JOURNAL OF VIROLOGY, July 1996, p. 4538–4548 Vol. 70, No. 7
0022-538X/96/$04.00+0

Constitutive Phosphorylation of the Vesicular Stomatitis Virus P Protein Modulates Polymerase Complex Formation but Is Not Essential for Transcription or Replication

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Received 1 February 1996/Accepted 11 April 1996

As a subunit of both the P-L polymerase complex and the P-N assembly complex, the vesicular stomatitis virus (VSV) P protein plays a pivotal role in transcription and replication of the viral genome. Constitutive phosphorylation of this protein is currently thought to be essential for formation of the P-L complex. We recently identified the three relevant phosphate acceptor sites in the VSV Indiana serotype P protein (R. L. Jackson, D. Spadafora, and J. Perrault, Virology 214:189–197, 1995). We now report the effects of substituting Ala at these acceptor sites on transcription reconstitution in vitro and replication of defective interfering virus (DI) templates in vivo. The singly substituted S60A, T62A, and S64A mutants and the doubly substituted S60A/T62A and T62A/S64A mutants, all of which retain some constitutive phosphorylation, were nearly as active as the wild type in both assays. Surprisingly, the nonphosphorylated S60A/S64A protein was also active in transcription (≥28%) and replication (≥50%) under optimal conditions. However, this mutant was much less active in in vitro transcription (≤5% of wild type) at low P concentrations (<27 nM). In addition, S60A/S64A required higher concentrations of L protein than did the wild type for optimal DI replication in vivo. DI replication efficiency and intracellular accumulation of L, P, and N proteins in the transfected system were very similar to those in VSV-infected cells. We conclude that P protein constitutive phosphorylation is not essential for VSV RNA synthesis per se but likely plays an important role in vivo in facilitating P multimerization and possibly P-L complex formation.

Transcription and replication of the 11-kb, negative-stranded vesicular stomatitis virus (VSV) genome require a virus-encoded polymerase complex that contains two subunits: the large L protein (241 kDa), which carries active sites for polymerization and accessory activities such as transcript capping and methylation, and the P protein (30 kDa), which is required for binding of the polymerase complex to the template. The latter consists of the viral genome tightly assembled with the nucleocapsid protein N (50 kDa). Transcription takes place sequentially along the N-RNA template beginning with the leader gene at the 3′ end and reinitiating at each gene junction. Replication requires coassembly of nascent strands by a P-N assembly complex and readthrough of all gene junctions by the P-L complex (for a review, see reference 1).

Several reports in the last few years have strongly implicated an essential role for P protein phosphorylation in VSV transcription. P protein produced in bacteria lacks phosphates and does not reconstitute transcription in vitro when mixed with purified L and N-RNA template unless first modified by casein kinase II (CKII) (2, 3, 17). The acceptor sites for this in vitro modification have been mapped to Ser-59 and Ser-61 in the New Jersey VSV serotype P protein (31) and to Ser-60 and Thr-62 in the Indiana serotype (17). Without phosphorylation of at least one of these sites, the bacterial P is unable to multimerize in vitro (15, 17) and, at least for the Indiana protein, cannot bind to L protein and N-RNA template (18). Whether the VSV P protein forms dimers, trimers, or perhaps even tetramers is still unresolved (15, 17, 18). In the case of the Sendai paramyxovirus, which transcribes and replicates its genome very much like VSV, Curran et al. (13) have recently provided evidence that P is a trimer.

Some studies suggest that CKII-mediated phosphorylation of the VSV P protein also takes place in the context of mammalian cells in vivo. Ala substitution at Ser-59 and Ser-61 in the New Jersey VSV P protein expressed in COS cells eliminates essentially all constitutive phosphorylation, and when this mutant construct is translated in wheat germ lysates, the protein is inactive in transcription reconstitution (31). Likewise, we showed recently that Ser-60 and Thr-62 of Indiana P are phosphorylated in vivo when expressed in BHK cells via the vaccinia virus-T7 system. In this case, Ser-64 must first be phosphorylated before Thr-62 is modified (21).

The picture which has emerged from the above-described studies is that the P protein must first be phosphorylated by cellular CKII before it can multimerize and in turn bind to L protein and the N-RNA template. However, at least two reports suggest that this phosphorylation is not as essential as now believed. When the N-terminal domain of the New Jersey VSV P protein was replaced by the acidic domain of β-tubulin, the protein produced in rabbit reticulocyte lysates was reported to be 65% as active as wild-type protein in reconstitution studies (11). Likewise, a Sendai virus P protein construct lacking a large portion of its N-terminal domain and at least 90% of its normal phosphate content was shown to be fully active for transcription reconstitution in vitro following expression in the vaccinia virus-T7 system and was 65% as active as the wild type for defective interfering virus (DI) replication in vivo (14).

Barik and Banerjee (4) have shown that following conversion of bacterial P0 to the P1 species using CKII in vitro, the latter can be further modified to a P2 species by a kinase associated with L protein. A similar conversion of P1 to P2 also takes place with the purified virion transcription complex in

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vitro (7) and to a more limited extent in vivo (12, 21). This cascade phosphorylation was proposed to be essential for transcription (4), but the need for the second phosphorylation has been called into question in other studies (7, 21, 23).

We have examined here the effects of Ala substitutions at the three Indiana P protein acceptor sites identified in our earlier study (21) on transcription reconstitution in vitro and DI replication in vivo. We find that lack of constitutive phosphorylation has only a modest effect on either transcription or replication. However, higher concentrations of these proteins are required, presumably to drive P-L complex formation. We also show that the amounts of L, P, and N proteins accumulating intracellularly in VSV-infected BHK cells are comparable to those obtained in the vaccinia virus-T7 expression system under conditions where lack of P constitutive phosphorylation has relatively little effect on VSV RNA synthesis.

(The findings described here were presented in part at the 14th Annual Meeting of the American Society for Virology at the University of Texas at Austin, 8 to 12 July 1995.)

MATERIALS AND METHODS

Cells, plasmid constructs, and transfections. BHK cells were grown to ~60 to 80% confluency in 5-cm-diameter tissue culture dishes in minimal essential medium containing 7% calf serum. Vaccinia virus VTF7.3 infection (multiplicity of infection = 10) and cotransfections with the indicated amounts of Indiana VSV P, N, and L plasmid constructs were carried out as before (21) using a cationic lipid mixture (dioleoyl-L-α-phosphatidylethanolamine and dimethyldioctadecylammonium bromide) prepared according to the method of Rose et al. (28). The wild-type and mutant P constructs, as well as the wild-type L plasmid, were described previously (8, 21). The wild-type N gene construct was obtained by subcloning the *Xho*I fragment from the pSV2neoRSVN plasmid (27), kindly provided by Lynn Puddington and Jack Rose, into the *Sal*I site of pGEM1 vector (Promega).

In vitro transcription assays. The in vitro transcription assay procedure was identical to that described previously (8) except as follows. Cells cotransfected with the amounts of P and L plasmids indicated below (salmon sperm DNA added to bring the total transfection amount to 15 or 20 μg) were incubated for 10 h at 37°C before extraction by the lysolecithin method in a total volume of 150 μl per 5-cm-diameter plate. Experiments reported in Fig. 1 and 2 also included 40 μg of 1-β-D-arabinofuranosylcytosine (AraC; Sigma) per ml in all solutions including the vaccinia virus VTF7.3 inoculum until the time of extraction. Transcription reactions were carried out as before, except they used 15- or 30-μl volumes and a higher proportion of cell extract (two-thirds) and also included 0.5 U of RNasin (Promega) per μl. The N-RNA template concentration in the experiments of Fig. 1 and 2 (~100 ng/15 μl) was slightly less than half that used previously.

