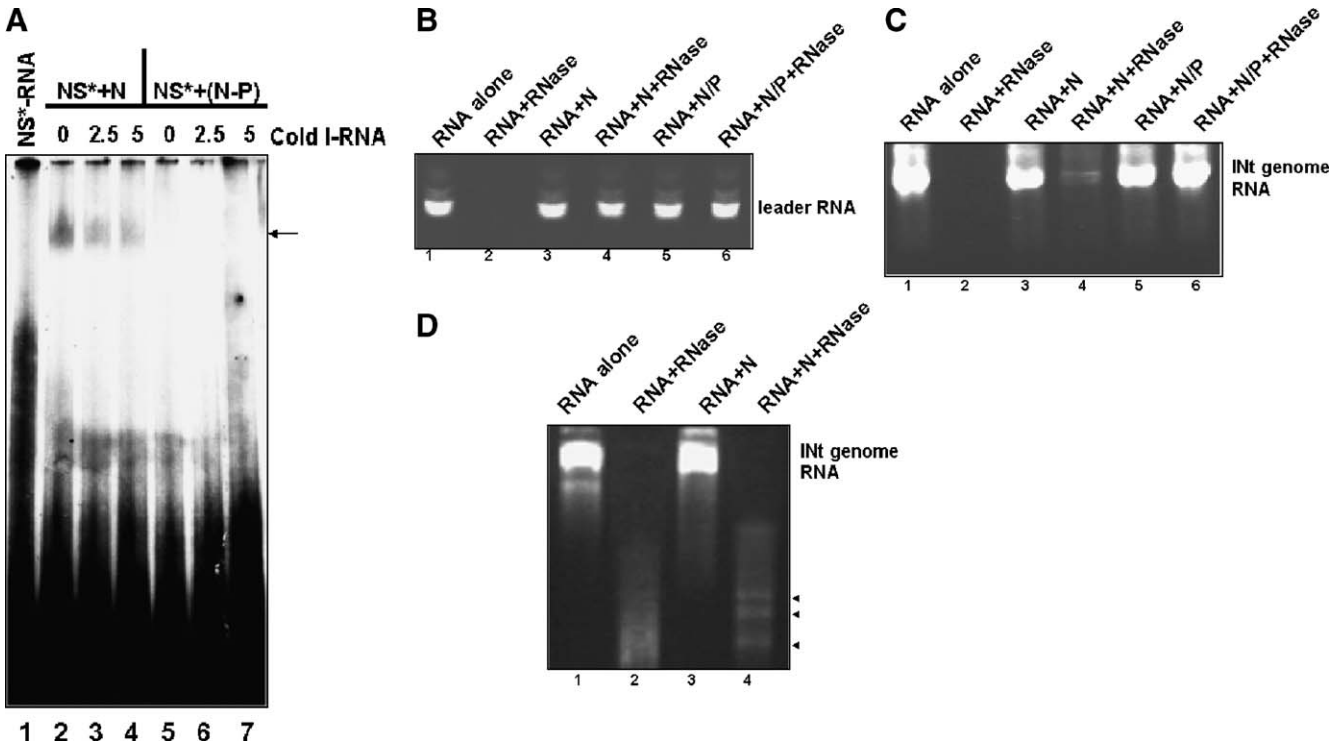

Fig. 8. N monomer–P complex encapsidates viral sequences in a specific yet processive manner. (A) P provides specificity to the RNA binding ability of N. NS*RNA (lane 1) was incubated in 1× binding buffer with DOC dialyzed N or N–P either alone (lanes 2 and 5, respectively) or in the presence of 2.5-fold (lanes 3 and 6) or 5-fold (lanes 4 and 7) molar excess of specific cold l-RNA before resolving the complexes through a 4% native gel. (B and C) RNase protection assay was performed with N (lanes 3 and 4) or N–P complexes (lanes 5 and 6) after DOC dialysis and in vitro synthesized leader RNA (B) or lN_t genome RNA (C). The RNA–protein complexes were allowed to form as usual. Subsequently, RNase (200 μg/ml final concentration) was added to the reaction mixture and the digestion was stopped by Proteinase-K addition after 15 min. The protected RNAs were viewed by ethidium bromide staining of the respective gels—10% urea PAGE for the l-RNA (B) and 1% agarose gel for lN_t genome RNA (C). A control RNA lane without protein and the corresponding digestion pattern are shown for both the cases (lanes 1 and 2, respectively). (D) The protected RNA products of lN_t genome RNA before and after interaction with DOC dialyzed N protein and subsequent RNase treatment were run on a 4% agarose gel and viewed similarly. Arrows correspond to the protected RNA products.

