

Identification of functional domains of phosphoproteins of two morbilliviruses using chimeric proteins

Paramananda Saikia · M. S. Shaila

Received: 2 October 2007 / Accepted: 8 April 2008 / Published online: 22 April 2008
© Springer Science+Business Media, LLC 2008

Abstract The paramyxovirus P protein is an essential component of the transcriptase and replicase complex along with L protein. In this article, we have examined the functional roles of different domains of P proteins of two closely related morbilliviruses, Rinderpest virus (RPV) and Peste des petits ruminants virus (PPRV). The PPRV P protein physically interacts with RPV L as well as RPV N protein when expressed in transfected cells, as shown by co-immunoprecipitation. The heterologous L–P complex is biologically active when tested in a RPV minigenome replication/transcription system, only when used with PPRV N protein but not with RPV N protein. Employing chimeric PPRV/RPV cDNAs having different coding regions of P protein in the minigenome replication/transcription system, we identified a region between 290 and 346 aa in RPV P protein necessary for transcription of the minigenome.

Keywords Rinderpest virus · Peste des petits ruminant virus · P protein · Transcription · Replication

Introduction

Paramyxoviruses contain a single stranded RNA genome encapsidated by nucleocapsid (N) protein forming a ribonuclease resistant ribonucleoprotein which serves as a template for transcription and replication. The paramyxovirus phosphoprotein (P) is a multifunctional protein that binds to both nucleocapsid (N) and large (L) protein and acts as a chaperone to keep the N protein in a soluble form for binding to the viral RNA and as a co-factor in the transcription complex. Another important function of the P protein is its interaction with the L protein to form the RNA dependent RNA polymerase [1]. The L-interacting domains of Rinderpest virus (RPV) P protein have been mapped to a carboxy terminal region encompassing amino acids 347–490 [2]. Furthermore, the presence of P protein has been shown to be important for the stability of L protein, since, P must be co-expressed with L to keep it stable [2]. Earlier work on RPV P–N interactions has shown that two separate domains, an amino terminal 1–59 amino acid and carboxy terminal amino acid region (316–346) of P are involved in P–N interaction [3]. Similarly, in Sendai virus, two separate domains (residues 345–412 and 479–568) within the carboxy terminal part of the protein are required for binding to the N protein [4]. In Measles virus, the carboxy terminal 40% has been shown to interact with the N protein [5].

Peste des petits ruminants virus (PPRV), which is closely related to Rinderpest virus, encodes a P protein of similar length. The PPRV P protein from infected cells migrated as 79 kDa protein on SDS-polyacrylamide gels although its calculated molecular weight is 60 kDa [6]. The aberrant mobility of P protein of negative-sense RNA viruses has been reported previously [5, 7]. The interaction of PPRV P protein with other viral proteins remains to be

P. Saikia · M. S. Shaila (✉)
Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India
e-mail: shaila@mcbliisc.ernet.in

Present Address:
P. Saikia
Department of Molecular Genetics/NE2-250, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, Ohio 44195, USA

determined. The alignment of PPRV P protein sequence with other available morbillivirus P proteins shows that residue 311–418 are more conserved than the amino terminal half [8]. The PPRV P protein shares 51% identity with RPV P protein sequence. Although the amino terminal sequence of the P protein is poorly conserved, their functions may be conserved in genome transcription and replication.

In the present article, functional interactions between L protein of RPV and P as well as N proteins of both viruses were investigated employing a RPV based minigenome system. Further, a series of chimeric plasmids were constructed, where defined domains of RPV P gene sequence coding for regions involved in P–P, P–N, and P–L interactions were replaced by corresponding domain sequences of PPRV P. In vivo functional interactions of chimeric P proteins with homologous and heterologous N protein together with RPV L in the minigenome replication/transcription have been tested to assess the functional conservation of the domains in the P protein of the two viruses.

Materials and methods

Cells and virus

A549, a human lung carcinoma cell line was obtained from ATCC, USA and maintained in DMEM supplemented with 10% New Born Calf Serum (NBCS) (GIBCO-BRL, USA). Vero and BSC 1 cells (used for maintenance and propagation of the VTF7.3) were obtained from National Center for Cell Science, Pune, India and maintained in DMEM supplemented with 10% Fetal Calf Serum (GIBCO-BRL, USA) at 37°C. *Spodoptera frugiperda* (Sf21) insect cells was obtained from National Center for Cell Sciences, Pune, India and was maintained in TC-100 medium (Life Technologies, USA) with 10% Fetal Calf Serum. VTF7.3, a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase in mammalian cells was obtained from Dr. Bernard Moss, NIH, USA [9]. Recombinant baculoviruses expressing wild type PPRV P and N proteins were generated in this study employing BAC-to-BAC cloning kit of GIBCO-BRL, USA. Recombinant baculovirus expressing wild type RPV L was generated as described before [2].

Plasmids

The RPV minigenome, pMBD8A and the cDNA clones of RPV N (pKSN1), and L (pPOL10) were obtained from Dr. M. Baron, Institute of Animal Health (IAH), Pirbright Laboratory, U.K. [10, 11]. The pRPS3 (pRSETB plasmid expressing full length PPRV P gene) was generated in this study. pRP6 (pRSETB plasmid carrying full length P gene)

and pGKSN1 (PPRV N gene in pRSETB) have been described earlier [12, 13].