N-RNA templates were purified as before (19) except for one additional chromatography step through an 8-ml column of Sepharose CL-6B (Sigma) in 10 mM Tris-Cl (pH 7.7)–1 mM EDTA followed by pelleting through a 50% glycerol cushion containing 20 mM Tris-Cl (pH 8.1)–100 mM NaCl–1 mM EDTA for 12 to 16 h at 40,000 rpm and 4°C in a Beckman SW50.1 rotor. The pellet was resuspended in 20 mM Tris-Cl (pH 8.1)–1 mM EDTA–2 mM dithiothreitol–10% glycerol and stored at −80°C. No P protein was detectable in these purified N-RNA templates by Western blot (immunoblot) analysis (not shown). Incorporation of [α-^{32}P]UTP and analysis of transcripts on agarose gels following glyoxal denaturation were described previously (8).

In vivo DI replication assays. The DI virus (DI 0.22, also called MS-T) was described previously and contains a copy-back genome of ~2,500 nucleotides derived from the 5′ end of the standard genome (26). DI particles (~20 ng of virus protein), purified free of infectious virus as before (26) and resuspended in minimal essential medium, were added directly to the 1-ml volume of liposome-DNA mixture 4 h after cotranfection with the indicated amounts of P, L, and N plasmids as described above (salmon sperm DNA added to bring the total transfection amount to 15 μg). An additional 1 ml of minimal essential medium was added after 30 min, and incubation at 37°C was continued for ~20 h. AraC was included in all solutions as described above.

Following the above incubation, cells were rinsed with saline and then lysed with 225 μl of 50 mM Tris-Cl (pH 7.8)–150 mM NaCl–0.5% Nonidet P-40 detergent. Lysates were centrifuged at 1,000 × *g* for 5 min at 4°C to remove nuclei, adjusted to ~1 mM $CaCl_2$, and then treated with 2.5 μg of micrococcal nuclease (Sigma) per ml at 37°C for 15 min to digest unencapsidated nucleic acids. EDTA and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were then added to final concentrations of 12 and 2.5 mM, respectively; this was followed by digestion in the presence of ~500 μg of proteinase K per ml and ~1% Sarkosyl detergent at 55°C for 10 min. Nucleic acids were then purified by phenol-chloroform extraction and ethanol precipitation and separated on agarose gels after glyoxal denaturation as described above for transcription products.

Northern (RNA) blot analysis was carried out by first transferring nucleic acids onto nylon membranes (Qiabrane Nylon Plus) by capillary elution overnight and then treating blots at 65°C for 20 min in 20 mM Tris base to reverse glyoxylation. The hybridization probe consisted of an [α-^{32}P]UTP-labelled T7 transcript containing positive sense sequences from the VSV L gene. The transcript was synthesized from a plasmid containing a portion of the wild-type L gene constructed by deleting the *Nsi*I restriction fragment (nucleotides 286 to 2559 of the L gene) from the full L gene-containing pGEM-LS1 plasmid (8). The T7 plus sense labeled probe was synthesized following linearization of the pGEM-$L_{\Delta Nsi}$ plasmid with *Hin*dIII, purified by spin column chromatography over G-50 Sephadex, and used directly for hybridization overnight at 65°C in a buffer containing 0.5 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, 7% sodium dodecyl sulfate (SDS), and 5 mg of bovine serum albumin per ml. The blot was washed first at room temperature and then at 65°C in 1× or 0.5× standard saline citrate–0.1% SDS for 1 to 2 h and then developed for film autoradiography. In all cases, the blot was also quantitated by PhosphorImager analysis (Molecular Dynamics).

VSV infections. In experiments comparing VSV infections with the vaccinia virus expression system, BHK cells were infected at a multiplicity of infection of 10 with the VSV Indiana Mudd-Summers virus isolate, with or without the same inoculum of DI used in the vaccinia virus-driven assay. In all cases, cells were lysed and analyzed for viral proteins and DI RNA synthesis as described above in the DI replication assay.

SDS-PAGE and Western blots. A small portion of the cell extracts used in transcription assays and those obtained from the replication assays before addition of $CaCl_2$ and micrococcal nuclease (see above) were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting as described previously using polyclonal antibody to gel-purified L, P, and N proteins (all kindly provided by Don Summers, University of California, Irvine). Estimates of viral protein amounts were calculated by comparison with parallel lanes containing purified virions which contain relative protein weights of 4.8, 6.2, and 30% P, L, and N, respectively (8, 33).

RESULTS

Effects of P protein acceptor site mutations on in vitro transcription reconstitution. Extracts from cells expressing VSV L and P proteins via the vaccinia virus-T7 system faithfully and efficiently reconstitute transcription when added to purified N-RNA templates in vitro (8). We recently showed that Ser-60, Thr-62, and Ser-64 together account for all constitutive phosphorylation of the P protein in this system (21). We next wanted to probe the role of this phosphorylation in transcription using the phosphate acceptor site mutants characterized in our previous study. Initial experiments revealed that transfection efficiencies had a strong effect on the transcription activity obtained with some of our mutants (see below). We therefore compared all mutant versus wild-type activities together in the same experiment with various amounts of input P plasmid. This enabled us to assess the effects of P protein concentration, which was monitored by Western blot analysis in each case.

When transfection conditions resulted in maximum P protein expression, data such as those shown in Fig. 1 and 2 were obtained. Transcription activity (incorporation of labeled UTP into VSV transcripts) remained more or less constant as the amount of input P plasmid was increased from 1 to 10 μg. The Western blot shows that P protein amounts increased as a function of plasmid input in all cases. Excess P protein was therefore not inhibitory for transcription under these conditions. For all single Ala-substituted mutants, transcription was affected only moderately compared with the wild type (Fig. 1). S60A, T62A, and S64A substitutions yielded 76, 71, and 63% of wild-type activity, respectively (average of all four P plasmid concentrations in each case). All these single-site-substituted mutants retain about one-half to two-thirds of wild-type P phosphate content, and because Thr-62 phosphorylation depends on prior modification of Ser-64, only Ser-60 retains phosphate in the S64A mutant (21). We therefore conclude that a lack of phosphorylation at any one of the three sites, or phosphorylation of Ser60 only, is sufficient to give about two-

