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Multimerization and transcriptional activation of the phosphoprotein (P) of vesicular stomatitis virus by casein kinase-II

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Casein kinase-II (CK-II) is a widely distributed protein kinase, which plays numerous roles in the regulation of transcription through modification of transacting transcription factors. Phosphorylation of vesicular stomatitis virus (VSV) P protein by CK-II was found to be both necessary and sufficient for transcriptional activation. Upon treatment of P by CK-II, activity was acquired faster ($t_{1/2}$ = 3.7 min) than were total phosphates ($t_{1/2}$ = 7.4 min). Stoichiometry was 2 mol phosphate/mol P, indicating activation by phosphorylation at either one or both of two independent sites. The sites were identified by substituting aspartate (D) residues at either S60 or T62, producing proteins that were partly active without phosphorylation, but were fully active at higher concentrations; CK-II added only a single phosphate group to each of these, and conferred full activity. P protein doubly substituted with D at S60 and T62 was fully active without phosphorylation, and was not a substrate for CK-II. Active P protein, whether CK-II treated or doubly substituted, was shown by gel filtration and crosslinking to exist as a discretely multimeric, probably tetrameric, structure. The singly substituted mutants were partly multimeric, becoming fully so after CK-II treatment. Phosphorylation by CK-II thus mediates the self-association of P into the multimeric, transcriptionally active form.

Key words: casein kinase II/multimerization/P protein/transcription activation/VSV

Introduction

Vesicular stomatitis virus (VSV) is a negative-stranded RNA virus of simple composition. A fully active transcription complex (the nucleocapsid) can be readily isolated from purified virions after disruption of the viral envelope with detergent. Nucleocapsids consist of three distinct, separable components: the N-RNA template, comprising N protein tightly wound around the genome; the L protein, which is the polymerase enzyme; and the phosphoprotein, P (formerly called NS), of unknown function. Full transcriptional activity can be reconstituted by recombining these three purified components. Transcription products consist of a short leader and five capped, polyadenylated mRNAs, which encode the five viral proteins. VSV transcription almost certainly initiates from a single site at or near the 3′ end of the genome, and it is characterized by attenuation, i.e. each successive gene is transcribed to about one-third the extent of the preceding one (Wagner, 1990). The combination of simple composition and complex behavior of this system makes it particularly suitable for structure–function analysis.

The phosphorylation requirements for transcriptional activity of P protein have been the subject of much study. P protein from virions or infected cells is always found in various phosphorylated forms (Wagner, 1990). Unphosphorylated recombinant P protein from bacteria was found to be transcriptionally inactive (Barik and Banerjee, 1992a,b). Activation required phosphorylation by a cellular protein kinase, which was purified and identified as casein kinase-II (CK-II; Barik and Banerjee, 1992b). This kinase plays a broad role in the regulation of transcription factors by phosphorylation (Hunter and Karin, 1992; Kretzschmar *et al.*, 1994).

Purified nucleocapsids, whether isolated from infected cells or virions, possess associated protein kinase activity that can phosphorylate viral P protein (Massey *et al.*, 1990; Barik and Banerjee, 1992a). Much of this activity co-purifies with L protein during standard purification on a phosphocellulose column. This has led to claims that L protein itself possesses P protein kinase activity, a conclusion based chiefly on the fact that L is specifically labeled by azido-ATP (Sanchez *et al.*, 1985; Hammond *et al.*, 1992). Previous work from our laboratory showed, however, that all detectable P protein phosphorylating activity could be removed from L protein by gel filtration, indicating that L-associated kinases (LAKs) were cellular in origin. Further separation indicated that LAKs consisted of at least two different protein kinases (Massey *et al.*, 1989, 1990).

Transcriptional activation of P has been claimed to be a two-step process, consisting of initial phosphorylation by CK-II followed by phosphorylation by LAKs (Barik and Banerjee, 1992a). In those experiments, however, LAKs were never removed from the L protein used to assay recombinant P for transcriptional activity. Hence, despite the fact that phosphates were added by LAKs during transcription, the relationship of that phosphorylation to transcriptional activation was not established. The requirement for sequential phosphorylation in transcription has been questioned by others (Beckes and Perrault, 1992).

In the present study, we have prepared recombinant P proteins possessing a poly(His) extension at their N-termini, thus permitting the facile purification of wild-type or mutant sequences by the same routine procedure. By correlating stoichiometry of phosphorylation by CK-II with transcriptional activity of purified P proteins, we have concluded that phosphorylation of either or both of two independent CK-II sites is both necessary and sufficient for transcriptional activation. This conclusion

was confirmed, and the sites identified, by substituting either or both S60 and T62 with negatively charged aspartate (D) residues, which created active, phosphate-free P proteins. Gel filtration and crosslinking studies indicated that transcriptionally active P proteins were always self-associated into a multimeric form.