Antibodies

Rabbit polyclonal antibody against bacterially expressed purified RPV P (12), RPV N, PPRV N [13], and RPV L [2], made previously in the laboratory, were used. Rabbit polyclonal antibody against bacterially expressed and purified PPRV P protein was generated in this study. Horse Radish Peroxidase conjugated goat anti-rabbit IgG was purchased from Bangalore Genei Pvt.Ltd., India.

Construction of chimeric cDNA

Chimeric RPV-PPRV P cDNA were constructed by using PCR and overlap extension to splice the genes together [14]. The chimeric gene constructs were then cloned into pRSET (A/B/C) expression vector under T7 promoter.

Co-immunoprecipitation

A549 cells were plated in 60 mm tissue culture dishes at a density of 2.5×10^6 cells in 5 ml of DMEM supplemented with 10% FCS (GIBCO-BRL, USA). When the cells were 70% confluent, infected with vaccinia virus VTF7.3 that express T7 RNA polymerase at a multiplicity of infection 3 and co-transfected with 5 µg of plasmid containing specific genes mentioned in each figure legends. At 24 h post-transfection, the cells were washed twice with ice-cold PBS and lysed in 100 µl of RIPA buffer (100 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 2 mM PMSF). The cell lysates (300 µg) were precleared by incubating with rabbit pre-immune serum and protein A-sepharose beads for 1 h at 4°C. Specific antibody was incubated with 10% suspension of protein A-sepharose beads for 1 h at 4°C. The beads were washed twice with RIPA buffer and added to the precleared supernatant. After 4 h of incubation at 4°C, the beads were washed twice with RIPA wash buffer (100 mM Tris-Cl pH 7.4, 300 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) and once with RIPA buffer. The precipitated proteins were detected by immunoblot with specific antibody. 10% SDS-PAGE was run to detect P and N protein while to detect L protein 6% SDS-PAGE was run.

In vivo interaction of PPRV P, N and RPV L proteins in insects cells

Sf 21 cells at 80% confluency (6.0×10^6) were infected singly, doubly, or triply with the recombinant baculovirus at m.o.i ratio of 1:5:5. After 72 h of infection, cells were

washed with PBS and lysed in hypotonic buffer (20 mM Tris pH7.5, 20 mM NaCl). After lysis, the extract was adjusted to a final concentration of 150 mM NaCl, 1 mM DTT, 5% glycerol, followed by centrifugation at 100,000g. The soluble supernatant (S100) was immunoprecipitated with either anti-PPRV N or P antibody and the interacting proteins were detected by western immunoblotting with anti-RPV L antibody.

In vivo replication/transcription assay

A549 cells in six well culture plate at a density of 1.0×10^6 were infected with VTF7.3 and subsequently transfected with RPV minigenome (pMDB8A), carrying the 3' regulatory sequence (leader region), transcription/replication start regions & 5' trailer sequences flanking a reporter gene (CAT) ORF driven by T7 promoter, plasmid DNA of pKSN1 (RPV N) or pGKSN1 (PPRV N), pRPS3 (PPRV P) or pRP6 (RPV P) and pPOL10 (RPV L). As described by Kaushik and Shaila (2004) CAT assay was performed after 36 h of post-transfection as per the manufacturer's instructions (Boeringer Maneheim).

RNA extraction and RT-PCR analysis

In order to distinguish the replication and transcription products of in vivo minigenome replication/transcription assay, total RNA was isolated from the cell lysates which were used for in vivo minigenome replication/transcription assay as mentioned above by TRIZOL reagent (Life Technologies) according to manufacturer protocol. In order to detect the transcription product, reverse transcription reaction (RT) was carried out with oligo (dT)₁₈ primer and PCR with CAT gene specific upstream (5' GGA TAT ACC ACC GTT GA 3'), and CAT gene specific downstream primer (5' GGG ACG GTG AGT AGC GTC ATG 3') whereas to detect the replication product, RT was carried out with CAT gene specific downstream primer and PCR with leader specific upstream (5' CTG GGT AAG GAT CGT TCT 3' nt 12–30) and CAT gene specific downstream primer (5' GGG ACG GTG AGT AGC GTC ATG 3'). RT was done using Superscript II (GIBCO-BRL) reverse transcriptase maintaining 0.5 µg of total cellular RNA/reaction.

Results

Formation of heterologous polymerase complex (P–L) between PPRV P and RPV L proteins

The amino acid sequences of RPV (accession no. CAA83178) and PPRV (accession no. CAJ01695) P protein were taken

from the EMBL database and different regions were aligned using ClustalW (data not shown). The results show that P proteins share only 51% sequence identity. The alignment of L protein sequence of RPV (accession no. CAA83183) and PPRV (accession no. CAJ01701) revealed that L proteins of the two viruses are highly conserved (71% identity). The P protein sequence is less conserved in the amino terminal half and exhibits greater degree of identity at the carboxy terminus [8]. The N⁰ binding domain at the amino terminus as well as the assembled N (NC) binding region at the carboxy terminus of RPV P protein has been mapped earlier [3]. The corresponding region on PPRV P protein shows 43% and 57% identity. In the L binding region (347–490 aa) [2], there is 71% identity between these two protein. The identity is higher (87%) in the amino terminal part of 347–490 aa region. Although homology of P proteins of RPV and PPRV is not very high, the region involved in the P–L complex is conserved. We determined whether PPRV P protein is able to form a polymerase complex with heterologous L protein (RPV L) given the greater degree of identity in the L binding regions of P proteins of two viruses. The PPRV P protein was co-expressed with RPV L in transfected cells and the polymerase complex in cell lysates were immunoprecipitated with anti-RPV P antibody. When co-expressed with PPRV P protein, the RPV L protein was co-immunoprecipitated with anti-RPV P antibody (Fig. 1). The results show that the regions of the L and P protein required for complex formation are functionally conserved in these closely related viruses.