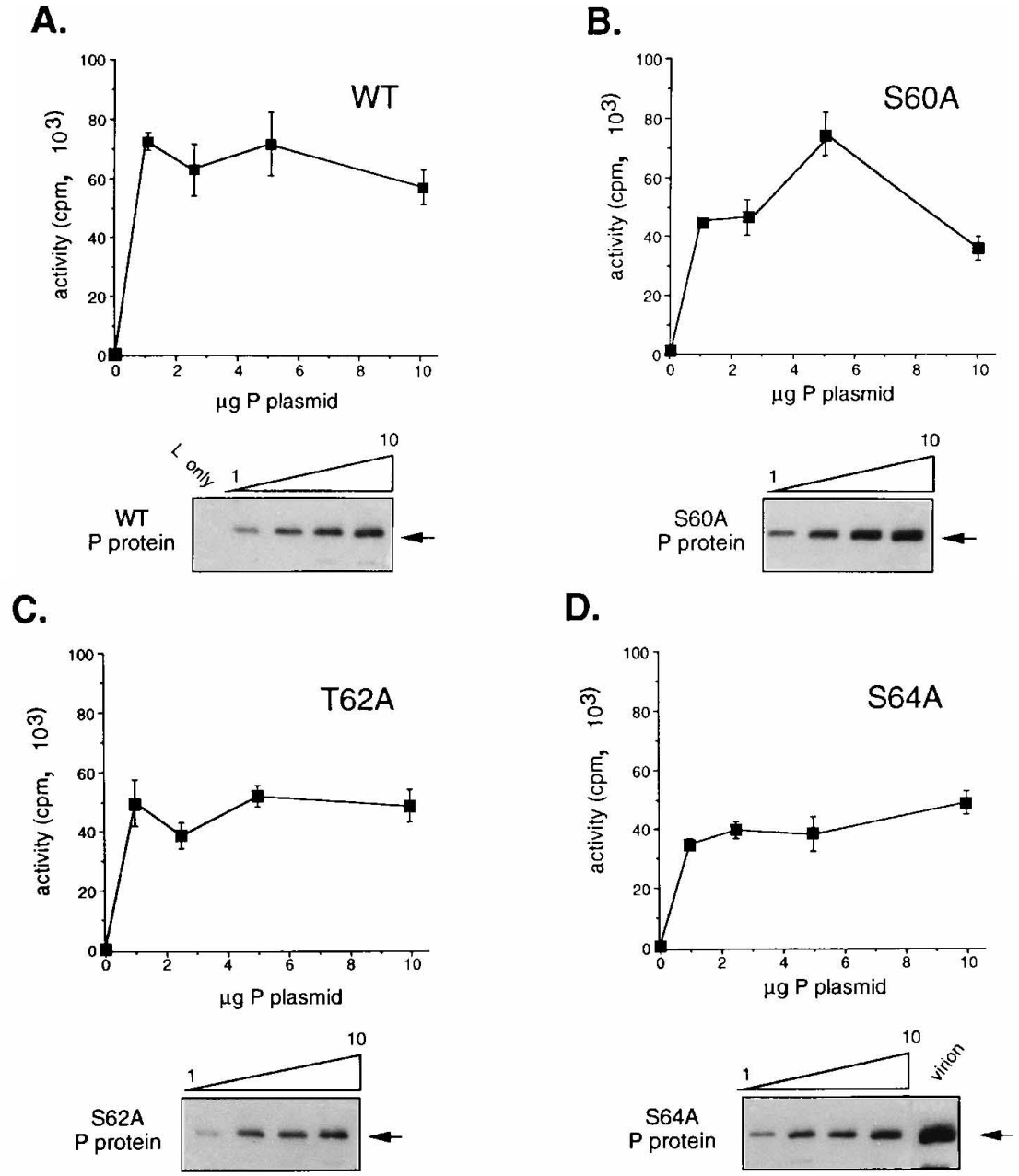

FIG. 1. Comparison of transcription activities in the presence of wild-type (WT) versus mutant P proteins containing Ala replacements at either S-60, T-62, or S-64. Cells were cotransfected with 10 µg of L plasmid and various amounts of P plasmid as indicated on the *x* axis. A Western blot of P protein in each extract is shown below each graph. Activity values refer to [α-^{32}P]UTP incorporation into VSV-specific transcripts (see Materials and Methods). The average (squares) and range (bars) of two independent determinations for each extract are shown.

thirds of wild-type transcriptional activity. These findings differ from those reported with the New Jersey P protein, in which mutation of either Ser-59 or Ser-61 to Ala eliminated all transcription activity (31).

In the case of the doubly substituted mutants (Fig. 2), both S60A/T62A and T62A/S64A also showed relatively high activity compared with the wild type, i.e., 65 and 70%, respectively. These double-site mutants retain about one-third the amount of phosphate (21), showing again that phosphorylation of only one acceptor site, in this case either Ser-64 or Ser-60, is sufficient to display about two-thirds of wild-type activity. The surprising result, however, was that the nonphosphorylated S60A/S64A mutant was also active in transcription, although less so than the other mutants (28% of wild-type activity). Transcription activity of all mutants was evaluated in several independent experiments over the course of several months. Consistent results were obtained in all cases except for S60A/S64A. That mutant showed far more variation than the others, with values ranging from 3 to 60% of wild-type values in six independent determinations. The reason for this variation will become apparent below. We also tested the triple S60A/T62A/S64A mutant, and it too showed significant but variable activity of up to 10% of the wild-type level (not shown).

It is important to point out that we observed no significant

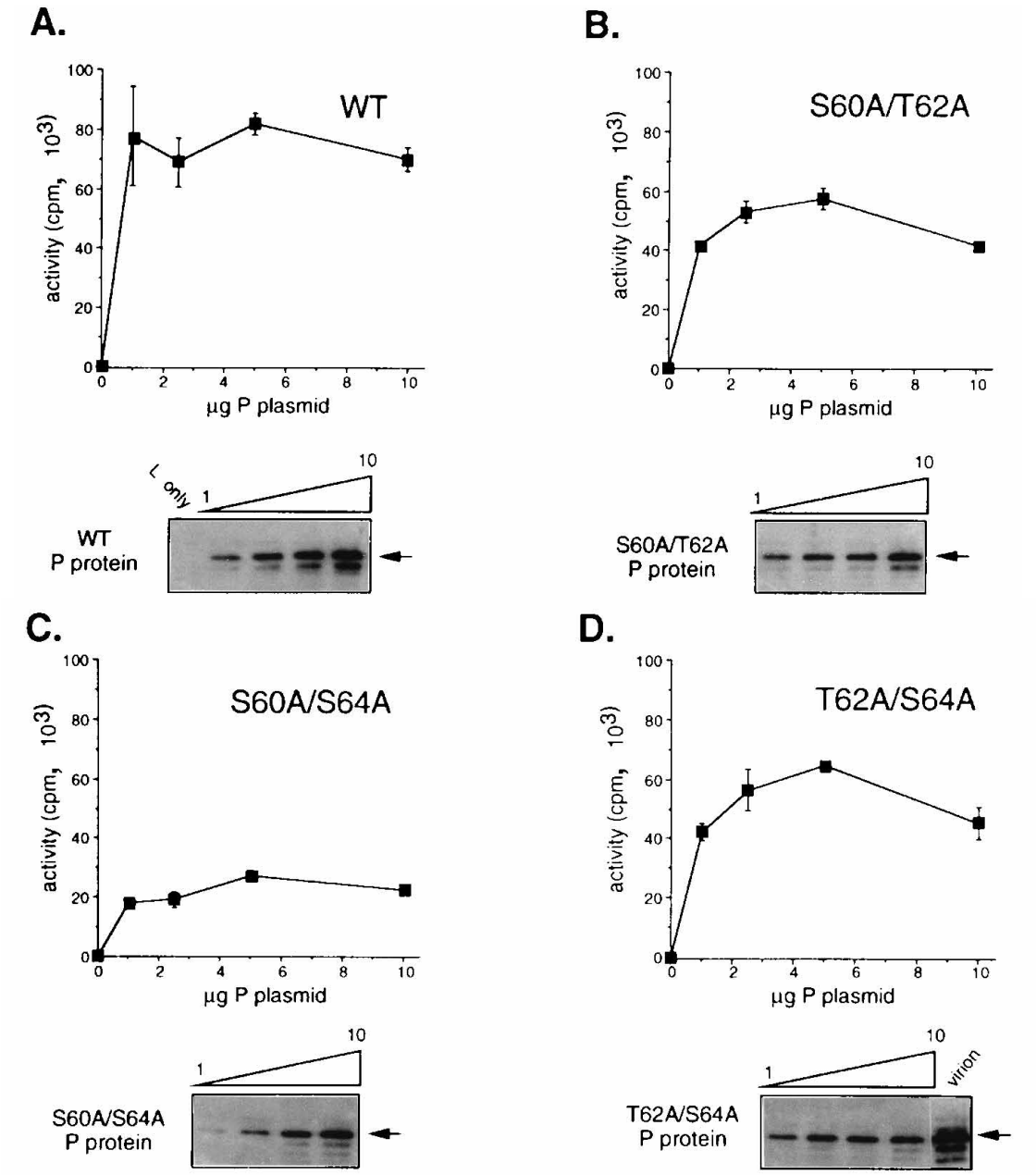

FIG. 2. Comparison of transcription activities in the presence of wild-type (wt) versus mutant P proteins containing double Ala replacements at either S-60 and T-62, S-60 and S-64, or T-62 and S-64 (see Fig. 1 legend for details). Small amounts of P2 and P3 species and/or degradation products can be seen below the major P protein band (21).

background transcription with extracts from cells transfected with L plasmid only (see Fig. 3 below). This negligible background depended on stringent purification of N-RNA template preparations to remove all detectable traces of wild-type P protein (see Materials and Methods). The activities reported here therefore reflect that of the recombinant P protein only.

