Arch Virol (2008) 153:615–626
DOI 10.1007/s00705-008-0034-9

ORIGINAL ARTICLE

Phosphorylation status of the phosphoprotein P of rinderpest virus modulates transcription and replication of the genome

Paramananda Saikia · M. Gopinath · M. S. Shaila

Received: 15 July 2007 / Accepted: 6 December 2007 / Published online: 29 January 2008

Abstract The phosphoprotein P of paramyxoviruses is known to play more than one role in genome transcription and replication. Phosphorylation of P at the NH_2 terminus by cellular casein kinase II has been shown to be necessary for transcription of the genome in some of the viruses, while it is dispensable for replication. The phosphorylation null mutant of rinderpest virus P protein, in which three serine residues have been mutated, has been shown earlier to be non-functional in an in vivo minigenome replication/transcription system. In this work, we have shown that the phosphorylation of P protein is essential for transcription, whereas the null mutant is active in replication of the genome in vivo. The null mutant P acts as a transdominant repressor of transcriptional activity of wild-type P and as an activator of replication carried out by wild-type P protein. These results suggest the phosphorylation status of P may act as a replication switch during virus replication. We also show that the phosphorylation null mutant P is capable of interacting with L and N proteins and is able to form a tripartite complex of L-(N-P) when expressed in insect cells, similar to wild-type P protein.

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

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

Introduction

The non-segmented negative-strand RNA genome of paramyxoviruses is tightly encapsidated by the nucleocapsid protein (N) to form a helical nucleocapsid [14, 26]. The N-RNA serves as a template for both mRNA synthesis and genome replication by the associated viral polymerase. In infected cells, the nucleocapsid core first directs transcription to generate mRNAs, catalysed by L in association with P protein, which are translated to produce viral proteins required for replication. During replication, a full-length complement (called antigenome) of the genomic RNA is synthesized, which gets encapsidated by the newly synthesized N protein, which in turn acts as the template for the synthesis of genomic nucleocapsid [25]. It is generally believed that the same polymerase complex performs both transcription and replication of the virus genome. However in vesicular stomatitis virus (VSV), two distinct polymerase complexes perform transcription and replication [17, 32]. The phosphoprotein of paramyxoviruses plays multiple roles in both transcription and replication, being an indispensable subunit of the viral polymerase complex. The P protein transactivates the L protein for transcription similar to what has been observed for eukaryotic transctivators. It interacts with the N protein, maintaining it in a form that is competent to support efficient RNA encapsidation during replication [10, 27]. It interacts with the L protein and stabilizes it against proteolytic degradation [5, 6, 10] and places the polymerase complex (L-P) on N-RNA template as L alone is unable to interact with the the N-RNA [18, 29]. The P protein is found in phosphorylated form inside the virion [8, 19].

The role of phosphorylation of P protein in viral transcription and replication is being studied in detail by several groups. The P protein of VSV, Chandipura virus,

respiratory syncytial virus (RSV) and measles virus (MeV) have been shown to be phosphorylated by CKII [7, 28, 12]. In VSV (NJ), phosphorylation of serine residues 59 and 61 within the acidic domains of P protein by recombinant Casein Kinase II is necessary for transcription activity [34], while the viral replication is not affected [31]. In hPIV3, addition of a pseudosubstrate specific for PKC-ζ, which phosphorylates P protein, completely inhibited the replication of the virus, thereby demonstrating the importance of phsophorylation of P protein [13, 20]. In Chandipura virus, it has been shown that phosphorylation is important for transcription but not for replication [3]. It has been demonstrated previously for rinderpest virus, that P protein is phosphorylated by cellular casein kinase II at three serine rersidues: S49, S88 and S151; phosphorylation at S49 and S88 is required for its function in the transcription and replication process, using a viral minigenome system for RPV [21]. This work did not clearly distinguish the effect of P protein phosphorylation on transcription versus replication. The present work has utilized both processes using a different approach. Virus-infected cells were transfected with phosphorylation null mutant P (Ptm) or wild-type P plasmid, and the synthesis of viral mRNA transcripts as well as replicated RNA was measured. When present in the same cell along with the wild-type P, the null mutant P behaves as a dominant negative repressor of transcription while allowing increased replication to occur with an increasing amount of null mutant P made in the cell. In addition, an in vitro transcription reconstitution system has also been used to study the effect of unphosphorylated P protein expressed in *E. coli* on transcription carried out by a suboptimal concentration of phosphorylated P.

Materials and methods

Cells and viruses

A549, a human lung carcinoma cell line was obtained from ATCC, USA, and maintained in DMEM supplemented with 10% newborn calf serum (NBCS) (GIBCO-BRL, USA). Vero and BSC 1 cells (used for maintenance and propagation of the VTF7-3) were obtained from the 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 were obtained from the National Center for Cell Sciences, Pune, India, and were maintained in TC-100 medium (Life Technologies, USA) with 10% fetal calf serum. A tissue-culture-adapted vaccine strain of RPV (RBOK, the original attenuated Kabete 'O' strain of RPV) was obtained from the Institute of Animal Health and Veterinary Biologicals, Bangalore, India. The virus was propagated in Vero cells as described previously [16]. VTF7-3, a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase in mammalian cells was a kind gift from Dr. Bernard Moss, NIH, USA [15]. A recombinant baculovirus expressing RPV N protein was generated using the cDNA clone of N (pKSN1; 1], employing a BAC-to-BAC cloning kit (GIBCO-BRL, USA). Recombinant baculoviruses expressing wild-type P and L were generated as described before [6]. The baculovirus expressing phosphorylation null mutant P was made previously in the laboratory (Rahman and Shaila, unpublished).

The oligonucleotides used in this study were purchased from Sigma-Genosys. For the detection of viral (+) sense N gene readthrough (2.5 kb), the PCR primers used were 5′ ATG GCT TCT CTC TTG AAG AGC TTA 3′ (N gene nt 1–24) and 5′ GAT CCT TGG TTT ATT GCC TGG A 3′ (P gene nt 729–750), viral leader-specific primer 5′ CTG GGT AAG GAT CGT TCT 3′ (nt 12–30) and bacterial chloramphenicol acetyl transferase (CAT) ORF-specific primer 5′ GGA TAT ACC ACC GTT GA 3′ (upstream) and 5′ GGG ACG GTG AGT AGC GTC ATG 3′ (downstream). The actin control primers used were 5′ GCG CCC CAG GCA CCA GGG 3′ (upstream) and 5′ AGG ATG GCA TGG GGG GGA 3′ (downstream).

Plasmids

The cloning of full-length P (pCMXP), phosphorylation triple mutant P (pCMX Ptm), N (pCMX N) and L (pCMX L) under a CMV promoter in the eukaryotic vector pCMXPL2 has been described [21]. The RPV minigenome, pMBD8A and the cDNA clones of N (pKSN-1), and L (pPOL10) were kind gifts from Dr. M. Baron, Institute of Animal Health, Pirbright Laboratory, UK [1, 2]. pRP6 (pRSETB plasmid carrying the full-length P gene) and Ptm, phosphorylation null mutant P gene cloned in the pRSETB vector have been described earlier [21]. For expression of null mutant P protein with a Flag tag, P mutant gene ORF was PCR amplified using a pRK8 plasmid carrying a P mutant gene generated earlier in the laboratory and cloned into pGEMT EASY Vector and subsequently subcloned into eukaryotic expression vector pFLAG-CMV^{TM}-4 (SIGMA) at the Not I and EcoRV sites.

