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Cooperative Binding of Multimeric Phosphoprotein (P) of Vesicular Stomatitis Virus to Polymerase (L) and Template: Pathways of Assembly

YUAN GAO AND JOHN LENARD*

Department of Physiology and Biophysics, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854-5635

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It was previously shown that the phosphoprotein (P) of vesicular stomatitis virus must undergo phosphorylation-dependent multimerization to become transcriptionally active. Phosphorylation at S-60 and/or T-62 by casein kinase II or substitution of these residues by D is required for multimer formation. We now find that substitution of either one of these residues by A prevents phosphorylation by casein kinase II and multimer formation. The binding of multimeric P to the other two transcriptional components of vesicular stomatitis virus (L protein and the N-RNA template) has been characterized by using P immobilized on beads through its poly(His) tag to facilitate recovery of bound complexes. Multimerization of P was absolutely required for binding to both L and template. Multimeric P combined with the polymerase enzyme (L) in a stoichiometric 1:1 complex, which bound to the N-RNA template much more strongly than multimeric P alone. Substitution of S-227 and S-233 by A residues had no effect on multimerization or binding of L to P but prevented binding of both P and L to template and abolished transcriptional activity. In contrast, substitution of these residues with D residues had no effect on template binding or activity. However, substitution at these sites by either D or A largely abolished phosphorylation by L-associated kinases, thus identifying S-227 and S-233 as the major sites targeted by these kinases and confirming that phosphorylation of P protein by L-associated kinases is without transcriptional effect.

Vesicular stomatitis virus (VSV) is a small negative-sense RNA virus that transcribes and replicates its genome entirely in the cell cytoplasm. VSV transcripts from the encapsidated 11-kb genomic RNA consist of a short (47-nucleotide) leader representing the extreme 3′ end of the genome and five capped, polyadenylated mRNAs which are synthesized sequentially, in decreasing amounts (4, 10). This entire transcriptional regimen can be carried out in a defined reaction mixture consisting of three purified viral components: the encapsidated genome, or N-RNA template; the 241-kDa RNA-dependent RNA polymerase, named L; and a 30-kDa phosphoprotein, P (4). This juxtaposition of simple composition and complex transcriptional behavior offers unique opportunities for study. We have been studying the role of P protein in this defined system by using bacterially expressed recombinant P possessing a His tag at its N terminus (6).

The precise function of the enzymatically inert P protein in the transcription reaction has remained a puzzle, but it is generally considered to act by binding together the other two components in a functional way (4). Studies of binding interactions between the isolated transcriptional components of VSV have produced results that are broadly consistent with this idea and have suggested that P protein binding to L and template are governed by different regions of the molecule (5, 8, 13, 14).

The P protein must be specifically phosphorylated in order to work. Thus, recombinant P protein produced in bacteria is inactive, requiring modification by a single kinase, casein kinase II (CK-II), for transcriptional activity (1). Activation consists of phosphorylation at two residues, S-60 and T-62, followed by spontaneous multimerization into a homotrimer or homotetramer (the transcriptionally active form), which can be recognized by its elution position upon gel filtration (6).

The discovery of point mutations that substitute completely for CK-II phosphorylation (6) and of others that abolish transcriptional activity (3, 15) has led to this study of the relationship between the ability of the P protein to bind to the other two transcriptional components and its phosphorylation, multimerization, and transcriptional behavior. The His tag, originally attached to bacterially produced P to aid in purification (6), was used in this study to immobilize P protein on beads, thus facilitating the isolation of complexes.

MATERIALS AND METHODS

Purification of VSV and viral proteins. Growth of VSV (Indiana serotype [VSV_{Ind}], Mudd-Summers strain) in BHK-21F cells, purification of intact virions, disruption and purification of transcribing viral nucleocapsids, and purification of the viral N-RNA template, L, and P protein were carried out as previously described (6).