Transcription activity of S60A/S64A is reduced at low P protein concentrations. Figure 3 shows a comparison of transcription products purified from reactions containing wild-type versus S60A/S64A P protein. No difference in either the size or ratio of products was discernible at all three plasmid inputs used. In the absence of P plasmid in the transfection (lane n), no products were visible (<1% by densitometry scanning). Not surprisingly, other Ala-substituted mutants also showed no difference in the profile of transcription products (not shown). Although a direct analysis of the small leader transcript was not done here, it is nonetheless clear that a nonphosphorylated P protein appears to function just like the wild type in most, if not all, aspects of transcription.

A closer look at Fig. 3 reveals an important difference from the results in Fig. 2. Transcription activity in this case increased with input plasmid. With wild-type P, activity with 1 µg of plasmid was about half that with 9 µg. With mutant S60A/S64A, however, only 5% as much activity was observed at the lowest input. These data suggest that P protein is limiting with low plasmid input, especially for the mutant. In fact, the max-

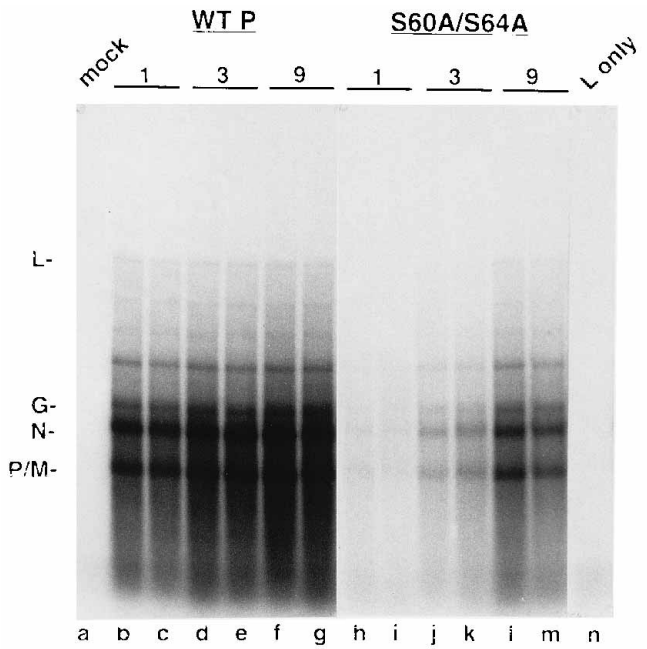

FIG. 3. Transcription products synthesized in vitro in the presence of wild-type (wt) versus nonphosphorylated S60A/S64A P protein. Duplicate transfections were carried out with 6 μg of L plasmid and various amounts of P plasmid (1, 3, or 9 μg). Purified ^{32}P-labelled products were denatured with glyoxal and analyzed by agarose gel electrophoresis (see Materials and Methods). Bands corresponding to L, G, N, P, and M viral mRNAs are indicated. Lane a, control obtained in the absence of P and L plasmids; lane n, result with L plasmid only.

imum level of P protein synthesized in this particular experiment, although similar for both mutant and wild type, was about threefold lower than in Fig. 2 (not shown). Moreover, the transcription reconstitution assay contained twice as much template (see Materials and Methods). More importantly, mutant P activity was proportionately far less relative to wild-type activity at the lowest P protein concentration. This phenomenon is illustrated in Fig. 4A, where mutant P protein activity is plotted relative to wild-type activity as a function of input plasmid. Several independent experiments in which P protein synthesis was relatively low showed the same phenomenon.

To determine whether the phenomenon was in fact due to a P protein concentration effect and not to some other aspect of transfection, we again measured transcription activity of the wild-type and S60A/S64A extracts used in Fig. 2, but this time as a function of dilution with mock extract. The same phenomenon was observed. A 10-fold dilution of wild-type P protein resulted in 34% as much activity as the undiluted extract, while the same dilution of S60A/S64A yielded only 5% as much activity. Mutant activity relative to that of the wild type decreased from 25 to 3%, as illustrated in Fig. 4B. This dilution effect suggests a lower efficiency of nonphosphorylated P-L complex formation when the concentrations of both P and L proteins are reduced. Very similar results were obtained when a constant amount of extract from cells expressing L protein only was mixed with decreasing amounts of extract from cells expressing P only expressing (not shown), which also reconstitutes transcription, albeit less efficiently than extracts from cells coexpressing the proteins (8, 9). We therefore conclude that the nonphosphorylated P protein mutant is significantly less active in transcription than the wild type when P concentration is limiting. This behavior is consistent with a concentration-dependent requirement for P multimerization as proposed by Gao and Lenard (17). Lack of constitutive phosphorylation could in addition have a modest effect on binding of the P multimer to L protein and/or binding of the P-L complex to N-RNA templates. Whatever the case, our results indicate that phosphorylation is not essential for these protein-protein and protein-template interactions and is also not required for the transcription process per se.

Effects of P protein acceptor site mutations on DI replication in vivo. To test whether constitutive phosphorylation of the P protein is necessary for replication, we used the same vaccinia virus-T7 expression system to drive DI replication in vivo as first described by Pattnaik and Wertz (25). In this assay, a plasmid encoding the N protein is also included in the cotransfection step since replication depends on coassembly of progeny strands by the P-N complex. DI replication was monitored by Northern blotting of encapsidated, nuclease-resistant

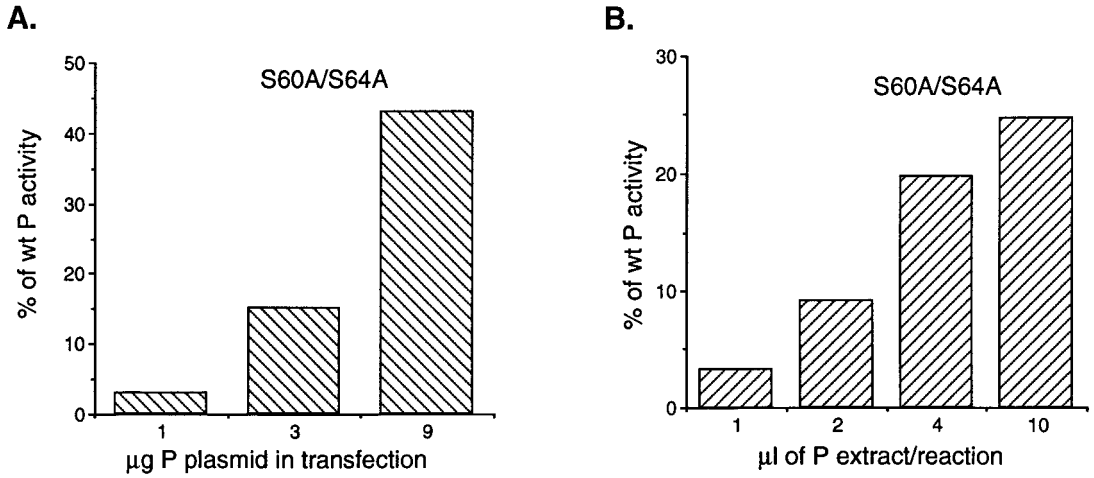

FIG. 4. Effect of P protein concentration on transcription activity of S60A/S64A relative to that of the wild type (wt). (A) Incorporation of [α-^{32}P]UTP label into VSV-specific transcripts in the presence of S60A/S64A P protein as a percentage of the wild-type level for each of the three inputs of P plasmid shown in Fig. 3. (B) Extracts used in Fig. 2C and containing equal amounts of P protein (wild type, 1 μg of input plasmid; S60A/S64A, 2.5 μg of input plasmid) were assayed for transcription activity either without dilution (10 μl/15-μl total reaction volume) or after dilution with mock extract.