protecting leader RNA (lanes 5–6) as N-oligomer (lanes 3–4), implying encapsidation of viral sequence by N–P complex in our assay condition. However, protein binding to these short transcripts (~50 nt) may prevent RNase action by multiple mechanisms, which could be independent of true enwrapment of bound RNA by protein units. Hence, we utilized the RNase protection assay to examine processive encapsidation of longer transcripts that resemble viral genome termini. To this end, we first cloned a 597 nt fragment from the 3′end of Chandipura genome under the control of T7 promoter in pGEM3z vector and in vitro synthesized (+) sense lNt genome RNA (Materials and methods). Subsequently, genome lNt RNA (Fig. 8C) was incubated with DOC dialyzed N (lanes 3–4) or N–P complex (lanes 5–6). RNA alone or RNA–protein complexes were treated with RNaseA and protected RNA was run on a 1% agarose gel. As depicted in Fig. 8C, the N oligomer was unable to protect mini-genome length RNA from RNase action (lanes 3–4). However, discrete bands of ~50 to 200 nucleotides were observed on 4% agarose gel after RNase treatment (Fig. 8D, lane 4), reflecting the ability of the N-oligomer to provide protection to RNA of finite length. Interestingly, proper encapsidation of genome lNt RNA in the presence of N–P complex into an RNase-resistant structure was revealed by its ability to offer complete protection of the genome like fragment (compare lanes 5 and 6, Fig. 8C). These results indicate that monomeric N protein present in a P-bound form retains two important characteristics of the true encapsidating macromolecule, namely, its ability to recognize a specific site on leader sequence (nucleation) and its power to ensure processive enwrapment of genome length RNA into an RNase-resistant form (elongation). Current analysis also indicates that oligomeric N binds to RNA nonspecifically and can protect bound RNA from RNase action only up to a finite length, in a way that is not dependent on true encapsidation.

Discussion

While one major function of N is to protect genome RNA from RNase action, processive encapsidation was proposed to modulate polymerase activity in the replication phase (Gubbay et al., 2001). How N protein recognizes critical sequences on nascent viral genome within infected cell and continues to progressively encapsidate a 11.2-kb genome RNA into a mature nucleocapsid structure remains poorly understood. N protein of Chandipura virus, similar to its VSV counterpart, lacks any known RNA recognition motif in its amino acid sequence, which implies possible involvement of novel RNA recognition modes in the encapsidation process. Although, the last five amino acids

of VSV nucleocapsid protein were shown to be required for genome encapsidation (Das et al., 1999), it is currently unclear how N protein acquires specific yet processive encapsidation capability. Nonetheless, self-association tendency presented difficulty in obtaining adequate amount of N for its biochemical characterization. Over-expression in *E. coli* resulted in a form of N protein that was insoluble except in high salt (Das and Banerjee, 1993). Although coexpression with P protein maintained N in a soluble form, it resulted in a higher order N–P complex that bound bacterial RNA nonspecifically (Green et al., 2000). We were able to optimize an expression condition for Chandipura virus N protein that allowed us to obtain homogeneous N protein in a soluble oligomeric form (Majumder et al., 2001). Although, our purified N preparation was free from any cellular RNA (Majumder et al., 2004), it had the ability to interact with nonspecific RNA in gel shift assays. We believe ion exchange-based purification protocol resulted in dissociation of any bacterial RNA from our N preparation during high salt elution steps. Consistent to this notion, we observed rapid dissociation of N–RNA complex upon salt treatment by EMSA although this interaction is stable when competed with unlabeled RNA, implying formation of a complex that is stabilized through ionic interaction. However, consistent with prior reports, this form of the N protein sedimented rapidly in density gradients as demonstrated in Fig. 2B and was able to form higher order complexes with leader RNA (Howard and Wertz, 1989). The leader RNA bound to oligomeric N is resistant to RNase action and sediments as a ~18 S particle similar to the authentic encapsidation complexes previously observed for VSV (Blumberg et al., 1981, 1983). When tested with a 597-nt long RNA that was identical to (+) ve sense genome fragment derived from the 5′end of the Chandipura genome intermediate (Figs. 8C and D), the oligomeric N, however, was incapable of encapsidating RNA into an RNase-resistant structure beyond a certain length (~50–200 nt). This observation prompted us to define the true encapsidating unit that can ensure specific yet processive encapsidation.