Preparation of mutant recombinant His-tagged P proteins. All constructs were derived from the P gene, originally cloned and sequenced by Hudson et al. (9) and kindly provided to us by Manfred Schubert. New mutant P protein genes used in this study were prepared in the GEMX-PΔ4 vector by the method of Kunkel et al. (11), as described previously (6). Sequences of the synthetic oligonucleotides used in their preparation are available upon request. P genes carrying the desired mutations were excised with *Xho*I, ligated into the pET-19b vector, and expressed in bacteria as previously described (6). The His-tagged mutant P protein was purified by binding the N-terminal His tag to a His-Bind resin column and releasing it with imidazole as previously described (6).

Binding assay. (i) Bead method. His-Bind resin (Novagen) was charged with $NiSO_4$ and equilibrated with transcription buffer (0.1 M NaCl, 5 mM $MgCl_2$, 50 mM Tris [pH 8.0]). The standard assay mixture (100 μl in transcription buffer) contained 20 μl of His-Bind resin, 2 μg each of viral L and recombinant P protein, and 5 μg of viral N-RNA template, except when specifically indicated. The mixture was incubated at 4°C for 1 h with constant shaking, centrifuged at 500 × *g* for 1 min, and washed three times with transcription buffer. Finally, the

* Corresponding author. Mailing address: Department of Physiology and Biophysics, UMDNJ/Robert Wood Johnson Medical School, 675 Hoes La., Piscataway, NJ 08854-5635. Phone: (908) 235-4784. Fax: (908) 235-5038. Electronic mail address: Lenard@umdnj.edu.

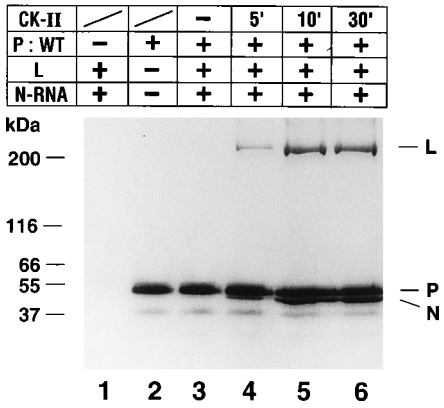


FIG. 1. Acquisition of binding activity by His-tagged wt P protein during phosphorylation by CK-II. Purified His-tagged wt P was treated with CK-II and ATP for 5, 10, or 30 min, as indicated. Aliquots were withdrawn at the indicated times and tested for the ability to bind L and N-RNA template in the bead assay. Binding activity was acquired as a result of phosphorylation by CK-II. The minor band migrating at ca. 38 kDa is a proteolytic degradation product of His-tagged recombinant P (Fig. 1C in reference 6).

His-tagged P protein, together with associated proteins, was eluted from the His-Bind resin with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 1 M imidazole and analyzed by SDS-PAGE. Densitometry was performed after staining with Coomassie blue, using a model 670 Bio-Rad densitometer.

(ii) Sedimentation method. The standard assay mixture defined above (lacking the His-Bind resin) was analyzed by ultracentrifugation in some experiments. After incubation, the mixture was sedimented in an SW50 rotor (Sorvall) at 45,000 rpm for 2 h through a 0.8-ml 30, 50, and 100% glycerol step gradient. The N-RNA template fraction, with associated proteins, was collected from the 50/100% interface, diluted, precipitated with acetone, and separated by SDS-PAGE.

Phosphorylation by CK-II and L-associated kinases. Phosphorylation by CK-II (Sigma) was carried out under exactly the same conditions as described previously (6). Phosphorylation by L-associated kinases was carried out under identical conditions with, as the kinase source in place of CK-II, 2 μg of L protein that had not been passed over Superose-6 (6), with 5 μg of P protein as the substrate.

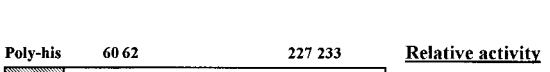
Other procedures. Transcription reactions and multimer analysis by gel filtration on a Superose-12 column were performed as previously described (6).

RESULTS

Phosphorylation of P by CK-II, or substitution of target residues by D, is essential for binding to L and N-RNA template. The relationship between transcriptional activation of wild-type (wt) recombinant P protein and its phosphorylation by CK-II was reported previously (6). Phosphorylation was complete at a stoichiometry of 2 phosphates per P protein. Transcriptional activation occurred more rapidly, however, reaching a maximum after only ca. 1 phosphate per P molecule had been introduced (6).

