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Phosphorylation within the Amino-Terminal Acidic Domain I of the Phosphoprotein of Vesicular Stomatitis Virus Is Required for Transcription but Not for Replication

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Phosphorylation by casein kinase II at three specific residues (S-60, T-62, and S-64) within the acidic domain I of the P protein of Indiana serotype vesicular stomatitis virus has been shown to be critical for *in vitro* transcription activity of the viral RNA polymerase (P-L) complex. To examine the role of phosphorylation of P protein in transcription as well as replication *in vivo*, we used a panel of mutant P proteins in which the phosphate acceptor sites in domain I were substituted with alanines or other amino acids. Analyses of the alanine-substituted mutant P proteins for the ability to support defective interfering RNA replication *in vivo* suggest that phosphorylation of these residues does not play a significant role in the replicative function of the P protein since these mutant P proteins supported replication at levels $\geq 70\%$ of the wild-type P-protein level. However, the transcription function of most of the mutant proteins *in vivo* was severely impaired (2 to 10% of the wild-type P-protein level). The level of transcription supported by the mutant P protein (P_{60/62/64}) in which all phosphate acceptor sites have been mutated to alanines was at best 2 to 3% of that of the wild-type P protein. Increasing the amount of P_{60/62/64} expression in transfected cells did not rescue significant levels of transcription. Substitution with other amino acids at these sites had various effects on replication and transcription. While substitution with threonine residues (P_{TTT}) had no apparent effect on transcription (113% of the wild-type level) or replication (81% of the wild-type level), substitution with phenylalanine (P_{FFF}) rendered the protein much less active in transcription (<5%). Substitution with arginine residues led to significantly reduced activity in replication (6%), whereas glutamic acid substituted P protein (P_{EEE}) supported replication (42%) and transcription (86%) well. In addition, the mutant P proteins that were defective in replication (P_{RRR}) or transcription (P_{60/62/64}) did not behave as transdominant repressors of replication or transcription when coexpressed with wild-type P protein. From these results, we conclude that phosphorylation of domain I residues plays a major role in *in vivo* transcription activity of the P protein, whereas *in vivo* replicative function of the protein does not require phosphorylation. These findings support the contention that different phosphorylated states of the P protein regulate the transcriptase and replicase functions of the polymerase protein, L.

Vesicular stomatitis virus (VSV) is a negative-strand RNA virus that carries an active RNA-dependent RNA polymerase within the virion core. The nucleocapsid template in the virion core consists of the 11,161-nucleotide (nt)-long genomic RNA tightly wrapped around by the nucleocapsid protein (N) and is the template for two distinct types of RNA synthetic processes (transcription and replication) by the associated viral polymerase (1, 2, 20). In infected cells, the nucleocapsid core first directs transcription to generate subgenomic mRNAs, which are translated to produce viral proteins required for replication. During replication, a full-length complement (called antigenome) of the genomic RNA is synthesized in the form of a nucleocapsid, which in turn acts as the template for the synthesis of genomic nucleocapsids. It is generally believed that the same polymerase performs both transcriptive and replicative functions of the virus.

The RNA polymerase of VSV is a complex of two proteins: the large protein, L, and the phosphoprotein, P. Several genetic and biochemical analyses have suggested that both proteins are required for the polymerase activity of the virus (18,

21, 44). While the L protein carries the catalytic center for polymerization of nucleotides during RNA synthesis, the P protein serves as an accessory protein that is required for the function of the L protein (1, 2). The P protein is multifunctional, playing key roles in both transcription and replication processes. It interacts with the N protein, maintaining the N protein in a form competent to support efficient RNA encapsidation during replication (47, 48, 54). It interacts with the L protein and stabilizes the protein from proteolytic degradation (9, 22). Furthermore, it interacts with specific nucleotide sequences at the termini of VSV genome, presumably for transcription and replication (29, 31). The P protein is found in different phosphorylated forms in infected cells as well as in the virion core, and different functional forms of P protein with different degrees of phosphorylation have been shown to exist (7, 14, 27, 28, 32, 41). A direct role of phosphorylation in P-protein function was provided by the studies of Chattopadhyaya and Banerjee (12), who demonstrated that phosphorylation within a specific domain regulates the function of P protein in transcription *in vitro*.

In the last few years, the role of phosphorylation of P protein in transcription has been the subject of intense investigation. Most of these studies were facilitated by the initial demonstration that expression of P protein of VSV New Jersey serotype in bacteria yields a completely unphosphorylated P protein (P0

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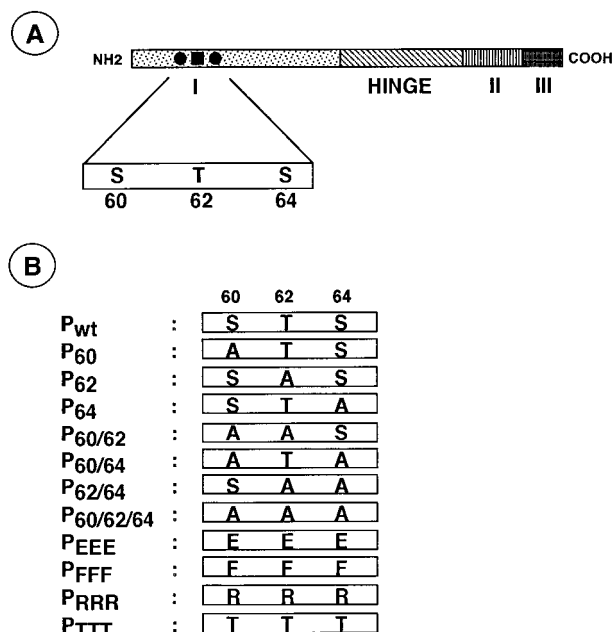


FIG. 1. (A) Domain structure of the P protein of VSV (Indiana serotype). The full-length P protein with the three functionally defined domains (I, II, and III) and the hinge region is shown. The phosphate acceptor sites within the domain I are shown by filled circles representing S-60 and S-64 and filled square representing T-60. (B) Various mutant P proteins with alanine (A), glutamic acid (E), phenylalanine (F), arginine (R), or threonine (T) substitutions at the phosphate-acceptor sites are shown.

form) which is transcriptionally inactive under in vitro transcription reconstitution conditions (3, 5). The bacterially expressed inactive P protein, however, becomes transcriptionally active if it is first phosphorylated by casein kinase II (CKII) (4, 26). By various mutagenesis and biochemical studies, the acceptor sites for CKII-mediated phosphorylation of P protein have been mapped to Ser-59 and Ser-61 for New Jersey serotype (51) and Ser-60, Thr-62, and Ser-64 for Indiana serotype of VSV (13, 50). Phosphorylation of these residues which are located in the amino-terminal acidic domain I of the protein (see Fig. 1 for domain structure) has been recently shown to mediate multimerization of the P protein that facilitates complex formation with the L protein (16, 24, 25). Although the mechanism by which phosphorylation modulates the activities of the P protein is not clear, it has been shown that such a modification of New Jersey P protein substantially increases the α -helical structures within the P protein (16), which may be required for multimer formation and interaction with the L protein.

