Phosphoprotein P of non-segmented negative-sense RNA viruses, also known as the polymerase subunit, is an essential component of the transcriptional complex. The P protein functions as a transcriptional activator of L protein (Barik & Banerjee, 1992a; Curran et al., 1994). The P protein seems to transactivate the L protein for transcription in a similar way to that observed for eukaryotic transactivators. Extensive biochemical and genetic studies on the P protein of vesicular stomatitis virus (VSV), which is a prototype negative-stranded RNA virus, have revealed that P protein is phosphorylated by a cellular kinase (CK) (Barik & Banerjee, 1991, 1992b; Gao & Lenard, 1995). This kinase has been identified as CKII. Recent studies have addressed the role of phosphorylation in the function of the P proteins of several non-segmented negative-stranded RNA viruses. The P proteins of VSV, Chandipura virus, respiratory syncytial virus (RSV) and measles virus (MeV) have been shown to be phosphorylated by CK II, and this phosphorylation step is essential for its transcriptional activation in RSV, VSV and Chandipura virus (Mazumder & Barik, 1994; Das et al., 1995; Chattopadhyay et al., 1997). In the case of canine distemper virus (CDV), the P protein is predominantly phosphorylated by protein kinase C-ζ (PKC-ζ) and CKII has minor activity; PKC-ζ mediated phosphorylation is essential for the virus replication (Liu et al., 1997). The P protein of Sendai virus has also been shown to be phosphorylated by PKC-ζ (Huntley et al., 1997).

RPV, a member of the morbillivirus subgroup of the Paramyxoviridae, is an important pathogen of wild and domestic bovids. The virus genome is a single-stranded, negative-sense RNA that is encapsidated by viral nucleocapsid protein N. The virus P and L proteins together constitute the RNA-dependent RNA polymerase; both P and L proteins are associated with the nucleocapsid to form the RNP core of the virus, which is the transcription complex of the virus. The transcription complex synthesizes, both in vivo and in vitro, at least seven mRNAs that are capped at the 5’ end and polyadenylated at the 3’ end (Ghosh et al., 1995).

The present work studied the role of phosphorylation of RPV P protein in virus transcription. The RPV P gene cDNA clone used in this study, p3-35, was isolated from an RPV RBOK vaccine strain-infected Vero cell cDNA library. RPV P gene was cloned into the bacterial expression vector pRSET B (Invitrogen), expressed as an N-terminal His-tag fusion protein and purified using Ni-affinity chromatography. Although the calculated molecular mass of the recombinant P protein is 61 kDa, it migrated as an 80 kDa protein when electrophoresed on a 10% SDS-polyacrylamide gel. The aberrant mobility of P protein of negative-sense RNA viruses has been reported previously (Emerson & Schubert, 1987; Huber et al., 1991).

Bacterially expressed P protein was found to be unphosphorylated. However, P protein was phosphorylated when expressed transiently in CV-1 cells under a vaccinia virus expression system and metabolically labelled with

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**Cellular casein kinase II-mediated phosphorylation of rinderpest virus P protein is a prerequisite for its role in replication/transcription of the genome**

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Phosphoprotein P of rinderpest virus (RPV), when expressed in *E. coli*, is present in the unphosphorylated form. Bacterially expressed P protein was phosphorylated by a eukaryotic cellular extract, and casein kinase II (CK II) was identified as the cellular kinase involved in phosphorylation. *In vitro* phosphorylation of P-deletion mutants identified the N terminus as a phosphorylation domain. *In vivo* phosphorylation of single or multiple serine mutants of P protein identified serine residues at 49, 88 and 151 as phospho-acceptor residues. The role of P protein phosphorylation in virus replication/transcription was evaluated using the RPV minigenome system and replication/transcription of a reporter gene *in vivo*. P protein phosphorylation was shown to be essential for *in vivo* replication/transcription since phosphorylation-null mutants do not support expression of a reporter gene. Transfection of increased amounts of phosphorylation-null mutant did not support minigenome replication/transcription *in vivo*.

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[32P]orthophosphate (data not shown). To confirm whether phosphorylation of RPV P protein occurs in vitro, purified RPV P protein was incubated with a CV1 cellular extract (0.5 μg), as a source of kinase, in the presence of [γ-32P]ATP (as described by Das et al., 1995) and was found to be phosphorylated (Fig. 1A). P protein did not show auto-phosphorylation in the absence of cell extract (data not shown). Cell extracts from other sources (HeLa, A549) also phosphorylated recombinant P protein (data not shown).

The cellular kinase involved in phosphorylation of P protein was identified using inhibitors known to inhibit two ubiquitous cellular protein kinases, CKII and protein kinase C (PKC). As shown in Fig. 1(A), the phosphorylation of P is inhibited completely in presence of heparin, a specific inhibitor of CKII, at a concentration of 5 μg ml⁻¹; staurosporine, a PKC inhibitor, did not affect the phosphorylation of P protein even at 400 nM. These results suggest that cellular CKII might be the kinase involved in RPV P protein phosphorylation.