Results

Casein kinase-II is both necessary and sufficient for transcriptional activation of P protein

Recombinant wild-type P was tested for transcriptional activity. Treatment with casein kinase-II (CK-II) led to activation of transcriptional activity to the same level as found for P protein purified from virions (Figure 1A). RNA products obtained using viral or recombinant P proteins were indistinguishable after separation on gels (not shown). Unphosphorylated recombinant P protein was essentially inactive. (The small amount of activity seen for this protein in Figure 1A is presumably due to traces of residual viral P remaining tightly bound to the purified N-RNA template; Canter *et al.*, 1993.) Thus, the poly(His) tag has no effect on transcriptional activity.

We inquired next into whether additional phosphorylation by LAKs was required for transcriptional activity, as has been claimed (Barik and Banerjee, 1992a). LAKs were completely removed from L protein by gel filtration, and additional cellular kinases associated with the N-RNA template were inactivated by brief heat treatment (Massey *et al.*, 1990). Activity of CK-II treated P protein was identical before and after these treatments (Figure 1B).

The activity of LAKs was assayed during transcription, by using [γ-^{32}P]ATP in the transcription assay in place of [^{3}H]uridine (see Materials and methods). Phosphorylation of P protein by LAKs during transcription was negligible in assays from which LAKs had been removed (Figure 1C, lane 2), but was appreciable when LAKs were present (Figure 1C, lane 1). Thus, in addition to being necessary, phosphorylation of P protein by CK-II is also sufficient for full transcriptional activity. Consistent with a previous observation (Barik and Banerjee, 1992a), unphosphorylated wild-type P protein was not a substrate for LAKs (Figure 1C, lane 3). This suggests that phosphorylation by CK-II induces major structural changes in P, sufficient to expose new phosphorylation sites.

Relationship between phosphorylation and transcriptional activation by CK-II

Since P protein contains several possible phosphorylation sites for CK-II, it was of interest to determine how many were phosphorylated, and which ones were actually needed for activation. Phosphorylation of P by CK-II reached a clear end-point at 2 mol phosphate/mol P protein (Figure 2A). Phosphorylation followed pseudo-first order kinetics up to >90% completion, exhibiting a half-time of 7.4 min under our conditions (Figure 2B). The rate of transcriptional activation induced by CK-II also followed pseudo-first order kinetics to >90% of full activation. Surprisingly, however, its rate was much faster, with a half-time of only 3.7 min. The kinetics of activation remained the same whether an optimal or a limiting amount of P protein was used in the transcription assay (Figure 2A and B). These results are consistent with a model in which