Following phosphorylation of bacterially expressed P protein (P0 form) in domain I by CKII, the P1 protein undergoes subsequent phosphorylation by L-associated kinase at two specific residues within domain II to generate P2 protein (4). It has recently been shown that the P1 and P2 proteins of Indiana serotype VSV are similar to the proteins previously designated NS1 and NS2, respectively (13). There exists a precursor-product relationship between P1 and P2 forms, and P2 forms are generated by phosphorylation of P1 at the two serine residues (S-226 and S-227) within the domain II (13). In addition, it has been shown recently that the P2 form can also be generated directly from the P0 form by L-associated kinase or N-RNA-associated kinase by phosphorylation of residues in domain II only (reference 13 and unpublished data from our laboratory).

The phosphorylation of these residues has been shown to be important for activation of transcription in vitro by the P protein of New Jersey serotype (12). However, studies with Indiana serotype P protein indicate that phosphorylation of domain II residues may not have a role in transcription in vitro (24, 40).

To address the role of phosphorylation within domain I of the P protein of VSV (Indiana serotype) in RNA transcription and replication in vivo, we have studied a panel of mutant P proteins containing single, double, or triple alanine or other amino acid substitutions at the three phosphate acceptor sites (S-60, T-62, and S-64) within amino-terminal acidic domain I. Our results show that while replication of defective interfering (DI) RNA in vivo remained largely unaffected by the phosphorylation status of the P protein, transcription activity of the mutant P proteins was significantly downregulated, indicating that phosphorylation of domain I residues is critical for P-protein function in transcription in vivo.

MATERIALS AND METHODS

Cell cultures and viruses. Baby hamster kidney (BHK-21) cells were grown as monolayer cultures in Eagle's minimal essential medium (MEM) containing 7.5% fetal bovine serum (FBS). Human 143B (thymidine kinase-negative) cells were also grown in MEM supplemented with 5% FBS. VSV (Indiana serotype, San Juan, and Mudd-Summers strains) were propagated in BHK-21 cells. Stocks of DI-T particles (37) were prepared as described previously (46). Recombinant vaccinia virus (vTF7-3) carrying the bacteriophage T7 RNA polymerase gene (23) was propagated in BHK-21 cells by infecting the cells at a multiplicity of infection of 0.1. Titers of stock vTF7-3 virus were determined by plaque assay in 143B cells.

Plasmids. Plasmids pN, pP, and pL, containing the coding sequences for the VSV (Indiana serotype, San Juan strain) N, P, and L proteins under the control of bacteriophage T7 RNA polymerase promoter in pGEM vectors (Promega Biotech, Madison, Wis.), have been described previously (46). The construction of the plasmid (pET-P) carrying a full-length cDNA clone of the P gene of VSV (Indiana serotype, Mudd-Summers strain) in pET-3a vector and the construction of various P-gene mutants used in the present study have been described recently (13). Plasmid pDI, carrying a cDNA copy of the DI-T genomic RNA under the control of T7 RNA polymerase promoter, has been described previously (45). The construction of the plasmid, p9BN, used for in vivo transcription studies has been described recently (39). Upon transcription by T7 RNA polymerase, p9BN generates a positive-sense antigenomic VSV RNA [9BN(+)] 1,618 nt long. In cells cotransfected with plasmids coding for VSV N, P, and L proteins and 9BN(+) RNA, the 9BN(+) RNA undergoes replication to generate the negative-sense genomic RNA [9BN(-)]. The 9BN(-) RNA is then transcribed by the VSV polymerase to synthesize a chimeric mRNA (NAL mRNA) about 1,470 nt long [excluding the poly(A) tail] containing the entire coding sequence of the N gene and a part of the L gene.

Virus infections and DNA transfections. The procedures used are essentially as described previously (46). Briefly, BHK-21 cells in 35-mm-diameter six-well plates or in 60-mm-diameter plates were grown to approximately 90% confluency. Cells were then infected with the recombinant vaccinia virus (vTF7-3) at a multiplicity of infection of 10. After adsorption at 37°C for 45 min, cells were washed twice in serum-free Dulbecco's modified MEM (DMEM) and subsequently transfected with various combinations of plasmids by using Lipofectin (Gibco/BRL, Bethesda, Md.). Medium from the transfected cells was removed at 4 to 5 h posttransfection, cells were washed twice in DMEM supplemented with 2% FBS and incubated with appropriate volume of the same medium. For DI particle infection to analyze DI RNA replication, washed cells at 4 to 5 h posttransfection were superinfected with 500 μ l of 1:500 dilution of stock DI particles for 45 min at 37°C. Following adsorption, cells were washed twice in serum-containing DMEM and incubated in 1.5 ml of the same medium containing [3 H]uridine as described below. In most experiments, unless specifically described in figure legends, 3 μ g of pN, 3 μ g of pET-P, 1 μ g of pL, and 5 μ g of p9BN or pDI plasmids were used in transfection of cells in 60-mm-diameter plates.