The above result was further confirmed by a similar kinase assay using recombinant CKII as the source of kinase (Fig. 1B) in the presence of [γ-32P]ATP. CKII-mediated phosphorylation was inhibited in the presence of the specific inhibitor heparin, and excess of a CKII peptide analogue of CKII substrate (RRRNNNTTNNN), which competes with P protein. There was no inhibition of phosphorylation in the presence of staurosporine or a non-specific N-peptide derived from sequences corresponding to the RPV nucleocapsid N protein. Taken together, these results strongly suggest that cellular CKII is involved in the phosphorylation of RPV P protein.

Phosphoamino acid analysis of the in vitro phosphorylated P protein identified a serine residue as the phospho-acceptor amino acid (data not shown). The potential CKII phosphorylation sites within the P proteins were mapped using the computer search program SCANPROSITE (Exasy, Prosite tool), within the putative consensus motif S/TXXD/E (where X can be any amino acid). Eight serine residues were identified that satisfy the n + 3 rule, where a serine at position n is followed by an acidic amino acid at position n + 3. Since acidic residues at positions n + 1 and n + 2 are known to be better targets for CKII, four serine residues at positions 49, 88, 151 and 180 were identified as the most likely CKII sites. Using different deletion mutants, the phosphorylated domain was mapped to the N-terminal 156 amino acid residues (data not shown). Hence, serine residues at positions 49, 88 and 151 were mutated to alanine, either individually or in combination, by site-directed mutagenesis in order to map the phospho-acceptor residues of RPV P protein further. The mutant P genes were cloned into a pRSET B vector. P protein was expressed in CV-1 cells by infecting them with 1 p.f.u. of T7 polymerase-expressing vaccinia virus (VT7-3, a kind gift from Bernard Moss, NIH, USA) and transfecting with plasmid-encoding wild-type (WT) or mutant P proteins. The relative expression of mutant P proteins was verified by Western blotting of immunoprecipitated cell extracts as described in the legend (Fig. 2A). The expression level of all the mutants was found to be similar to that of the WT P protein. The phosphorylation status of each mutant in eukaryotic cells was analysed by labelling the transfected cells with [32P]orthophosphate. The results (Fig. 2B) indicated that different mutants are phosphorylated to different extents, except for phosphorylation-negative double mutants.
The approximate percentage phosphorylation of all the mutants compared to WT was calculated by quantifying the phosphorylation signal intensities using a phosphorimager and values are shown below each lane in Fig. 2(B). Alteration of single serine residues reduced phosphorylation by approximately 30–60%. Presence of either Ser49 or Ser88 appears to be critical for phosphorylation, as mutants S49/151A and S88/151A are partially phosphorylated, whereas in mutant S49/88A, phosphorylation is completely abolished. Although phosphorylation of S88/151A appears to be very much less, it was variable (10–28% of WT) in different experiments.

In vitro phosphorylation of bacterially expressed mutant P proteins by cellular extract or recombinant CKII yielded a similar phosphorylation profile. CKII may be the only kinase involved in P protein phosphorylation, as the removal of all three CKII sites abrogates phosphorylation completely in vivo. However, the role of virus L protein in the additional phosphorylation of P protein could not be ruled out as Sendai virus and VSV L proteins have been shown to possess P protein kinase activity (Einberger et al., 1990; Barik & Banerjee, 1992b).

P protein function was analysed using the RPV plasmid-based minigenome system, as described previously (Baron & Barrett, 1997). The minigenome used contains the CAT reporter gene flanked by the minimal cis-acting RPV genomic termini. Plasmids encoding the minigenome (pMD88A) and the N, P and L proteins are described in the above reference. All constructs were transcribed from T7 promoters after transfection into A549 cells infected with 1 p.f.u. per cell of recombinant vaccinia virus expressing T7 RNA polymerase. Addition of plasmids encoding the RPV P, N, L proteins into cells together with the minigenome resulted in replication and transcription of the minigenome, and hence production of CAT protein. Replacing RPV P plasmid with various mutant P plasmids resulted in a reduced level of CAT expression (Fig. 3A). The extent of phosphorylation of each of the P mutants

Fig. 2. Phosphorylation analysis of site-directed mutants. CV-1 cells were infected with VTF7-3 and subsequently transfected with wild-type (WT) or mutant P protein-expressing vector. The transfected cells were labelled with [32P]orthophosphate for 16 h and cell lysates were immunoprecipitated with an anti-P protein antibody. After washing, the immunoprecipitated samples were electrophoresed on a 10% SDS-polyacrylamide gel and autoradiographed. (A) Western blot of immunoprecipitated samples with anti-P protein antibody. (B) Autoradiogram showing the phosphorylation status of mutants. Lane 1, WT; lane 2, S49A; lane 3, S88A; lane 4, S151A; lane 5, S49/S88A; lane 6, S88/151A; lane 7, S49/151A; and lane 8, S49/88/151A. Percentage phosphorylation of each mutant has been calculated taking the phosphorylation of WT P protein as 100%.