Antibodies

Rabbit polyclonal antibody against bacterially expressed purified RPV P [21] and RPV L [6], made previously in the laboratory, were used.

Infection-transfection

For infection-transfection experiments, A549 cells grown in DMEM supplemented with 10% FCS in a 35-mm dish were transfected at 90% confluency with different concentrations of pCMX constructs of wild-type and P mutants using Fugene 6 (Roche) in 1 ml OPTI-MEM medium. At 12 h post-transfection, cells were infected with rinderpest virus at an m.o.i of 5. Cells were harvested 36 h after infection, and the total RNA was isolated.

RT-PCR analysis

Twelve or 36 h postinfection, A549 cells were washed twice with PBS and trypsinized, and total RNA was isolated using Trizol reagent (Life Technologies). Reverse transcription was carried out using oligo $(dT)_{18}$ (SIGMA) for the transcription product and a P-gene-specific 3′ primer for the replication product using Superscript (GIBCO-BRL) reverse transcriptase maintaining 5 μg of total cellular RNA per reaction.

For semi-quantitative analysis, preliminary experiments were done to determine the optimum PCR cycle number within the linear range of amplification for each gene being measured. RT products were subjected to PCR with different primer pairs corresponding to the following: (1) N-gene-specific upstream and downstream primer to detect the N-gene-specific transcript, (2) N-gene-specific upstream and P-gene-specific downstream primer to identify the replication product. The RT-PCR products were electrophoresed on a 1% agarose gel and visualized by staining with ethidium bromide.

Co-immunoprecipitation

For P-L immunoprecipitation, A549 cells were plated in 60-mm tissue culture dishes at a density of 5×10^6 cells in 10 ml of DMEM supplemented with 10% FCS (GIBCO-BRL, USA). When the cells were 70% confluent, cells were transfected with 5 μg of wild-type P or phosphorylation null mutant P plasmids in combination with the same amount of either wild-type N or L plasmid (pCMX constructs). 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 whole-cell lysate (300 μg) was precleared by incubating with rabbit pre-immune serum and protein sepharose beads for 1 h at 4°C. Rabbit anti-N or anti-L antibody was incubated with a 10% suspension of protein-A Sepharose beads for one hour 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. SDS-PAGE loading dye was added to the beads, and the sample was boiled for 5 min before loading onto a 10% SDS-Polyacrylamide gel.

In vivo interaction of P or P mutant, N and 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 an 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, pH 7.5, 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 $100{,}000 \times g$. The soluble supernatant (S100) was subjected to co-immunoprecipitation with either anti P or anti N antibody, and the interacting proteins were detected by western immunoblotting with anti-L antibody.

In vivo minigenome replication transcription product analysis

The in vivo minigenome replication/transcription were carried out as described previously [2]. Total RNA was isolated 36 h after transfection and analysed. To detect the transcription product, RT-PCR was carried out with a CAT ORF-specific upstream primer and an oligo $(dT)_{18}$ downstream primer. For the replication product, RT-PCR was carried out with a leader-RNA-specific upstream and CAT ORF-specific downstream primer.

Partial purification of polymerase (L-P) complex from insect cells

Partial purification of L-P complex from insect cells was carried out according to Curran [9] with some modifications. Sf21 cells (1×10^7) were co-infected with recombinant baculoviruses expressing RPV L and RPV P at an moi of 2 and 10, respectively. Cells were collected 96 h after infection and washed once in PBS. Cells were suspended in 4 ml of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.5% Triton X 100, 2 mM DTT, 5 mM $MgCl_2$ and 1X protease inhibitor cocktail. After lysis, the clarified lysate was layered onto a linear 5–20% glycerol gradients prepared in buffer containing

100 mM HEPES-KOH (pH 8.0), 150 mM NH_4Cl, 5 mM magnesium acetate, and 1 mM DTT. Gradients were centrifuged at 29,000 rpm in an SW41 rotor for 36 h at 4°C. Gradient fractions (1.2 ml) were collected from the top of the tube. Fractions containing active L-P complex were analyzed by western blotting detection of L and P proteins and co-immunoprecipitation analysis (data to be published elsewhere).

Reconstitution of in vitro RNA synthesis

Polymerase-free RPV genomic N-RNA was isolated from virus-infected cells by cesium chloride density gradient centrifugation as per Curran [9]. Viral transcription was reconstituted in a 50-µl total volume with buffer containing 100 mM HEPES-KOH (pH 8.0), 150 mM NH_4Cl, 5 mM $MgCl_2$, 1 mM DTT, 40 U/ml of creatine phosphokinase, 1 mM creatine phosphate, 1 mM each of ATP, CTP, GTP and spermidine, 50 µM of UTP and 10 µCi of α-^{32}P-UTP 25U of human placental RNase inhibitor, 5 µg of actinomycin D per ml, 2 µg of N-RNA (with respect to N protein) template, 6 µg of glycerol gradient fraction containing L-P complex and the indicated amounts of unphosphorylated P protein (wild-type P_0 or Ptm purified from *E. coli*) or phosphorylated P (P purified from insect cells or *E. coli* P_0 phosphorylated in vitro with CKII). The transcription reaction was carried out at 30°C for 3 h. The mRNA products were subjected to Northern blot with antisense probe against N-mRNA.

Results

Phosphorylation null mutant P protein forms P-L, P-N_0 and N-P-L complexes

Phosphorylation of at least two of the three (S49 & S88) serine residues located at the N-terminal part of the P protein of RPV has been shown to be necessary for its in vivo function, using a minigenome replication/transcription system [21]. The impairment of the P function could be at the level of formation of complexes with L protein or with unassembled N_0 (encapsidation complex). Therefore, formation of P-L and P-N_0 complexes was assessed in vivo using the phosphorylation null mutant P or wild-type P plasmids with either L or N plasmids by transfecting A549 cells in combination or alone. Proteins in the cell extracts were immunoprecipitated with N antibody or L antibody, and proteins were separated electrophoritically. The proteins were immunoblotted with P antibody. The result shown in Fig. 1a, b demonstrates that the phosphorylation null mutant P interacts with both L and N_0 protein and thus forms complexes which are necessary to carry out transcription and replication. The complexes so formed are specific, since triple mutant P (having 3 serine mutated to alanine) when expressed alone is not immunoprecipitated either with N antibody or L antibody. In the VSV system, it has been shown that P forms a complex with L (transcription complex) and a separate tripartite complex L + P + N_0 (replication complex), which performs replication [17]. The existence of these two distinct complexes