Metabolic labeling and analysis of proteins. To examine the expression of P protein, cells transfected with pET-P plasmids were incubated in methionine-free DMEM at 18 h posttransfection for 45 min at 37°C and then labeled with 20 μ Ci of [35 S]methionine per ml of the same medium for 2 h. In pulse-chase experiments, after labeling with [35 S]methionine, cell monolayers were washed twice with medium containing a 100-fold molar excess of unlabeled methionine and incubated for different lengths of time in the same medium containing unlabeled methionine. Cell monolayers were then washed twice in cold phosphate-buffered saline, scraped into phosphate-buffered saline, and collected by centrifugation in a microcentrifuge for 1 min at 4°C at 18,000 \times g. The cell pellets were resuspended in radioimmunoprecipitation assay buffer, and cytoplasmic

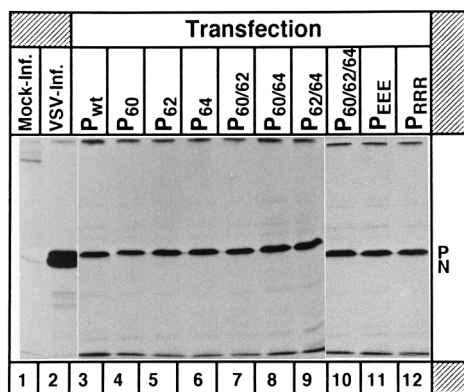


FIG. 2. SDS-PAGE analysis of wt and mutant P proteins expressed in transfected cells. BHK-21 cells in 35-mm-diameter wells of six-well plates were infected with vTF7-3 and subsequently transfected with 1.5 μ g of each of the plasmids indicated above the lanes. Transfected cells were labeled with [35 S]methionine. Cell extracts were prepared; labeled P proteins were immunoprecipitated with anti-P (Indiana serotype, Mudd-Summers strain) antibody, separated by SDS-PAGE, and detected by fluorography. Lanes 1 and 2 show the immunoprecipitated proteins from mock-infected and VSV (Mudd-Summers strain)-infected cells, respectively. The P and N proteins in VSV-infected cells, which form complexes, are often coimmunoprecipitated by either anti-N or anti-P antibody and are seen as two intensely labeled and closely migrating protein bands in lane 2.

extracts were prepared as before (46). P proteins were immunoprecipitated by using a rabbit anti-P (Indiana serotype, Mudd-Summer strain) antibody, separated by polyacrylamide gel electrophoresis (PAGE) in 10% polyacrylamide gels (33), and subsequently detected by fluorography (10).

In vivo labeling of RNA replication and transcription products and gel electrophoretic analysis. For DI RNA replication studies, transfected cells superinfected with DI particles were labeled with 25 μ Ci of [3 H]uridine per ml overnight (14 to 15 h). When replication was studied in pDI-transfected cells, cells at 16 to 18 h posttransfection were treated with 15 μ g of actinomycin D per ml of DMEM for 45 min at 37°C prior to labeling with [3 H]uridine in the presence of actinomycin D for 6 h. After labeling, cytoplasmic extracts were prepared and nucleocapsids containing newly synthesized and labeled RNAs were immunoprecipitated with anti-VSV antibody as described earlier (46). The RNAs recovered from immunoprecipitated nucleocapsids by phenol-chloroform extraction were electrophoresed in an acid agarose-urea gel as described before (38, 46) and detected by fluorography (35).

For transcription analysis, transfected cells at 14 to 16 h posttransfection were treated with actinomycin D and labeled with [3 H]uridine as described above. Total RNAs from cytoplasmic extracts were recovered by extraction with phenol and chloroform and analyzed by electrophoresis and fluorography as described above. To determine the normalized levels of transcription supported by the mutant P proteins, we took into consideration the relative levels of replication and transcription supported by each mutant and the following formula: normalized levels of transcription = $100 \times (\text{relative levels of transcription} / \text{relative levels of replication})$.

RESULTS

Expression and stability of wt and mutant P proteins in transfected cells. Since the cDNAs encoding the wild-type (wt) and mutant P proteins of VSV (Indiana serotype, Mudd-Summers strain) had been subcloned into pET-3a vector under the control of the T7 RNA polymerase promoter (13), we first wanted to determine the levels of expression of these proteins in cells transfected with these plasmids. Accordingly, BHK-21 cells infected with vTF7-3 were transfected with plasmids encoding wt or mutant P proteins. Expression of P protein in these cells was analyzed by metabolic labeling with [35 S]methionine, immunoprecipitation with excess anti-P antibody to quantitatively recover the expressed P proteins, and subsequent sodium dodecyl sulfate (SDS)-PAGE. Results (Fig. 2) show that all of the mutant P proteins were expressed at levels similar to that of the wt P protein. The levels of expressions were comparable to the levels of expression of the P protein of

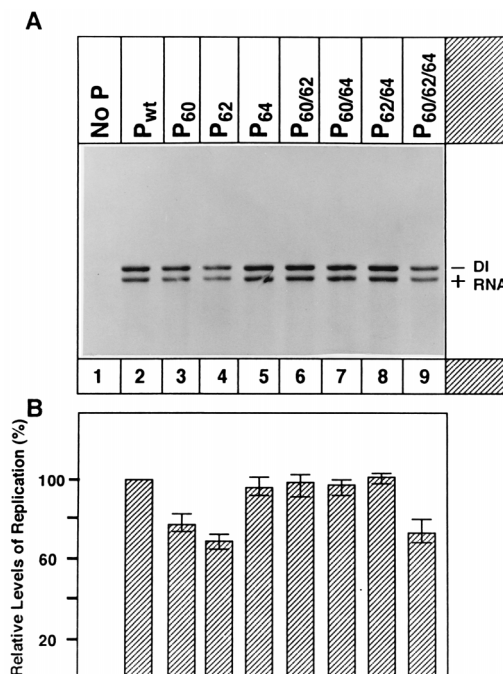


FIG. 3. (A) In vivo replication of DI RNA supported by the wt P or various mutant P proteins. BHK-21 cells in 60-mm-diameter plates were infected with vTF7-3 and subsequently transfected with pN, pL, and either wt P or mutant P plasmids as described in Materials and Methods. After DI particle superinfection and [3 H]uridine labeling, cell extracts were prepared, nucleocapsids were immunoprecipitated, and the labeled RNAs recovered from the nucleocapsids were analyzed by electrophoresis in an agarose-urea gel. – and + represent genomic and antigenomic RNAs, respectively, of the DI particle. (B) Quantitative analysis of relative levels of DI RNA replication supported by each of the mutant P proteins. The histograms represent the average and the range of relative levels of replication supported by various mutant P proteins. The data were obtained by densitometric scanning of band intensities of the fluorogram shown in panel A and those from two other similar experiments.