Biological activity of heterologous (P–L) polymerase complexes

We next examined whether the heterologous polymerase (P–L) complex formed between PPRV P and RPV L is biologically active by using a RPV minigenome replication-transcription system as described earlier [11]. CAT expression from the minigenome was measured in the lysate of cells that co-expressed PPRV P, PPRV N and RPV L (PPR). The results shown in Fig. 2 indicate that the PPRV P-RPV L complex recognizes the RPV transcription initiation and termination signals to produce CAT transcripts. In the absence of RPV L, no CAT expression was detected (RRX). The CAT expressions mediated by PPRV P-RPV L complex were, however, 63% of that when homologous proteins (RRR) were expressed. The lower activity may be due to the use of RPV minigenome. Cells expressing RPV P-RPV L displayed 50% CAT expression when PPRV N was used (PRR), while less than 10% CAT expression was detected when PPRV P was expressed (RPR). As we have used a RPV minigenome, RPV P-RPV L complex recognizes PPRV N to form the encapsidation complex but PPRV P-RPV L cannot function with RPV N. This indicates that homologous encapsidation complex is

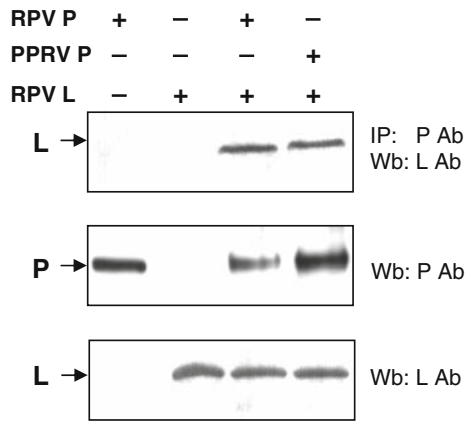


Fig. 1 Formation of complexes by P and L protein expressed from cDNA in cultured cells. A549 cells were infected with VTF7.3 and transfected with plasmid containing RPV L and either RPV P or PPRV P gene. After 24 h post-transfection, cell lysates were prepared in 100 μ l of lysis buffer and 300 μ g of total lysates were immunoprecipitated with anti-RPV P antibody (P-Ab) and precipitated protein analyzed by immunoblot with antibody against RPV L protein (L-Ab). Same protein extracts were used for direct immunoblot with RPV P (middle panel) and RPV L antibody (lower panel). The positions of the migration of the proteins are indicated by arrows

equally important for the biological function of the heterologous P–L complex, since in this minigenome system, replication of the minigenome occurs first [11], which depends upon the presence of encapsidation complex of unassembled N and P.

We examined the interaction of PPRV P with RPV N (N^0) and RPV P with PPRV N^0 protein. The results show that complex formation can occur between PPRV P and RPV N^0 as well as RPV P and PPRV N^0 (Fig. 3). We also analyzed the formation of a tripartite complex of PPRV N–PPRV P–RPV L, which has been shown to function as replication complex in VSV [15] by co-infecting baculoviruses in insect cells expressing all three proteins together. Sf21 cell lysates (300 μ g) infected with single or combination of the recombinant baculoviruses were subjected to immunoprecipitation with either anti-PPRV N or anti-PPRV P antibody and the co-precipitated RPV L protein was detected by immunoblotting. The P as well as N antibody can co-precipitate the RPV L protein either from L–P or L–P–N expressing extracts. Interestingly, L protein failed to interact with the N protein in the absence of P protein. Thus it seems that RPV L protein does indeed form a tripartite complex composed of PPRV P and PPRV N (Fig. 4) only when all three proteins are co-expressed in insect cells.

The domains on RPV P protein interacting with itself, unassembled N and assembled NC as well as with L protein have been identified [2, 3, 16] therefore, it was of interest to find if the corresponding regions in PPRV P

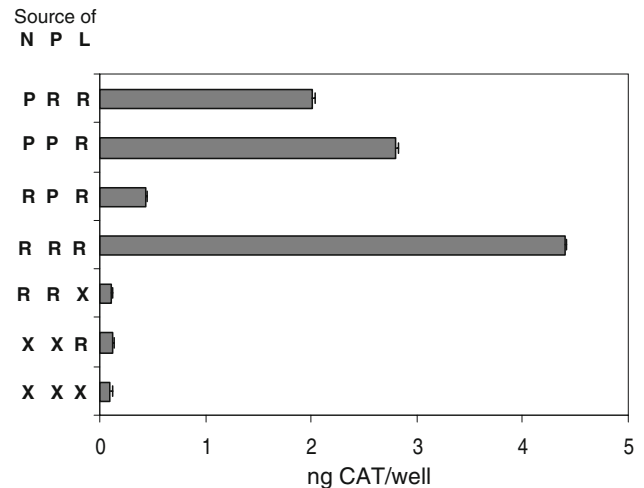


Fig. 2 Biological activity of polymerase complex composed of PPRV P and RPV L proteins. A549 cells were infected with VTF7.3 and transfected with 100 ng of RPV L containing plasmid, 1 μ g of P and N plasmids of either RPV or PPRV and 1 μ g of RPV minigenome plasmid. Cells were lysed in 100 μ l of lysis buffer and 50 μ g of total protein/well were tested for CAT protein expression by CAT ELISA (Borehinger Manneheim). The means and standard deviations of three independent transfection experiments are given