Figure 1 shows the acquisition of L and N-RNA template binding by wt recombinant P protein with increasing time of incubation with CK-II. Unphosphorylated P was unable to bind to either component. Significant binding of wt P to both components was generated by 5 min of phosphorylation and reached a maximum by 10 min, or about the time that maximum transcriptional activity was acquired under the same conditions in earlier experiments (6).

The two sites of P protein phosphorylation by CK-II were previously identified as residues S-60 and T-62 (6). Substitution of these residues individually with D produced mutants (S60D and T62D, respectively) that were partially transcriptionally active without modification (Fig. 2). Each mutant protein accepted 1 phosphate per P protein upon treatment with



Wild type	S T	S S	0	100[a]
S60A	A T	S S	9	ND[b]
T62A	S A	S S	5	ND
S60A/T62A	A A	S S	12	ND
S60D	D T	S S	39	95
T62D	S D	S S	40	93
S60D/T62D (DDSS)	D D	S S	93	ND
DDAA	D D	A A	16	ND
DDDD	D D	D D	96	ND

FIG. 2. Schematic diagram of the mutants used in this study. The activities of each mutant before and after activation by CK-II in a defined transcription assay (6) are listed. a, 100 equals 14.9 nmol UTP/mg of protein/h; b, ND, not determined.

CK-II, which resulted in full activation. Substitution of D at both residues produced S60D/T62D, a fully active phosphate-free mutant, the "pseudo-wt," which was not a substrate for CK-II (6). On the other hand, work by others had shown that substitution of either or both of the equivalent residues in VSV_{NJ} by A caused transcriptional inactivation (15), an observation that we have extended to VSV_{Ind} (Fig. 2). It was therefore of interest to learn whether these A mutants could be phosphorylated by CK-II. Results are shown in Fig. 3. As was reported previously, substitution of either S-60 or T-62 by D produced proteins that were phosphorylated by CK-II to about half the extent of the wt, while the doubly substituted mutant S60D/T62D was not a substrate (6). Substitution of either S-60 or T-62 with A, on the other hand, blocked phosphorylation by CK-II at the unmodified site (Fig. 3). Evidently, the A substitutions, unlike the D substitutions, altered the region around the CK-II substrate sites sufficiently to prevent reaction. As expected, all three A-substituted proteins were monomeric as judged by their elution position from a Superose-12 column (data not shown).

The ability of each of these mutants to bind to L and N-RNA template was determined. As shown in Fig. 4, the binding ability of each mutant correlated closely with its transcriptional activity as listed in Fig. 2: S60D and T62D each bound modestly before phosphorylation by CK-II and acquired full binding capacity afterward, the pseudo-wt S60D/T62D possessed full binding capacity (Fig. 4A), and all three A mutants possessed no detectable binding ability (Fig. 4B).

It was possible that the binding site on the pseudo-wt S60D/T62D, by which it binds to the other transcriptional compo-

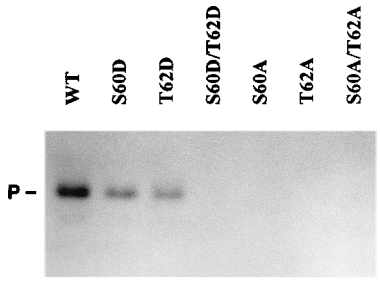


FIG. 3. Phosphorylation of His-tagged wild-type P protein and several mutants by CK-II. Each purified protein was reacted with [γ-^{32}P]ATP and CK-II as described previously (6). Identical amounts of each protein were then run on SDS-PAGE and visualized by autoradiography.