VSV (Indiana serotype, San Juan strain) under transfection conditions similar to those described previously (46). From pulse-chase experiments, we determined that more than 80% of the wt and mutant P proteins were stable for at least 6 to 8 h after synthesis (data not shown). Furthermore, we found that in cotransfected cells, all of the viral proteins were expressed at comparable levels (data not shown).

Replication of DI RNA in cells expressing mutant P proteins. Previous work from our laboratory showed that cells expressing the N, P, and L proteins from transfected plasmids support high levels of replication and amplification of DI RNA (46). We used this system to determine the ability of the mutant P proteins to support DI RNA replication. BHK-21 cells infected with vTF7-3 were transfected with plasmids encoding the N, L, and wt or mutant P proteins. Following superinfection with DI particles, replication of DI RNA in these cells was analyzed by [3 H]uridine labeling, immunoprecipitation of labeled nucleocapsids, and agarose-urea gel electrophoresis of RNAs recovered from the nucleocapsids. Results from a representative experiment is shown in Fig. 3A. All the mutant proteins supported high levels of replication of DI RNA (lanes 3 to 9) compared to the wt protein (lane 2).

A quantitative analysis of the levels of replication of DI RNA was performed by densitometric scanning of fluorograms from three independent experiments and is shown in Fig. 3B. The levels of DI RNA replication supported by the mutant P proteins were at least 70% of that of the wt P protein. In some

experiments, replication levels supported by the mutant P proteins were comparable to that of the wt P protein. From these experiments, we conclude that phosphorylation of the residues (S-60, T-62, and S-64) within the domain I of the P protein has little or no effect on replication of DI RNA.

It should be noted that the foregoing experiments were performed with the replicating DI RNA templates provided by the superinfecting DI particles. When the experiments were carried out with DI RNA provided by transfection of a plasmid carrying a cDNA copy of DI RNA as previously described (45), similar results were obtained (data not shown).

Analysis of the ability of mutant P proteins to support transcription in vivo. We next examined the ability of the mutant P proteins to support transcription in vivo. To perform these experiments, we used a plasmid (p9BN) containing a cDNA fragment coding for a transcription- and replication-competent VSV RNA template. The cDNA contained sequences from the 3' and 5' termini of the VSV genome, the entire N gene, and a small part of the L gene (39). The RNA generated from the cDNA by T7 RNA polymerase is a positive-sense antigenomic RNA of VSV which must first be replicated to generate the genomic RNA to be used as a template for transcription. In initial experiments, we determined that the levels of replication of the antigenomic RNA to generate the genomic RNA template correlated well with the levels of replication obtained with DI RNA (data not shown) and thus provided the basis for using plasmid p9BN to directly assess the effects of the mutations in P protein in in vivo transcription of VSV RNA.

To determine the effect of mutations in P protein on transcription, BHK-21 cells were infected with vTF7-3 and then cotransfected with plasmids encoding the N, L, and wt or mutant P proteins and plasmid p9BN. Transfected cells were treated with actinomycin D at 16 h posttransfection to block further synthesis of RNA from transfected DNA templates. Transcription in these cells was then analyzed by examining the synthesis of NAL mRNA in the presence of actinomycin D and [³H]uridine. A fluorogram of total labeled RNAs synthesized under these conditions and separated in an agarose-urea gel is shown in Fig. 4A. Results show that the mutant P proteins supported various levels of transcription relative to the wt P protein. The level of transcription supported by P₆₀ mutant (lane 3) was about 40% of the wt P protein level (lane 2). The other single-site substitution mutants (lanes 4 and 5) or the double mutants (lanes 6 to 8), however, supported levels of transcription that were about 3 to 10% of the wt P protein level. The triple mutant in which all phosphate acceptor sites in domain I have been substituted for alanines did not support detectable levels of transcription (lane 9). Upon longer exposure of the fluorogram, we detected transcription products whose levels were, on best estimation, 2 to 3% of that of the wt P protein. This low level of transcription directed by the P_{60/62/64} mutant was consistently observed in three independent experiments. The levels of expression of the wt and mutant P proteins in these experiments were comparable (similar to those seen in Fig. 2); therefore, low levels of transcription supported by the mutant proteins were not due to the levels of expression of the mutant proteins.

A quantitative determination of the normalized levels of transcription supported by the mutant P protein was performed. These data were obtained by taking into consideration the relative levels of replication and transcription supported by each mutant protein as described in Materials and Methods. It should be noted that in these in vivo transcription experiments, the plasmid used generates the antigenomic positive-sense RNA of a VSV minigenome, which must first undergo replication to generate the negative-sense genomic RNA as the

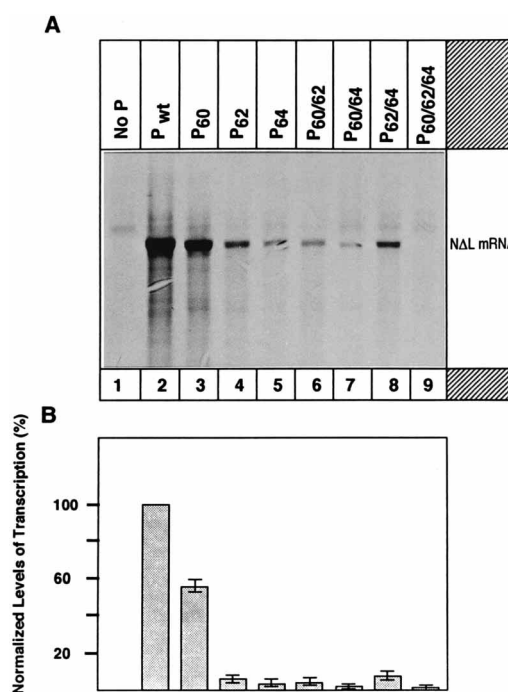


FIG. 4. (A) Analysis of in vivo transcription supported by various P mutants. BHK-21 cells in 35-mm-diameter wells of six-well plates were infected with vTF7-3 and then cotransfected with 1.5 μ g of pN, 0.5 μ g of pL, 2.5 μ g of p9BN, and 1.5 μ g of pET-P plasmids coding for the wt or various mutant P proteins. At 14 to 16 h posttransfection, cells were treated with actinomycin D and labeled for 6 h with [³H]uridine. Total RNAs were extracted from cytoplasmic extracts and analyzed by electrophoresis in an agarose-urea gel. NAL mRNA represents the transcription product generated by VSV RNA polymerase in the transfected cells under the experimental conditions used. (B) Quantitative determination of the normalized levels of transcription. The data were obtained by scanning of the band (NAL mRNA) intensities in the fluorogram shown in panel A and those from two other similar experiments and taking into consideration of the relative levels of replication and transcription as described in Materials and Methods. The average and the range of normalized levels of transcription supported by each mutant relative to the wt P protein are shown.