protein are functionally conserved or not. In order to test this, seven different chimeric P constructs having different regions of PPRV P replaced by RPV P gene were generated. The seven constructs as shown in Fig. 5 (upper panel) were generated by overlap extension PCR and the sequences were verified by sequencing the plasmid DNA. Expression of the chimeric P proteins were tested by western immunoblotting with anti-RPV P antibody using A549 cell lysates (50 μ g) infected with recombinant vaccinia virus and transfected with 5 μ g of chimeric P plasmid (Fig. 5, lower panel).

We next examined whether the chimeric proteins can interact with PPRV N and RPV L protein by coimmunoprecipitation. Figure 6a shows that all chimeric P proteins were immunoprecipitated by anti-PPRV N antibody to a different extent when co-transfected PPRV N plasmid. This demonstrated the conservation N^0 interacting domain(s) on PPRV P protein. Similarly RPV P antibody was also able to immunoprecipitate RPV L protein in the lysates of cells co-transfected with RPV L and chimeric P plasmids to a different extent (Fig. 6b).

Since the physical interacting domains on PPRV P for both N and L are conserved, we next examined the functionality of chimeric P proteins in vivo using the RPV minigenome system. The chimeric P constructs were co-transfected with RPV N along with RPV L and RPV minigenome constructs and the expression of CAT reporter gene was measured. When the source of N protein was RPV, the chimeras 2 and 3 showed no activity while

Fig. 3 In vivo interaction of heterologous P and N proteins. A549 cells were infected with VTF7.3 and transfected with 5 µg of each plasmid expressing RPV N, RPV P or PPRV P (a) and PPRV N, RPV P or PPRV P (b). After 24 h of post-transfection, cell lysates were prepared in 100 µl of lysis buffer and 300 µg of lysates were immunoprecipitated with anti-RPV P antibody (P-Ab), the immunoprecipitates were subjected to electrophoresis on 10% SDS-Polyacrylamide gels and western immunoblotted with either RPV N (left panel) or PPRV N antibody (right panel) after transferring the protein to nitrocellulose membrane. Cell lysates (50 µg) were also directly subjected to immunoblotting with RPV P (middle panel) and N antibody (lower panel). The positions of the migration of the proteins are indicated by arrows

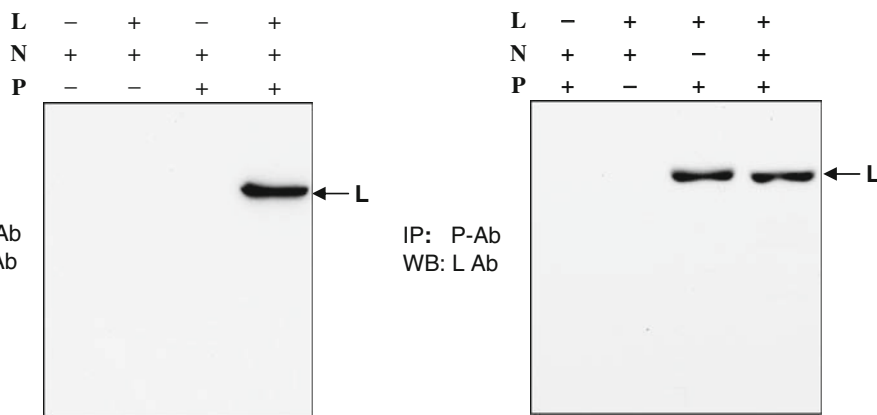
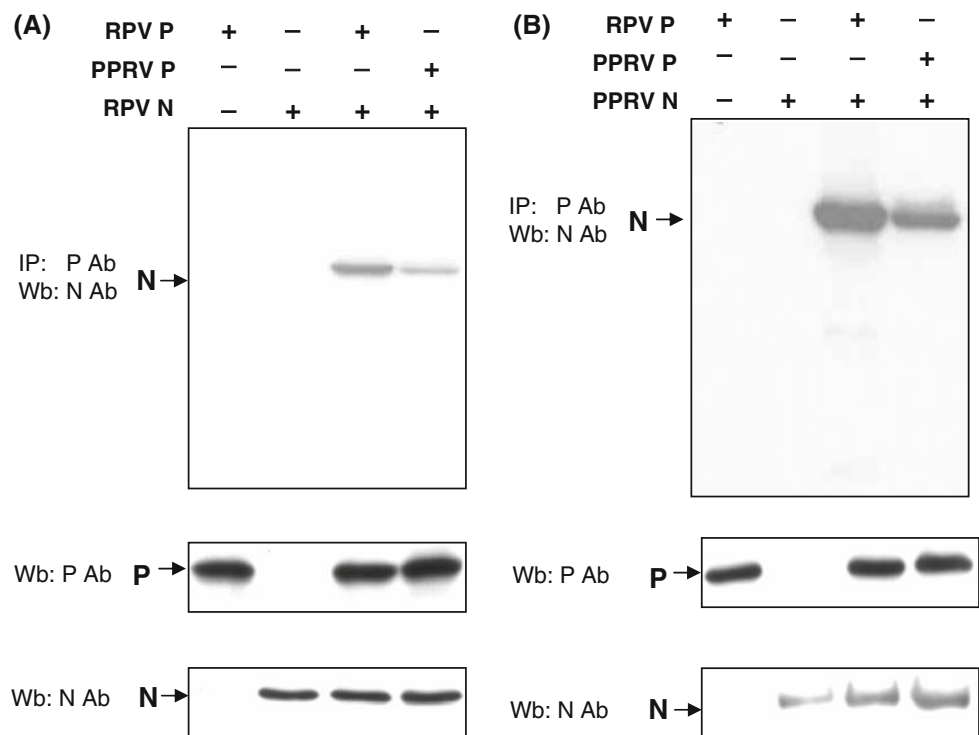