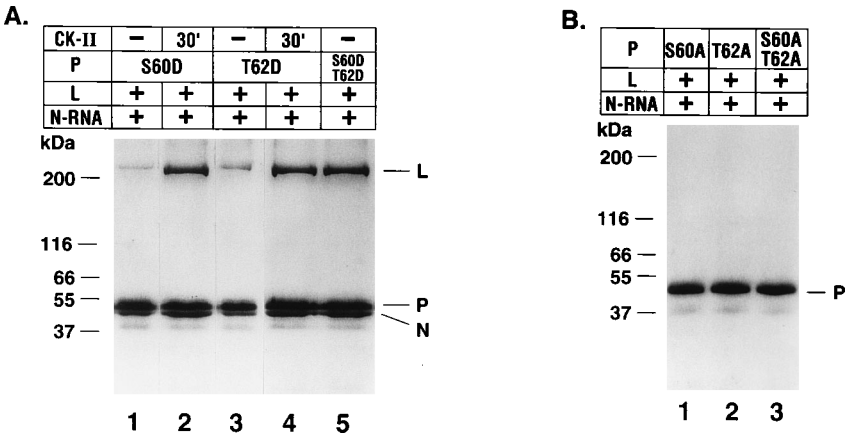


FIG. 4. Binding activity of various P protein mutants in the bead assay. (A) Mutants S60D, T62D, and S60D/T62D. S60D and T62D were tested before (lanes 1 and 3) and after (lanes 2 and 4) phosphorylation of the unmodified residue by CK-II. (B) Mutants S60A, T62A, and S60A/T62A. No binding activity is detectable for any of the A-containing mutants. The minor band migrating at ca. 38 kDa is a proteolytic degradation product of His-tagged recombinant P (Fig. 1C in reference 6).

nents, differed from that of fully phosphorylated viral P. To test this possibility, a competition experiment was carried out between the two proteins. To a fixed amount of the S60D/T62D mutant, immobilized on Ni beads by its His tag, was added fixed amounts of the other transcriptional components, together with increasing amounts of purified viral P. Any components bound to viral P would be removed during the washing steps, since it lacks the His tag. Results are shown in Fig. 5. Viral P competed efficiently with the immobilized pseudo-wt, with the midpoint occurring at 1 to 2 μg of viral P, i.e., at about equimolar amounts of viral and mutant P. This experiment provides strong evidence that the mode of binding of the other transcriptional components to S60D/T62D is identical to that of fully phosphorylated viral P.

Formation of P-L and P–N-RNA template complexes. In all of the above experiments, the ratio of L to N-RNA template to recombinant P in each reaction was fixed, and it was observed that the relative amounts of L and N-RNA template bound to P were invariant; in no instance was preferential binding of one component or the other observed. It was therefore of interest to test the binding of each component separately. Results of the bead assay are shown in Fig. 6A. Both CK-II-activated wt P and pseudo-wt S60D/T62D were tested, with identical results. When L protein was added alone, binding occurred to the same extent as when it was added together with N-RNA template. The template, however, was unable to bind in this assay in the absence of L. If L was added first, the beads thoroughly washed, and the complex then reincubated with N-RNA template, the full complement of template was bound.

The observation that P alone could not bind the N-RNA template in the bead assay appeared to contradict several previous reports, in which saturable binding was observed (5, 8, 13, 14). In those studies, however, different methods were used to isolate complexes: immunoprecipitation with anti-N antibodies or sedimentation of N-RNA templates after incubation. We therefore examined the amount of P bound to N-RNA templates by a sedimentation assay, as originally done by Mellon and Emerson (13). Results are shown in Fig. 6B. Binding of multimeric P to template was observed in this assay, in agreement with previous reports. However, the finding that the immobilized P-L complex holds the N-RNA templates on the beads (and thus "binds" them in this assay) while immobilized P alone does not shows that attachment of the P-L complex to the template is significantly stronger than is attachment of P alone.

The stoichiometry of the L-P complex was explored by incubating a fixed amount of immobilized pseudo-wt P with increasing amounts of L protein (Fig. 7). Saturation was reached at a P/L molar ratio of 3.6:1, corresponding to a 1:1 ratio of L to multimeric P.

