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VIROLOGY

Virology 307 (2003) 372–385

www.elsevier.com/locate/yviro

Leader RNA binding ability of Chandipura virus P protein is regulated by its phosphorylation status: a possible role in genome transcription-replication switch

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Received 31 July 2002; returned to author for revision 13 September 2002; accepted 29 October 2002

Abstract

The molecular events associated with the transcriptive and replicative cycle of negative-stranded RNA viruses are still an enigma. We took Chandipura virus, a member of the *Rhabdoviridae* family, as our model system to demonstrate that Phosphoprotein P, besides Nucleocapsid protein N, also acts as a leader RNA-binding protein in its unphosphorylated form, whereas CKII-mediated phosphorylation totally abrogates its RNA-binding ability. However, interaction between P protein and leader RNA can be distinguished from N-mediated encapsidation of viral sequences. Furthermore, P protein bound to leader chain can successively recruit N protein on RNA while itself being replaced. We also observed that the accumulation of phosphorylation null mutant of P protein in cells results in enhanced genome RNA replication with concurrent increase in the viral yield. All these results led us to propose a model explaining viral transcription-replication switch where Phosphoprotein P acts as a modulator of genome transcription and replication by its ability to bind to the nascent leader RNA in its unphosphorylated form, promoting read-through of the transcription termination signals and initiating nucleocapsid assembly on the nascent RNA chain.

Keywords: Leader RNA; Phosphoprotein; Transcription; Replication; Chandipura virus

Introduction

All members of the recently classified virus order Mononegavirales contain a nonsegmented, negative-stranded, 11- to 19-kb-long RNA genome that is tightly wrapped in a nucleocapsid structure (Bishop, 1971). Vesicular stomatitis virus (VSV), the prototype *Rhabdovirus*, remains one of the best-studied models because of the availability of in vitro (Barik and Banerjee, 1992; Patton et al., 1984; Peluso and Moyer, 1988) and in vivo (Lawson et al., 1995; Pattnaik et al., 1992; Whelan et al., 1995) systems for assaying transcription and replication. VSV genome RNA codes for five viral genes (N, P, M, G, L) and a short leader RNA (I) complementary to the 3′ end of the genome (Banerjee, 1987). The nucleocapsid protein N protects the genome from degradation. The large protein L (~240 kDa), and the major phosphoprotein P (~29 kDa) constitute the viral RNA polymerase, where L is the catalytic subunit and P is the cofactor (Banerjee and Barik, 1992). Contransfection of the plasmids encoding these three proteins (N, L, and P) along with cDNAs to the viral genomes or minigenomes in host cells demonstrated that these three viral proteins are necessary and sufficient for transcription and replication of the virus (Pattnaik et al., 1992, 1997).

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doi:10.1016/S0042-6822(02)00093-4

The VSV life cycle has two major steps. In the first step, the viral RNA polymerase transcribes the viral genome. The viral polymerase, packaged within the virion particle, initiates transcription at the 3′ end of the RNA genome, resulting in the synthesis of a short (~50 bases) leader RNA and separate monocistronic mRNAs with progressive attenuation of transcription at each intergenic junction (Abraham and Banerjee, 1976; Iverson and Rose, 1981). The transcription of the viral genome does not require synthesis of new protein, although it largely depends on the phosphorylated form of P protein (Banerjee, 1987). The second step is the replication of the viral genome, which needs synthesis of viral proteins (Wertz and Levine, 1973). In the replication mode, the RNA polymerase transcribes the same template to produce a polycistronic, positive-strand RNA complementary to the whole genome, which serves as template for subsequent synthesis of progeny genome RNAs (Banerjee, 1987; Wertz and Levine, 1973). Despite detailed analysis of these two steps, the molecular event that leads to the transition from transcription to the replication mode is largely unknown.

One of the molecules that has been implicated in the transcription–replication switch is the viral N protein. The intracellular concentration of N protein modulates the transition of the polymerase action from transcription to replication by encapsidating the nascent leader RNA and thereby suppressing the intergenic transcription termination signals (Blumberg et al., 1981; Wertz et al., 1998). However, N protein tends to form inactive aggregates in the absence of P protein and associates nonspecifically with cellular RNA (Blumberg et al., 1983; Sprague et al., 1983). The inactive aggregated form of N can bind to viral leader RNA but is unable to encapsidate the genome RNA (Blumberg et al., 1983). The accumulation of a critical amount of the replication-competent form of N protein is believed to be controlled by the phosphoprotein P. The phosphoprotein P through its C-terminus interacts (Takacs et al., 1993). and keeps the N protein in a soluble and active form (Gupta and Banerjee, 1997; Howard and Wertz, 1989). N–P protein complexes have been observed in VSV-infected cells (La Ferla and Peluso, 1989; Masters and Banerjee, 1988; Richardson and Peluso, 1996) and it was proposed that the formation of this complex might be one of the ways to regulate two events of RNA synthesis (Abraham and Banerjee, 1976). Infected cell extract immunodepleted of N–P complex with an antibody against P protein was unable to support replication in an in vitro assay (Richardson and Peluso, 1996). In the absence of P protein, N interacts with nonspecifically with the phosphate backbone of RNA. However, in the presence of P protein N interacts with a specific sequence of the leader RNA (Keene et al., 1981; Masters and Banerjee, 1988). All these reports strongly suggest a possible auxiliary function of P protein in viral replication.

In this article we have investigated the role of P protein in transcription–replication switch using Chandipura virus (CHP) as a model system. This rhabdovirus was isolated in India and closely resembles VSV (Bhatt and Rodrigues, 1967; Gadkari and Pringle, 1980; Masters and Banerjee, 1987). Sequence at the 3′ end of the CHP genome shows remarkable homology up to first 18 bases with that of both serotypes of VSV (Giorgi et al., 1983). Previously we have shown that phosphorylation by Casein Kinase II (CKII) at single serine residue (ser62) in the N-terminus of P protein is required for its activity in transcription reconstitution assay (Chattopadhyay et al., 1997). Here we used in vitro synthesized CHP leader RNA to study its interaction with unphosphorylated (P_0) or CKII-phosphorylated (P_1) forms of P protein and also its interaction with N protein (Majumder et al., 2001). The relevance of the results obtained from the in vitro RNA-binding assays was tested in vivo by allowing the CHP virus to multiply within Vero cells, harboring a performed pool of wild-type or phosphorylation-negative mutant of P protein. As a further support we have also performed virion-mediated in vitro transcription assays under the same conditions. This article presents a demonstration of a probable regulatory role of P protein phosphorylation in modulating a transcription–replication transition.

Results

Unphosphorylated P protein binds to the leader RNA in vitro

The treatment of VSV-infected cell extract with phosphatase or its inhibitor results in an increase or inhibition of full-length RNA synthesis and subsequent secondary transcription, respectively (Chuang et al., 1997; Richardson and Peluso, 1996). Work on rabies virus major phosphoprotein N suggested that the phosphoprotein regulates virus transcription and replication via its ability to interact with the leader RNA (Yang et al., 1999). We questioned whether the CHP major phosphoprotein P interacts with the cognate leader RNA. To this end we cloned the CHP leader gene and trailer region and performed gel retardation assay to score the interaction between recombinant unphosphorylated P protein (P_0) and leader RNA (I). Fig. 1A shows the binding ability of P_0 toward leader RNA in a background of 100-fold molar excess of yeast tRNA (lane 5) where nearly the entire input leader RNA formed a slower migrating complex, whereas neither BSA nor bacterial extract lacking P protein interacted with the leader sequence (lanes 3 and 4) in the same assay condition. The complex formation was completely prevented in the presence of 100-fold molar excess of cold leader RNA showing specificity of interaction (lane 6). We have also excluded the possibility of involvement of 12-nt vector-derived sequence present in the leader transcript in the complex formation by incubating labeled nonviral control RNA having the same sequence in its 5′ terminal with or without P_0 (lanes 7 and 8). We have also carried out an antibody supershift experiment using antibody against P protein that confirms the shifted complex