Previous studies with VSV-defective interfering particle revealed that the 5′terminal 36 nucleotides and 3′terminal 51 nucleotides of the genome are sufficient for VSV-DI replication, encapsidation and budding of the virus like particles (Pattnaik et al., 1995). In vitro assembly of synthetic nucleocapsid identified the first 19 nt from the 5′end of the (+) sense RNA to be necessary and sufficient for processive encapsidation (Moyer et al., 1991). The same set of studies revealed that heterologous sequences, when fused downstream to the first 19 authentic nucleotides, supported encapsidation as efficiently as viral sequences, demonstrating compromised specificity in the ensuing encapsidation process although enwrapment reportedly follows a distinct pattern that requires one N molecule per 9 nucleotides of RNA (Thomas et al., 1985). We utilized a dissociating detergent, deoxycholate, to disrupt oligomeric N into monomeric form (Fig. 2) and dissected specific as well as general RNA recognition modes of N. Gel filtration and sucrose density gradients showed that the monomeric N forms 1:1 RNA-bound complex in the presence of DOC that is distinguishable from higher order complexes. However, as depicted in Fig. 5B, the most intriguing RNA binding property of monomeric N is its specificity towards viral leader sequence, particularly the first ~20 nt of the (+) ve sense genome. Interestingly, the trailer sequence, representing the 5′terminus of (−) ve sense genome and which is complementary to and has high sequence homology with leader RNA, was also recognized specifically by the monomer. The monomeric N bound to specific nucleation site on the first half of the leader RNA was unable to recruit additional N molecules to form encapsidation complexes in the presence of DOC, revealing discrete requirement for N–N association in the ensuing encapsidation process. To the best of our knowledge, this is the first biochemical demonstration of intrinsic RNA binding specificity of a rhabdoviral monomeric N protein. This RNA binding specificity of N is, however, compromised upon formation of N-oligomer (Fig. 8A). Moreover, these results indicate that encapsidation process might be a biochemically separable biphasic event; the initiation or nucleation phase involves specific recognition of conserved sequences present in the first half of the leader chain while processive encapsidation or elongation phase ensures enwrapment of heterogeneous sequences into the nucleocapsid particle.

Phosphoprotein P acts as an N-specific chaperone by preventing nascent N protein from growing into insoluble aggregates (Majumder et al., 2004). Moreover, nonspecific RNA binding ability of N was suppressed in the presence of P, though the mechanism remained unclear (Masters and Banerjee, 1988b). We developed an in vitro system that allowed us to form complexes between P protein and monomeric N, as well as to test the RNA binding specificity of such complexes. We noticed that such a complex prevented N from binding to nonspecific RNA unlike a higher order N–P complex isolated in a bacterial RNA-bound form (Green et al., 2000). One critical difference between N–P complex studied by Green et al. and our group is, however, the different subunit composition; bacterially isolated coexpressed N–P complex is an assembly of decameric N as compared to the N monomers in the N–P complex of our preparation (Fig. 7). Thus, it seems likely that P protein modulates RNA binding specificity of N by keeping it in a monomeric form, therefore, playing an essential role in ensuring supply of replication competent or in other words encapsidation-competent N during the viral replication stage. N-oligomer, once formed, apparently retains its ability to interact with nonviral RNA even in the presence of P protein, which is unable to either disrupt this oligomer assembly or mask the nonspecific RNA recognition domain. Interestingly, Fig. 8 showed that the N monomer–P complex, unlike DOC-treated N, not only interacted but also formed higher order encapsidation complexes with leader RNA. Furthermore, N monomer -P complex and not N-oligomer, showed the ability to enwrap and protect minigenome length viral RNA in vitro, thus resembling the behavior of a true encapsidating entity in vivo.

Based on our analysis, we provided a refinement of the existing hypothesis to explain mechanistic pathway involved in the encapsidation process. In this model, N protein as such, possesses specific RNA recognition domain in its primary structure, while oligomerization alters the RNA binding surface

of each monomer unit to impart a nonspecific RNA binding property for N-oligomer. Oligomeric N, stabilized through N–N association possibly into a pentamer assembly, is capable of enwrapping short RNA of viral or cellular origin into an encapsidation-like structure so as to deny nuclease accessibility, as shown previously by our group (Majumder et al., 2001). The stable oligomer while bound to RNA was unable to cooperatively recruit additional oligomer units on the leader chain. However, N protein present in a monomeric form can recognize specific *cis*-acting signal in the leader chain, a prerequisite for nucleation. Subsequently, N protein polymerization on RNA chain relies on a substitution reaction whereby P protein is released from N monomer–P complex as N associates with the phosphate backbone of the RNA, protecting it from RNase action. However, inhibition of N–N association resulted in abrogation of this elongation phase suggesting critical involvement of N–N association either prior or simultaneous to N–RNA binding. Accordingly, we noticed cooperativity in the N monomer–leader RNA interaction when encapsidation was studied in vitro in the absence of detergent (unpublished data). Additional interactions between RNA bound and free N during further recruitment of N moiety in the adjacent sequences primarily generate an altered RNA binding surface that may recognize a broad spectrum of RNA sequences, a prerequisite for processive encapsidation or elongation phase. While a fast reversible interaction between N and P protein ensures supply of monomeric N protein requirements during nucleation process, N–N as well as N–RNA association traps additional N molecule in an RNA-bound form in the elongation phase.