RNA using a positive sense probe to detect negative sense L gene sequences present within the copy-back DI genome. Our initial experiments confirmed that the ratio of input L, P, and N plasmids was critical for obtaining optimal replication, as noted by Pattnaik and Wertz (25). A very low input of L plasmid was sufficient to reach maximum DI replication (125 ng versus 6 to 10 μg for transcription). Again, to ensure valid comparisons, each mutant was analyzed in parallel with the wild type over a wide range of P plasmid concentrations in the presence of optimal amounts of L and N plasmids. Note also that all replication assays detailed here were carried out in the presence of AraC (see Materials and Methods). This DNA synthesis inhibitor had no effect on VSV transcription reconstitution but had a marked stimulatory effect on DI replication (30).

Figure 5 shows representative results obtained with two of the single-substitution mutants and two of the double-substitution mutants. Replication activity with wild-type P protein displayed a relatively sharp optimum between 0.6 and 1.3 μg of input P plasmid in all cases. S60A and S64A mutants showed profiles very similar to that of the wild type, with relative peak values of 86 and 83%, respectively (Fig. 5A and B). The other single-site mutant, T62A, also behaved similarly (not shown). In the case of the double mutants, S60A/T62A was 70% as active as the wild type near or at its optimum but was 23 and <5% as active at 2.5 and 5 μg of input P plasmid, respectively. T62A/S64A showed a profile similar to that of the wild type and was 64% as active (not shown). In stark contrast to these results, the nonphosphorylated S60A/S64A mutant displayed only minimal activity, with a maximum of 3.6% compared with the wild type (Fig. 5D).

Western blots showed comparable amounts of P protein in mutants versus the wild type (Fig. 5). The amount synthesized increased with input plasmid and reached saturation at about 1.3 to 2.5 μg of input plasmid in all cases. Optimum replication therefore ensued when P protein levels were slightly below the maximum level. It is not clear why replication is inhibited at higher inputs of plasmid, although the slight excess of P protein could conceivably be responsible. All mutants were also tested in the absence of AraC with similar results, except for overall lower activities (not shown). We therefore conclude that phosphorylation of either Ser-60 or Ser-64 alone is sufficient to yield at least two-thirds of the DI replication activity seen with the wild type, similar to the effects on transcription described above. However, in contrast to transcription, the nonphosphorylated S60A/S64A mutant appeared to be almost completely inactive under these conditions.

Increased expression of L protein rescues DI replication in the presence of the S60A/S64A P protein. The results described above showed that the nonphosphorylated S60A/S64A protein could in fact form a transcriptionally active P-L complex but could only do so at relatively high concentrations of P protein. Since our DI replication assays employed very low levels of input L plasmid, we reasoned that a reduced affinity between P and L protein, in addition to putative effects on P multimerization, might explain the lack of S60A/S64A activity. Alternatively, the replication process might be more dependent on constitutive phosphorylation, either for formation of a unique P-L complex or for formation of the P-N assembly complex. To explore these possibilities, we first tested the effects of increasing L protein expression in the DI replication assay (Fig. 6A). As before, no significant activity was detected with the mutant P protein with a low input of L plasmid (125 ng) whereas optimum activity was observed with wild-type P. However, a fourfold increase in L plasmid rescued S60A/S64A activity to 27% of the wild-type optimum, which remained at this same level up to 5 μg of input plasmid. Replication activity with wild-type P remained near the optimal level from 125 ng up to 1 μg of input L and then decreased to ~30% with 5 μg of input. Independent experiments repeatedly showed an optimum near or at 125 ng of input L plasmid with wild-type P, while the nonphosphorylated P always required at least fourfold higher L inputs to reach its optimum (not shown).

The Western blot analysis in Fig. 6A sheds light on important aspects of DI replication dependence on L and P proteins. As a function of plasmid dose, L protein increased about four- to fivefold in the presence of wild-type P and reached a plateau at about 1 μg of input plasmid. Thus, a substantial increase in L protein was not inhibitory for DI replication. In the presence of the mutant P protein, L protein accumulation was identical to that seen with wild-type P at the lowest doses of L plasmid (125 and 250 ng), but no further increase took place with higher inputs of plasmid. The maximum level obtained was therefore about two- to threefold lower than with the wild type. As expected, P and N protein amounts were identical and constant in both sets of transfections with up to 1 μg of L plasmid (not shown). Maximum replication activity with S60A/S64A coincided with reaching the highest level of L protein, suggesting that, in this case, it is limiting. Similar results were reproducibly obtained in several independent experiments. The lower accumulation of L protein in the presence of S60A/S64A corroborates independent studies showing that coexpression with the mutant S60A/S64A protein stabilizes only 40 to 60% of L protein from degradation in vivo, in contrast to full protection with wild-type P (9, 10).

The results in Fig. 6A indicate that P constitutive phosphorylation is not essential for replication, as in the case for transcription. However, the nonphosphorylated protein requires a higher concentration of L protein for activity, suggesting that phosphorylation somehow facilitates P-L complex formation. Whether this is due to limiting amounts of a putative S60A/S64A multimer in vivo, a reduced affinity between the nonphosphorylated multimer and the L protein, or both is not yet clear (see Discussion). In any case, if one accounts for the lower L protein accumulation, the nonphosphorylated P-L complex once formed is possibly about half as active as the wild type. This same consideration may also apply to the transcription activity of the analogous P-L complex reported in Fig. 2C. The triple mutant also appeared to be active in replication, although significantly less than S60A/S64A, as observed in the transcription assays (not shown). Note that we also verified that coexpression of N and L proteins with S60A/S64A under optimal replication conditions did not change the phosphorylation status (not shown). Enhanced expression of L protein, or binding of S60A/S64A to L or N protein, therefore does not uncover any new sites of phosphorylation. Our earlier study also showed that S60A/S64A remains unphosphorylated when coexpressed with saturating amounts of L protein (21).

Figure 6A also shows a threefold inhibition of replication at the highest levels of L plasmid, but only in the presence of wild-type P. However, accumulation of both P and N proteins was reduced about two- and threefold at 3 and 5 μg of input L plasmid, respectively (not shown). Why high inputs of L plasmid would reduce expression from the others is not clear. Little or no such effect was observed with high inputs of P plasmid, while L accumulation was affected only moderately at the highest inputs of N plasmid (see below). In agreement with the findings of Pattnaik and Wertz (25), we observed a much sharper DI replication optimum in the absence of AraC (30). The data in Fig. 6A, however, suggest that excess L protein per se is not deleterious for DI replication.