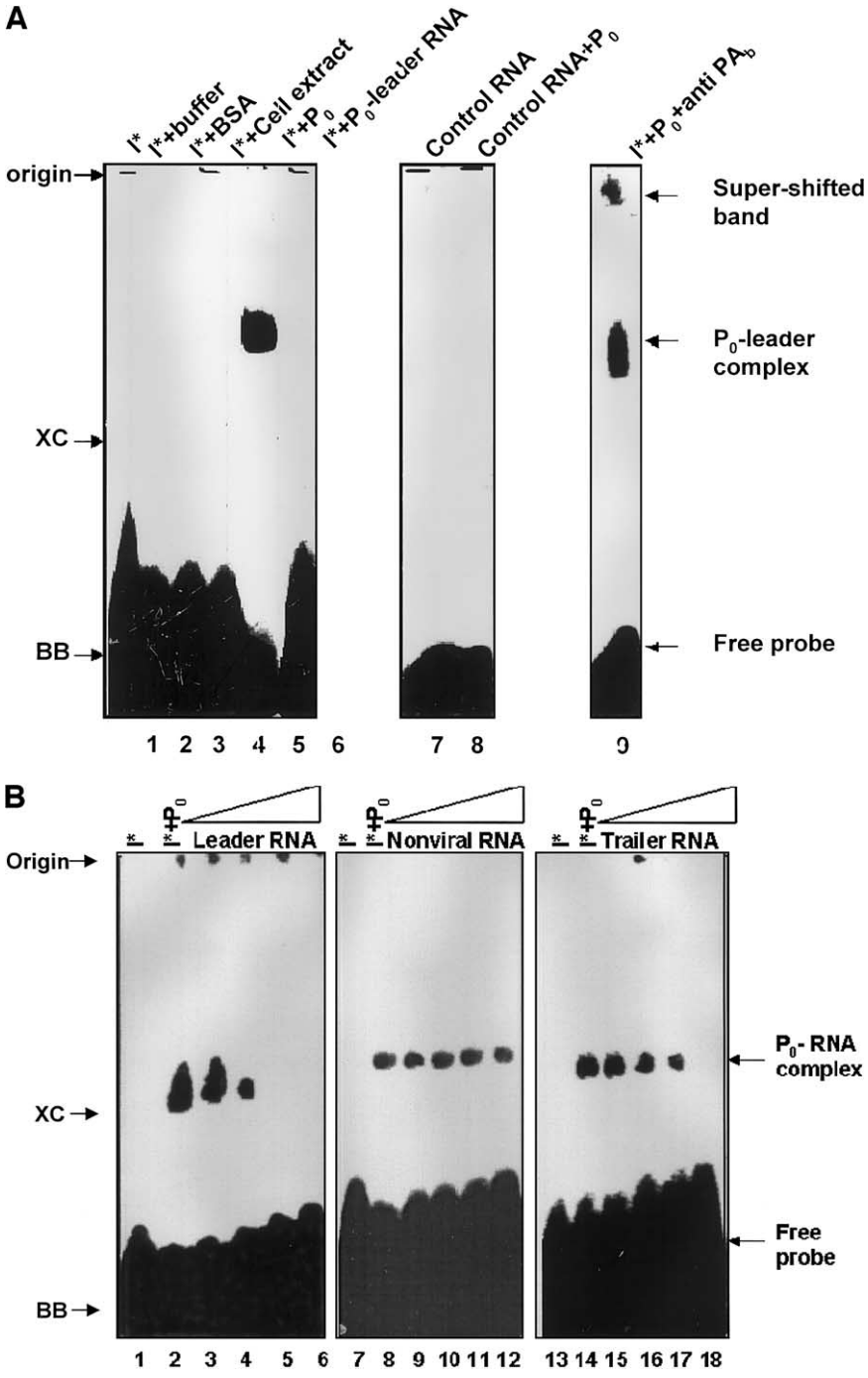

Fig. 1. Phosphoprotein P in its unphosphorylated form interacts reversibly with leader RNA. (A) The RNA binding ability of P protein in its unphosphorylated form was tested by gel retardation assay (GRA). Three nanograms of labeled leader RNA (I*) (60,000 cpm) was incubated in binding-phosphorylation buffer containing 100-fold molar excess of yeast tRNA either alone (lane 2) or in the presence of 3 μg of BSA (lane 3) or 5 μg of BL21 DE3 cell extract (lane 4) or 3 μg of bacterially expressed P protein (P_0) (lanes 5 and 6) at 37°C for 15 min. Lane 6 contains 100-fold molar excess of cold leader RNA as competitor. A pGEM-4Z vector-derived nonviral RNA used as a control (lanes 7 and 8) in the RNA binding assay. In antibody supershift experiments anti-P antibody was added to the reaction mixture at the completion of I^*P_0 complex formation with further incubation for 15 min (lane 9). Complexes were resolved onto 6% native gel. BB and XC correspond to Bromphenol blue and xylene cyanol. (B) Reversibility and specificity of RNA binding activity of P protein was tested by GRA. P_0 (350 ng) was incubated with 3 ng of I* for 15 min as described earlier to allow the complex to form. The preformed complex was then challenged with buffer (lanes 2, 8, and 14) or with equal (lanes 3, 9, and 15), twofold (lanes 4, 10, and 16), fivefold (lanes 5, 11, and 17) or 10-fold (lanes 6, 12, and 18) molar excess of cold leader RNA, nonviral RNA or trailer RNA, respectively. The reaction mixtures were further incubated for 15 min before resolving on native gel.

to be P specific (lane 9). This evidence led us to conclude that unphosphorylated P protein specifically interacts with the viral leader RNA in vitro.

Next we asked whether the P_0–leader RNA interaction is reversible in nature. To address that, preformed P_0–I complex was challenged with an increasing amount of cold leader RNA and the entire reaction mixture was incubated for another 15 min (Fig. 1B, lanes 3–6). The cold RNA competed out P_0-bound labeled leader RNA and fivefold molar excess of cold RNA resulted in total displacement (Fig. 1B, lane 5), whereas a pGEM-4Z vector-derived nonviral RNA of similar size had no effect (Fig. 1B, lanes 9–12). Our reversibility data suggest this interaction to be temporal in nature in an in vivo situation. By immunoprecipitating N–P complex from VSV-infected cell extract, Peluso and Moyer (1988) showed that though this complex is essential for the viral replication, only the N protein of this complex remains associated with the replication product. The reversible nature of P_0–leader RNA complex is consistent with this observation. Negative-stranded progeny genome RNAs are synthesized from the positive-stranded full-length template during replication. In this step RNA synthesis initiates from the complementary trailer sequence that has a very close homology (70%) with the leader RNA (Leppert et al., 1979; Nichol and Holland, 1987). So we tested the ability of trailer sequence to reverse P_0–leader RNA interaction. Our result showed that it can compete the leader RNA–P_0 binding (Fig. 1B, lanes 15–18) but less efficiently than leader RNA. This further confirms P_0–leader RNA interaction to be a sequence-specific phenomenon.

Phosphorylated P does not interact with leader RNA

VSV and CHP P proteins have been shown to require CKII-mediated phosphorylation at specific N-terminal sites for their activity in transcription in vitro (Barik and Banerjee, 1992; Chattopadhyay et al., 1997). However, phosphorylation of P protein is not essential for in vitro replication (Pattnaik et al., 1997). As P protein is present in a variety of phosphorylated forms in VSV-infected cells and N–leader RNA interaction of rabies virus varies with the phosphorylation status of N, we looked for the effect of phosphorylation of P on its ability to interact with leader RNA. Bacterially expressed P protein was phosphorylated with CKII as described earlier (Chattopadhyay et al., 1997) until all the P_0 is converted to P_1. This phosphorylated protein was used in the RNA-binding assay. Fig. 2A shows that when phosphorylated by CKII, P protein can no longer bind to the leader RNA (lane 6). A point mutant (S62A) of P protein lacking CK II phosphorylation site when tested for its RNA-binding ability showed similar affinity toward leader sequence as P_0 (lane 2). The results indicate that the function of P associated with its leader RNA-binding ability might be separable from its role in transcription.