Consistent with this model, it was observed that N formed multiple complexes with P (Davis et al., 1986; Masters and Banerjee, 1988a) and the slowest sedimenting form was implicated as the substrate for replication and encapsidation (Peluso and Moyer, 1988). This model allowed us to explain the previously found intimate relationship between encapsidation, N protein aggregation, P-mediated specificity in N protein action as well as requirement of coexpressed P protein in viral replication process. Although, additional viral peptides, such as M protein or host factors, may play an essential role in maturation of encapsidation complexes into a higher order condensed RNP particle during late stages of virion assembly, it seems that defined viral components, N and P, are necessary and sufficient for presenting genomic sequences into an RNase-resistant form. Mutagenic analysis on N protein to dissect oligomerization, P binding as well as RNA binding will shed further light on the precise steps in Chandipura virus assembly pathway. Accordingly, screening of natural or synthetic small molecules that disrupts interaction between monomeric N with its cognate RNA sequence may provide novel therapeutic intervention in viral infection process.

Materials and methods

Materials

Oligonucleotides used in this study were purchased from Operon Technologies, USA. The column materials used for ion exchange or gel filtration chromatography were from Amersham Biosciences (Uppsala, Sweden). All restriction enzymes were from NEB. Protein molecular weight standards were purchased from Sigma (St. Louis, MO). Cell culture reagents were from Gibco-BRL (Gaithersburg, MD). Radioactive biomolecules were purchased from BRIT, India. All other chemicals were of analytical grade.

Cell culture and virus

CHP virus (Strain 1653514) was purified from BHK-21 cells grown in DMEM supplemented with 5% calf serum by infection with virus at an m.o.i. of 0.1 (Basak et al., 2003).

Cloning of a 597-nt fragment (lNt) from the 3′end of the viral genome

Genomic RNA was isolated from purified Chandipura virus using Tripure reagent (Roche) according to manufacturer's protocol. Viral RNA (500 ng) and sense primer (25 pmol) (3′ 1chp1-5′-aga*gaattc*acgaagacaaaaaaaccatttaaacgattatat-3′) that anneals to the 3′termini of the viral genome were heated together at 85 °C for 10 min and immediately placed on ice. Subsequently, reverse M-MuLV transcriptase (Roche) was added in cDNA synthesis buffer and the mixture was incubated at 42 °C for 1 h. RT product was used for PCR amplification with Pwo polymerase (Roche) and 3′1chp1-DJC2 primer set according to manufacturer's instructions and the products were analyzed in a 1% agarose gel. DJC2 corresponds to N gene downstream primer (5′-tttata*ggatcc*tcatgcaaagag-3′). The leader to N PCR product was digested with *Eco*RI to give an lNt fragment (encompassing the leader sequence – intragenic gene junction – and N gene coding sequence up to the 541st nucleotide) that was cloned into an *Eco*RI linearized pGEM3z vector. Positive clones were confirmed through restriction digestion.

Bacterial expression and purification of N and P proteins

N protein was over-expressed from pET3a NC clones in *E. coli* (BL21DE3) cells and purified from the soluble cell lysate fraction by anion exchange chromatography using Mono-Q FPLC column with a sodium chloride gradient of 200 mM to 700 mM in TET buffer (Majumder et al., 2001). Purification of recombinant P protein was carried out essentially as mentioned earlier (Basak et al., 2003). All recombinant proteins were concentrated with Centricon protein concentrators (Amicon) and checked in SDS-PAGE. Quantitations were made with BIO-RAD DC assay reagents.