template for transcription. Thus, when the transcription activities of these mutants were calculated from the scanning of the band intensities of the transcription product (NAL mRNA), we also considered the relative levels of replication supported by these mutant P proteins. The average normalized levels of transcription supported by these mutants determined in this manner are shown in Fig. 4B. These results show that mutations in the phosphate acceptor sites within the domain I of the P protein severely impair its ability to function efficiently in transcription in vivo. Phosphorylation of T-62 and S-64 residues appear to be more critical than that of the S-60 residue in transcription. Taken together, these data strongly suggest that phosphorylation within domain I is important for transcription activity of the P protein.

Increasing amounts of P_{60/62/64} expression do not rescue transcription in vivo. Data from Fig. 4 show that relative levels of transcription supported by most of the mutant P proteins (except P₆₀) were very low compared to the wt P protein level. In these experiments, a fixed amount of plasmid encoding wt or mutant P proteins was used in transfection. Our earlier results (shown in Fig. 2) suggested that comparable amounts of P proteins were being synthesized in transfected cells under these conditions and that the P proteins remained stable for at least 6 to 8 h after synthesis. Therefore, the low levels of transcription supported by these mutant P proteins were not

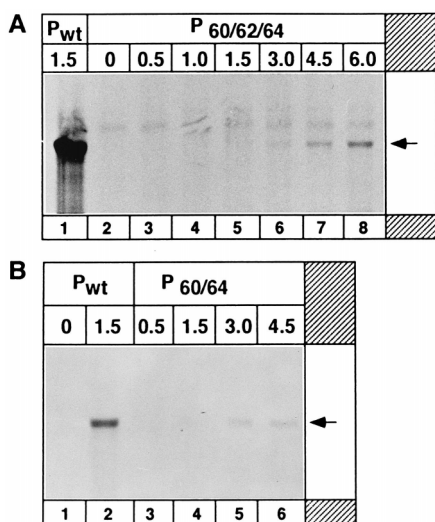


FIG. 5. (A) In vivo transcription supported by various amounts of $P_{60/62/64}$ protein. Transcription analysis was performed as described in the legend to Fig. 4A, but various amounts (micrograms) of $P_{60/62/64}$ plasmid DNA (as shown above the lanes) were used in transfection. The arrow indicates the product (NΔL mRNA) of transcription. (B) Transcription supported by various amounts of $P_{60/64}$. The experiment was performed as described for panel A with the indicated amounts (micrograms) of $P_{60/64}$ plasmid used in transfection.

due to their relative instability. It is possible that the mutant proteins assume different structural conformations (as a result of loss of some or all of the phosphorylation sites in domain I) that are not competent to support efficient transcription. Alternatively, these mutant proteins may have lost the ability to efficiently oligomerize and/or interact with the L protein, which may depend on the concentration or the amount of the mutant protein in transfected cells. Therefore, it may be possible to rescue transcription by expressing increasing or decreasing amounts of mutant P proteins in transfected cells.

To address such a possibility, we chose to use $P_{60/62/64}$ since transcription with this mutant was most severely impaired. Results shown in Fig. 5A suggest that decreasing the amount of $P_{60/62/64}$ protein expression (by decreasing the amount of $P_{60/62/64}$ plasmid during transfection) resulted in almost undetectable levels of transcription (lanes 3 and 4). When the $P_{60/62/64}$ expression was increased, the level of transcription gradually increased (lanes 6 to 8). With 6 μ g of transfected $P_{60/62/64}$ plasmid (lane 8), the level of transcription was 7 to 8% of the level of transcription supported by 1.5 μ g of transfected wt P plasmid (lane 1). Further increase in the amount of $P_{60/62/64}$ plasmid in transfection did not result in increased levels of transcription (data not shown). It should be noted that with increasing amounts of the plasmid transfected (up to 9 μ g), a corresponding increase in the P-protein expression was observed (data not shown), which is consistent with our previous observation (46) and those obtained recently by Spadafora et al. (50).

It has been observed recently that the P mutant ($P_{60/64}$) with alanine substitution at residues 60 and 64 in domain I, when expressed in vivo, does not contain phosphorylated T-62 and is not phosphorylated (13, 30). This result was interpreted to suggest that phosphorylation of T-62 is dependent on prior phosphorylation of S-64 (30). However, under in vitro phosphorylation conditions, the mutant protein could be readily phosphorylated at T-62, which led to the suggestion that the mutant protein is phosphorylated at T-62 when expressed in

vivo but perhaps is selectively dephosphorylated by the phosphatase(s) present in the cell extracts (13). When this mutant protein was tested for its ability to support transcription under in vitro conditions, it was found to be much less active ($\leq 5\%$ of the wt P level) at low concentrations but was active (about 28% of the wt P level) at higher concentrations of the P protein (50). We therefore wished to determine if this mutant protein could rescue transcription in vivo with increased concentrations of the protein. Accordingly, in vivo transcription activity of the mutant protein was analyzed in cells expressing various amounts of the protein. Results in Fig. 5B show that a slight increase in transcription in vivo with increasing amounts of $P_{60/64}$ was observed. However, the level of transcription (8 to 10%) was significantly below that obtained with wt P protein. Taken together, results shown in Fig. 5 suggest that increasing amounts of the mutant P proteins ($P_{60/64}$ and $P_{60/62/64}$) do not rescue their in vivo transcription activities.