Considering the regulatory role of CKII-mediated phosphorylation of P protein on its RNA-binding ability, we

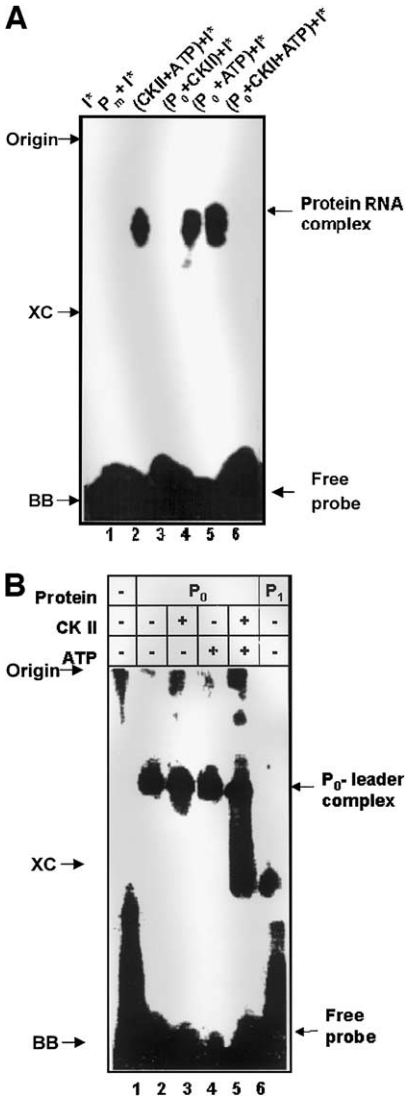

Fig. 2. Phosphorylated P does not interact with leader RNA. (A) Three micrograms of bacterially expressed P protein was either incubated with CK II (Fig. 2A, lane 4) or with ATP (lane 5) or with both CKII and ATP (lane 6) for 1 h in binding-phosphorylation buffer in 15 μl reaction volume. For lane 3, CKII and ATP were incubated together for the same period of time omitting P protein. Three nanograms of I* (60,000 cpm) was added to each set followed by further incubation for 15 min at 37°C. In lane 2, 3 μg of bacterially expressed P_m was incubated with I* for 15 min. Reaction mixtures were resolved onto 6% native gel. (B) To show phosphorylation as the key event modulating RNA-binding ability of the P protein, we have incubated 3 μg of P_0 with 3 ng of I* (60,000 cpm) to allow the RNA–protein complex to form. The complex was then further incubated for another 15 min either alone (lane 2) or with CKII (lane 3) or with ATP (lane 4) or with both CKII and ATP (lane 5). For lane 6, P protein prephosphorylated by incubating with CKII and ATP for 15 min was incubated with I* for another 15 min. Finally the reaction mixture was resolved into 6% native gel.

asked whether P_0 bound to leader RNA can be utilized by CKII as its substrate. To address this possibility we allowed P_0–I (both being unlabeled) complex to form, keeping reaction components in equal molar ratio that assures most of the protein to be present in an RNA-bound state. We have

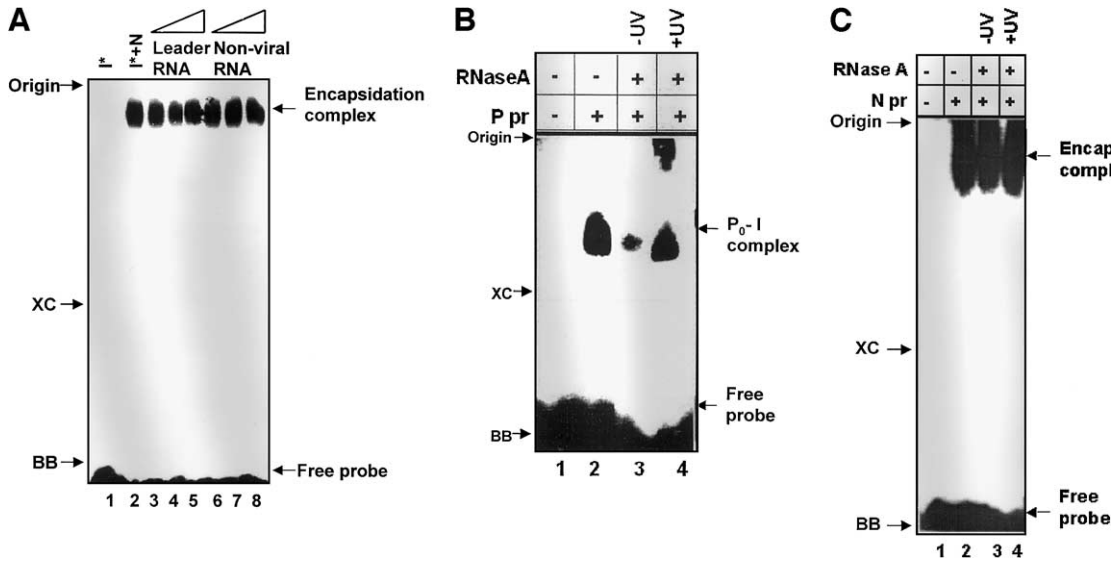

Fig. 3. P_0–leader RNA interaction is different from encapsidation of RNA by N protein. (A) N encapsidates leader RNA in an irreversible manner. Three micrograms of N protein was incubated with I* (60,000 cpm) and encapsidation complex thus formed was challenged with either buffer alone (lane 2) or with equal (lanes 3 and 6), 5-fold (lane 4, 7), 10-fold (lane 5, 8) molar excess of cold leader RNA or nonviral RNA, respectively. The reaction mixture was incubated for another 15 min before resolving onto 4% native gel. (B) For RNase protection assay 3 μg of P_0 was incubated with 6 ng of labeled leader RNA (120,000 cpm) for 15 min as described allowing complex formation. The complex was then either directly loaded on gel (Fig. 4A, lane 2) or treated with RNase A (lane 3) or UV-crosslinked and treated with RNase A (lane 4) before loading onto 6% native gel. (C) The above RNase protection experiment has been repeated with 3 μg of N protein in place of P_0, keeping all the parameters constant except that the reaction mixtures were resolved into 4% native gel.

incubated this preformed complex with CKII and radiolabeled ATP and the reaction mixture was resolved in SDS–PAGE followed by autoradiography. Our observation suggests that P_0 bound to leader RNA could be phosphorylated by CKII as efficiently as P_0 alone (data not shown). To find out the effect of phosphorylation of P_0 on bound leader RNA, we incubated labeled leader RNA–P_0 complex with CKII and ATP and resolved the reaction mixture on native gel. We found phosphorylation by CKII not only prevents P from binding to the leader RNA but also releases leader RNA from the complex. When preformed P_0–I complex is phosphorylated, the protein–RNA interaction becomes weak and the complex gradually breaks down to form a faster migrating smear in 6% native gel (Fig. 2B, lane 5) (Nagai and Mattaj, 1994). This observation indicates that reversible binding of P with leader RNA can also be achieved by CKII-mediated phosphorylation of P. However, incubation with CKII or ATP alone (lanes 3 and 4) does not disrupt the RNA–P_0 complex, emphasizing phosphorylation as the key event modulating RNA-binding property of P.

P_0–leader RNA interaction is different from encapsidation of RNA by N protein

A major function of rhabdoviral nucleocapsid protein is to encapsidate viral genome into a tight nuclease-resistant structure (Dillon and Gupta, 1988). Unlike phosphoprotein–leader RNA interaction that is reversible in nature, we observed N-mediated encapsidation of genome termini to be irreversible in practical consideration. Instead of 300 ng of P_0 that has been used to test reversibility (Fig. 1B), 3 μg of N protein was used in the binding reaction to mimic viral system where N is present in a stoichiometrically higher amount than P. Bacterially expressed soluble N protein when incubated with labeled leader RNA forms an encapsidation complex (Fig. 3A, lane 2). This leader RNA–N protein complex, when challenged with either an increasing gradient of cold leader RNA (Fig. 3A, lanes 3–5) or an unlabeled nonviral RNA (lanes 6–8), did not release bound leader probe. Although N protein of negative-stranded RNA viruses renders the genome RNA resistant to nucleases, it does not protect the bases from methylation (Keene et al., 1981). To define the nature of P_0–leader RNA complex, we incubated this complex with RNase A and analyzed on 6% non-denaturing gel. Fig. 3B demonstrates that P_0 could not protect the leader RNA from nuclease action (lane 3), but when the same complex was treated with UV to cross-link the protein with the bases of RNA, the complex becomes nuclease resistant (lane 4). Under the same experimental condition when N protein is incubated with leader RNA it forms a nuclease-resistant complex irrespective of UV cross-linking (Fig. 3C, lanes 3 and 4). This suggested that in contrast to the N protein, P protein presumably interacts with the ribonucleoside bases of the leader RNA, keeping the phosphate backbone available for nuclease action. It may also be due to the reversible nature of leader RNA–P_0 complex whereby the released RNA is being degraded by RNase A. Nonetheless, this temporal nuclease sensitive interaction of P_0 with leader sequence can be distinguished from encapsidation mediated by N protein.

