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Research paper

# Characterization of the chandipura virus leader RNA–phosphoprotein interaction using single tryptophan mutants and its detection in viral infected cells

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ABSTRACT

The phosphoprotein (P protein) of the chandipura virus (CHPV), a negative strand RNA virus, is involved in both transcription and replication of the viral life cycle. Interaction between the P protein and the viral leader (le) RNA under *in vitro* conditions has been previously reported for CHPV and other negative strand RNA viruses such as the rinderpest virus (RPV). However, till date, the region of the P protein involved in le RNA binding remains undefined. Moreover, the *in vivo* occurrence of this interaction has not been studied before. Here, we have characterised the P protein–le RNA interaction, using single tryptophan mutants of the P protein. The CHPV P protein contains two tryptophan residues located at amino acid position 105 and 135 respectively. Our previous study showed that Trp 135 is located in a buried region within a less polar environment whereas Trp 105 is more solvent-exposed. In this study we have used steady state and time resolved fluorescence spectroscopy at 298 K to show that the buried tryptophan (Trp 135) is involved in the interaction with the le RNA and the more solvent exposed Trp 105 is only slightly perturbed during this interaction. We also show that Trp 135 is responsible for the dimerization of the CHPV P protein. In addition, we have been able to demonstrate for the first time that the P protein–le RNA interaction is detectable in CHPV-infected Vero-76 cells and this interaction is augmented during the replication phase of the viral cycle.



## 1. Introduction

The chandipura virus (CHPV) is an important human pathogen implicated in violent encephalitis attacks in various parts of the Indian subcontinent [1]. It is an RNA virus of the family Rhabdoviridae, order *Mononegavirales* (MNV). This family includes several important human, animal and plant pathogens like the rabies virus, vesicular stomatitis virus (VSV), Isfahan virus, potato yellow dwarf virus, etc [2]. MNV viruses share similar genomic and structural organizations and the molecular regulations of their replication and transcription events are also comparable [2].

Essentially, the single-stranded, negative-sense RNA genome is tightly encapsidated by the viral nucleocapsid protein (N) to form a helical ribonucleoprotein complex (RNP) [3,4]. This encapsidated genome serves as the template for both replication and transcription and encodes five proteins, in the order: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large protein (L) [5]. The 3′ end of the genome encodes a 49 nt transcribed but non-translated leader (le) RNA, while the 5′ end comprises of a non-transcribed 46 nt trailer sequence (t) [5]. For viruses belonging to the *Vesiculovirus* genera, like the rabies virus (RV), vesicular stomatitis virus (VSV) and chandipura virus (CHPV), the viral RNA synthesis is carried out by the viral RNA dependent RNA polymerase (RdRp) complex, which consists of the large protein (L) and the phosphoprotein (P) [5,6]. The P protein has been proposed to play multiple functions during the replication cycle of this class of viruses. It has long been established to function as a cofactor of the viral RNA polymerase, forming a heterodimeric complex with the L polymerase [6,7]. The P protein also acts as an N-specific chaperone to maintain it in a monomeric form which is competent for encapsidation of the viral RNA [8,9]. In addition to this, the P protein loads the L polymerase onto the RNP template by

*Abbreviations:* CHPV, chandipura virus; VSV, vesicular stomatitis virus; MNV, *Mononegavirales*; P protein, phosphoprotein; CKII, casein kinase II; le RNA, leader RNA; EMSA, electrophoretic mobility shift assay; Trp, tryptophan (W); Phe, phenylalanine (F); Ser, serine; RNP, ribonucleoprotein complex; RdRp, RNA dependent RNA polymerase; SEC, size exclusion chromatography; CD, circular dichroism; RIP, RNA immunoprecipitation; RT-PCR, reverse transcription-polymerase chain reaction.

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binding to both the L and the N proteins [6]. However, little is known about the regulatory functions of this protein, and its role in the RNA polymerization process may not have been fully understood.

Two RNA synthesis events occur after entry of the viral genome RNA into the host cell, namely, transcription of viral mRNAs and replication of the complete viral genome [7]. During transcription, progressive termination at each gene junction produces the positive sense leader RNA and five mRNAs corresponding to the five viral genes. After sufficient viral proteins have been synthesised, most importantly the N protein, the polymerase switches from its transcriptase mode to the replicase mode. During replication, the gene junction termination signals are ignored to produce a positive strand, polycistronic RNA complementary to the whole genome which serves as the template for the subsequent synthesis of the progeny genome RNAs [7]. Till date, the molecular events leading to this transition of polymerase activity from transcription to replication have remained unclear. The role of the N protein in encapsidating the nascent RNA during replication but not during transcription has been suggested to play a role in this switch [10]. However, there is little evidence that can confirm the role of the N protein as a sole mediator for this switching event.

In chandipura virus, specific interaction between the P protein and the positive sense leader transcript (le RNA) has been proposed to play a crucial role in this transition [11,12]. It has been demonstrated that the CHPV P protein, in its unphosphorylated form ($P_0$) interacts specifically with the le RNA, and this $P_0$–le RNA complex may have a role in viral replication [11,12]. It has been demonstrated that the phosphorylation of the P protein at Ser-62 ($P_1$) by cellular casein kinase II entirely abrogates its ability to bind the le RNA [10]. This has been envisaged as one of the probable mechanisms that drive the switching of the polymerase action in this class of viruses [5,12]. Among other related viruses, the rinderpest virus (RPV) P protein has been shown to bind specifically to the plus sense leader RNA depending on its phosphorylation status [13]. Further, the rabies virus major phosphoprotein has been proposed to interact with the leader RNA to regulate transcription and replication [14].