Substitutions at S-227 and S-233 implicate the C-terminal region of P in binding to N-RNA template and identify sites of phosphorylation by L-associated kinases. Previous studies had shown that substitution of two residues close to the C terminus of P protein—either S-236 or S-242 of VSV_{NJ} P—by A resulted in a substantial loss of transcriptional activity (3). Substitution of both residues by A caused almost complete inactivation and also prevented phosphorylation by L-associated kinases (3). Since our previous work had demonstrated that phosphorylation by CK-II of only residues S-60 and T-62 of P protein was sufficient for full transcriptional activation (6), we wished to investigate further the role of these important C-terminal S residues.

For this purpose, two new mutant forms of P were prepared, based on the pseudo-wt S60D/T62D (also called DDSS in Fig. 2). This mutant was further substituted at positions 227 and 233 with either D or A residues (to yield mutants DDDD and DDAA, respectively, in Fig. 2). S-227 and S-233 in VSV_{Ind} P

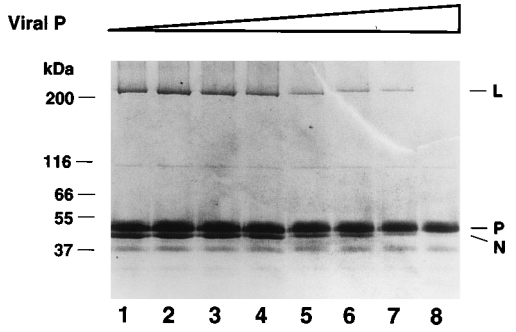


FIG. 5. Competition for binding between recombinant His-tagged pseudo-wt P (S60D/T62D) and viral P. To fixed quantities of the normal components of the bead assay (resin, His-tagged P, L, and N-RNA template [see Materials and Methods]) were added the following quantities of viral P protein: 0 μg (lane 1), 0.2 μg (lane 2), 0.4 μg (lane 3), 1 μg (lane 4), 2 μg (lane 5), 4 μg (lane 6), 10 μg (lane 7), and 20 μg (lane 8). The minor band migrating at ca. 38 kDa is a proteolytic degradation product of His-tagged recombinant P (Fig. 1C in reference 6).

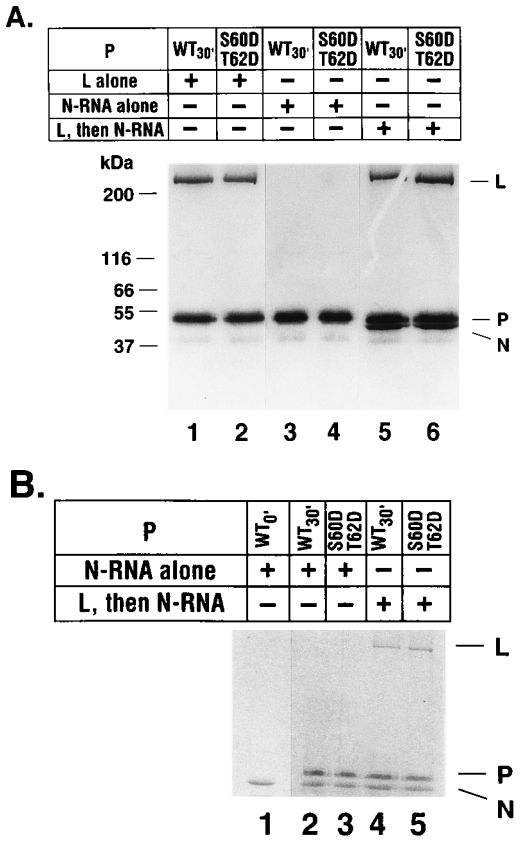


FIG. 6. (A) Sequential binding of L and N-RNA template to His-tagged wt and pseudo-wt P by using the bead method. L protein bound to CK-II activated wild-type P ($WT_{30'}$, lane 1) or S60D/T62D (lane 2) in the absence of template, but template was not retained by these bead-immobilized proteins in the absence of L (lanes 3 and 4). Prior binding of L to immobilized P allowed retention of subsequently added template (lanes 5 and 6). (B) Both CK-II activated wild-type P (lanes 2 and 4) and S60D/T62D (lanes 3 and 5) bound to templates in comparable amounts in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of L, as measured by the sedimentation method. Unphosphorylated wt P did not bind to template as measured either by the sedimentation method (lane 1) or by the bead method (cf. Fig. 1, lane 3). The minor band migrating at ca. 38 kDa is a proteolytic degradation product of His-tagged recombinant P (Fig. 1C in reference 6).