N protein can encapsidate P_0 bound leader RNA

One of the prevailing models suggests that transcription–replication switch in *Vesiculovirus* is regulated by intracellular concentration of N protein (Blumberg et al., 1981). It was postulated that encapsidation of nascent RNA chain by de novo synthesized N protein not only protects progeny genome molecules from cellular RNases but also provides an antitermination signal required to read through the intergenic sequences (Blumberg et al., 1981; Leppert and Kolakofsky, 1980; Patton et al., 1984). Nucleation site for this N-mediated encapsidation process is believed to reside on first 14 bases of leader chain (Blumberg et al., 1983), whereas we demonstrated P_0 also interacts with the positive-sense leader RNA. To verify whether P_0 binding interferes with the subsequent encapsidation process, we looked at the ability of N protein to encapsidate leader RNA that is already present in a P_0 bound form. To test this idea, preformed P_0–RNA complex was incubated with an increasing amount of recombinant N protein. Fig. 4A shows while N alone can encapsidate leader RNA giving rise to a slower migrating encapsidation complex (lanes 7–10), it is equally efficient to enwrap leader RNA bound to P_0 with a gradual disappearance of P_0–I complex upon increase in N concentration (lanes 3–6). The efficiency of P_0–leader RNA complex encapsidation was comparable to the N-mediated encapsidation of naked leader RNA. Preformed P_0–N complex was found to be equally efficient in encapsidating the leader RNA (data not shown). UV-induced covalent crosslinking between P_0 and bound leader RNA eliminates the temporal nature of their interaction. We observed this UV-crosslinked P_0–I complex when incubated N protein could not be utilized for encapsidation as there was no reduction in the intensity of the shifted band corresponding to I–P_0 complex with an increase in nucleocapsid protein concentration (Fig. 4B, lanes 2–5). Moreover, Western blot analysis of the encapsidation complexes formed at the expense of leader–P_0 complex (Fig. 4A, lanes 3–6) with anti-P antibody could not demonstrate the presence of P protein (data not shown). These results indicate that although P_0 and N protein share a common target for binding, namely leader sequence, prior binding of P_0 to leader chain does not interfere with the subsequent encapsidation process and P protein is displaced from the leader chain during recruitment of N on RNA, exploiting the temporal nature of leader RNA and P_0 interaction in this process.

Overexpression of unphosphorylated P enhances replication

Higher affinity of P_0 to the leader RNA as compared to that of phosphorylated protein and the ability of N protein to encapsidate P_0 bound leader RNA indicate that phosphorylation of P with CKII may have a regulatory role in controlling transcription–replication transition. In this proposal binding of P_0 to nascent leader chain brings about the replicative phase where the polymerase read-through gene boundaries, eventually synthesizing a complete (+)-sense genome. These (+)-sense replication intermediates act as a

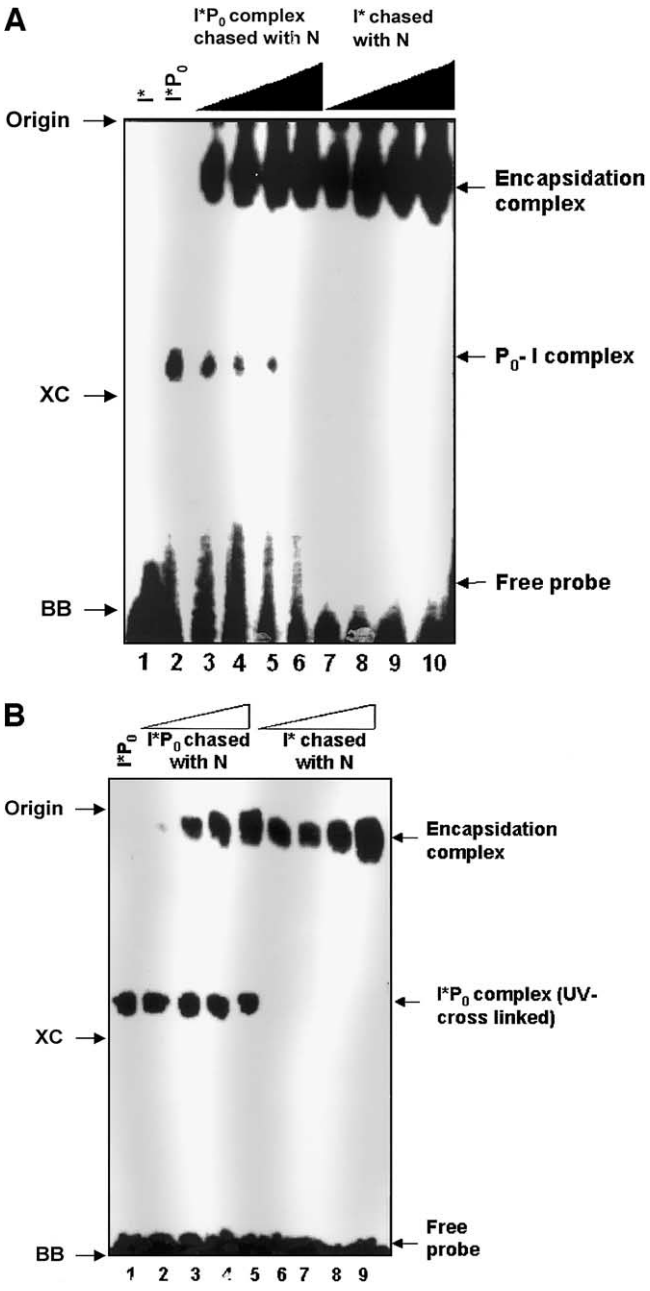

Fig. 4. N protein can encapsidate leader RNA bound to P protein. (A) To show the potentiality of RNA–P_0 complex to act as substrate of N protein mediated encapsidation 3 μg of P_0 was incubated with I* for 15 min allowing the complex formation. This preformed complex was then either directly loaded onto 4% native gel (lane 2) or incubated with 1 μg (lane 3), 2 μg (lane 4), 3 μg (lane 5), or 4 μg (lane 6) of N protein for another 15 min. For lanes 7–10 identical amounts of N protein as in lanes 3–6 were incubated with I* for 30 min. The reaction mixture was finally resolved onto 4% native gel. (B) To find out the role of P_0 bound to leader chain in recruiting N protein for encapsidation, we incubated I* (60,000 cpm) and 3 μg of P_0 together, allowing complex formation and then UV cross-linked P_0–I complex. This cross-linked species (lanes 2–5) or simply UV-treated RNA (lanes 6–9) was incubated with 1, 2, 3, or 4 μg of N protein for another 15 min before finally resolving onto native gel.