Synthesis of RNA probes

Positive sense leader RNA, 65 nt long nonviral RNA, trailer RNA, l–1 (1– 21 nucleotides of the leader RNA) and l–2 RNA (24–45 nucleotides of the l-RNA) used in gel shift assays were obtained as mentioned earlier (Basak et al., 2003, 2004). The unrelated pET3a RNA used in the competition gel shift assay

was in vitro synthesized from pET3a vector linearized with *Nde*I using T7 RNA polymerase and was similar in length and base composition to leader RNA (A–T composition: 66% in comparison to 69% of leader). In vitro synthesized RNA was eluted from urea-polyacrylamide gels, precipitated twice with ethanol and suspended in RNase free water. Radioactivity was measured in liquid scintillation counter. RNAs used in this study were quantified spectrophotometrically by their absorbance at 260 nm. The positive sense lNt genome RNA used in the RNase sensitivity assay was synthesized from pGEM3z/lNt construct linearized with *Xba*I using T7 RNA polymerase (Bangalore Genei, India). For primer extension, the leader gene was cloned into pGEM4z vector using overlapping primers as mentioned (Basak et al., 2003). The reverse primer, 3′lchp2-T7, contained a 16-nt tag of the T7promoter sequence at its 5′end just after the *Hin*dIII site (5′-aga*aagctt*tatagtgagtcgtattgtgttcgtgtactactatataatcgtttaa-3′). pGEM4z/CHPl-T7 was linearized with *Hin*dIII and the RNA was in vitro synthesized using SP6 RNA polymerase (Roche). Cold RNA was gel eluted and precipitated, before using in binding reactions with protein.

Electrophoretic mobility shift assay (EMSA)

EMSA of radiolabeled leader, nonviral or trailer RNA (60,000 cpm) with N protein was carried out in binding buffer as mentioned earlier (Basak et al., 2003) or first the N protein was treated with various concentrations of deoxycholate (DOC) or NP40 in binding buffer for 10 min before incubating with RNA.

For reversibility gel shift analysis, labeled probe was incubated with protein. The preformed complexes were then chased with indicated amount of unlabeled RNAs for 10 min and then loaded onto the gels. Competition assay was performed similarly, except that indicated unlabeled RNA was added to protein prior to radiolabeled probe addition. For the RNase or Proteinase protection experiments, first the N–RNA complexes were allowed to form and then treated with RNase or Proteinase at a final concentration of 60 μg/ml. The incubation was carried out for additional 10 min before resolving through gels. All the EMSA experiments were run on 4% native gels containing 5% glycerol, at 4 °C in 1× TAE.

Gel filtration analysis

Gel exclusion chromatography was carried out in a Sephacryl S-300 column (45 × 1.5 cm) equilibrated with binding buffer (10 mM Tris, 40 mM KCl, 1 mM DTT, 10 mM $MgCl_2$ and 100 mM NaCl) at 4 °C. Sample loading volumes were kept at 300 μl and constant flow rate (0.2 ml/min) was maintained by a peristaltic pump. Void volume of the column was determined by monitoring the elution profile of blue dextran (20 ml). To determine the oligomeric status of N protein, ion exchange-purified N protein either directly or after treating with 1%DOC for 30 min at RT was loaded onto the pre-equilibrated column at a concentration of 0.4 mg/ml. Protein elution was monitored by taking O.D. 280 values of the 1 ml fractions collected and plotted against their respective elution volumes. For determination of stoichiometry of RNA to protein in the initiation and encapsidation complexes, N protein either as it is or after pretreatment with 1%DOC was incubated with radiolabeled leader RNA in binding buffer for 30 min prior to loading onto the column pre-equilibrated in the same buffer at 4 °C. One-milliliter fractions were collected. We ensured that the peaks of either RNA or N protein alone did not coincide with the RNA–protein peaks in either case. Elution of RNA was monitored by Cerenkov counting and the radioactivity present in each fraction, expressed in counts per minutes (cpm), was plotted against elution volumes (ml). Five independent gel filtration experiments were carried out for each set. The column was calibrated by running protein standards of known molecular weights-Ferritin (450 kDa), Catalase (232 kDa), BSA (67 kDa) and Ovalbumin (45 kDa). Standard protein elution was expressed as K_{av} and plotted against respective log molecular weight values. K_{av} was calculated from the formula: $(V_e - V_o) / (V_t - V_o)$, where V_e is the eluted volume, V_o is the void volume and V_t is the total volume. Molecular weights of the DOC treated and untreated oligomeric forms of N protein as well as the RNA–protein complexes were determined from the calibration curve.