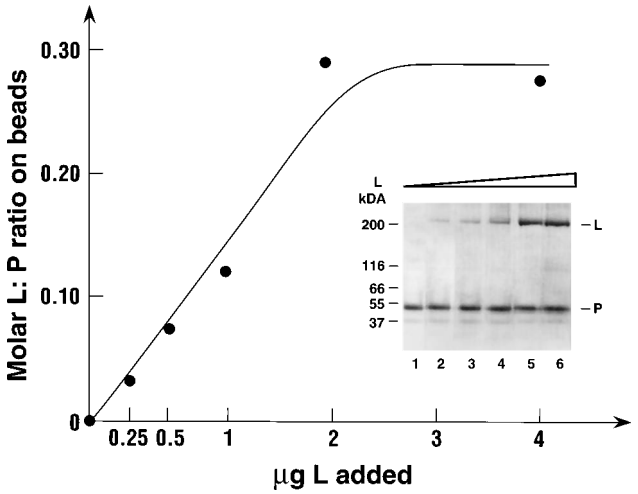


FIG. 7. Stoichiometry of L binding to His-tagged pseudo-wt P. To 1 μg of S60D/T62D P immobilized on beads were added increasing amounts of L, and the amount of L retained on the beads was determined as described in Materials and Methods. The amounts of P and L bound were measured by densitometry of the gel (inset) and quantitated by comparison with standards containing known amounts of each protein (not shown). Amounts of L protein added: 0 μg (lane 1); 0.25 μg (lane 2); 0.5 μg (lane 3); 1 μg (lane 4); 2 μg (lane 5); and 4 μg (lane 6).

protein are homologous to S-236 and S-242, respectively in VSV_{NJ} P (7). DDAA was found to be transcriptionally inactive, in agreement with a previous report on the effects of A substitutions at these positions in wt P of VSV_{NJ} (3). The DDDD mutant retained full activity, however, as did DDSS under conditions where no phosphorylation occurred (Fig. 2). Gel filtration showed that all three mutants were multimeric, however (data not shown).

The three mutant proteins DDSS, DDDD, and DDAA were compared in a binding assay. All three mutants bound L protein normally, but only DDSS and DDDD were able to bind template; the transcriptionally inactive mutant DDAA had lost this ability, as measured both by the bead assay (Fig. 8A) and by the sedimentation assay (Fig. 8B). These findings implicate residues 227 and 233 in template binding and support previous suggestions that the C-terminal region plays an important role in this process (5, 8, 14).

It is especially noteworthy that L protein failed to bind to template when the mutant DDAA was tested in the sedimentation assay (Fig. 8B, lane 2) but associated normally with this P protein mutant when tested in the bead assay (Fig. 8A, lane 2). It must be remembered that the bead assay measures binding to P protein while the sedimentation assay measures binding to N-RNA template. Thus, the L-P complex forms normally with DDAA but the next step, i.e., binding of this complex with template, cannot occur. L protein cannot bind to template alone; it requires the presence of a template-binding P protein, in confirmation of a previous report (13).

Finally, the phosphorylation of these mutants by L-associated kinases was investigated. DDSS could be phosphorylated by L-associated kinases, as observed previously for CK-II-activated wt P protein (2, 6). Phosphorylation was decreased by >90% in both the DDDD and DDAA mutants, however (Fig. 9). This finding confirms a previous report on the effect of A