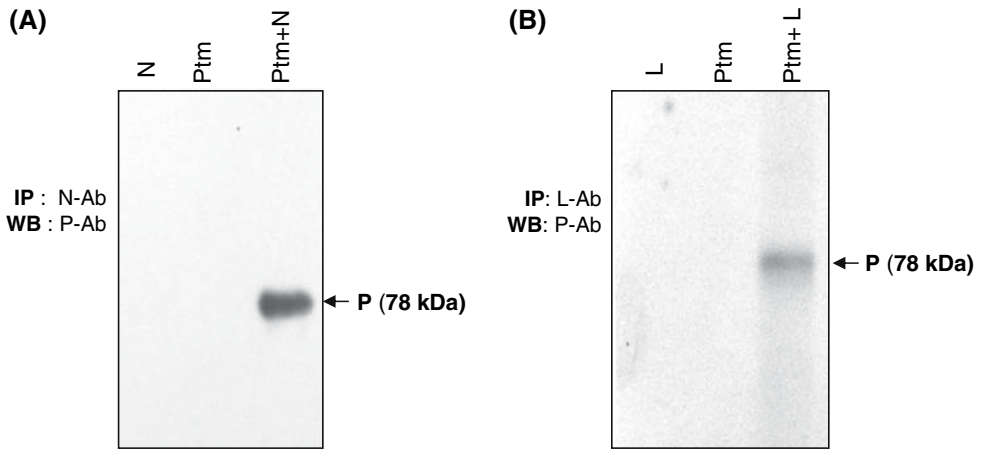

Fig. 1 In vivo interaction of P mutant with N and L protein. A549 cells were co-transfected with recombinant pCMX plasmids expressing mutant P (Ptm) with either N or L protein. 300 µg of lysate was immunoprecipitated with anti-N antibody (**a**) or anti-L antibody (**b**) and the immunoprecipitate electrophoresed on 10% SDS-PAGE and western immunoblotted with anti-RPV P antibody after transferring the protein to a nitrocellulose membrane. *Lanes Ptm + N* and *Ptm + L* depict proteins immunoprecipitated with the anti-N or anti-L antibody from extracts of cells transfected with Ptm plasmid and either N or L plasmid. The positions of the P protein are indicated by *arrows*. As a control, only Ptm-, N- and L-transfected cell lysate was immunoprecipitated with anti-N and anti-L antibody and immunodetected with anti-P antibody

in VSV-infected cells has also been demonstrated, and these complexes were responsible for transcription or replication activity, but not both [32]. Therefore it was of interest to look for the presence of an L-P-N complex in RPV and to test if the null mutant P has the ability to form such a complex. To test this, recombinant baculoviruses expressing L, P and phosphorylation null mutant P were employed [6, Rahman and Shaila, unpublished), and a recombinant baculovirus capable of expressing RPV N protein in insects cells was generated. This virus expressed authentic RPV N protein (Mr 62 kDa) in *Sf21* cells (data not shown). Insects cells (*Sf21*) were co-infected with these recombinant baculoviruses, and the S-100 extracts from infected cells were immunoprecipitated with ether N or P antibody and western immunoblotted with L antibody. The results shown in Fig. 2a, b demonstrate that the tripartite complex is formed when L, P and N proteins are co-expressed in the same cell. It has been shown earlier [17] that L by itself cannot form a complex with N protein, and the interaction is through P protein. Expression of L alone or with N does not result in its immunoprecipitation with N antibody (Fig. 2a, lane L & L-N) whereas co-expression of all three proteins result in the immunoprecipitation of the L + P + N complex, which was shown by immunoblotting with anti-L antibody (Fig. 2a, lane L-N-P). This result was also obtained using P antibody for immunoprecipitation and western blotting with L (Fig. 2b) and N antibody (Fig. 2c). When this experiment was repeated with the null mutant P, it retained the ability to form the complex of L-P-N (Fig. 3a, b). Therefore, the null mutant P is not defective in forming either the P-L or N-P-L complex required for transcription and replication, respectively.

Effect of phosphorylation null mutant P protein on viral transcription and replication

Virus transcription and replication in infected cells were measured in the presence of transiently expressed phosphorylation null P protein by RT-PCR analysis of specific products of transcription and replication employing specific primers described in "Materials and methods". As can be seen from Fig. 4a, lane 5, the transcription of N gene by the viral polymerase was significantly lower (35%) in the infected cells transiently expressing the phosphorylation-null mutant, compared to the amount of N transcript made by the virus polymerase in the presence of transiently expressed wild-type P protein (over and above the wild-type P expressed in infected cells). In contrast, the synthesis of plus-sense antigenomic RNA as measured by RT-PCR was greatly increased (300%) in cells expressing the phosphorylation null mutant (Fig. 4b, lane 12). There was no difference in the levels of expression of a house keeping cellular

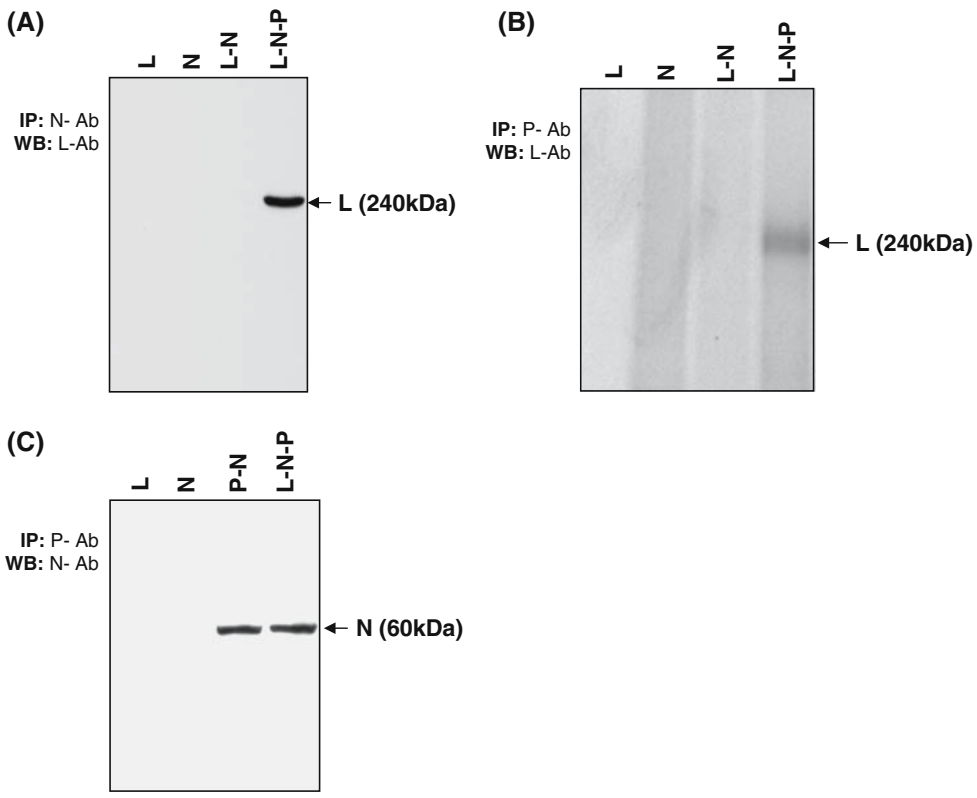

Fig. 2 Tripartite complex formation by rinderpest virus N, P and L proteins. Immunoprecipitation of Sf21 soluble (S100) extracts. Cells expressing different viral proteins either alone or in combination (marked on *top* of each lane) were immunoprecipitated either with anti-N antibody (**a**) or anti-P antibody (**b, c**), followed by SDS-PAGE and western blotting using anti-L (**a, b**) or anti-N (**c**) antibody. The positions of the L and N proteins are indicated by *arrows*