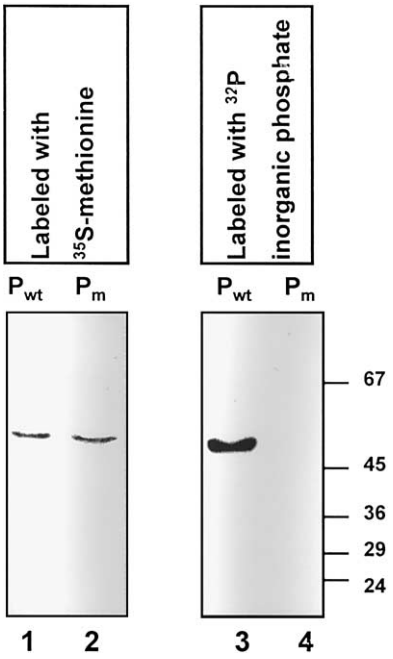

Fig. 5. Expression profile and phosphorylation status of wild-type and mutant P protein in transfected VERO cells. To test the expression of P_{wt} and P_m proteins and their phosphorylation status, transfected Vero cells were metabolically labeled with [^{35}S]methionine (lanes 1 and 2) or with ^{32}P-inorganic phosphate (lanes 3 and 4). P protein was immunoprecipitated from cell extract with anti-P antibody and subjected to 10% SDS–PAGE followed by autoradiography. Protein molecular mass markers are noted at the right in kilodaltons.

template for further round of replication producing (−)-sense progeny genomes that upon packaging bud out as mature virion particles. We wanted to test this hypothesis by comparing the virus yield, levels of transcription, and replication in infected cells overexpressing either the wild-type P (P_{wt}) or P protein lacking the CKII phosphorylation site (P_m). The expression of P_{wt} and P_m proteins in the transfected Vero cells was confirmed by [^{35}S]methionine labeling (Fig. 5, lanes 1 and 2). To make sure that P_m is indeed not phosphorylated, cells expressing P_m were labeled with ^{32}P and immunoprecipitated with anti-P antibodies. Unlike the wild-type P, there was no ^{32}P-labeling of the mutant P (Fig. 5, lanes 3 and 4). To find out the effect of unphosphorylated P protein in virus life cycle, Vero cells were transfected with plasmid bearing P_{wt} or P_m gene, followed by infection with CHP virus as described under Materials and methods. The virus titer was measured 24 h postinfection. Transient expression of P_m enhances the viral yield by *almost* 100-fold as compared to the cells expressing wild-type P (Fig. 6).

When the relative level of viral transcription was measured by analyzing the amount of N-transcript present in the infected cells using RT-PCR assay, it was found to be higher in cells overexpressing wt P protein compared to the cells overexpressing mutant P (Fig. 7, lanes 4 and 5). This observation validates our earlier in vitro result demonstrating indispensability of CKII-mediated phosphorylation of P protein in viral transcription (Chattopadhyay et al., 1997). In contrast when we compared the RT-PCR products representing both transcription and replication, we surprisingly noticed that cells expressing P_m had more products compared to that with P_{wt} (Fig. 7, lanes 9 and 10). As P_m does not support viral transcription (Chattopadhyay et al., 1997), this increase in the amount of RT-PCR products can only be due to enhanced replication. This result suggests that the phosphorylation-defective mutant of P boosts up viral replication, providing support to our hypothesis.

To further substantiate the observation that phosphorylation-defective P protein favors viral replication over transcription, we did Northern analysis of the total RNA at different time points after infection and probed for a viral-replication product. We repeatedly found that within 4 h of infection a considerable amount of replication products accumulate in cells expressing P_m compared to that expressing that P_{wt} (Fig. 8A, lanes 2 and 3). The trend continued in the later time points with more replication products in the P_m population as compared to the P_{wt}-expressing cells (Fig. 8A, lanes 5 and 6 and lanes 8 and 9, respectively, and Fig. 8B). So, comparing the transcription and the transcription–replication products together, we infer that the cell population expressing P_m supports more replication than the wt P.

Leader-sized RNA synthesis by lysed virus is decreased in presence of P_m

To mimic our in vivo results, we carried out in vitro transcription assay with detergent-disrupted CHP virus (Colonno and Banerjee, 1978) in the presence of exogenously added P_m or P_1. Lysed virus retains all the requisite machinery to initiate RNA synthesis starting from the 3′ end of the genome and produces discrete ~50-nt-long leader-sized RNA transcript (lane 1, Fig. 9A and B). We observed that with increasing concentrations of P_m (S62A) there was a gradual decrease in the discrete leader-like RNA synthesis (Fig. 9A, lanes 2–5). This inhibitory effect of P_m provides in vitro support to the transdominant negative effect of P protein in its unphosphorylated form on viral transcription (Das et al., 1995; Mazumder and Barik, 1994). However, P_1 showed no such effect on viral RNA synthesis in this assay (Fig. 9A, lane 6; Fig. 9B, lane 3). Interestingly, we found that P_m alone when used in excess (~3 μg/rection) can result in synthesis of heterogeneous population of higher molecular weight RNA of 80–200 nt (Fig. 9B, lane 2) at the expense of discrete leader-sized RNA. These results suggest that under the assay conditions employed, an excess amount of P_m inhibits the synthesis of leader-sized RNA and increases the synthesis of larger products which may correspond to readthrough at the leader-N junction. During replicative phase antitermination at the gene boundaries eventually leads to synthesis of full-length genome molecules. Thus our in vitro data were found to be in accordance with the in vivo results showing more replication with P_m.

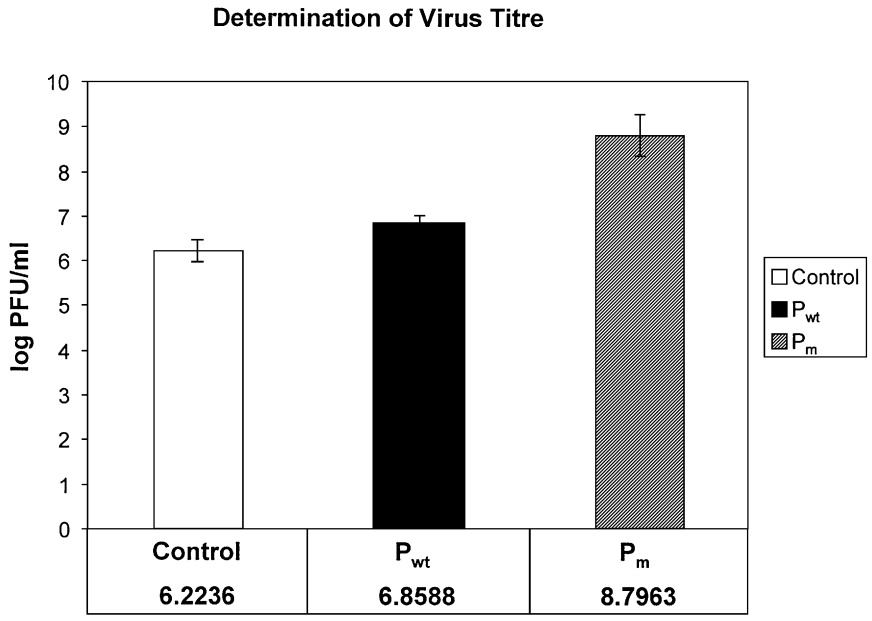

Fig. 6. Effect of P_{wt} or P_m protein on virus yield. To understand the effect of P protein phosphorylation on viral yield, Vero cells were transfected with either vector alone or with recombinant clones expressing P_{wt} and P_m proteins. Successively, transfected cells were infected with Chandipura virus and 24 h postinfection virus titer was measured for different sets by plaque assay.

Discussion

The mechanism underlying the regulation of transcription and replication of negative-stranded RNA viruses has been elusive for many years (Ball and Wertz, 1981). This differential regulation is believed to be due to the binding of nascent RNA by the nucleocapsid protein which may act as an attenuator of the mRNA processing steps during transcription, thus allowing the readthrough of the intragenic regions with concomitant synthesis and encapsidation of the progeny genome RNA (Banerjee and Barik, 1992). Ongoing synthesis of N protein is required not only for initiation but also for continuation of genome replication (Banerjee, 1987). Being the major nucleocapsid protein to provide protection to genome RNA against nuclease action as well as the most abundant protein of the virus, it is not surprising that replication of the virus will be dependent on its synthesis. Moyer showed that in the Sendai virus system purified NP protein is necessary and sufficient for RNA synthesis and encapsidation from preinitiated templates that is for

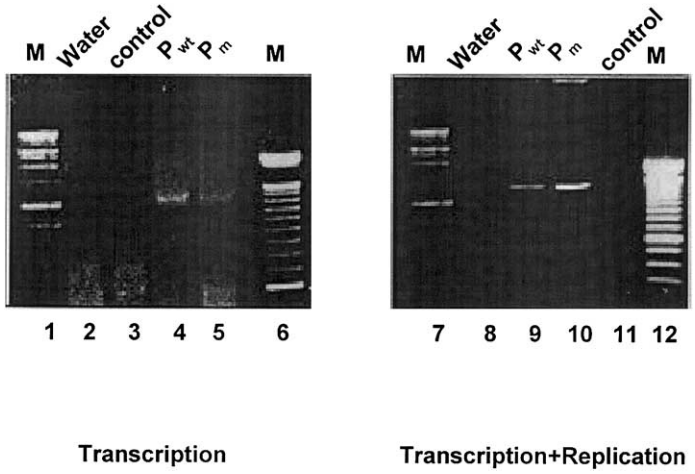

Fig. 7. RT-PCR assay. Total RNA was isolated from cells either not transfected and not infected (control, lane 3, and lane 11) or from cells transfected with construct expressing P_{wt} or P_m protein and infected with CHP Virus. RT-PCR was done in two sets as described under Materials and methods. RT-PCR products reflecting either relative level of viral N gene-specific transcript (lane 4 and lane 5) or level of both viral N gene specific transcripts and viral genome RNA (lanes 9 and 10) was analyzed in 1% agarose gel. Lanes 2 and 8 correspond to water control used in PCR. Used as DNA molecular weight markers were 1 Kb DNA ladder (lanes 1 and 7) or 100 bp DNA ladder (lanes 6 and 12).