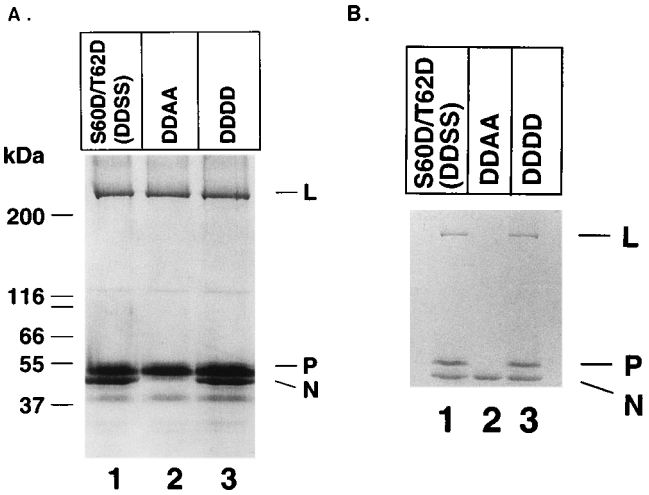


FIG. 8. Binding activity of the mutants DDSS, DDAA, and DDDD in the bead assay (A) or the sedimentation assay (B). DDAA lacks N-RNA template-binding activity in both assays. The minor band migrating at ca. 38 kDa is a proteolytic degradation product of His-tagged recombinant P (Fig. 1C in reference 6).

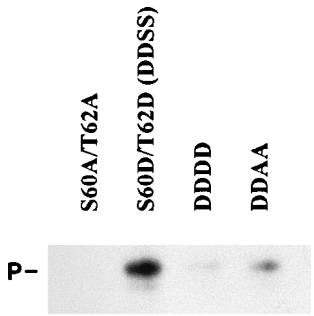


FIG. 9. Phosphorylation of P protein mutants by L-associated kinases. Each mutant was reacted with L-associated kinases and [γ-^{32}P]ATP (6). Identical amounts of each product were run on SDS-PAGE and visualized by autoradiography.

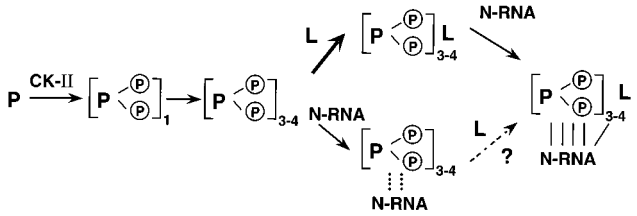


FIG. 10. Pathways of assembly of the VSV transcription complex.

substitution at these positions (3) and, together with our previous finding that viral P contains 4 to 5 phosphates per P molecule (6), identifies S-227 and S-233 as the major targets of the L-associated kinases.

DISCUSSION

The present study has elucidated several properties of the interaction between P protein, the polymerase enzyme L, and the N-RNA template. First, binding of P to the other components requires multimerization, as does transcriptional activation (6). The ability to form a ternary complex (L-P-template) thus reflects the functional requirements for transcription. The relationship between complex formation and activity has been in question ever since Mellon and Emerson determined that L and P binding to template became saturated at a level far higher than could be accounted for by the formation of transcription complexes (13). However, the absolute correlation in our experiments between the transcriptional activity of various P protein mutants (Fig. 2) and their ability to bind to the other components (Fig. 1, 4, 6, and 8) provides strong evidence that this binding is relevant to transcriptional function.

Defective P protein mutants were unable to bind for two different reasons. Those possessing A substitutions at S-60 and/or T-62 were not substrates for CK-II (Fig. 3) and could not multimerize; they bound neither L nor template (Fig. 4B). On the other hand, a mutant possessing A substitutions at S-227 and S-233 multimerized and bound L protein normally, but the complex was unable to bind template (Fig. 8). This is consistent with previous studies of deletion mutants, which found that the C-terminal portion of P is involved in template binding while L binding is governed chiefly by the N-terminal portion (5, 8, 14).

Mellon and Emerson found that L protein could not bind to template in the absence of P (13). Others have observed that more P protein binds to template in the presence of L (8, 14). We have now provided a connection between these two observations by showing that P forms a stoichiometric complex with L (Fig. 7), which then binds to template more strongly than does P alone (Fig. 6A).

When the binding data of Mellon and Emerson (13) are corrected for the actual molecular weights of P and L (which were unknown at the time), saturation occurred at an L/P ratio of 1:3.3, in close agreement with the value of 1:3.6 determined in Fig. 7. It is therefore likely that all of the binding to template seen by Mellon and Emerson was of L-P complexes, consistent with the inability of L to bind to template in the absence of competent P (Fig. 8B). When the binding data of reference 13 are further corrected to 1,258 N molecules per template (16), saturation is found to occur at about 65 L-P complexes per template, or 1 complex per 19 N molecules.