Replication and transcription supported by mutant P proteins substituted with other amino acids. Results in Fig. 3 to 5 show that mutant proteins with alanine substitutions at the three phosphate acceptor sites (S-60, T-62, and S-64) in domain I can support high levels of replication in vivo whereas transcription activity in vivo of these mutants is severely impaired. It is possible that these alanine substitutions induce a conformation in the mutant protein that has no effect on the replicative function of the protein but may not be compatible for its transcription function. To determine whether other amino acid substitutions at these three sites allow for generation of functional P proteins, we generated a set of mutant P proteins with substitution of a number of different amino acids: P_{EEE} , containing glutamic acid (negatively charged residue); P_{FFF} , containing phenylalanine (hydrophobic residue with an aromatic ring); P_{RRR} , containing arginine (positively charged residue); and P_{TTT} , containing threonine (polar residue similar to serine).

The ability of each of these mutant P proteins to support replication of DI RNA was analyzed as described above. Results of such an analysis (Fig. 6A) show that the mutant proteins supported DI RNA replication to various degrees. Replication supported by P_{EEE} and P_{FFF} (lanes 4 and 5) was approximately 40% of that of the wt P protein (lane 2). P_{TTT} mutant supported replication (lane 7) at a level similar to that for $P_{60/62/64}$ mutant (lane 3), which was approximately 75% of the wt P protein level. The level of replication supported by P_{RRR} mutant (lane 6) was significantly below the wt level. Upon longer exposure of the gel, it was possible to detect replication products, which represented approximately 5% of the level of replication supported by the wt P protein. Increasing amounts of P_{RRR} did not increase the level of replication of DI RNA significantly (data not shown). Thus, these results show that the P protein with other amino acid (except arginine) substitutions at positions 60, 62, and 64 can still function relatively well in supporting RNA replication in vivo.

We next examined the ability of these mutant proteins to support transcription in vivo. Transcription activity of these mutant P proteins was analyzed as described before. Results of this experiment are shown in Fig. 6B. P_{EEE} (lane 4) and P_{TTT} (lane 7) proteins supported significantly high levels of transcription, whereas transcription activity of P_{FFF} and P_{RRR} mutants was at a level below detection (lanes 5 and 6). Upon longer exposure of the fluorogram, it was still not possible to detect transcription products supported by these mutants. A quantitative measurement of the relative levels of replication and normalized levels of transcription supported by these mutants was performed from the data shown in Fig. 6A and B and from two other similar experiments, using the formula as de-

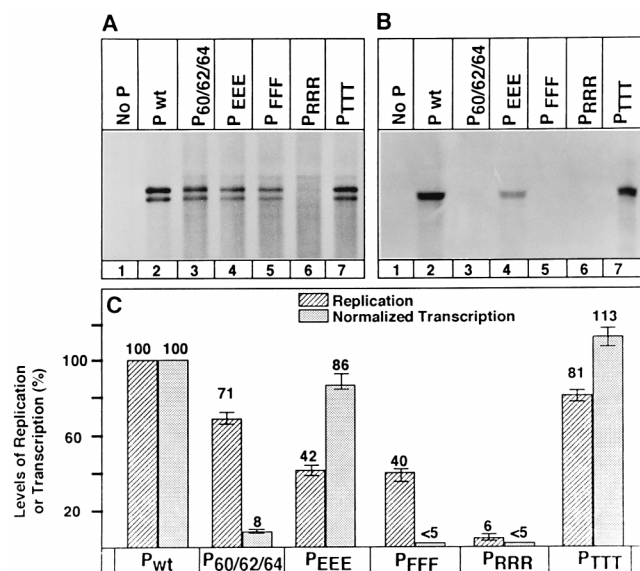


FIG. 6. Replication and transcription supported by mutant P proteins substituted for other amino acids. (A) Replication assay performed as described for Fig. 3A with the mutant P proteins indicated above the lanes. (B) Transcription analysis with the indicated mutants, using the assay described for Fig. 4A. (C) Quantitative determination of the levels of replication and normalized levels of transcription supported by the mutants. The relative level of replication supported by each of the mutants was obtained by taking the average of the scanning data from three separate experiments. The normalized level of transcription supported by the mutants was also obtained by taking the average of the scanning data from three separate experiments and taking into consideration the relative level of replication and transcription supported by each mutant as described in Materials and Methods.

scribed in Materials and Methods. The results are shown in Fig. 6C. We determined that the P_{EEE} and P_{TTT} mutants were almost (86 and 113%, respectively) as active as the wt P protein in transcription in vivo. It should be noted that the P_{TTT} mutant is phosphorylated in vitro by recombinant CKII as well as by the CKII purified from BHK cells. The phosphate-to-protein ratio for P_{TTT} is similar to that of the wt P protein (data not shown), indicating that the threonine residues in P_{TTT} are phosphorylated. The level of transcription supported by P_{FFF} and P_{RRR} could not be determined definitely in the absence of any detectable transcription products even upon very long exposure of the gel. Since P_{FFF} supports replication at a level similar to that for P_{EEE}, transcription at a level of $\geq 5\%$ could have been detected for this mutant. Although P_{RRR} supports replication only to 5 to 6% of the wt level, transcription at a level $\geq 5\%$ of the wt level also could have been detected for this mutant. From these results, we estimate that both P_{FFF} and P_{RRR} support transcription at a level $< 5\%$ of the wt P protein level.

From these experiments, we conclude that while substitution with glutamic acid or threonine residues at the phosphate acceptor sites in domain I of the P protein does not affect the in vivo transcription activity of the P protein, substitution of phenylalanine, arginine, or alanine residues at these sites renders the protein significantly inactive in transcription.