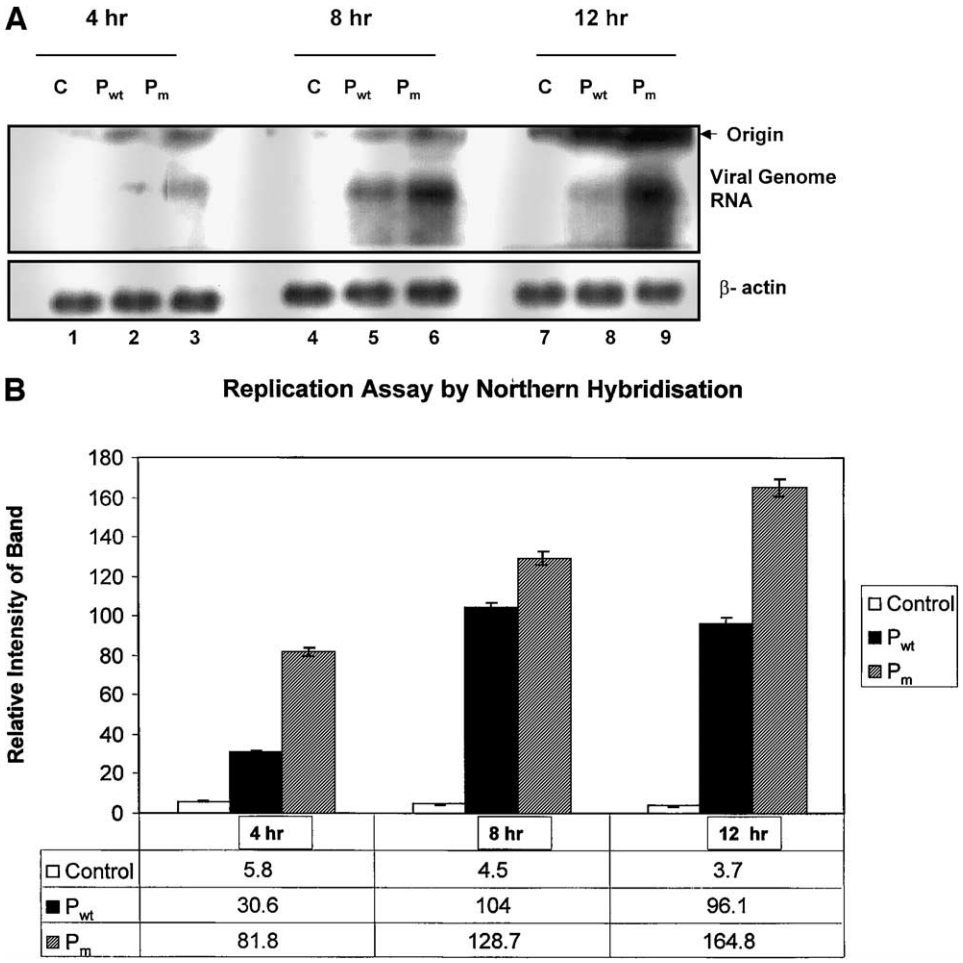

Fig. 8. Northern blot analysis. (A) Total RNA was isolated at three different time points (4, 8, and 12 h postinfection) from cells either not transfected and infected (control, lane 1, lane 4, and lane 7) or from cells transfected with construct expressing P_{wt} (lane 2, lane 5, and lane 8) or P_m (lane 3, lane 6, and lane 9) and infected with CHP virus and probed for viral genome RNA and β-actin mRNA. (B) Autoradiogram presented in (A) was scanned; band intensities were quantitated using Matlab software version 5.1.0.421. Relative intensity of band was plotted as a percentage increase in band intensity corresponding to viral genome RNA to that of β-actin for each lane, reflecting relative level of replication in different sets. Data presented here are an average over three independent experimental sets (one shown in A and two additional sets).

elongation reaction but alone cannot initiate RNA replication and encapsidation. Another viral component, in addition to the NP protein, appears to be required for the initiation of encapsidation, since the soluble protein fraction of the infected but not uninfected cells did support DI-H genome replication (Baker and Moyer, 1988). A number of biochemical studies reveal that when synthesized alone N protein tends to form inactive aggregates and the P protein makes it soluble as well as specific toward viral RNA sequences (Masters and Banerjee, 1988).

In this article, we have presented a correlative study showing that the unphosphorylated form of P protein has specificity of binding leader RNA which could be abolished by CKII-mediated phosphorylation. We have also demonstrated that phosphorylation null mutant P_m favors the synthesis of full-length RNA and finally proposed a modification to the existing hypothesis explaining transcription–replication switch.

Initial indication about the involvement of distinct forms of P protein in replication and transcription was provided with the isolation of a temperature-sensitive mutant having a lesion in the P gene that cannot replicate but transcribes at a nonpermissive temperature (Unger and Reichmann, 1973). A monoclonal antibody, raised against a form of P protein that is not phosphorylated at some sites, is capable of inhibiting replication but not transcription (Richardson and Peluso, 1996). Using phosphatase inhibitor, Chuang et al. (1997) suggested hyperphosphorylation of P prevents replication. The possibility of P as a leader RNA-binding protein was provided by Keene et al. (1981). P protein could act as a cofactor necessary for the initiation of encapsidation, shown in the Sendai virus system (Baker and Moyer, 1988). It was also demonstrated that the protein requirement for initiation and elongation is different. There are several possible explanations for the difference in the response observed. The P protein may play a role in stabilizing and activating the nucleocapsid protein for the initial binding to nascent RNA.

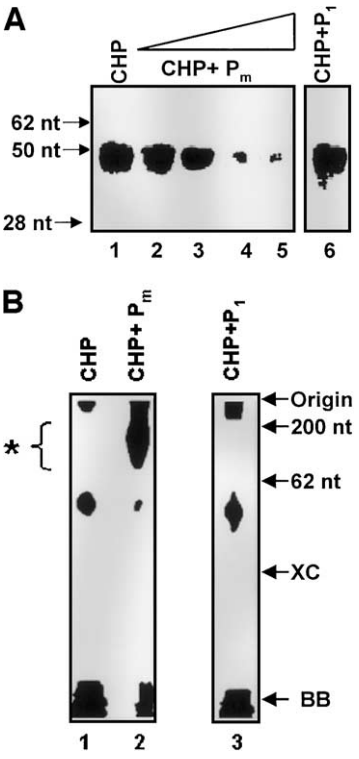

Fig. 9. In vitro transcription assay with detergent disrupted virus. (A and B) To test the effect of exogenously added P_m on viral transcription, we incubated detergent-disrupted virion in transcription buffer either alone (lane 1, A and B) or in the presence of 0.25, 0.5, 1, 1.5 (lanes 2, 3, 4, and 5, respectively, A), or 3 μg of P_m (lane 2, B) as described under Materials and methods. As an internal control 1.5 or 3 μg of P_1 was included in the assay mixture (lane 6, A, and lane 3, B, respectively). Reaction mixture was resolved on 20% polyacrylamide gel containing 7 M urea. *Transcription products of 80–200 nt in length (B). RNA molecular mass markers (Promega) are noted beside the autoradiograms.