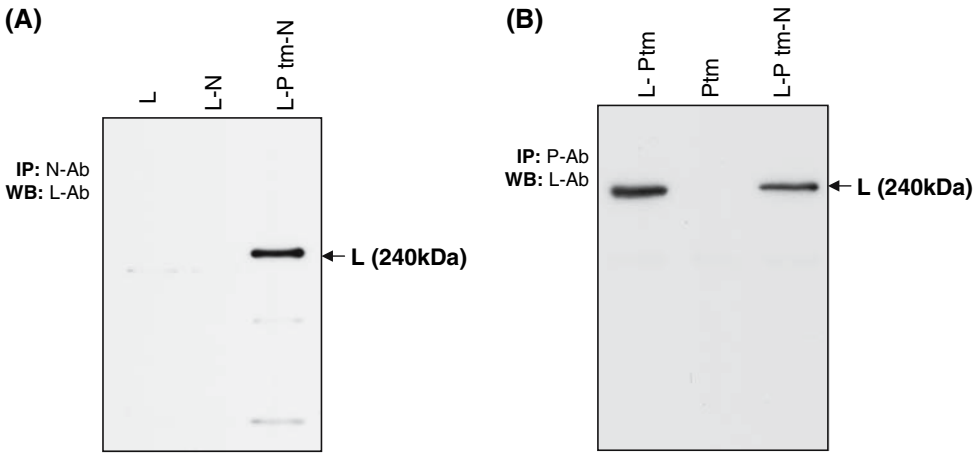

Fig. 3 Tripartite complex formation with N and L proteins by null mutant P. Immunoprecipitation of Sf21 soluble (S100) extracts. Cells expressing different viral proteins either alone or in combination, (marked on *top* of each lane), were immunoprecipitated either with anti-N antibody (**a**) or anti-P antibody (**b**), followed by SDS-PAGE and western blotting using anti-L antibody. The positions of the L protein are indicated by *arrows*

gene, actin, which served as a control for the amount of RNA used for RT-PCR (bottom panel of Fig. 4a, b). The transcription of other genes such as F also was decreased in infected cells in the presence of transiently expressed null mutant P protein (data not shown). Since the phosphorylation null mutant P protein modulates transcription and replication of the RPV genome in vivo, we tested whether the plasmid-derived P tm protein interacts with the N protein expressed by the virus in infected cells. As shown in Fig. 4c, when the lysate from cells infected with rinderpest virus and subsequently transfected with phosphorylation null mutant P plasmid was immunoprecipitated with anti-N antibody and immunodetected the mutant P with anti-FLAG antibody, plasmid-derived Ptm protein showed interaction with viral N, indicating that mutant P has not lost its ability to interact with the viral proteins. A similar result was seen with the L protein expressed by the virus in infected cells (data not shown).

When the above experiments were repeated with varying concentrations of the triple mutant plasmid DNA for transfection of infected cells, a dose-dependent decrease in the synthesis of viral mRNA (Fig. 5a) and a dose-dependent increase in the replication of (+) sense viral RNA (Fig. 6a) were seen. This effect is clearly due to increased expression of the triple mutant protein P as seen on the western blot (bottom panel of Fig. 5a).The wild-type P protein expression was almost constant compared to the triple mutant P protein (middle panel, Fig. 5a). The increase in the replication of the virus genome was observed with increasing amounts of mutant P protein expression in infected cells (Fig. 5b). Thus, the phosphorylation null mutant exhibits a dual effect on transcription and replication; the mutant behaves as a dominant negative effector for virus transcription and as an activator for replication, indicating that it works as a dual-function switch.

Phosphorylation-null mutant P protein participates in minigenome replication in vivo

The null-mutant P retained its ability to interact with L and N_0 protein in vivo and is able to form the tripartite complex. Furthermore, in the presence of wild-type P, it inhibited transcription in virus-infected cells in a dose-dependent manner, while the reciprocal effect was seen on replication of viral RNA in vivo. Together with the previous observation [21] that in vivo minigenome replication/transcription is inhibited in the presence of mutant P protein, we tested its effect on the replication step of the minigenome system. RT-PCR analysis of the replication product using specific primers described in "Materials and methods" showed that a 740-bp genomic sense CAT RNA is made in the cells (Fig. 7a) transfected with the mutant P along with N, L and minigenome plasmids. The amount of replication product made in the cells increased as a function of the concentration of transfected null mutant P (Fig. 7b). These results confirmed that the earlier results [21] are due to a block at the transcription step (which follows the replication step), and the replication step is unaffected. To further substantiate this, RNA from the same in vivo minigenome replication/transcription system was subjected to reverse transcription with oligo $(dT)_{18}$ and PCR with CAT-specific 5′primer and oligo $(dT)_{18}$ primer. The data shown in Fig. 7c indicates that transcription is blocked at any concentration of null mutant P plasmid used for transfection. A similar result was observed when we analysed RT-PCR products of N-gene-specific transcripts with purified viral RNP (data not shown).

In order to provide unequivocal evidence for the inhibition of transcription by unphosphorylated P (*E. coli* expressed) or phosphorylation null mutant P, an in vitro reconstituted transcription system was employed (Gopinath and Shaila, to be published elsewhere).