Fig. 3. Effect of P protein phosphorylation on minigenome replication/transcription. (A) A549 cells were infected with VTF7-3 and subsequently transfected with plasmids expressing L, N and wild-type (WT) or mutant P proteins and the minigenome plasmid. The cells were harvested 24 h post-transfection and CAT ELISA was performed according to the manufacturer’s protocol. CAT expression with WT P plasmid has been taken as 1 U. (B) As (A), except that a different ratio of WT/phosphorylation-null mutant P was transfected along with other plasmids. The numbers on the x-axis denote the molar ratio of triple mutant (TM) and WT P constructs cotransfected.
correlated well with their effect on minigenome replication/transcription. It was observed that the phosphorylatable mutants (S\(^{49A}\), S\(^{88A}\), S\(^{151A}\), S\(^{49/88A}\) and S\(^{49/88/151A}\)) support replication/transcription, whereas cells transfected with phosphorylation-null mutants, e.g. S\(^{49/88A}\) and S\(^{49/88/151A}\), had CAT expression below 10% when compared to WT. Since CAT expression from the minigenome system in the absence of P is approximately 10% of expression in the presence of WT P (data not shown), mutants S\(^{49/88A}\) and S\(^{49/88/151A}\) can be considered completely defective in minigenome replication/transcription. Together, these results indicate that Ser\(^{49}\) and Ser\(^{88}\) are critical for virus replication/transcription, whereas the alteration of Ser\(^{151}\) did not show any effect. While all three serines are involved in phosphorylation, only Ser\(^{49}\) and Ser\(^{88}\) seem to be important for virus replication. Hence for the S\(^{49A}\) and S\(^{88A}\) mutants, although there are two residues in the protein still available for phosphorylation, reduction in CAT expression is greater as Ser\(^{151}\) does not appear to be important. Even higher amounts (up to fivefold excess) of the mutant P plasmid did not rescue the defect, suggesting that phosphorylation may be a prerequisite for the replication/transcriptional activity of P protein. The direct role of P protein phosphorylation on virus transcription has already been observed in RSV (Mazumder & Barik, 1994) and rhabdoviruses such as VSV and Chandipura virus (Takacs et al., 1992; Pattnaik et al., 1997; Mathur et al., 1997; Chattopadhyay et al., 1997).

The effect of coexpression of WT and phosphorylation-null triple mutant P (TM) on the expression of the reporter gene in the RPV minigenome was investigated. Both plasmids were mixed in different ratios and cotransfected along with N, L and CAT reporter plasmids. The results showed that CAT expression was nearly twofold higher in cells that were cotransfected with the WT and mutant P than in cells transfected with WT alone (Fig. 3B). This increase in CAT expression was not due to an increased concentration of either WT or mutant P plasmid DNA, since there was no effect on CAT expression when individual plasmids were used in the minigenome replication/transcription assay at varying concentrations. There was no CAT expression in cells transfected with the phosphorylation-negative mutant alone. This experiment was repeated using all the mutants with the WT P plasmid, and a higher level of expression of CAT reporter gene was observed with S\(^{49/88A}\) in addition to S\(^{49/88/151A}\) (data not shown). The enhancement in CAT expression in the presence of WT together with phosphorylation-null mutant of RPV P indicates a definite role played by the unphosphorylated P protein, which might be present in the infected cells in low amounts as a consequence of phosphatase action to control phosphorylation events. It is known that CAT expression occurs by a two-step process in which replication of the T7 transcript is followed by transcription, leading to CAT expression (Baron & Barrett, 1997). Therefore, it is possible that the phosphorylation-null mutant might cause enhanced replication of the minigenome, which in turn leads to the synthesis of more mRNA, and results in translation of increased amounts of CAT protein. This enhanced CAT expression might also be due to the additive effect of dominant WT P protein action on transcription of the minigenome, combined with the increase in replication of the minigenome due to the presence of unphosphorylated mutant P protein in the cells functioning through an unknown mechanism. The increase in CAT activity was similar at different ratios of WT and mutant plasmids, which may be due to higher levels of expression of P proteins in transfected cells than the desired optimum ratio of WT to mutant proteins. It might be possible to achieve this optimum ratio if limiting amounts of P plasmids are used for transfection, which was not tested in the present work. Recently, it has been reported for Chandipura virus, a virus closely related to VSV, that unphosphorylated P protein binds to leader RNA leading to enhanced virus replication (Basak et al., 2003). Unphosphorylated RPV P protein also shows specific binding to its leader RNA in vitro, which is abolished upon in vitro phosphorylation (to be published elsewhere). The present work on RPV P protein provides the first report on the role of phosphorylation of a morbillivirus P protein in replication/transcription of the virus genome.

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