We took the advantage of using recombinant P free from other viral proteins and purified leader RNA synthesized by in vitro transcription to demonstrate the binding of unphosphorylated P protein to leader RNA. P proteins of VSV as well as CHP have been shown to form multimers when phosphorylated by CKII (Chattopadhyay et al., 1997; Gao and Lenard, 1995). Total attenuation of leader RNA-binding ability after phosphorylation suggests that either increase in the net negative charge prevents the interaction with acidic leader RNA or phosphorylation-mediated conformational alteration (Raha et al., 1999) and/or multimerization (Richardson and Peluso, 1996) may mask the RNA-binding domain. Recently, phosphorylation of the major phosphoprotein N of rabies virus has been shown to modulate its leader RNA encapsidation (Yang et al., 1999). Cuesta et al. (2000) reported that the structural phosphoprotein M2-1 of hRSV is a leader RNA-binding protein and this ability is regulated by phosphorylation as has been observed in the case of CHP P protein in the present work.

P_0–leader RNA interaction that is reversible moderately fast in manner can also be reverted by CKII-mediated phosphorylation. Temporal nature of P_0–leader RNA interaction advocates a possible regulatory role in the transition point of viral life cycle. Although N protein successfully encapsidates the P_0–I complex, P_0 was found to be absent in the final nucleocapsid assembly. A prerequisite of reversible character of P_0–leader RNA interaction in this encapsidation process and the ability of P_0–N complex to bind leader sequence suggest that P_0 bound to leader RNA may assist in the recruitment of N on nascent chain while itself being replaced. Interestingly, one of the established functions of P protein is to provide specificity to the RNA-binding ability of N protein where CKII phosphorylation of P protein was found to be dispensable (Masters and Banerjee, 1988).

P proteins of two extensively studied serotypes of VSV (NJ and IND) contain multiple domain I phosphorylation sites, whereas P protein of CHP is unique in this regard, having a single phosphorylation site in domain I. This single-site mutated protein P_m binds to the leader RNA similar to its unphosphorylated wild-type counterpart. To test the relevance of the RNA-binding ability of P protein in the viral context, we used wild-type virus instead of DI particle to infect cells expressing P_{wt} or P_m Interestingly it was found that the P_m does not facilitate transcription in vivo, supporting our earlier in vitro observation (Chattopadhyay et al., 1997). It has been reported that though phosphorylation within N terminal domain of P protein by CKII is required for transcription, it can be compromised for replication. P protein without CKII-mediated phosphorylation can form trimeric complexes with L and N protein, which can support replication in vitro (Gupta and Banerjee, 1997; Pattnaik et al., 1997). In line with these reports we found an increase in replication product with the expression of P_m as compared to P_{wt}. To explain more replication with P_m. We propose a modified model for transcription–replication switch where binding of P_0 to the nascent leader RNA initiates the replicative mode either by signaling the polymerase complex to readthrough the gene junctions or by promoting N-mediated encapsidation leading to antitermination. A gradual decrease in the leader RNA synthesis in the presence of P_m in virion-mediated in vitro transcription assay along with the production of some readthrough transcripts suggest that P_0 bound to nascent leader chain alone may bring about some unspecified changes in the polymerase complex, resulting in a change in its function from transcription to replication, although failure to obtain full-length replication product in absence of N protein in this in vitro assay underscores the requirement of a pool of de novo synthesized N protein in viral replication. In our in vivo experiment this requirement was satisfied by N protein synthesized from viral genome. All these results together suggest that P_0 binding to the leader RNA sequence to be the rate-limiting step in the transcription–replication switch, though synthesis of full-length genome remains dependent on continuous production and recruitment of N on nascent chain either independently or with the help of leader-bound P_0.

In a natural situation a mutant P does not exist and one

Table 1
Sequence of primers used in this study

Name of primers	Sequence
3′l chp1	agagaattcacgaagacaaaaaaaccat*ttaaacgattatat*
3′l chp2	agaaagcttgtgttcgtgtactacta*tataatcgtttaa*
5′t chp1	tttgaattcatgttg*tgtacaatttaatcgtataactggttttgttt*
5′t chp2	ataaagcttacgaaga*aaacaaaaccagttatacgattaaattgtaca*
DJC1	tttatacatatgagttctcaagta
DJC2	tttataggatcctcatgcaaagag

may question the existence of any unphosphorylated form of P in the infected cell. P_0 may be formed as a short-lived intermediate (Wertz, 1983) by the action of any phosphatase in the infected cell or a subset of newly synthesized P can remain in unphosphorylated state in equilibrium with a phosphorylated counterpart in the infected cell. This modified model explains some of the early observations by other groups and is consistent with similar findings in related viruses. Chuang et al. (1997) have proposed that switch from transcription to replication of the genome is coupled to assembly on nascent chains and involves a functional change in the P–L polymerase complex. The transcription-defective P mutants can support efficient replication of DI RNA in vivo (Das et al., 1997). Pattnaik et al. (1997) have proposed that replication complex and transcription complexes are different and distinct in nature and CKII-mediated phosphorylation of P favors the formation of transcription complex, whereas only LAK-phosphorylated P favors the formation of replication complex. Though Pattnaik et al.'s model cannot explain why the polymerase composed of L, P_0, and N replicates better than it transcribes, our result could provide an explanation for this observation. Use of mutants disrupting different functions of P protein such as RNA binding, oligomerization, and replication will provide further insight about the roles of phosphorylated and unphosphorylated P protein in transcription and replication.

Materials and methods

Cell culture and virus

CHP virus (Strain 1653514) was purified from baby hamster kidney (BHK-21) cells (Masters and Banerjee, 1987). All protein quantitations were made with Bio-Rad DC protein assay reagent. Oligonucleotides used in this study were purchased from Operon Technologies, USA.

Cloning of leader gene and 5′trailer region of CHP virus

To clone 49-nt-long leader RNA gene (I) of CHP virus, two complementary primers 3′lchp1 and 3′lchp2 (Table 1) encompassing the entire leader sequence was designed. *Eco*R1 or *Hin*dIII restriction site was incorporated in each primer. PCR was done with these two primers and amplification product was restriction digested and cloned into pGEM-4Z vector (Promega). A 46-nt-long 5′-end trailer region of the genome (t) was amplified by PCR using a pair of overlapping primers 5′tchp1 and 5′tchp2 (Table 1) and cloned similarly into pGEM-4Z vector. Sequences of both recombinant clones were found to be identical with published sequences of Chandipura genome (Nichol and Holland, 1987).

Expression of P_{wt} and P_m protein in Escherichia coli

P protein or P_m (S62A) of CHP virus was overexpressed in *E. coli* (BL21DE3) cells from pET-3a PC or pET-3a PCM plasmids, respectively, as described earlier (Chattopadhyay et al., 1997), except that cells were induced with 500 μM IPTG at 20°C for 8 h to maximize production of the proteins in soluble form. Recombinant P protein was purified from bacterial lysate by Q-Sepharose (Pharmacia Biotech) anion-exchange column (Raha et al., 1999). Fractions containing the purified proteins were pooled together and concentrated through Centricon (Amicon).

In vitro phosphorylation of P protein

Recombinant P protein (3 μg) was phosphorylated in vitro with 1 unit of CKII purified from rabbit reticulocyte (Hathaway and Traugh, 1983) in binding-phosphorylation buffer (10 mM Tris, 40 mM KCl, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 100 μM ATP) at 37°C for 1 h (Chattopadhyay et al., 1997). Phosphorylated form of P protein was used either directly in binding reaction or purified by gel filtration chromatography (Pharmacia Biotech) from the reaction mixture. To phosphorylate P bound to leader RNA, 3 μg of P_0 and 3 ng of radiolabeled leader RNA (I*) was incubated together in binding phosphorylation buffer at 37°C for 15 min and then the complex was further incubated with 1 unit of CKII and 100 μM ATP at 37°C for 15 min. Resultant reaction mixture was directly loaded onto 6% native polyacrylamide gel as described below.