DI replication dependence on N protein concentration is

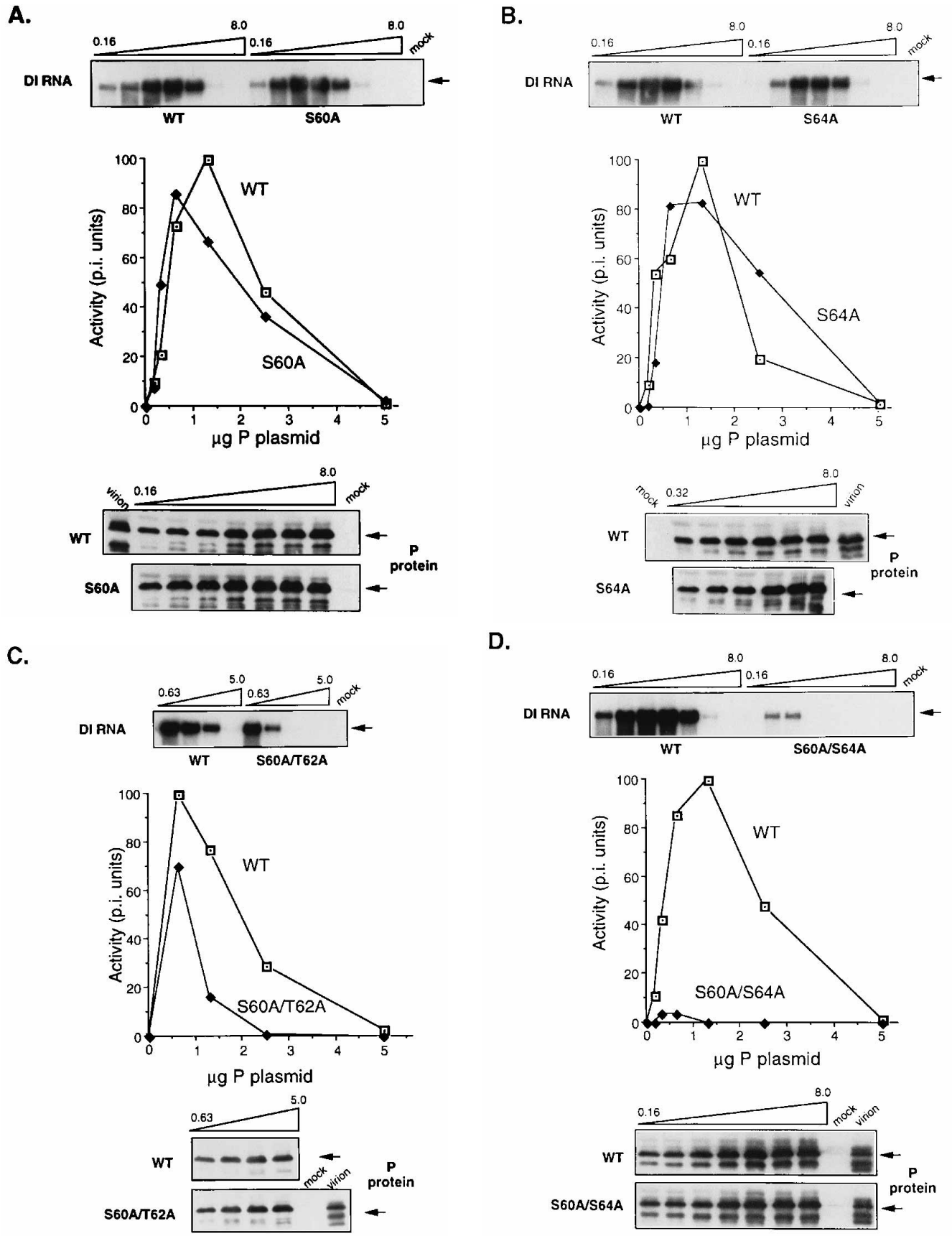

FIG. 5. In vivo DI replication activity in the presence of wild-type (wt) versus mutant P proteins. Autoradiographs of the Northern blots are depicted at the top. Activity values shown in the graphs were determined by PhosphorImager quantitation of the ^{32}P blot. Parts A to D represent separate experiments comparing the wild type with a single P mutant as a function of input P plasmid. In all cases, constant amounts of input L plasmid (125 ng) and N plasmid (2.5 µg) were used. P protein levels are shown in the Western blots below each graph. A single protein gel was used to analyze all samples (wild-type, mutant, and virion lanes) for each experiment except for part D, where wild-type and mutant samples were loaded on two separate gels and processed together. Virion lanes contained 3 µg (A to C) or 1 µg (D) of purified virus.

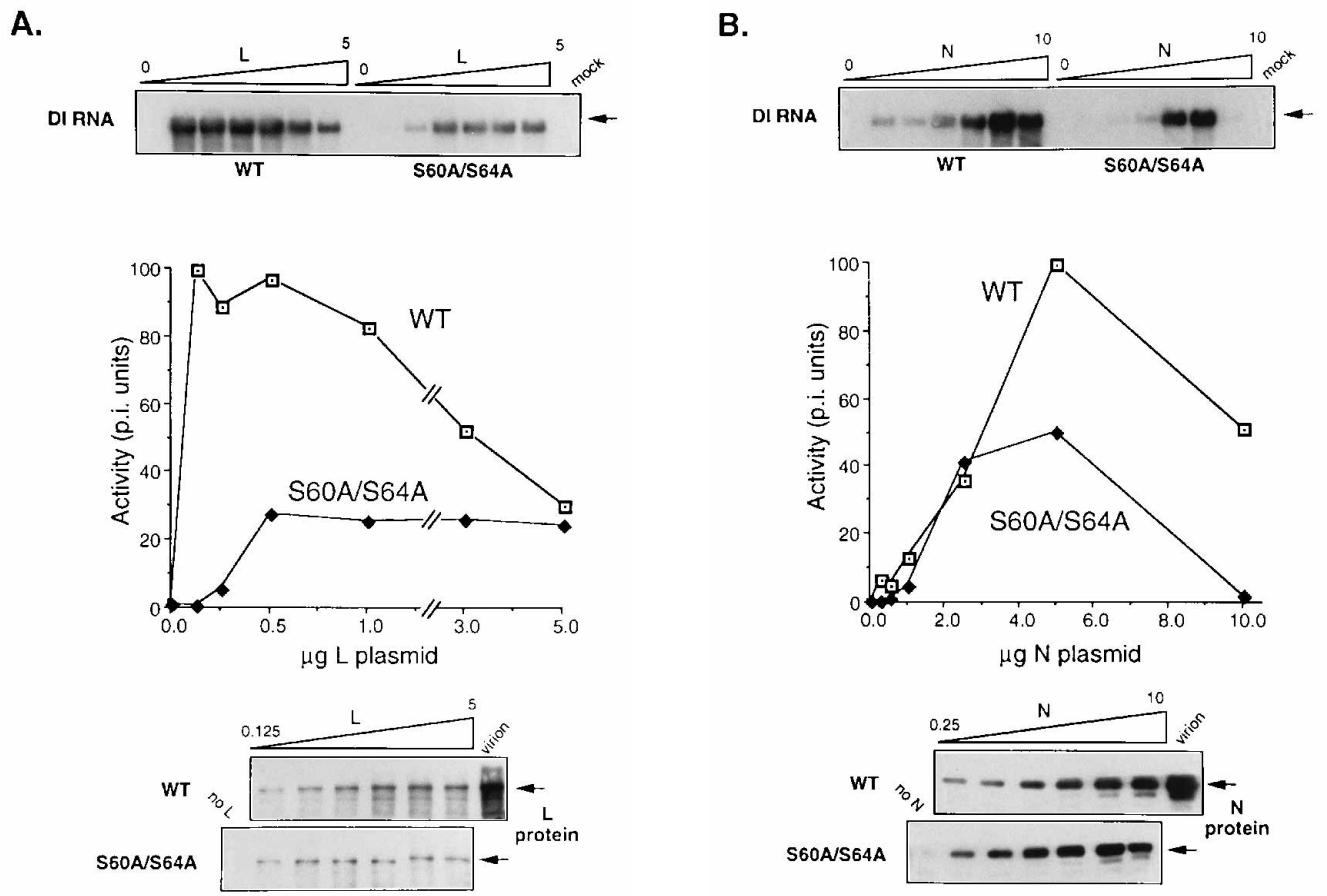

FIG. 6. Increased expression of L protein rescues in vivo DI replication activity in the presence of S60A/S64A P protein. (A) Effect of increasing L plasmid input; (B) effect of increasing N plasmid input. P plasmid inputs were 1.3 μg for the wild type (wt) and 0.64 μg for S60A/S64A in both cases. N plasmid input was 2.5 μg in part A, while L plasmid input was 3 μg in part B. Western blots were analyzed as described for Fig. 5A to C.

similar in the presence of wild-type and S60A/S64A proteins. To assess whether lack of phosphorylation also affects P interaction with N protein, we measured DI replication in the presence of wild-type or S60A/S64A P protein (with a 3 μg of input L plasmid) as a function of increasing amounts of input N plasmid. Peak replication activity in this case was observed at similar input levels of N plasmid (5 μg) for both mutant and wild-type P (Fig. 6B). S60A/S64A activity was equal to wild-type activity at 2.5 μg of input N plasmid (the level used in Fig. 5) and 50% as high at 5 μg of input N plasmid. The Western blot shows that N protein accumulation reached the same saturation level in concert with peak activities in both cases. P protein levels remained constant at all levels of input N plasmid and were essentially the same for mutant and wild-type P (not shown). These results therefore indicate that constitutive phosphorylation of P protein likely has no major effect on its affinity for soluble N protein nor on the activity of the resulting P-N assembly complex.