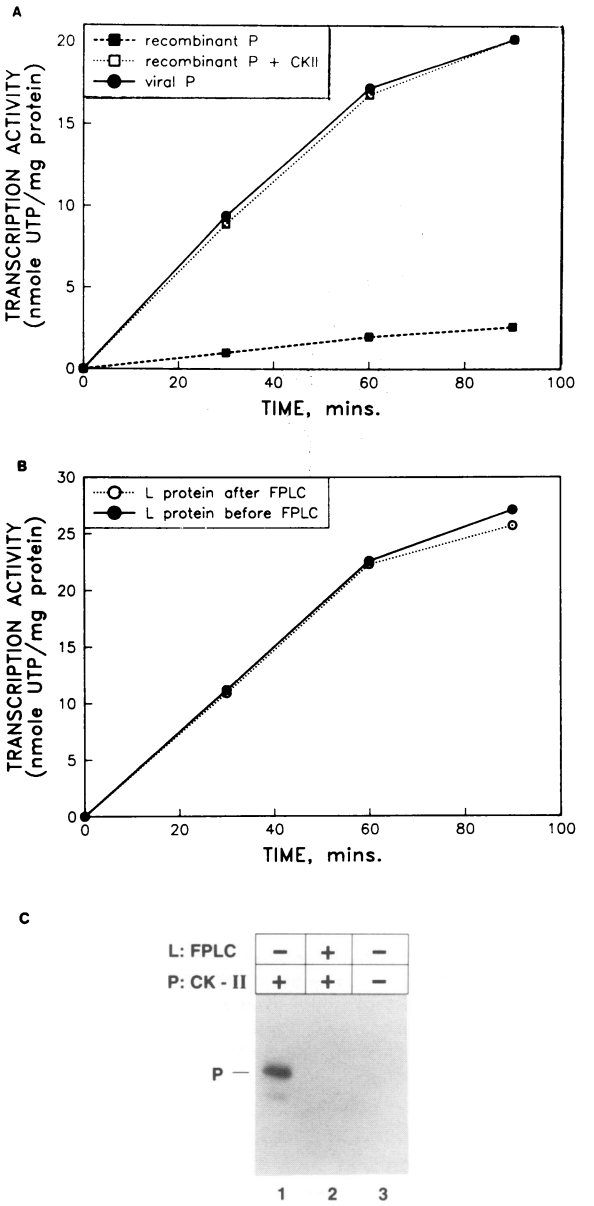

Fig. 1. (**A**) Viral transcription supported by wild-type recombinant P protein before (■) and after (□) treatment with CK-II, compared with transcription supported by viral P protein (●). (**B**) Viral transcription supported by wild-type recombinant P protein after CK-II treatment, before (●) and after (○) removal of LAK from L protein by gel filtration, and removal of N-RNA template-associated kinases by heating at 60°C for 30 min. (**C**) Radioautogram of a gel showing: lane 1, phosphorylation of wild-type, CK-II activated P protein by LAK during transcription; lane 2, lack of phosphorylation of wild-type CK-II activated P protein during transcription, after removal of LAKs; lane 3, lack of phosphorylation of wild-type P protein before activation by CK-II.

phosphorylation occurs at two equivalent and independent sites, with activation resulting from phosphorylation at either or both of these.

An alternative model to explain the kinetic data was considered, in which phosphorylation at the two sites was ordered and sequential, with the first phosphorylation being rate limiting and required for the second. This has been found for another phosphorylated protein, eEF-2 (Redpath *et al.*, 1993). If that were the case for P, only

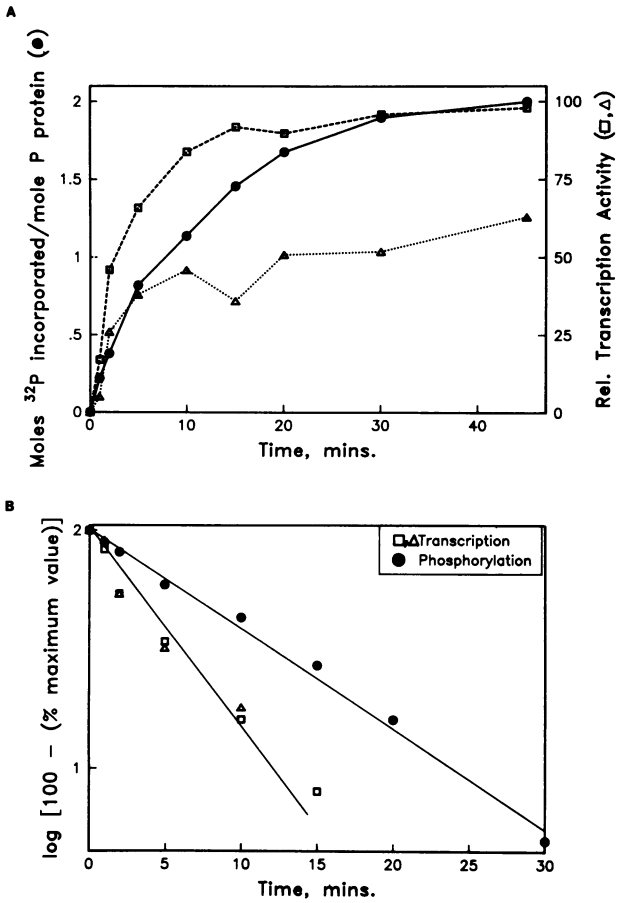

Fig. 2. (**A**) Time dependence of acquisition of phosphates (●) and transcriptional activity (□,△) by recombinant P upon treatment with CK-II. Transcriptional activity was assayed as described in Materials and methods using either 1 μg (□) or 0.5 μg (△) per assay tube. (**B**) Data from (A), plotted as a pseudo-first order reaction.

one singly phosphorylated form could exist, and that one might never be present in a significant amount, so that the doubly phosphorylated form might be the only active species. This possibility was ruled out, however, by showing that the kinetics of activation remained unchanged when the amount of P protein present in the assay became rate limiting for transcript formation (Figure 2A and B). It was concluded, therefore, that the first model was probably correct: transcriptional activation resulted from phosphorylation at one or both of two independent sites.

The stoichiometry of phosphorylation was confirmed using laser desorption mass spectrometry (LDMS; Table I). The mass added to recombinant P by CK-II was 147 Da, equivalent to 1.84 phosphate groups, in good agreement with the value determined by ^{32}P incorporation (Figure 2A and Table II). In contrast, viral P was more heavily phosphorylated, possessing excess mass equivalent to 4.88 phosphates. Thus, viral P possesses two or three more phosphate groups than are added by CK-II. These are presumably acquired in the infected cell, through the actions of LAKs or other cellular protein kinases. They appear to be completely irrelevant to transcriptional activity, however, neither increasing nor decreasing it (Figure 1A).