Expression of N gene in E. coli

N protein was expressed from pET-3a NC clones in *E. coli* (BL21DE3) cells in a soluble monomer form and pu-

rified from bacterial lysate by anion exchange chromatography using Mono Q column (Pharmacia Biotech) as described (Majumder et al., 2001).

Synthesis of different RNA probes

Positive-sense leader RNA (I) and genome-sense trailer RNA (t) was synthesized in vitro from either 3′leader-Chp/pGEM-4Z linearized with *Hin*dIII or 5′trailer-Chp/pGEM-4Z linearized with *Eco*R1 using SP6 RNA polymerase or T7 RNA polymerase (BM), respectively, according to the manufacturer's protocol. Leader RNA synthesized in in vitro transcription reaction has 12-nt vector-derived fusion at the 5′ end of the 49-nt-long leader RNA sequence, whereas t-RNA bears a 13-nt vector-derived sequence at the 5′ end of 46-nt-long viral sequence. Sixty-five-nucleotide-long nonviral RNA used as control in this study, bearing same 12-nt sequence at 5′ termini as present in leader transcript, was synthesized by transcribing *Hin*dIII-digested pGEM-4Z vector with SP6 RNA polymerase. To obtain radiolabeled RNA [α^{32}P]UTP (BRIT) was included in the transcription reaction. In vitro synthesized RNA was eluted from the gel, precipitated twice with ethanol, and suspended in RNase-free water. Radioactivity was measured in liquid scintillation counter.

Gel retardation assay (GRA)

Leader RNA probe was incubated with recombinant P (unphosphorylated or phosphorylated in vitro with CKII), P_m (S62A), or N protein in binding buffer (10 mM Tris, 40 mM KCl, 1 mM DTT, 10 mM $MgCl_2$, and 100 mM NaCl) for 15 min at 37°C in a total volume of 15 μl. Native gel-loading dye was added to the reaction mixture (final 3% Ficoll, 0.1 mM EDTA, 0.025% Bromphenol blue, and 0.025% xylene cyanol) and finally the complex was resolved on a 6 or 4% native polyacrylamide gel containing 5% glycerol, run at 4°C in 1× TAE. The gel was dried and exposed to X-ray film. To test reversibility in the supershift experiment different amounts of unlabeled RNA as indicated in figure legends or 1 μg of anti-P antibody was added to the reaction mixture at completion of RNA binding. Samples were incubated for another 15 min before resolving onto gel. To test the specificity-labeled pGEM-4Z-derived nonviral control RNA was incubated in binding buffer either alone or in the presence of P_0 and loaded onto the gel. To show N-mediated encapsidation of P_0–I* complex, performed complexes either directly or after UV cross-linking were titrated with different amounts of N protein. The entire reaction mixture was incubated for another 15 min before resolving onto 4% native gel.

RNase protection assay

Recombinant P or N protein was incubated with I* (120,000 cpm). The RNA–protein complex was UV cross-linked (Majumder et al., 2001) and treated with RNAse A at a concentration of 60 μg/ml for 15 min at 37°C. Native gel loading dye was added to the reaction mixture before loading into 6 or 4% native polyacrylamide gel. Gel was dried and exposed to X-ray film.

Expression of P_{wt} and P_m (S62A) protein of CHP virus in vero cells

Full-length P_{wt} and P_m (S62A) genes of CHP virus were subcloned in eukaryotic expression vector pCMXPL2 under control of CMV promoter. Vero cells grown in minimal essential medium supplemented with 5% fetal calf serum in 35-mm six-well plates were transfected at their 90% confluency with 1 μg of pCMXPL2, pCMXPL2-P_{wt}, or pCMXPL2-P_m (S62A) using lipofectamine (GIBCO-BRL). Medium from transfected cells was removed at 24 h posttransfection and subsequently cells were treated for immunofluorescence assay accordingly using anti-CHP antibody to follow the expression of viral proteins. The maximum level of expression was found to occur after 12–14 h posttransfection in all cases. For metabolic labeling 20 h posttransfection medium was aspirated out and methionine-free MEM or phosphate-free MEM supplemented with ^{35}S-labeled methionine or ^{32}P-labeled inorganic phosphate, respectively, was added. After 4 h of incubation, cells were collected and lysed in RIPA buffer followed by immunoprecipitation of P protein from cell extract with anti-P antibody. Immunopellet was resolved in 10% SDS–PAGE followed by autoradiography.

Transfection-infection

As expression of foreign protein reaches a peak at 12 h posttransfection for transfection-infection assay, we have selected this time point for infecting transfected cells with CHP virus. For transfection-infection experiments Vero cells were grown and transfected as discussed earlier and 12 h posttransfection cells were infected with CHP virus at a m.o.i. of 1. Cells were frozen and thawed 24 h postinfection to release virus particles and virus yield was measured by plaque assay.

RT-PCR assay

Twelve hours postinfection Vero cells were washed twice with PBS and trypsinized, and total RNA was isolated using Tripure Reagent (GIBCO-BRL). Reverse transcription was done simultaneously with oligo$(dT)_{18}$ and N gene-specific upstream primer in a single tube using Superscript II (GIBCO-BRL) reverse transcriptase maintaining 0.5 μg of total cellular RNA/reaction.

For semiquantitative analysis preliminary experiments were done to determine the optimum PCR cycle number within the linear range of amplification for each gene being measured. RT product was subjected to PCR reaction with

different primer pairs corresponding to the following: (i) N gene-specific upstream primer (Table 1) and oligo$(dT)_{18}$ to detect N gene-specific transcript and p53 (Intron 6)-specific upstream and downstream primers (Masters and Banerjee, 1987). (ii) N gene-specific upstream and downstream primers (Table 1) (Majumder et al., 2001) to identify N mRNA and replication product simultaneously and upstream and downstream primers for p53 gene (Intron 6). The RT-PCR products were electrophoresed on 1% agarose gel and visualized by staining with ethidium bromide.

Northern blot analysis

Vero cells were transfected and infected with CHP virus as described previously. Total RNA was isolated from infected cells at regular time intervals postinfection and run in 1% MOPS agarose gel. RNA was Northern transferred to Nylon membrane, UV cross-linked, and hybridized with N gene-specific mRNA sense riboprobe generated simultaneously from pET3a-NC by in vitro transcription and with β-actin gene-specific probe as a control. The β-actin gene-specific probe was synthesized with the Random Priming Kit (Boehringer Manheim) according to the manufacturer's protocol using [α^{32}P]dCTP (BRIT). Membrane was washed, air-dried, and exposed to X-ray film. Autoradiogram was scanned and band intensities were quantitated with Matlab version 5.1.0.421 and corrected for β-actin gene product.

In vitro transcription reconstitution

In vitro reconstitution of viral transcription was done with detergent-disrupted CHP virus as described (Colonno and Banerjee, 1978). Briefly, 0.1 mg/μl of purified virus was incubated in reconstitution buffer containing 0.05% Triton N101 (50 mM Tris–HCl pH 8.0, 0.1 M NaCl, 5 mM $MgCl_2$, 4mM DTT) for 15 min at 37°C. This detergent-disrupted virus was further incubated in the same buffer supplemented with 30 μM UTP, 1 mM ATP, CTP, and GTP, 200 μCi [α^{32}P]UTP in 200 μl reaction volume for 2 h at 30°C. Indicated amounts of P_m or P_1 were added to different sets at this time point. Reaction was terminated with 0.1% SDS, 25 μg/ml yeast t-RNA (GIBCO-BRL), followed by incubation with 200 μg/ml proteinase K (GIBCO-BRL) for 30 min at 37°C. Phenol-chloroform extraction was done before ethanol precipitation. The pellet was dissolved and loaded in 20% polyacrylamide gel containing 7 M urea, run in 1× TBE. The gel was dried and exposed to X-ray film.

Acknowledgments

We thank Anjan DasGupta, University of Calcutta, India; Kaushik Si, for critical discussion and help in preparation of the manuscript. Soumen Basak and Amitabha Majumder acknowledge CSIR and UGC, Govt. of India, for providing Junior Research Fellowship. We acknowledge S. Roy Choudhury of Indian Institute of Chemical Biology, India, for DNA sequencing. This work was supported by DST, Govt. of India.

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