Fig. 4 Expression and association of recombinant proteins in Sf21 cells. Sf21 Cells were grown in 150 cm² tissue culture flasks and infected singly, doubly or triply with recombinant baculovirus containing PPRV P, N and RPV L genes. Seventy-two hours post-infection, the cells were harvested and soluble S-100 extracts were

made. 300 µg of cell lysates were immunoprecipitated with either PPRV N or PPRV P antibody and precipitated proteins were detected by western immunoblotting with RPV L antibody. The positions of the migration of L protein are indicated by arrows

chimera 1, 4, 5, 6, and 7 showed reduced CAT expression in comparison with wild type RPV P (Fig. 7a). With PPRV N (Fig. 7b) chimera 1 retained 100% of activity of wild type PPRV P. A significant finding was that chimera 2 and 3 were non functional in both combinations. This loss of activity may be due to the replacement of the P multimerization domain with that of PPRV P or due to the misfolding of the chimeric protein.

Since in this minigenome system, the expression of reporter gene represents combined replication and transcription processes, we investigated which of these steps is blocked when

chimera 2 and 3 are used. Therefore, RT-PCR analysis of the RNA made in the minigenome assay was performed using primers that distinguish replication product from transcription. A CAT ORF specific sense primer in combination with oligo (dT)₁₈ primer can amplify cDNA corresponding to minigenome transcription produced by L protein assisted by P, whereas leader RNA specific primer in combination with CAT specific downstream primer can amplify cDNA corresponding to minigenome replication product. Data shown in Fig. 8a, b demonstrates that while all chimeric P proteins are active in replication, chimeras 2 and 3 are unable to transcribe the

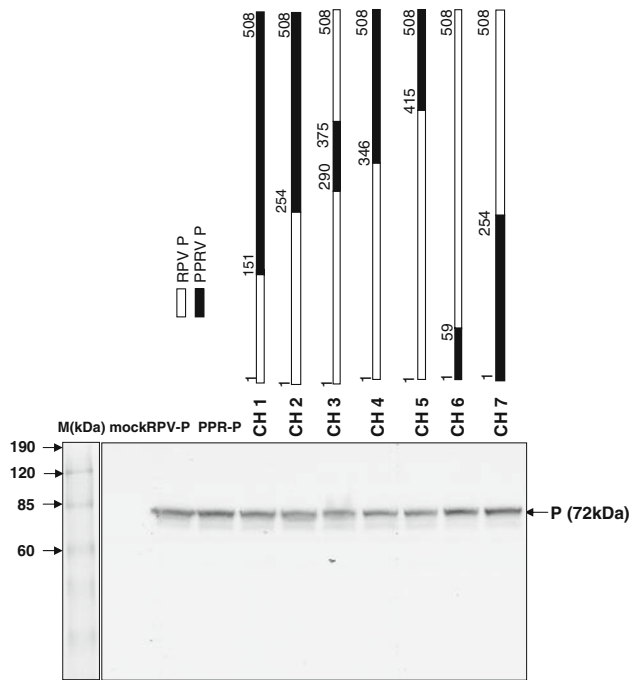


Fig. 5 Schematic diagrams of the chimeric P proteins of RPV and PPRV used in this study (upper panel). Expression of chimeric P protein in A549 cells. A549 cells were infected with VTF7.3 and transfected with 5 μ g of plasmid containing chimeric P constructs. Cells were lysed in 100 μ l of lysis buffer and 50 μ g of cell lysates were loaded into SDS-PAGE and the expression of chimeric proteins were detected by immunoblotting with RPV P antibody. As control RPV and PPRV P gene plasmids were also transfected and detected their expression by western blot (Lane; RPV P and PPRV P)

replicated RNA. Thus, the block is at the level of transcription. Further, these results rule out the earlier possibility of misfolding of chimeric proteins being the cause of functional inactivity.