Activity in the presence of wild-type P was down 50% at the highest level of input N plasmid (10 μg), while it was essentially nil with S60A/S64A. DI replication reproducibly showed greater sensitivity to excess N plasmid with mutant P in several independent experiments. L protein accumulation was about twofold lower with 10 μg of N plasmid (not shown). On the basis of the data in Fig. 6A, lower L protein accumulation would likely affect formation of the P-L complex more severely with mutant P.

Viral protein accumulation is similar in transfected versus VSV-infected cells. One obvious concern in probing protein function with transfected constructs is whether intracellular concentrations reflect physiological levels. We therefore compared L, P, and N protein accumulation obtained under optimal conditions in the vaccinia virus-T7 expression system with that seen in cells coinfected with VSV standard helper virus and DI. The experiment shown in Fig. 7 was carried out with matched cell monolayers in parallel and differed only in time of harvest, i.e., 18 h post-DI addition for transfected cells versus 12 h postcoinfection with helper VSV and DI. PhosphorImager quantitation of the Northern blot (top) indicated that the transfected system replicated DI 51% as well as the natural infection. Remarkably, the Western blot analysis showed comparable amounts of P and N proteins synthesized in both cases. This must also be the case on a per-cell basis since, with the same vaccinia virus expression system, transfection with a T7 promoter–β-galactosidase gene construct routinely resulted in a majority of BHK cells (≥80%) staining positive for β-galactosidase activity (not shown).

The transfection system, however, accumulated much less L protein (below the detection level of this particular blot) than the natural infection (Fig. 7). But a low input of L plasmid (125 ng) was used in this comparison. Nonetheless, since DI replication took place very efficiently, it appears that natural infections accumulate more L protein than is necessary for this

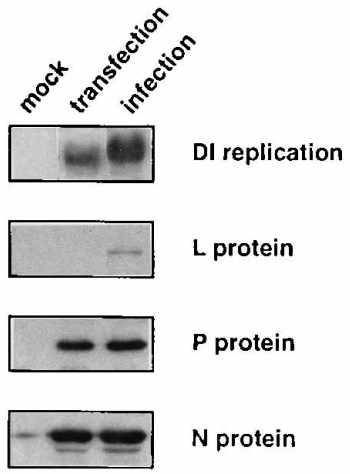

FIG. 7. Comparison of DI replication efficiency and viral protein accumulation in VSV-infected versus transfected cells. Transfection lanes show the results of an in vivo DI replication assay using optimal amounts of input plasmids (125 ng of L, 2.5 μg of N, and 0.64 μg of wild-type P). Infection lanes correspond to the same number of cells infected with VSV standard helper virus and the same DI inoculum and processed in parallel (see text). The Northern blot autoradiograph is shown at the top, and Western blots for all three viral proteins are shown below.

process, at least in the case of BHK cells. In a repeat of this experiment, using a 1-μg input of L plasmid, DI replication efficiency was equal to that of the wild type and all three proteins, L, P, and N, accumulated to the same extent in both systems. These results clearly show that the transfected system reflects physiological concentrations of these proteins.

Absolute amounts of L, P, and N proteins synthesized in transfected versus VSV-infected were estimated by comparison with lanes containing known amounts of purified virions which were included in all of our Western blot analyses. Table 1 shows the range of values obtained as a function of plasmid input (micrograms per $\sim 1.5 \times 10^6$ transfected cells) from the data shown in Fig. 5 and 6, and these are compared with estimates derived from BHK cells infected with standard VSV (no DI) and harvested at 10 h postinfection). The accuracy of these estimates is likely no better than twofold, but they confirm that the intracellular protein levels from the natural infection are well within the range that gives optimal DI replication in the vaccinia virus-T7 expression system. The amounts obtained for the natural infection correspond to a P/N/L molar ratio of about 30:10:1. The highest level of protein expression achieved here is comparable in efficiency to the uppermost levels reported previously for a vaccinia virus-based system (16).

An estimate of P and L concentrations in our transcription assays also reveals important information. For the assays in Fig. 1 and 2, the P protein concentration ranged from ~0.8 to ~6.3 μg/ml (~27 to 210 nM) as a function of input P plasmid. L protein remained constant at about 4.0 μg/ml (~17 nM) in the presence of wild-type P and about 3.0 μg/ml with S60A/S64A. Since transcription activity remained roughly the same over this range of P protein (Fig. 2) but showed sensitivity on further dilution (Fig. 4B), we infer that the apparent equilibrium constant for the P monomer-to-multimer reaction, and binding of the multimer to L protein, must be less than 27 nM with respect to S60A/S64A and even lower for wild-type P. The nonphosphorylated bacterial P protein was reportedly unable to multimerize at a concentration of 10 μg/ml and proved to be inactive in transcription reconstitution in vitro (17). If S60A/S64A protein also requires multimerization for activity, then its ability to do so appears to be significantly greater than that of its bacterial analog.

TABLE 1. Viral protein accumulation in the vaccinia virus-T7 expression system versus VSV-infected cells

Cellular expression	Amt of protein accumulated (μg/1.5 × 10^6 cells)[a]		
	L protein	P protein	N protein
Transfected	1.0–5.0	6.0–24	2.4–18
VSV infected	~2.2	~8.0	~4.7

[a] The ranges of values for the transfected samples correspond to the lowest and highest levels of plasmid inputs from Fig. 5 and 6. Estimates were obtained from Western blots by comparison with lanes containing known amounts of purified virions.

DISCUSSION

The most important conclusion from our work is that constitutive phosphorylation of the VSV P protein is not necessary for transcribing the viral genome in vitro or for replicating DI in vivo. The S60A/S64A P mutant, which lacks competent acceptor sites for this modification, was substantially active in both assays (Fig. 2C and 6B). Our conclusion is at odds with the one reached in several recent studies in which this phosphorylation was deemed essential for VSV transcription in vitro using either Indiana or New Jersey serotype P protein (2–4, 17, 18, 31). We offer two possible explanations for these opposing conclusions. Almost all previous studies employed a bacterial source of nonphosphorylated wild-type P protein. Conformational differences between bacterially synthesized P and the Indiana S60A/S64A mutant expressed here in the vaccinia virus-T7 system are possible. A bacterial origin might conceivably make the protein more dependent on phosphorylation to achieve proper folding. However, this fails to explain why New Jersey P mutants with Ala-substituted acceptor sites were inactive for transcription in vitro when wheat germ extracts were used to synthesize P protein (31). A second possibility, which we believe explains at least some of the differing interpretations, is suggested by the concentration-dependent behavior of the S60A/S64A mutant documented here. Compared with wild-type P, the nonphosphorylated mutant lost much of its activity when assayed at low (<27 nM) P concentrations (Fig. 4). A similar effect on in vitro transcription reconstitution was recently reported for the P protein of respiratory syncytial virus, a member of the family *Paramyxoviridae*. When the respiratory syncytial virus P protein lacked phosphate at either one of two acceptor sites, it showed virtually no activity for transcription reconstitution in vitro at low P protein concentrations but recovered full activity at high concentrations (5).