Previous *in-vitro* characterization of the CHPV $P_0$–le RNA interaction using the Electrophoretic Mobility Shift Assay (EMSA) revealed that the P protein forms two stoichiometrically different complexes with *in-vitro* transcribed leader RNA in an electrophoretic mobility shift assay (EMSA) [12]. The lower migrating complex (complex I) was found to be a 1:1 association between the protein and the RNA, while the higher migrating complex II comprised of two molecules of the P protein associated with one molecule of the leader RNA. Further investigation into the oligomerization potential of the P protein revealed that it undergoes concentration dependent oligomerization and maintains a monomer–dimer–tetramer equilibrium *in-vitro* [12]. At low concentrations (300 nM or below) it exists solely as a monomer population, while, increasing the concentration gradually results in an increase of the dimer population and at 1500 nM, only dimers exists [12]. Under monomeric concentrations, P protein binds specifically to the 3′ stem-loop motif of the leader RNA (24–45 nt) to form complex I [12]. At higher concentrations, dimers of the protein bind to the first 21 nt of the leader transcript, forming complex II [12]. Based on these observations, Basak et al. proposed a model in which these dual bindings of $P_0$ to the nascent leader transcript regulate the switch of the polymerase to the replication mode [12]. However, the precise domain of the P protein responsible for this interaction remains unidentified. Moreover, the possibility of two leader RNA interacting sites on the P protein, involved in interacting with the two distinct P-binding sites on the leader RNA, also needs to be investigated. Although the leader RNA–P protein interaction can be observed by biochemical and biophysical techniques *in vitro*, the occurrence of this interaction in the *in vivo* milieu in CHPV infected cells still needs validation. If this interaction is observable in infected systems, then its role in the infection cycle also warrants further investigation.

To address these questions, here, we have shown for the first time that P–le RNA interaction occurs within viral infected cells and this interaction is prominent during the replication phase of the viral cycle. To further characterize the P–le RNA interaction, tryptophan fluorescence quenching, time resolved fluorescence and circular dichroism studies have been undertaken. Using single Trp mutants of P, it has been possible to show that Trp 135 plays an important role in both dimerization of the P protein and complex formation with the leader RNA. This is the first evidence that amino acids positioned around residue 135 of the P protein is instrumental in formation of the $P_0$–leader RNA complex. Our data also indicates that this interaction of the leader RNA with the $P_0$ protein induces a structural change in the protein, implication of which in the viral life cycle has been discussed.

## 2. Materials and methods

### 2.1. Production and purification of $P_{Wt}$ and its tryptophan mutants

For the present study $P_{Wt}$, $P_{W105F}$, $P_{W135F}$ were produced and purified using the same procedure as described previously [15] except for one change. Triton-X100 was not used in the purification as it interferes with fluorescent spectroscopic analysis.

### 2.2. Synthesis of leader RNA

The 49 nt long positive sense leader RNA (le RNA) was synthesized *in vitro* as described previously by Basak et al. [12]. Briefly, CHPl/pGEM-3Z clone linearized with HindIII, and *in vitro* transcription performed using T7 RNA polymerase by standard protocols. The *in vitro* synthesized RNA was eluted from 10% Urea-PAGE, phenol:chloroform extracted, precipitated twice with ethanol and resuspended in DEPC treated water. RNA used in this study was quantified spectrophotometrically by its absorbance at 260 nm, using its calculated extinction coefficient, $\varepsilon = 672{,}000\ M^{-1}\ cm^{-1}$.

### 2.3. Fluorescence quenching studies of leader RNA binding

Leader RNA binding to wild type and mutant P proteins was monitored by tryptophan fluorescence quenching with a Hitachi F-7000 spectrofluorimeter equipped with spectrum addition and subtraction facility. All fluorescence experiments were carried out at 298 K using the correct mode of instrument. The excitation wavelength was 295 nm with a band pass of 10 nm, and the emission spectrum was monitored from 305 nm to 400 nm with a band pass of 5 nm. Inner filter effects have been eliminated in all the emission measurements. Wild type and mutant P proteins were kept at a concentration of 300 nM and 1500 nM, and titrated with increasing concentrations of leader RNA. Assays were done in 500 μl of binding buffer consisting of 10 mM Tris–HCl (pH 8.0) containing 100 mM NaCl, 40 mM KCl, 5 mM $MgCl_2$, and 5% Glycerol in DEPC treated milliQ water. Readings were taken in a cuvette with a 1 cm path length. Raman light scattering from the water and any background fluorescent emission from the buffer was corrected by subtracting the fluorescence of a buffer only solution that was at the equivalent RNA and salt concentrations as in the titration, with fluorescent signal of the sample. Values were further corrected for volume and inner filter effect. Previously it has been established that the leader RNA and P protein interaction reaches completion within 15 s [12]. Thus, after each addition during titration, we