Sucrose density gradients

Each 10 ml 10–60% sucrose step gradients was prepared in gradient buffer (50 mM Tris (pH 8), 1 mM EDTA, 0.1% TritonX-100 and 150 mM NaCl). Samples of ion exchange-purified N protein (0.4 mg/ml) before and after treatment with 1% DOC were layered on top of the gradient in a volume not exceeding 150 μl. The gradients were centrifuged for 16 h at 32,000 rpm in an SW Ti 40 Beckman rotor at 4 °C. Following centrifugation, 0.5-ml fractions were collected from the bottom of the gradients. The proteins from each alternate fraction were precipitated by acetone and run in (30:0.8) 10% SDS-PAGE. Proteins were visualized by silver staining. Sedimentation coefficients were determined by comparing with protein standards-Carbonic anhydrase (2.9 S), BSA (4.6 S), Catalase (11.4 S) and Ferritin (18 S), run in parallel gradients and detected identically. For determination of the two N–RNA complexes, first the complexes were allowed to form as described in gel filtration and fractionated through sucrose gradients (in binding buffer). Protein was detected through silver staining while the radiolabeled RNA was visualized through autoradiography. For the N–P experiments, DOC-treated N proteins were dialyzed over night against gradient buffer in the absence or presence of equimolar amount of P protein. The resulting protein complexes in both cases were run in gradients. Proteins were detected in 10% SDS-PAGE composed of 30:0.4 acrylamide:bisacrylamide for separation of N and P, which migrates anomalously to a 50-kDa position in 30:0.8 SDS gels.

Toeprinting assay

Toeprinting analysis of the initiation complex was performed with + sense leader RNA and primer PE1 (5′-tatagtgagtcgtatt-3′), 5′end-labeled with T4 Polynucleotide Kinase (Promega,

WI, USA) and γ-^{32}P ATP (4500 Ci/mmol) according to the manufacturer's instructions. Reaction components were assembled in a similar manner to the gel shift assay with minor variations. Briefly, 0.2 μg of leader RNA was incubated in binding buffer with 1.5–2 μg of N protein or with 1% DOC-treated N at different concentrations (0.5–2 μg) for 30 min at 37 °C in a total volume of 10 μl. Incubation was continued at 65 °C for 5 min after addition of end-labeled PE1 (10 pmol). Initiation complex formation was analyzed by primer extension using MMuLV reverse transcriptase at 42 °C for 30 min. The cDNA products were mixed with denaturing dye (95% deionized formamide, 0.025% (w/v) bromophenol blue, 0.025% xylene cyanol, 5 mM EDTA and 0.025% SDS), boiled at 95 °C for 5 min and resolved through an 8% sequencing gel. The products were compared with the ladder generated on the leader RNA with the same primer. Sequencing reactions were carried out in a similar manner where only ddNTPs (Promega) instead of dNTPs were used on the leader RNA template.

RNase sensitivity assay

Leader and lNt RNAs, in vitro transcribed from pGEM3zCHPl and pGEM3z/lNt plasmids, respectively, were used as templates for encapsidation by N protein. RNA (5–7.5 μg) was used for encapsidation studies with 50–100 μg N protein. N protein used for this assay was incubated with 1%DOC and DOC was dialyzed out in the absence and presence of P protein as mentioned earlier. Preformed complexes were digested with RNaseA at a final concentration of 200 μg/ml for 15 min at 37 °C. The RNase reaction was quenched by adding Trypsin (100 μg/ml) and the digested products were extracted by phenol–chloroform and precipitated in 100% ethanol. After resuspension in RNase free water, the protected leader and lNt genome RNAs were viewed by ethidium bromide staining of 10%urea PAGE or 1% and 4% agarose gels, respectively.

Acknowledgments

RB acknowledges CSIR, Govt. of India, for providing Senior Research Fellowship. SB thanks Alexander Hoffmann, UCSD, USA for his support. We acknowledge Siddhartha Roy of the Indian Institute of Chemical Biology, India for discussions and critical comments; Rajarshi Ghosh, AECOM, USA and Arpita Bhattacharya, Calcutta University, India for critical reading of the manuscript. This work was supported by DST, Govt. of India.

References

Abraham, G., Banerjee, A.K., 1976. Sequential transcription of the genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. 73, 1504–1508.

Arankalle, V.A., Shroti, S.P., Madhukar, W.A., Hanumaih, P.S., Dattatraya, M. A., Chandra, G., 2005. N and P gene-based analysis of Chandipura viruses, India. Emerging Infect. Dis. 11, 123–126.

Banerjee, A.K., 1987. Transcription and replication of rhabdoviruses. Microbiol. Rev. 51, 66–87.

Banerjee, A.K., Barik, S., 1992. Gene expression of vesicular stomatitis virus genome RNA. Virology 188, 417–428.