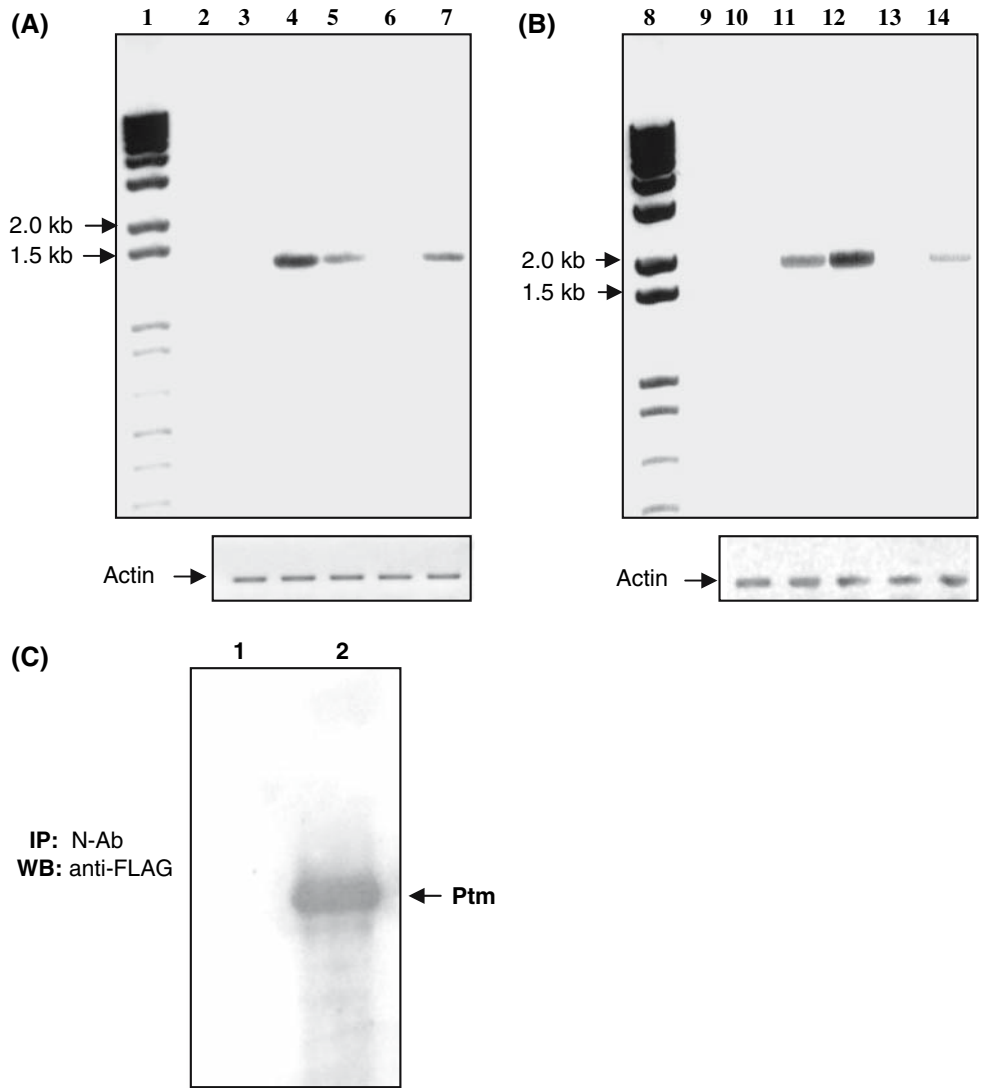

Fig. 4 Effect of null mutant P expression on virus transcription and replication in infected cells. Total RNA was isolated from cells which were not infected or transfected (*lane 3* and *lane 10*), only infected (*lane 7* and *lane 14*), only transfected with wild-type P (*lane 6* and *lane 13*), infected with RPV and transfected with either construct expressing P wt (*lane 4* and *lane 11*) or phosphorylation null mutant P proteins (*lane 5* and *lane 12*). RT-PCR was done in two sets as described in "Materials and methods". RT-PCR products reflecting viral N-gene-specific transcripts of 1.6 kb (**a**) or a viral genome RNA replication product of 2.5 kb in size corresponding to N-P read-through RNA (**b**) were electrophoresed on a 1% agarose gel. As a control for the amount of RNA used for RT-PCR, cellular actin (364 bp) was amplified using actin-specific upstream and downstream primers (*bottom panel* of **a**, **b**). *Lanes 2* and *9* were controls (no cDNA). *Lanes 1* and *8* represent 1 kb plus DNA ladder. The results are representative of three independent experiments. c A549 cells were infected with rinderpest virus and transfected with triple mutant P plsmid (*lane 2*) or wild-type P plasmid (*lane 1*). 12 hr post-transfection, cell lysate were prepared, and the complex was immunoprecipitated by N antibody and western blotted with anti-FLAG antibody, which specifically detects the Ptm protein

The transcription of the N gene was monitored in this system in the presence of increasing concentrations of bacterially expressed P protein or in vitro-phosphorylated recombinant P protein, or phosphorylated insect-cell-expressed P protein or phosphorylation null mutant P protein employing N-RNA from virus-infected cells and a suboptimal concentration of recombinant L-P complex (linear range), which was partially purified from insect cells infected with recombinant L and P baculoviruses. The N-gene-specific transcript was analysed by Northern blot hybridization using ^{32}P-UTP-labelled negative-sense RNA probe specific for the N gene. Representative data from such analysis are shown in Fig. 8a. Band intensities were quantified from three independent experiments and plotted against the indicated concentrations of P protein exogenously added to the reaction mixture (Fig. 8b). Addition of phosphorylated P protein in the reconstitution reaction containing L-P complex and N-RNA results in 2.5 times more transcription (Fig. 8a, lanes 2–5) compared to the reaction mix where no supplemental P was added (Fig. 8a,

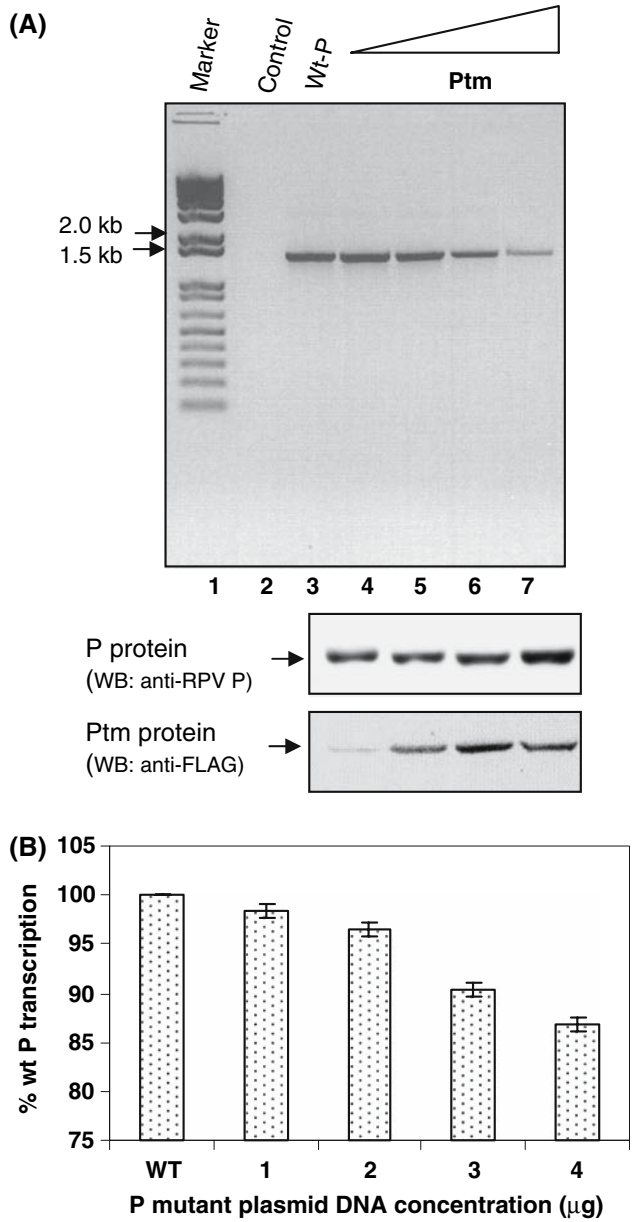

Fig. 5 Effect of increasing concentration of null mutant P DNA on virus transcription. Total RNA was isolated from cells infected with RPV and transfected with either wild-type P (*lane 3*) or different concentrations of plasmids expressing phosphorylation null mutant P. RT-PCR was carried out as described in "Materials and methods". **a** RT-PCR products reflecting the N-gene-specific transcript in the presence of 1, 2, 3 and 4 μg (*lanes 4, 5, 6* and *7*, respectively) of phosphorylation null mutant plasmid DNA were analyzed on 1% agarose gel. *Lane 2* corresponds to control for cDNA. DNA molecular weight marker (1 kb plus DNA ladder) loaded into *lane 1*. The transient expression of phosphorylation null mutant protein with 4 different concentrations of plsmid (1–4 μg) were detected by western immunoblotting with anti-FLAG antibody (*bottom panel*) and wild-type P expression by anti-RPV P antibody (*upper panel*). **b** The band intensities of the transcription products were quantitated using Image Gauge software version 2.54 (Fuji Film). Data presented here are an average of three independent experimental sets