Discussion

Phosphoprotein P of non segmented negative-sense RNA viruses is an essential component of the viral RNA polymerase composed of P and L proteins. In this study, we have analyzed the functional domains of P protein of two closely related viruses RPV and PPRV. The RPV polymerase complex (P–L) was biologically functional to the extent of 50% with the PPRV N protein. This suggests that the domains of the P protein that are involved in the functional interaction with nucleocapsid are conserved between these two viruses. Previous studies using deletion mutants have revealed that the nucleocapsid protein binding domain of the RPV P protein is composed of two separate regions, amino terminal 1–59 aa and carboxy terminal 316–347 aa [3]. In addition to the nucleocapsid-binding site, the sequence responsible for L binding is also

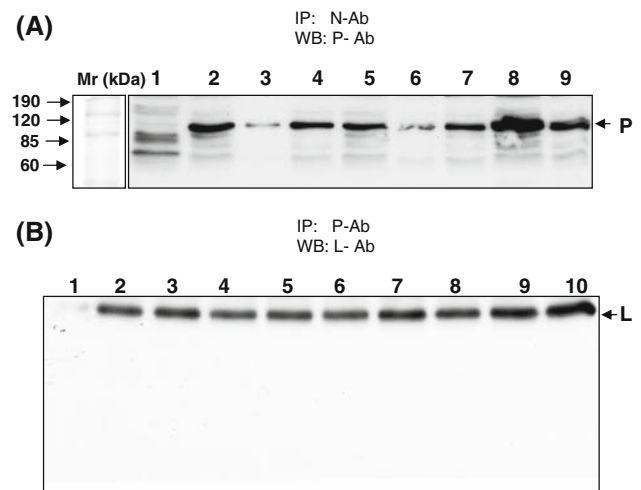


Fig. 6 Interaction of chimeric P with PPRV N (a) and RPV L (b) proteins. A549 cells were infected with VTF7.3 and cotransfected with 5 μ g of chimeric P and either PPRV N (pGKSN1) or RPV L (pPOL10) plasmids. After 24 h, cell lysates were prepared and 300 μ g of total lysates were immunoprecipitated with PPRV N (a) or RPV P antibody (b). The precipitated proteins were detected by immunoblotting with RPV P (a) or RPV L (b) antibody. In Fig. 6a, Lane 1: only wild type RPV P plasmid transfected lysates; lane 2: Co-transfected lysates of PPRV P and PPRV N. Lane 3, 4, 5, 6, 7, 8 and 9 corresponds to the co-transfected lysates of chimera 1, 2, 3, 4, 5, 6 and 7 with PPRV N, respectively. In Fig. 6b, Lane 1 only RPV L plasmid transfected lysates; Lane 2 represents co-transfected lysates of RPV P + RPV L; Lane 3 represents the co-transfected lysates of PPRV P + RPV L and Lane 4, 5, 6, 7, 8, 9 and 10 correspond to co-transfection of RPV L with chimera 1, 2, 3, 4, 5, 6 and 7 plasmids respectively

conserved in the P protein of RPV and PPRV; this sequence conservation is reflected in the finding that P and L protein of these viruses formed heterologous polymerase complexes, which are active together with PPRV N protein. It was previously shown that a region encompassing amino acids 347–490 of the RPV P protein is responsible for binding to the L protein [2]. The RPV and PPRV P proteins are well conserved in this region (70% identity) [8]. The high homology of P protein in this region is an agreement with the present finding that P and L proteins of PPRV and RPV form heterologous P–L complex. Further, the heterologous P–L complex is active in the minigenome system to drive the replication and transcription of the minigenome. For replication, formation of encapsidation complex of P–N⁰ is necessary. While the complex of PPRV P–RPV L is active in replication, it requires a homologous encapsidation complex formed with PPRV N protein. A tripartite complex of N–P–L has been shown in VSV to function in the replication of the genome [15]. In this study we have also demonstrated formation of a heterologous tripartite complex of PPRV P and N protein with RPV L protein.

The polymerase complex composed of RPV L and PPRV P was not biologically active with RPV N but active

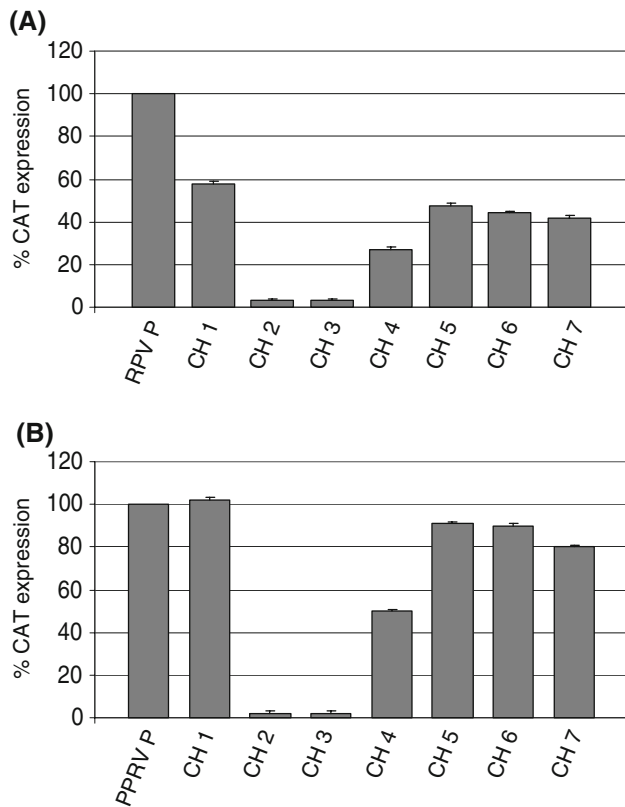


Fig 7 Biological activity of chimeric P proteins. **(a)** Chimeric P constructs along with RPV N and RPV L in combinations were expressed from cloned genes by the vaccinia virus T7 system in A549 cells that were transfected with RPV minigenome (pMDB8A). Activities of cell lysates were quantified by using CAT ELISA (Borehinger Manneheim). The Chimeric P proteins used are indicated by the chimeric constructs number (CH1, CH2, CH3, CH4, CH5, CH6 and CH7). As a positive control, RPV P was used in place of chimeric P construct along with RPV N and RPV L. Data presented here are an average of three independent transfection experiments. **(b)** Same as described above but used PPRV N construct instead of RPV N. As a positive control, PPRV P was used in place of chimeric P construct along with PPRV N and RPV L