Mutant P proteins do not act as transdominant repressors of transcription or replication. Since some of the mutant P proteins are found to be significantly defective in supporting replication and/or transcription, we wished to determine whether these mutant P proteins, when coexpressed along with

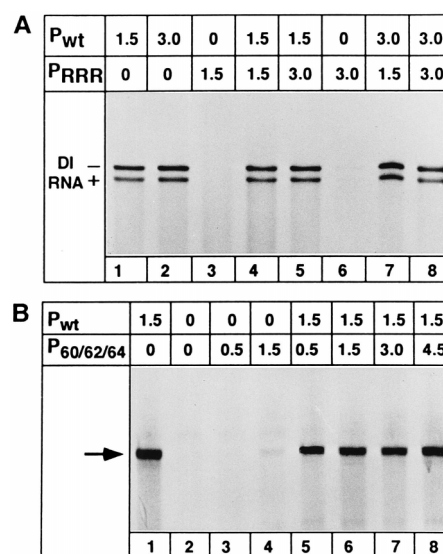


FIG. 7. Effect of coexpression of wt P protein and mutant P proteins on replication and transcription. (A) For replication assay, various amounts (micrograms) of plasmid encoding wt P or mutant P_{RRR} (as indicated above the lanes) were transfected either individually or together, and DI RNA replication in these transfected cells was analyzed as described for Fig. 3A. — and + represent genomic and antigenomic DI RNAs. (B) For transcription analysis, various amounts (micrograms) of plasmids encoding wt P or P_{60/62/64} were transfected either individually or together, and transcription of NΔL mRNA (indicated by the arrow) in these cells was studied as described for Fig. 4A.

the wt P protein, will interfere with the normal functioning of the wt P protein and therefore act as transdominant repressors. For these studies, we chose P_{RRR}, which supports replication at approximately 5% of the wt P protein, and P_{60/62/64}, which supports transcription at about 2 to 3% of the wt level.

To examine whether P_{RRR} acts as a transdominant repressor in replication supported by the wt P protein, replication of DI RNA in cells coexpressing both P_{RRR} and the wt P protein was analyzed. Results shown in Fig. 7A demonstrate that P_{RRR} protein alone supports very low levels of replication (lanes 3 and 6), further confirming the results shown in Fig. 6A. However, when P_{RRR} is coexpressed along with the wt P protein, the level of replication supported by the wt P protein was not decreased (lanes 4, 5, 7, and 8). Furthermore, increasing P_{RRR} expression by increasing the amount of the plasmid in transfection did not result in any measurable inhibition of RNA replication supported by the wt P protein. These results, therefore, indicate that the P_{RRR} protein does not act as a transdominant repressor to inhibit the function of the wt P protein in replication in vivo.

To determine whether P_{60/62/64} acts as a transdominant repressor of transcription in vivo supported by the wt P protein, we analyzed transcription in cells coexpressing the P_{60/62/64} and the wt P proteins. Results of this experiment are shown in Fig. 7B. As can be seen from this figure, very low levels of transcription were observed for P_{60/62/64} (lane 4). The level of transcription supported by a constant amount of the wt P protein in the presence of increasing amounts of P_{60/62/64} (lanes 5 to 8) was comparable to the level of transcription supported by the same amount of the wt P protein alone (lane 1). The inability of P_{60/62/64} protein to inhibit transcription in vivo supported by the wt P protein demonstrates that the mutant P protein does not act as a transdominant repressor of transcription in vivo.

DISCUSSION

The phosphoprotein P of VSV 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 the N protein to form N-P complexes that are required for encapsidation of nascent RNA chain during replication (34, 47). In virus-infected cells as well as in the virion core, the P protein has been shown to exist in multiple phosphorylated forms (7, 14, 27, 28, 32, 41). In this study, we examined the role of phosphorylation within the amino-terminal acidic domain I of the P protein of VSV (Indiana serotype) in transcription and replication under *in vivo* conditions. Using a panel of mutant P proteins in which the phosphate acceptor sites were replaced with alanines or other amino acids either individually or in combination, we analyzed the ability of each of the mutant proteins to support replication of DI RNA *in vivo* and transcription of VSV mRNA *in vivo*. From the results documented here, we conclude that although phosphorylation of P protein in the domain I residues is not essential for replication function of the protein, this modification is critical for its transcriptional activity. Using a similar panel of mutant P proteins, Spadafora et al. recently concluded that phosphorylation of the domain I residues of the Indiana serotype P protein does not play a major role in DI RNA replication *in vivo* (50). Our results presented in this report confirm their results of *in vivo* DI RNA replication studies.

Over the last several years, we and others have been studying the role of phosphorylation of P protein, using *in vitro* transcription reconstitution systems. The conclusions drawn from these studies have been somewhat conflicting. Recent studies suggest that phosphorylation of residues within the domain I of the P protein of Indiana serotype by CKII is essential for its *in vitro* transcription function (24–26). However, recently it was also shown that phosphorylation *per se* is not essential for transcription *in vitro*, and the authors proposed that such a modification may enable the protein to efficiently multimerize and interact with the L protein (50). These conclusions were based on the observation that the double mutant S60A/S64A (same as the P_{60/64} mutant in our study) could rescue transcription to about 28% of the wt P protein at high concentrations. Under the *in vivo* transcription conditions used here, however, we were unable to detect significant levels of transcription in assays using either P_{60/62/64} or P_{60/64} (Fig. 4 and 5). The inherent difference between the *in vivo* transcription assay used by us and the *in vitro* reconstitution assay used by Spadafora et al. (50) may explain the discrepancy in the results of these studies. It is possible that the S60A/S64A mutant does not result in a completely nonphosphorylated form of the protein. Although it was suggested that the T-62 residue is not phosphorylated in this mutant (30, 50), phosphorylation and subsequent dephosphorylation of this residue could not be ruled out. In fact, this may appear to be the case, since we have recently shown that this mutant can be phosphorylated *in vitro* (13). It is noteworthy that recently we have expressed L protein and the mutant P proteins either separately or together in COS cells by using a simian virus 40-based vector and have carried out transcription reconstitution *in vitro* with the cell extracts (41a). The results from these studies indicate that P_{60/62/64} mutant is barely (5 to 10%) active in supporting transcription, confirming our *in vivo* transcription results presented here. Thus, phosphorylation within domain I seems to be important for transcription function of the P protein.

Regardless of the type of transcription system used, it is clear from several studies that multimerization of P protein is

critical for its transcriptional activation (16, 24, 25). The multimerization of P has been shown to be absolutely required for interaction with the L protein as well as the N-RNA template. Furthermore, phosphorylation of domain I residues by CKII has been shown to be required for self-association of P into multimeric structures (16, 24, 25). Therefore, based on these results, it has been proposed that transcriptional activation of P protein occurs as a result of phosphorylation by CKII leading to multimerization and interaction with the L protein. It is possible that under the *in vitro* transcription conditions (50), the S60A/S64A mutant protein without phosphorylation assumes a conformation that favors low levels of multimer formation to support the observed level (28%) of transcription. However, we tend to favor the view that in a more relevant *in vivo* transcription condition used in our experiments, the unphosphorylated protein assumes a conformation unfavorable for multimer formation and therefore is transcriptionally inactive. The mechanism by which phosphorylation leads to multimerization is not known; however, phosphorylation of domain I residues by CKII has been shown to significantly increase the α -helical content of the P protein of New Jersey serotype VSV (16). Whether the increased α -helical content of the protein leads to a conformation favorable for multimerization or not remains to be determined.