Gao and Lenard (17) have provided strong evidence that the Indiana serotype bacterial protein must first be phosphorylated by CKII before it can multimerize to a transcriptionally active form. Multimerization to a homotrimer or homotetramer was deemed absolutely required for binding to both L and N-RNA template (18). The properties of the S60A/S64A mutant documented here are also in keeping with a requirement for concentration-dependent multimerization. However, our findings suggest that constitutive phosphorylation, rather than being essential for this process, more likely increases the rate of P protein self-association. Our conclusion is consistent with an earlier report showing that the New Jersey P protein produced in bacteria appears to multimerize (possibly to a dimer) with-

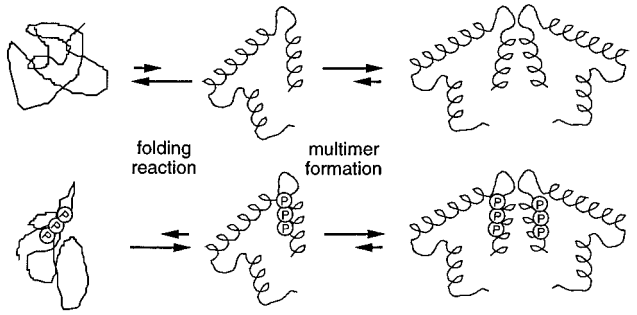

FIG. 8. Proposed role of constitutive phosphorylation in facilitating P protein multimerization. Both nonphosphorylated (top) and phosphorylated (bottom) P proteins are assumed to consist of an equilibrium between a less structured monomer form(s) (left) and a more structured form (middle). The latter species is presumed to be the one capable of multimerization. Both types of multimers (right), phosphorylated and nonphosphorylated, are assumed to possess similar secondary and tertiary structures since both are active in VSV RNA synthesis. Phosphorylation would then serve to shift the equilibrium to the structured form by facilitating the initial folding reaction. Multimers are drawn here as dimers for illustration purposes; the number of subunits and which domain of the protein is involved in multimerization are still unresolved (see text).

out prior phosphorylation, but only at high P concentrations (6). More recently, Das et al. (15) showed that the α-helical content of this New Jersey P protein, measured from its circular dichroism spectrum, increases in concert with phosphorylation-induced multimerization. If a conformational change in P protein is in fact necessary for multimerization, then our findings lead us to speculate that phosphorylation accelerates folding of the protein monomer to a structured form which is then capable of multimerizing (Fig. 8). This proposed chaperone-like function does not preclude additional roles for this phosphorylation, such as facilitating P interaction with L protein, as discussed below. It should be emphasized, however, that the unstructured P form with a low α-helical content has so far been described for a bacterial source of the protein; it remains to be seen whether this form is also produced in eukaryotic cells.

We have not yet mapped which domain of the VSV P protein is responsible for multimerization. For paramyxovirus P proteins, a small region (residues 314 to 411 in Sendai virus) appears sufficient for trimerization, and on the basis of computer predictions, a coiled-coil interaction has been proposed (13). The authors also noted that a helical region of ~30 residues near the very N termini of four of five rhabdovirus P sequences examined, including VSV, showed an elevated coiled-coil potential, albeit of lower significance than that found in paramyxoviruses. If this N-terminal domain is in fact responsible for VSV P multimerization, its accessibility on the surface of the protein could conceivably be governed by global conformational changes modulated by constitutive phosphorylation. Sendai virus P is also constitutively phosphorylated, and the main site was recently identified as Ser-249 (7a). However, the effects of this modification on the trimerization reaction using the whole P protein have not been examined.

Our results suggest that a nonphosphorylated P-L complex is not quite as active as that from the wild type. Maximum transcription activity was ~28% of the wild-type level with P in excess (Fig. 2C), and DI replication activity was ~27% of the wild-type level with an optimum input of L plasmid (Fig. 6A) and reached ~50% when both L and N plasmid inputs were optimized (Fig. 6B). Since lower levels of L protein accumulated in the mutant samples (about one-third to one-half as much in the replication assays), the specific activity of the nonphosphorylated complex could conceivably be similar to that of the wild type. In any case, a lack of constitutive phosphorylation had similar effects on both transcription and replication, supporting the notion that the same P-L complex is involved in both processes. Moreover, these results show that the P-N complex likewise does not require P constitutive phosphorylation for its assembly activity. In fact, varying the N protein concentration in the DI replication assay had a similar effect in the presence of S60A/S64A versus wild-type P (Fig. 6B), arguing that the affinity between the P and N proteins was also not significantly affected by phosphorylation. A recent study using a two-hybrid mammalian system similarly concluded that a lack of phosphorylation also had no effect on association between VSV P and N proteins (32).

In contrast to N protein, a slight lowering of L protein concentration (about twofold) dramatically reduced DI replication activity in the presence of the nonphosphorylated P protein, while activity with the wild type remained at the maximum (Fig. 6A). This suggest that the affinity of the S60A/S64A multimer for L protein is reduced by lack of phosphorylation. However, it is also possible that S60A/S64A P multimerization is incomplete in vivo. Judging solely from the in vitro transcription assays, multimer formation should not be limiting in vivo, because the P concentration is much higher (≥50 μM). One complicating factor, however, is that VSV P protein coexpression stabilizes L protein against degradation (9). A similar phenomenon has also been documented for three different viruses of the *Paramyxoviridae* family (20, 24, 29). For unknown reasons, coexpression with S60A/S64A stabilizes only 40 to 60% of the L protein (10), which accounts for the lower L protein accumulation noted here in both transcription and replication assays. This raises the possibility that P-L complex formation is not sufficient for stabilization.

It is noteworthy that a three- to fourfold excess of L protein was not inhibitory in the DI replication assay (Fig. 6A), in contrast to earlier findings using this vaccinia virus expression system (25). This, however, was true only when our assays were carried out in the presence of AraC. The basis of the AraC effect is not entirely clear but is probably related to its potent stimulatory effect on DI replication in this vaccinia virus expression system (30).

Since the S60A/S64A mutant lacks all constitutive phosphorylation (21), it provides the most sensitive test of the role of this modification, but we also tested P mutants that retain some of this phosphate. All displayed at least two-thirds of wild-type activity under optimal conditions (Fig. 1, 2, and 5) and showed minimal differences in protein concentration dependence in vitro compared with wild-type P protein (10). Modification of either Ser-60 or Ser-64 alone is therefore sufficient to make the protein behave much like the wild type, although more-sensitive measures of P-P and P-L interactions could well reveal some differences. Phosphorylation of Thr-62 alone could not be tested; we showed previously that it depends on prior phosphorylation of Ser-64 (21).

Under optimal conditions, the vaccinia virus expression system replicated DI as efficiently as VSV-helper-infected BHK cells and accumulated comparable amounts of P, N, and L proteins at the same ~30:10:1 molar ratio (Fig. 7; Table 1). L protein appeared to be in excess in both cases, since the transfected system replicated DI nearly as well with fourfold less L protein (Fig. 7). Pattnaik and Wertz (25) reported that the vaccinia virus system replicated DI 8- to 10-fold better than VSV-infected HEp-2 cells while synthesizing roughly similar amounts of P and N proteins. The optimal P/N/L molar ratio in their case was 200:200:1. The different host cells employed and the presence of AraC may account for the differences.

Since P protein phosphorylation is ubiquitous in nonseg-

mented negative-strand viruses, it likely serves an essential function under at least some circumstances. This may not be the case in highly permissive host cells, such as BHK cells, which accumulate high concentrations of viral proteins. We plan to introduce the S60A/S64A mutation into an infectious cDNA clone to test this possibility, as this is now feasible with VSV (22, 34). Nonetheless, even a small change in the ratio of different viral protein complexes could conceivably have dramatic effects on viral RNA synthesis. The modification could also play a role in other aspects of viral multiplication not measured in our assays. Whatever the case may be, there are obviously many situations in which viral protein expression is limited, because of either virus mutations or host factor modulation. By facilitating P-L complex formation under these conditions, constitutive P phosphorylation likely serves a very important role.

Lastly, the findings reported here point to possible pitfalls in assessing the phenotypes of viral protein mutants under a single set of transfection conditions when such proteins form complexes with themselves and/or other proteins. For example, if we had assessed the DI replication phenotype of the S60A/S64A mutant only under conditions optimal for the wild type, i.e., low levels of L plasmid, we might have been tempted to conclude that phosphorylation is required for DI replication but not transcription. Despite its more laborious nature, the approach we have taken here provides a clearer definition of mutant phenotype.

ACKNOWLEDGMENTS

We thank Katherine Rhodes for critical reading of the manuscript.

This work was supported by Public Health Service grant AI-21572 from the National Institutes of Health (J.P.).

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