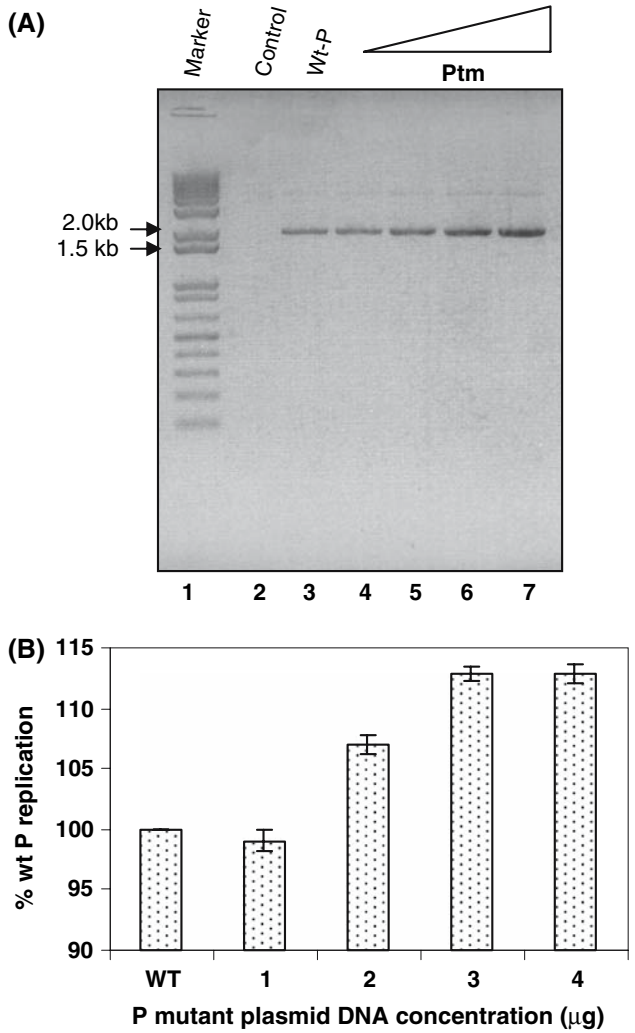

Fig. 6 Effect of increasing concentration of null mutant P DNA on virus replication. Total RNA was isolated from cells infected with RPV and transfected with either wild-type P (*lane 3*) or different concentrations of plasmids expressing phosphorylation null mutant P. RT-PCR was carried out as described in "Materials and methods". **a** RT-PCR products reflecting replication product in presence of 1, 2, 3 and 4 μg (*lanes 4, 5, 6 and 7*, respectively) of phosphorylation null mutant plasmid DNA were analyzed on a 1% agarose gel. *Lane 2* corresponds to the control for cDNA and *lane 3* corresponds to only the infection control. DNA molecular weight marker (1 kb plus DNA ladder) loaded into *lane 1*. **b** The band intensities of the replication products were quantitated using Image Gauge software version 2.54 (Fuji Film). Data presented here are an average of three independent experimental sets

lane 1). In agreement with the in vivo transdominant inhibition of transcription by the phosphorylation null mutant (Ptm), addition of either Ptm or unphosphorylated *E.coli* P (P_0) protein inhibited the transcription by 50% (Fig. 8a, lanes 6–13) in a concentration-dependent manner. Further confirmation for the role of P phosphorylation in activating viral transcription was observed by the ability of

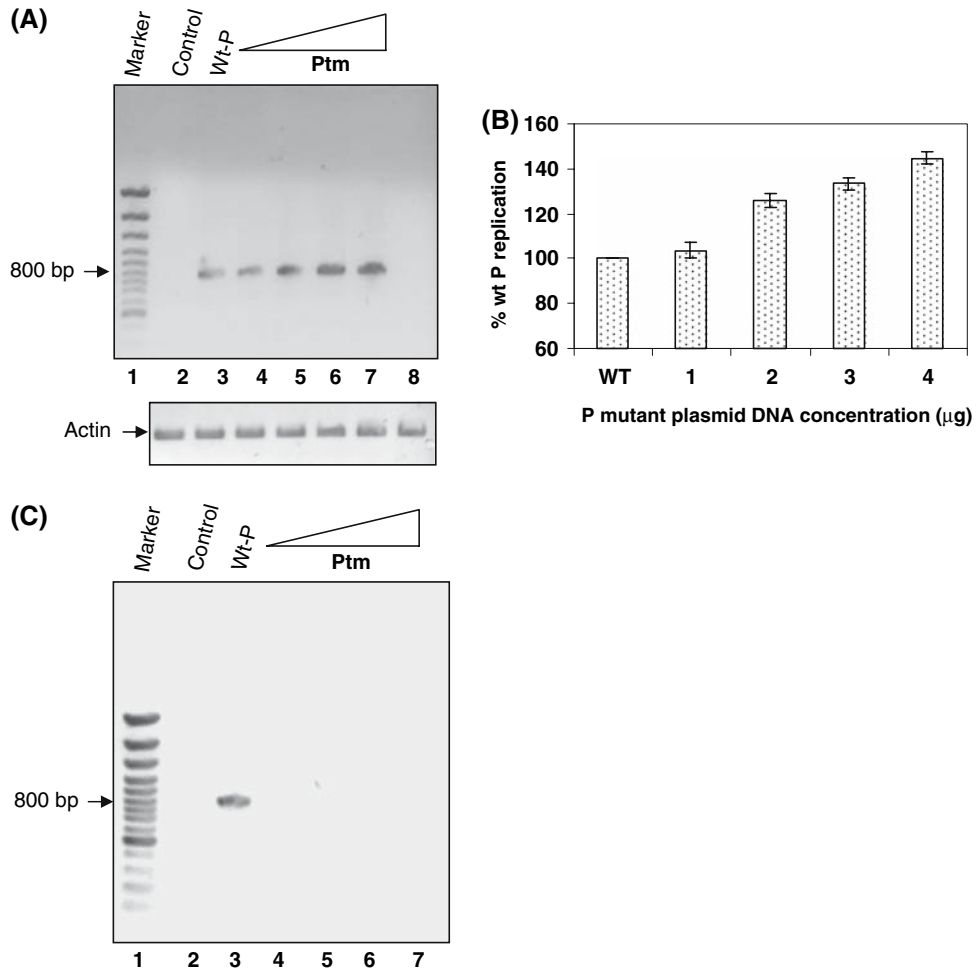

Fig. 7 In vivo minigenome replication function of null mutant P. A549 cells in 35-mm plates were infected with VTF7.3 (1 p.f.u per cell) and subsequently transfected with 1 μg of N (pKSN1), 100 ng of L (pPOL10) and either 1 μg of wild-type P (*lane 3*) or 1, 2, 3, 4 (*lanes 4, 5, 6*, and *7*, respectively) μg of phosphorylation null mutant plasmid along with 1 μg of RPV minigenome, pMDB8A plasmid. Total RNA was isolated 36 h after transfection. a RT-PCR was carried out to detect the minigenome replication product of 740 bp in size using leader-specific 5′ primer and CAT-gene-specific 3′ primers. DNA molecular weight marker (100 bp DNA ladder, *lane 1*). In *lane 2*, there is no addition of P and N plasmids along with the minigenome, and *lane 8* corresponds to the product in the absence of N, P and L plasmids. **b** The band intensities of the replication products were quantitated using Image Gauge software version 2.54 (Fuji Film). Data presented here are an average of three independent experimental sets. **c** RT-PCR analysis of in vivo minigenome transcription. Total RNA isolated from the same cells as described above and RT-PCR was carried out to detect the minigenome transcription products of 780 bp in size using CAT-specific upstream primer and oligo $(dT)_{18}$ downstream primer. DNA molecular weight marker was (100 bp DNA ladder, *lane 1*). *Lane 2* corresponds to the PCR product in the absence of L plasmid. The result is representative of three independent experiments

in vitro-phosphorylated *E. coli* P to augment viral transcription more than twofold (Fig. 8a, lanes 14–17).