with PPRV N. Differences in binding of P protein to assembled N protein (nucleocapsid) could be the key to the function of P in replication. Using Sendai virus minigenome system, it was shown that hPIV-1 P and Sendai virus L complex is biologically active [17]. Further employing the hPIV-1/Sendai chimeric P genes, it was shown that all the chimeric P proteins are active with Sendai virus L protein, indicating the conservation of P–L interaction domains in the P proteins of the two viruses. In contrast, Smallwood and Moyer [18] have shown heterologous complex formation between hPIV-3 L and Sendai virus P proteins which did not function in transcription either hPIV-3 or Sendai virus genome. Brown et al. [19] have shown replication/transcription of Measles virus minigenome by the heterologous combination of Measles virus N, P and RPV L protein to the extent of 41% compared to

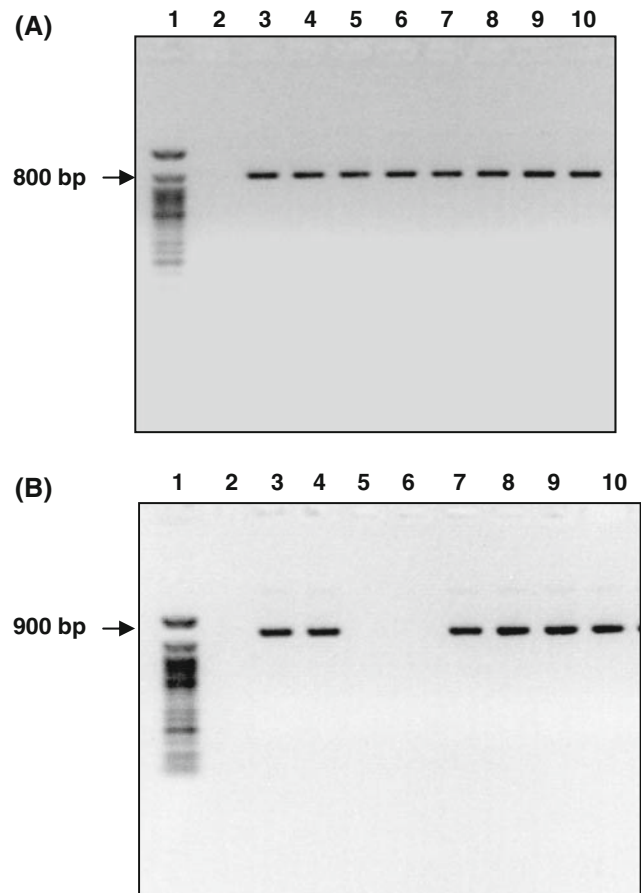


Fig. 8 RT-PCR analysis of Rinderpest virus minigenome replication/transcription product in presence of chimeric P protein. Total RNA was isolated from the cell lysates co-transfected with RPV minigenome, plasmids expressing RPV L and PPRV N proteins along with either PPRV P or chimeric P constructs. RT-PCR was done in two sets as described under materials and methods. RT-PCR products reflecting replication product of 800 bp **(a)** or transcripts of 850 bp in size **(b)** were electrophoresed on a 1% agarose gel. Lane 1: 50 bp DNA ladder; lane 2: PCR of total RNA isolated from infected transfected cells without doing RT; Lane 3: with PPRV P; Lane 4–10: in presence of chimera 1–7, respectively. The results are representative of three independent experiments

homologous complex (LPN). Bailey et al. [22] have recently shown that PPRV P does not function in the minigenome replication/transcription system, when used with RPV L and RPV N protein genes, while, in the present work, we detect a small level of CAT gene expression (less than 10%). This difference could be due to the use of P gene from Nigeria 75/1 strain (present work) and PPRV turkey 2000 strain employed by Bailey et al. whose sequences show 91% identity at protein level.

The strategy of producing chimeric proteins from homologous, structurally related protein is a better approach for mapping functional regions or residues on a protein, which is less likely to be affected by incorrect folding than the conventional deletion approach. In the

recent past, this strategy has been used to analyze functional domains of proteins from viruses like RSV and hPIV-1 [17, 20, 21]. In the present study, functional nucleocapsid binding domains of PPRV P protein involved in the transcription and replication process of virus minigenome were analyzed by using chimeric P protein of two closely related viruses RPV and PPRV.

All the chimeric P proteins interact with PPRV N protein, thus demonstrating the conservation of N⁰ interacting domains between the P proteins of the two viruses. Further, as expected, all chimeric P proteins interacted with RPV L. When the functional conservation of domains was tested in the RPV minigenome assay, chimeras 2 & 3 did not express CAT protein either with RPV N or PPRV N and they exhibited a block at the level of transcription and not replication, which is the first step in this minigenome system. An examination of the regions in the chimeras (Fig. 5) reveals that a region between 290 and 346 aa on RPV P is important and it may be required for binding to a cellular protein necessary for transcription to occur. This region is different in the two viruses. It would be interesting to test chimeras 2 & 3 in combination with PPRV L for biological activity.