Basak, S., Raha, T., Chattopadhyay, D., Majumder, A., Shaila, M.S., Chattopadhyay, D.J., 2003. Leader RNA binding ability of Chandipura virus P protein is regulated by its phosphorylation status: a possible role in genome transcription–replication switch. Virology 307, 372–385.

Basak, S., Polley, S., Basu, M., Chattopadhyay, D., Roy, S., 2004. Monomer and dimer of Chandipura virus unphosphorylated P-protein binds leader RNA differently: implications for viral RNA synthesis. J. Mol. Biol. 339, 1089–1101.

Bhatt, S., Rodrigues, F.M., 1967. Chandipura virus: a new arbovirus isolated from India from patients with febrile illness. Ind. J. Med. Res. 55, 1295–1305.

Blumberg, B.M., Kolakofsky, D., 1981. Intracellular vesicular stomatitis virus leader RNAs are found in nucleocapsid structures. J. Virol. 40, 568–576.

Blumberg, B.M., Leppert, M., Kolakofsky, D., 1981. Interaction of VSV leader RNA and nucleocapsid protein may control VSV genome replication. Cell 23, 837–845.

Blumberg, B.M., Giorgi, C., Kolakofsky, D., 1983. N protein of vesicular stomatitis virus selectively encapsidates leader RNA in vitro. Cell 32, 559–567.

Brown, F., Bishop, D.H., Crick, J., Francki, R.I., Holland, J.J., Hull, R., Johnson, K., Martelli, G., Murphy, F.A., Obijeski, J.F., Peters, D., Pringle, C. R., Reichmann, M.E., Schneider, L.G., Shope, R.E., Simpson, D.I., Summers, D.F., Wagner, R.R., 1979. Rhabdoviridae. Report of the Rhabdovirus Study Group, International Committee on Taxonomy of Viruses. Intervirology 12, 1–7.

Chattopadhyay, D., Raha, T., Chattopadhyay, D.J., 1997. Single serine phosphorylation within the acidic domain of Chandipura virus P protein regulates the transcription in vitro. Virology 239, 11–21.

Chuang, J.L., Perrault, J., 1997. Initiation of vesicular stomatitis virus mutant polR1 transcription internally at the N gene in vitro. J. Virol. 71, 1466–1475.

Das, T., Banerjee, A.K., 1993. Expression of the vesicular stomatitis virus nucleocapsid protein gene in *Escherichia coli*: analysis of its biological activity in vitro. Virology 193, 340–347.

Das, T., Chakraborti, B.K., Chattopadhyay, D., Banerjee, A.K., 1999. Carboxy-terminal five amino acids of the nucleocapsid protein of vesicular stomatitis virus are required for encapsidation and replication of genome RNA. Virology 259, 219–227.

Davis, N.L., Wertz, G.W., 1982. Synthesis of vesicular stomatitis virus negative-strand RNA in vitro: dependence on viral protein synthesis. J. Virol. 41, 821–832.

Davis, N.L., Arnheiter, H., Wertz, G.W., 1986. Vesicular stomatitis virus N and NS proteins form multiple complexes. J. Virol. 59, 751–754.

Green, T.J., Macpherson, S., Qiu, S., Lebowitz, J., Wertz, G.W., Luo, M., 2000. Study of the assembly of vesicular stomatitis virus N protein: role of the P protein. J. Virol. 74, 9515–9524.

Gubbay, O., Curran, J., Kolakofsky, D., 2001. Sendai virus genome synthesis and assembly are coupled: a possible mechanism to promote viral RNA polymerase processivity. J. Gen. Virol. 82, 2895–2903.

Gupta, A.K., Banerjee, A.K., 1997. Expression and purification of vesicular stomatitis virus N–P complex from *Escherichia coli*: role in genome RNA transcription and replication in vitro. J. Virol. 71, 4264–4271.

Howard, M., Wertz, G., 1989. Vesicular stomatitis virus RNA replication: a role for the NS protein. J. Gen. Virol. 70, 2683–2694.

Iseni, F., Barge, A., Baudin, F., Blondel, D., Ruigrok, R.W.H., 1998. Characterization of rabies virus nucleocapsids and recombinant nucleocapsid-like structures. J. Gen. Virol. 79, 2909–2919.

Iseni, F., Florence, B., Blondel, D., Riogrok, R.W.H., 2000. Structure of the RNA inside the vesicular stomatitis virus nucleocapsid. RNA 6, 270–281.

Iverson, I.E., Rose, J.K., 1981. Localized attenuation and discontinuous synthesis during vesicular stomatitis virus transcription. Cell 23, 477–484.