Discussion

The phosphoprotein of paramyxoviruses is a multifunctional protein. It is an essential component of the viral RNA polymerase complex (P-L) and is thought to mediate the interaction of the L protein with the viral nucleocapsid template for transcription and replication. It also interacts with N protein to form an N-P complex, which is required for encapsidation of the nascent RNA chain during replication [24, 25]. In the virus-infected cells as well as in the virion core, the P protein has been shown to exist in multiple phosphorylated forms [4, 8]. In the present work, the role of phosphorylation of P protein of rinderpest virus in transcription and replication has been examined in vivo and in vitro. It has been shown earlier that the phosphorylation-negative mutant of the P protein of rinderpest virus in which all three phosphate acceptor sites (S44, S89 & S151) were replaced with alanine is not able to carry out its function in vivo, as tested using a minigenome replication/transcription assay [21]. Semiquantative RT-PCR analysis

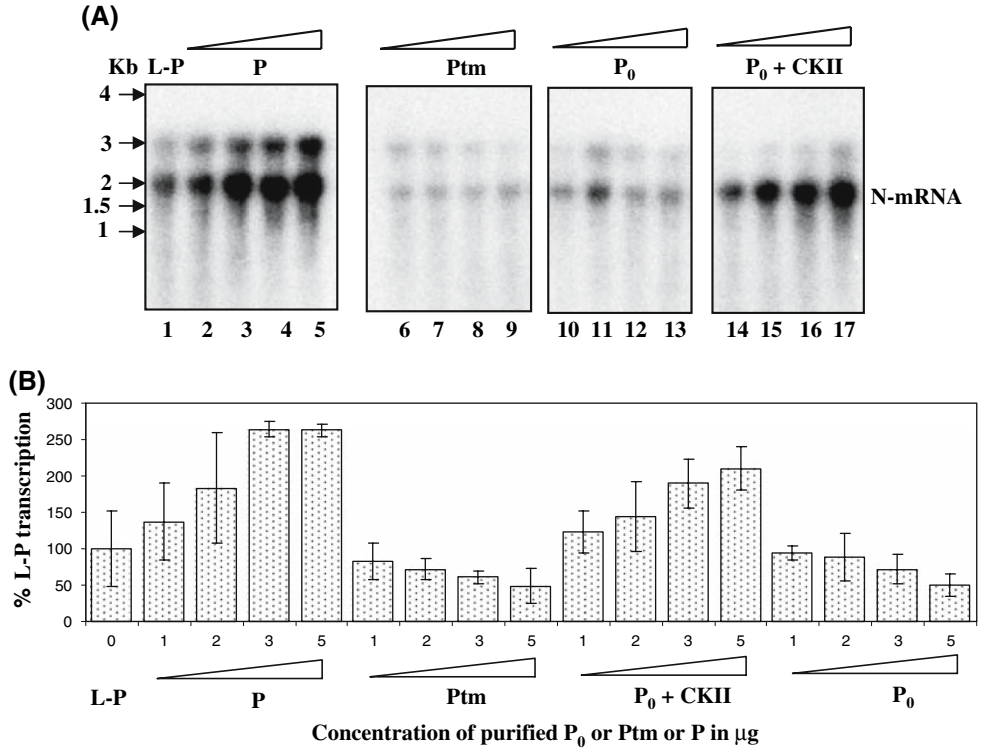

Fig. 8 Effect of P phosphorylation in in vitro-reconstituted transcription. Viral transcription was reconstituted in vitro with a suboptimal amount of L-P complex (6 μg) and 2 μg of N-RNA in the absence (L-P) or presence of increasing amounts (1, 2, 3 and 5 μg) of either phosphorylated P (P from insect cells or *E . coli* P_0 phosphorylated in vitro with CKII) or unphosphorylated P (wild-type or Ptm purified from *E. coli*) as mentioned in "Materials and methods". **a** Transcription products were extracted with phenol:chloroform and subjected to northern blotting with radioactive labeled N-mRNA-specific antisense probe made in vitro. N-mRNA was detected by subjecting the blot to autoradiography. **b** Band intensities were quantitated using Multiguage version 2.3 (Fuji Film) in terms of arbitrary units and plotted against the indicated concentrations of exogenously added P protein. L-P refers to the reaction mix where no exogenous P or P_0 was added. The band intensity for the reaction mix with L-P alone was considered as 100% transcription

of viral mRNA as well as replicated products in cells infected with virus and transfected with phosphorylation null mutant P or wild-type P plasmid showed that phosphorylation of P protein is critical for transcriptional activity but not essential for replication of the genome. In VSV, employing either a replicating DI genome with support plasmids or a transfected DI genome with support plasmids of L, N and Pwt, or P mutant genes for in vivo replication or a VSV minigenome with support plasmids for in vivo transcription, Pattanik et al. [31] have shown that phopshorylation of domain I residues has little or no effect on replication of DI RNA, while phosphorylation within domain I is important for transcription activity of the P protein. These authors further showed that the domain I null mutant P, when co-expressed with wt P protein, did not inhibit in vivo transcription or replication, thereby showing that null mutant P is not a transdominant repressor. In contrast to these results, the null mutant P protein of RPV acts like a transdominant repressor of transcription in vivo. When expressed along with viral P (wt) in cells (Fig. 4a) and with increasing concentration of null mutant P plasmid used for transfection of infected cells (Fig. 5a), there was progressive inhibition of transcription, whereas a reciprocal effect was seen on replication, leading to increased replication as a function of increased mutant P expression (Fig. 6a). Similar results were obtained in an in vitro reconstitution system with L-P complex and viral genomic N-RNA in which addition of Ptm or unphosphorylated P (P_0) results in inhibition of transcription, and this is reversed when in vitro-phosphorylated P is added to the reaction (Fig. 8a, b). Phosphorylation null mutant P may be acting in a dual fashion—as a transdominant repressor of transcription and as a transdominant activator of replication—when present in the same cell. This novel property supports the view that P protein is a candidate to function as a transcription-replication "switch" instead of the long-held role hypothesized for the N protein [22, 23].

In infected cells, phosphorylated P protein may get dephosphorylated by a virus-induced phosphatase in a temporal fashion. This may result in the accumulation of unphosphorylated P protein. The monomeric units of this protein can then easily exchange with those of

phosphorylated P protein [33], thereby rendering it inactive in transcription function. As the concentration of unphosphorylated tetrameric P increases, the transcription process gets inhibited. However, it is not clear how unphosphorylated P protein could function as a transdominant activator of replication of genomic RNA. Further work is needed to demonstrate a role for unphosphorylated P protein in replication. An in vitro-reconstituted replication system may provide an explanation for this tantalizing observation.