Acknowledgments We thank Dr. T. Barrett and Dr. M. Baron, Institute for Animal Health, Pirbright laboratory, UK for providing PPRV P gene construct, the RPV minigenome, L and N gene constructs. This work was partly supported by the Indian Council for Medical Research under Center for Advanced studies in Molecular Medical Microbiology.

References

1. R.A. Lamb, D. Kolakofsky, Paramyxoviridae the virus and their replication, in *Fields virology*, 4th edn., ed. by B.N. Fields, D.M. Knipe, P.M. Howley (Lippincott-Raven, Philadelphia, 2001), pp. 1305–1339
2. A. Chattopadhyay, M.S. Shaila, *Virus Genes* **28**, 169–178 (2004). doi:[10.1023/B:VIRU.0000016855.25662.95](https://doi.org/10.1023/B:VIRU.0000016855.25662.95)
3. D. Shaji, M.S. Shaila, *Virology* **258**, 415–424 (1999). doi:[10.1006/viro.1999.9740](https://doi.org/10.1006/viro.1999.9740)
4. K.W. Ryan, A. Portner, *Virology* **174**, 515–521 (1990). doi:[10.1016/0042-6822\(90\)90105-Z](https://doi.org/10.1016/0042-6822(90)90105-Z)
5. M. Huber, R. Cattaneo, P. Spielhofer, C. Orvell, E. Norrby, M. Messerli, J.C. Perriard, M.A. Billeter, *Virology* **185**, 299–308 (1991). doi:[10.1016/0042-6822\(91\)90777-9](https://doi.org/10.1016/0042-6822(91)90777-9)
6. A. Diallo, T. Barret, P.C. Lefevre, W.P. Taylor, *J. Gen. Virol.* **68**, 2033–2038 (1987)
7. S.U. Emerson, M. Schubert, *Proc. Natl. Acad. Sci. USA* **84**, 5655–5659 (1987). doi:[10.1073/pnas.84.16.5655](https://doi.org/10.1073/pnas.84.16.5655)
8. M. Mahapatra, S. Parida, B.G. Egziabher, A. Diallo, T. Barrett, *Virus Res.* **96**, 85–98 (2003). doi:[10.1016/S0168-1702\(03\)00176-X](https://doi.org/10.1016/S0168-1702(03)00176-X)
9. T.R. Fuerst, E.G. Niles, F.W. Studier, B. Moss, *Proc. Natl. Acad. Sci. USA* **83**, 8122–8126 (1986). doi:[10.1073/pnas.83.21.8122](https://doi.org/10.1073/pnas.83.21.8122)
10. M.D. Baron, T. Barrett, *J. Gen. Virol.* **76**, 593–602 (1995)
11. M.D. Baron, T. Barrett, *J. Virol.* **71**, 1265–1271 (1997)
12. R. Kaushik, M.S. Shaila, *J. Gen. Virol.* **85**, 687–691 (2004). doi:[10.1099/vir.0.19702-0](https://doi.org/10.1099/vir.0.19702-0)
13. S. Mitra-Kaushik, R. Nayak, M.S. Shaila, *Virology* **279**, 210–220 (2001). doi:[10.1006/viro.2000.0698](https://doi.org/10.1006/viro.2000.0698)
14. R.M. Horton, H.D. Hunt, S.N. Ho, J.K. Pullen, L.R. Pease, *Gene* **15**, 61–68 (1989). doi:[10.1016/0378-1119\(89\)90359-4](https://doi.org/10.1016/0378-1119(89)90359-4)
15. K.A. Gupta, D. Shaji, A.K. Banerjee, *J. Virol.* **77**, 732–738 (2003). doi:[10.1128/JVI.77.1.732-738.2003](https://doi.org/10.1128/JVI.77.1.732-738.2003)
16. A. Rahaman, N. Srinivasan, N. Shamala, M.S. Shaila, *J. Biol. Chem.* **279**, 23606–23614 (2004). doi:[10.1074/jbc.M400673200](https://doi.org/10.1074/jbc.M400673200)
17. T. Bousse, T. Takimoto, T. Matrosovich, A. Portner, *Virology* **283**, 306–314 (2001). doi:[10.1006/viro.2001.0881](https://doi.org/10.1006/viro.2001.0881)
18. S. Smallwood, S.A. Moyer, *Virology* **318**, 439–450 (2004). doi:[10.1016/j.virol.2003.09.045](https://doi.org/10.1016/j.virol.2003.09.045)
19. D.D. Brown, F.M. Collins, W.P. Duprex, M.D. Baron, T. Barrett, B.K. Rima, *J. Gen. Virol.* **86**, 1077–1081 (2005). doi:[10.1099/vir.0.80804-0](https://doi.org/10.1099/vir.0.80804-0)
20. H. Zhou, X. Cheng, H. Jin, *J. Virol.* **77**, 5046–5053 (2003). doi:[10.1128/JVI.77.9.5046-5053.2003](https://doi.org/10.1128/JVI.77.9.5046-5053.2003)
21. H.L. Stokes, A.J. Easton, A.C. Marriott, *J. Gen. Virol.* **84**, 2679–2683 (2003). doi:[10.1099/vir.0.19370-0](https://doi.org/10.1099/vir.0.19370-0)
22. D. Bailey, L.S. Chard, P. Dash, T. Barrett, A.C. Banyard, *Virus Res.* **126**, 250–255 (2007). doi:[10.1016/j.virusres.2007.01.015](https://doi.org/10.1016/j.virusres.2007.01.015)