It is interesting that the phosphate acceptor sites in domain I reside in a region that contains a large number of acidic residues (58 DDSDTESEPE 67). Phosphorylation of S and T residues (shown in boldface) may increase the net negative charge within this region such that the protein can assume a conformation compatible for multimerization and subsequent transcriptional activation. Indeed, substitution of S and T residues with aspartic acid (negatively charged) residues results in a protein that multimerizes and functions well in transcription (25), whereas substitution with alanine leads to a protein that does not multimerize and remains transcriptionally inactive (24, 25). Our results (Fig. 6) provide further credence to this hypothesis. Substitution with a different negatively charged residue (glutamic acid) resulted in a protein that was almost (86%) as active as the wt protein, whereas substitution with a positively charged residue (arginine) led to a transcriptionally inactive (<5%) protein. Furthermore, substitution with threonine (which is phosphorylated by CKII [data not shown]) also led to a protein that was at least as active (113%) as the wt protein in transcription. From these results, it appears that increase in net negative charge in this region of the protein as a result of phosphorylation is crucial for its transcriptional activation.

The observation that multimerization of P protein mediated by phosphorylation within domain I is required for interaction with L protein and subsequent transcriptional activation whereas the nonphosphorylated form of the protein cannot multimerize but can function as well as its phosphorylated counterpart in replication raises one interesting possibility as to the molecular structure of the transcriptase and the replicase of the virus. Since the L protein must interact with the P protein to carry out these functions, we propose (Fig. 8) that the monomeric form of a subset of P protein (P0 or P2), by interacting with L protein directly or indirectly, forms a complex that functions as a replicase. An association with the soluble N-P complex may be important for the replicase to function. On the other hand, the L protein, by interacting with a phosphorylation-dependent multimer of P1 protein, forms a complex that functions efficiently as a transcriptase (Fig. 8). The composition of the P multimer is uncertain; however, dimeric structures of P New Jersey serotype (16) and probably tetrameric structures of P Indiana serotype (25) have been

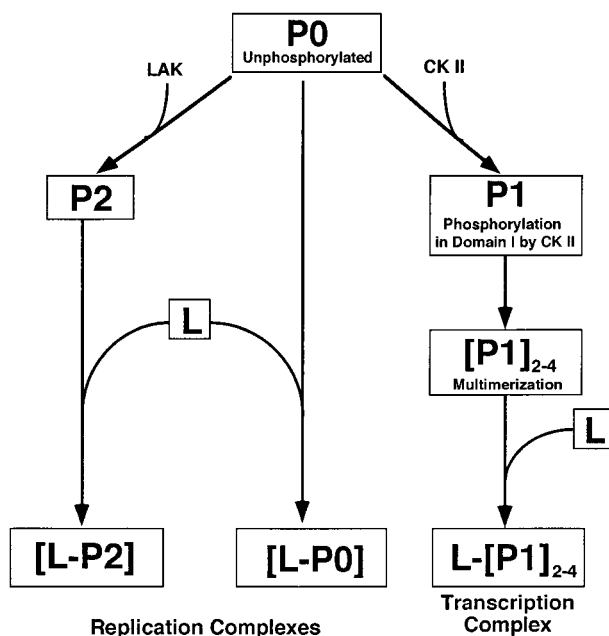


FIG. 8. Proposed structures for replication and transcription complexes of VSV based on the phosphorylation status of the P protein. P0, P1, and P2 are differentially phosphorylated forms of the P protein. L is the large subunit of the polymerase complex. LAK, L protein-associated kinase.

recently documented. Thus, it seems that different phosphorylated forms of the P protein (P0, P1, and P2) may form discrete complexes with the L protein and regulate the function of the latter during transcription and replication. It is noteworthy that for many negative-strand RNA viruses, the P protein is phosphorylated (6, 8, 15, 17, 19, 42, 43, 53), and phosphorylation has been shown to modulate the transcription activity of the protein (6, 8, 17, 43). It remains to be seen whether the phosphorylation status of the P proteins of these viruses has such distinct effects on transcription and replication as seen with VSV.

Supporting evidence for distinct forms of P protein involved in replication and transcription was initially provided by the identification of a temperature-sensitive mutant of VSV with a lesion in the P gene that could transcribe but not replicate the genome at the nonpermissive temperature (52). Recently, Richardson and Peluso (49), using a panel of monoclonal antibodies, have provided data that the P protein that functions in replication is distinct from the one that functions in transcription. A monoclonal antibody that reacts with P protein which is not phosphorylated at some sites has been shown to inhibit genome replication without affecting transcription (49). These results provide further support for another recent observation that hypophosphorylated form of the P protein functions in replication (11). The results presented in this report and the data from previous studies described above strongly suggest that structurally and functionally distinct forms of P protein are responsible for transcription and replication functions of the polymerase complex.

The inability of P_{60/62/64} and P_{RRR} to act as transdominant negative mutants of transcription or replication *in vivo* indicates that these mutant proteins perhaps do not interact with the wt P protein or the L protein. This observation was unexpected since the phosphorylation-defective P mutant of New Jersey serotype VSV acts as a transdominant negative mutant

and inhibits the activity of the wt P protein in an *in vitro* transcription reconstitution system (16). It will be interesting to find out whether other mutant P proteins act as transdominant repressors.

In summary, our results suggest that phosphorylation within the amino-terminal acidic domain I of the P protein appears to be critical for the transcription function of the protein whereas such a modification is not necessary for its replication function. Based on these results, we propose that the L protein of VSV manifests its transcriptase and replicase activities by complexing with differentially phosphorylated forms of the P protein. With the availability of infectious molecular clones of VSV (36, 55), it will be interesting to analyze the effects of these mutations in P protein in transcription and replication in the context of infectious VSV.

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