Since it has been demonstrated in the VSV system that the transcription complex is associated with cellular proteins, and the tripartite complex of N-P-L, which performs the replication, is devoid of cellular proteins [32], it is tempting to speculate that unphosphorylated P protein has an additional role in inhibition of transcription by modulating the activity of one of the cellular proteins needed for transcription. This may then act as a signal for the replication complex to proceed with replication with the genome RNA. Since the replication complex is devoid of cellular proteins, its function may not be dependent on the phosphorylation status of the P protein.

It would be very useful to identify the cellular proteins associated with the transcription complex in RPV and to investigate the role played by unphosphorylated P protein in modulating the cellular protein in such a way that transcription does not proceed.

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

References

1. Baron MD, Barrett T (1995) Sequencing and analysis of the nucleocapsid (N) and polymerase (L) genes and the terminal extragenic domains of the vaccine strain of rinderpest virus. J Gen Virol 76:593–602
2. Baron MD, Barrett T (1997) Rescue of rinderpest virus from cloned cDNA. J Virol 71:1265–1271
3. Basak S, Raha T, Chattopadhyay D, Majumdar A, Shaila MS, Chattopadhyay DJ (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
4. Bell JC, Prevec L (1985) Phosphorylation sites on phosphoprotein NS of vesicular stomatitis virus. J Virol 54:697–702
5. Canter DM, Perrault J (1996) Stabilization of vesicular stomatitis virus L polymerase protein by P protein binding: a small deletion in the C-terminal domain of L abrogates binding. Virology 219:376–386
6. Chattopadhyay A, Shaila MS (2004) Rinderpest virus RNA polymerase subunits: mapping of mutual interacting domains on the large protein L and phosphoprotein P. Virus Genes 28:169–178
7. Chattopadhyay DJ, Raha T, Chattopadhyay D (1997) Single serine phosphorylation within the acidic domain of Chandipura virus P protein regulates the transcription in vitro. Virology 239:11–19
8. Clinton GM, Burge BW, Huang AS (1979) Phosphoprotein of vesicular stomatitis virus: identity and interconverson of phosphorylated forms. Virology 99:84–94
9. Curran J (1996) Reexamination of the Sendai virus P protein domains required for RNA synthesis: a possible supplemental role for the P protein. Virology 221:130–140
10. Curran J, Pelet T, Kolakofsky D (1994) An acidic activation like domain of the Sendai virus P protein is required for RNA synthesis and encapsidation. Virology 202:875–884
11. Curran J, Marq JB, Kolakofsky D (1995) An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. J Virol 69:849–855
12. Das T, Schuster A, Schneider-Schaulies S, Banerjee AK (1995) Involvement of cellular casein kinase II in the phosphorylation of measles virus P protein: identification of phosphorylation sites. Virology 211:218–226
13. De BP, Gupta S, Gupta S, Banerjee AK (1995) Cellular protein kinase C isoform zeta regulates human parainfluenza virus type 3 replication. Proc Natl Acad Sci USA 92:5204–5208
14. Fitch JT, Gibbs AJ (1970) Observation on the structure of the nucleocapsid of some paramyxoviruses. J Gen Virol 6:141–150
15. Fuerst TR, Niles EG, Studier FW, Moss B (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc Natl Acad Sci USA 83:8122–8126
16. Ghosh A, Joshi VD, Shaila MS (1995) Characterization of an in vitro transcription system from rinderpest virus. Vet Microbiol 44:165–173
17. Gupta KA, Shaji D, Banerjee AK (2003) Identification of a novel tripartite complex involved in replication of vesicular stomatits virus genome RNA. J Virol 77:732–738
18. Horikami SM, Moyer SA (1995) Structure, transcription, and replication of measles virus. Curr Top Microbiol Immunol 191:35–50
19. Hsu CH, Kingsbury DW (1982) NS phosphoprotein of vesicular stomatits virus: subspecies separated by electrophoresis and isoelectric focusing. J Virol 42:342–345
20. Huntley CC, De BP, Murray NR, Fields AP, Banerjee AK (1995) Human parainfluenza virus type 3 phosphoprotein: identification of serine 333 as the major site for PKC zeta phosphorylation. Virology 211:561–567
21. Kaushik R, Shaila MS (2004) Cellular casein kinase II-mediated phosphorylation of rinderpest virus P protein is a prerequisite for its role in replication/transcription of the genome. J Gen Virol 85:687–691
22. Kingsbury DW (1974) The molecular biology of paramyxoviruses. Med Microbiol Immunol (Berl) 160:73–83
23. Kolakofsky D, Vidal S, Curran J (1991) The paramyxovirus. In: Kingsbury DW (ed) Plenum, New York, pp 215–233
24. La Ferla FM, Peluso RW (1989) The 1:1 N-NS protein complex of vesicular stomatitis virus is essential for efficient genome replication. J Virol 63:3852–3857
25. Lamb RA, Kolakofsky D (2001) Paramyxoviridae: the virus and their replication. In: Fields BN, Knipe DM, Howley PM (eds) Fields virology, Lippincott-Raven, Philadelphia, pp 1305–1339
26. Lamb RA, Mahy BW, Choppin PW (1976) The synthesis of sendai virus polypeptides in infected cells. Virology 691:16–31
27. Masters PS, Banerjee AK (1988) Complex formation with vesicular stomatitis virus phosphoprotein NS prevents binding of nucleocapsid protein N to nonspecific RNA. J Virol 62:2658–2664

28. Mazumder B, Barik S (1994) Requirement of casein kinase II-mediated phosphorylation for the transcriptional activity of human respiratory syncytial viral phosphoprotein P: transdominant negative phenotype of phosphorylation-defective P mutants. Virology 205:104–111
29. Mellon MG, Emerson SU (1978) Rebinding of transcriptase components (L and NS proteins) to the nucleocapsid template of vesicular stomatitis virus. J Virol 27:560–567
30. Mitra-Kaushik S, Nayak R, Shaila MS (2001) Identification of a cytotoxic T cell epitope on the recombinant nucleocapsid protein of rinderpest and peste des petits ruminants viruses presented as assembled nucleocapsid. Virology 279:210–220
31. Pattnaik AK, Hwang L, Li T, Englund N, Mathur M, Das T, Banerjee AK (1997) Phosphorylation within the amino-terminal acidic domain I of the phosphoprotein of vesicular stomatitis virus is required for transcription but not for replication. J Virol 71:8167–8175
32. Qanungo KR, Shaji D, Mathur M, Banerjee AK (2004) Two RNA polymerase complexes from vesicular stomatitis virus-infected cells that carry out transcription and replication of genome RNA. Proc Natl Acad Sci USA 101:5952–5957
33. Rahaman A, Srinivasan N, Shamala N, Shaila MS (2004) Phosphoprotein of the Rinderpest virus forms a tetramer through a coiled coil region important for biological function. J Biol Chem 27922:23606–23614
34. Takacs AM, Barik S, Das T, Banerjee AK (1992) Phosphorylation of specific serine residues within the acidic domain of the phosphoprotein of vesicular stomatitis virus regulates transcription in vitro. J Virol 66:5842–5848