Leppert, M., Rittenhouse, L., Perrault, J., Summers, D.F., Kolakofsky, D., 1979. Plus and minus strand leader RNAs in negative strand virus-infected cells. Cell 18, 735–747.

Li, T., Pattnaik, A.K., 1997. Replication signals in the genome of vesicular stomatitis virus and its defective interfering particles: identification of a sequence element that enhances DI RNA replication. Virology 232, 248–259.

Majumder, A., Basak, S., Raha, T., Chowdhury, S.P., Chattopadhyay, D., Roy,

S., 2001. Effect of osmolytes and chaperone-like action of P-protein on folding of nucleocapsid protein of Chandipura virus. J. Biol. Chem. 276, 30948–30955.

Majumder, A., Bhattacharya, R., Basak, S., Shaila, M.S., Chattopadhyay, D.J., Roy, S., 2004. P-protein of Chandipura virus is an N-protein specific chaperone that acts at the nucleation stage. Biochemistry 43, 2863–2870.

Marriott, A.C., 2005. Complete genome sequence of Chandipura and Isfahan Vesiculoviruses. Arch. Virol 150 (4), 671–680.

Masters, P.S., Banerjee, A.K., 1988a. Resolution of multiple complexes of phosphoprotein NS with nucleocapsid protein N of vesicular stomatitis virus. J. Virol. 62, 2651–2657.

Masters, P.S., Banerjee, A.K., 1988b. Complex formation with vesicular stomatitis virus phosphoprotein NS prevents binding of nucleocapsid protein N to nonspecific RNA. J. Virol. 62, 2658–2664.

Moyer, S.A., Smallwood-Kentro, S., Haddad, A., Prevec, L., 1991. Assembly and transcription of synthetic vesicular stomatitis virus nucleocapsids. J. Virol. 65, 2170–2178.

Pattnaik, A.K., Ball, L.A., LeGrone, A., Wertz, G.W., 1995. The termini of VSV DI particle RNAs are sufficient to signal RNA encapsidation, replication, and budding to generate infectious particles. Virology 206, 760–764.

Patton, J.T., Davis, N.L., Wertz, G.W., 1983. Cell-free synthesis and assembly of vesicular stomatitis virus nucleocapsids. J. Virol. 45, 155–164.

Patton, J.T., Davis, N.L., Wertz, G.W., 1984. N protein alone satisfies the requirement for protein synthesis during RNA replication of vesicular stomatitis virus. J. Virol. 49, 303–309.

Peluso, R.W., 1988. Kinetic, quantitative, and functional analysis of multiple forms of the vesicular stomatitis virus nucleocapsid protein in infected cells. J. Virol. 62, 2799–2807.

Peluso, R.W., Moyer, S.A., 1988. Viral proteins required for the in vitro replication of vesicular stomatitis virus defective interfering particle genome RNA. Virology 162, 369–376.

Qanungo, K.R., Shaji, D., Mathur, M., Banerjee, A.K., 2004. Two RNA polymerase complexes from vesicular stomatitis virus-infected cells that carry out transcription and replication of genome RNA. Proc. Natl. Acad. Sci. U.S.A. 101, 5952–5957.

Rao, B.L., Basu, A., Wairagkar, N.S., Gore, M.M., Arankalle, V.A., Thakare, J. P., Jadi, R.S., Rao, K.A., Mishra, A.C., 2004. A large outbreak of acute encephalitis with high fatality rate in children in Andhra Pradesh, India, in 2003, associated with Chandipura virus. Lancet 364, 869–874.

Richardson, J.C., Peluso, R.W., 1996. Inhibition of VSV genome RNA replication but not transcription by monoclonal antibodies specific for the viral P protein. Virology 216, 26–34.

Thomas, D., Newcomb, W.W., Brown, J.C., Wall, J.S., Hainfeld, J.F., Trus, B. L., Steven, A.C., 1985. Mass and molecular composition of Vesicular Stomatitis Virus: a scanning electron microscopy analysis. J. Virol. 54, 598–607.

Wertz, G.W., 1983. Replication of vesicular stomatitis virus defective interfering particle RNA in vitro: transition from synthesis of defective interfering leader RNA to synthesis of full-length defective interfering RNA. J. Virol. 46, 513–522.

Wertz, G.W., Levine, M., 1973. RNA synthesis by vesicular stomatitis virus and a small plaque mutant: effects of cycloheximide. J. Virol. 12, 253–264.

Whelan, S.P., Wertz, G.W., 2002. Transcription and replication initiate at different sites on the vesicular stomatitis virus genome. Proc. Natl. Acad. Sci. U.S.A. 99, 9178–9183.