The bead assay was used to reveal the increased binding affinity to template of P-L complexes compared with that of P protein alone. Thus, bead-immobilized P-L complexes retained templates throughout the necessary washes and low-speed centrifugations of the procedure (see Materials and Methods) and thus "bound" the templates in this assay; bead-immobilized P multimers alone did not. When templates were incubated with free P multimers, however, significant amounts of P were "bound" (i.e., cosedimented) in the absence or presence of L, in agreement with previous reports (5, 8, 13, 14).

A resolution of the apparent discrepancy between the two assays becomes apparent when it is recalled that the bead assay identifies proteins bound to P while the sedimentation assay identifies those bound to template. Further, the P protein bound to the template is small whereas the template bound to the bead-immobilized P is large and flexible. Random segmental motions of the template could easily generate kinetic energies many times greater than that of each individual P-template bond; bonds would thus be broken. Whenever energy generated by random kinetic motions exceeded that of all the bonds holding the template to the bead, the template would fall off. As a possible additional factor, the density of His tag-binding sites on the surface of each bead (which is much larger than a template) might be low, thus sterically limiting the maximum number of bonds holding the template to the bead. On the other hand, each P multimer, being much smaller than a template, generates less kinetic energy and thus requires less binding energy (i.e., lower affinity) to remain stably associated with the template during cosedimentation.

By comparing the results from the two assays, two conclusions were reached: (i) binding of P to template can occur either in the presence or in the absence of L; but (ii) binding of the P-L complex to template is significantly stronger than binding of P alone. Formation of the ternary complex thus involves cooperative interactions between P, L, and template.

Two possible pathways of assembly of transcriptionally active ternary complexes are suggested by these conclusions and are shown schematically in Fig. 10. This paper provides evidence for the top pathway and for the possibility of the first step in the bottom pathway.

It is noteworthy that no binding of monomeric P to template was found, even when the sedimentation method was used. This seemed surprising, since binding to template involves the C-terminal region (5, 8, 14) (Fig. 8), which might be expected to remain unmodified during multimerization. It is possible that binding of each P monomer to template (presumably via a single N molecule) is inherently weak and acquires higher

affinity through the multivalency of the multimer. This would make possible a kind of "walking" down the template by the complex, in which one subunit at a time could come loose from the template without destabilizing the multimer. It could then rebind to another N subunit further downstream on the template without dissociating or disorienting the entire complex. An alternative possibility is that multimerization creates the template-binding site by conformational rearrangement.

One further point of interest in these studies may be noted. It is often assumed that replacement of a phosphorylatable S or T residue in a protein by A produces the equivalent of a stable unphosphorylated form whereas replacement with D produces the equivalent of a stable phosphorylated form. In the case of P protein, we have found the actual situation to be considerably more complicated. Replacement of one or both of the essential phosphorylatable residues, S-60 and T-62, with D does indeed produce a protein that closely resembles the corresponding phosphorylated form (6). However, replacement of either residue with A yields a perturbed protein, which differs from the unphosphorylated form in that the remaining unsubstituted residue is no longer a substrate for CK-II. The situation at S-227 and S-233, on the other hand, is different again. Phosphorylation of these residues (by L-associated kinases) is without effect on transcriptional activity; i.e., either serines or serine phosphates are compatible with full activity, as shown by the finding that L-associated kinases can be completely removed from a fully active transcription reaction, leaving these residues unmodified (6, 12). Consistent with this, replacement of these residues by D residues is compatible with full function (Fig. 2). On the other hand, replacement with A residues inactivates the protein by rendering it incapable of attaching to template. Thus, serines, serine phosphates, and aspartates are all equally and fully acceptable at these positions whereas alanines are not and phosphorylation is irrelevant. The situation is doubtless different for other phosphorylated proteins, but P protein serves as an example that such substitutions for the purpose of studying phosphorylation effects should be used with caution.

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