Preliminary localization of the radiolabeled phosphates added to P by CK-II was obtained by specific cleavage with 10% acetic acid (Matsudaira, 1990). Partial cleavage occurred at the single D−P bond, between residues 78 and 79, yielding an N-terminal fragment of 12 kDa [including the poly(His) tag] and a C-terminal fragment of 21 kDa (Figure 3, lane 2). Radioautography revealed that the radiolabel was present exclusively in the N-terminal fragment of the cleaved material (Figure 3, lane 1).

Identification of phosphorylated residues by aspartate (D) substitution

We next sought to identify the two residues that were phosphorylated by CK-II using D substitution. In studies of many proteins of very different kinds, replacement of phosphorylatable residues by D or E has produced mutants which functionally resembled the protein's phosphorylated form (e.g. Huang and Erikson, 1994). Previous work with P protein of VSV_{NJ} showed that substitution of S by A at either or both of only two adjacent CK-II consensus sites caused complete loss of transcriptional activity; substitution at other sites in the N-terminal region had little or no effect (Takacs *et al.*, 1992). The equivalent sites in this clone from VSV_{IND} (Hudson *et al.*, 1986) occur at positions 60 and 62:

55 60 62 67
QAADDSDTESEPE

Replacement of either one of these residues with D yielded a protein which possessed partial transcriptional activity when assayed at the usual level (1 μg), but which was fully active at a higher concentration (10 μg; Table II). Reaction products separated on gels were indistinguishable from those made using viral P (not shown).

Treatment of either mutant with CK-II yielded a fully active form. Stoichiometry of phosphorylation of both single mutants was just half that of wild-type P, i.e. one phosphate group was introduced (Figure 4 and Table II).

The double mutant, substituted with D at both positions 60 and 62, possessed full transcriptional activity without phosphorylation. This protein was not a substrate for CK-II (Figure 4 and Table II).

Multimer formation by transcriptionally active forms of P protein

In order to examine the self-associating properties of the various forms of P protein characterized above, they were subject to gel filtration on a Superose-12 column, in the same buffer used to assay transcription. Results are shown in Figure 5. Unphosphorylated wild-type P eluted as a monomer at ~30 kDa (Figure 5); interestingly, it did not exhibit the anomalous behavior seen on SDS gels, where it migrates with an apparent mol. wt of ~50 kDa. Treatment with CK-II induced a dramatic shift, causing the protein to elute at ~120 kDa, i.e. most likely as a tetramer. The single mutant S60D showed partial multimerization before treatment with CK-II, and complete multimerization, like the phosphorylated wild-type, after phosphorylation. The other single mutant, T62D, behaved similarly (not shown). The double mutant S60D/T62D required no phosphorylation for complete multimerization (Figure 5). In contrast, the corresponding inactive A mutant, S60A/T62A, eluted as a monomer (not shown). The tendency of these proteins

Table I. LDMS analysis of P protein phosphorylation

Source of P	Treatment with CK-II	Molecular weight (Da)			Phosphates per molecule[a]
		Calculated	Found	▲	
Viral	–	30 000[b]	30 390	+390[c]	4.88
Recombinant	–	33 253[b,d]	33 307	+54[c]	[0]
	+		33 454	+147[e]	1.84

[a] 80 Da mass added/phosphate, based on ▲.
[b] Assuming N-terminal methionine removal and acetylation (viral) or formylation (recombinant).
[c](Observed mass) − (calculated mass).
[d] Includes poly(His) tag and intervening residues.
[e] Mass added by treatment with CK-II.

to self-associate spontaneously into multimers thus correlates completely with their transcriptional activity. The partial multimerization of the single mutants accounts well for the observation that larger amounts of these proteins are needed for full activity, supporting the idea that the multimer is the active form (Table II).

These results were confirmed by crosslinking studies. As shown in Figure 6, the ability of the various forms of P protein to be crosslinked by low concentrations of crosslinker parallels completely the results from gel filtration (Figure 5) and transcriptional activity (Figure 2 and Table II). Thus, wild-type P cannot be crosslinked in its unphosphorylated state (Figure 6, lane 1), but becomes progressively more crosslinkable into a discrete, slower migrating band, as phosphorylation by CK-II proceeds (Figure 6, lanes 2–4). Both single mutants, S60D and T62D, are partially crosslinked in the unphosphorylated form (Figure 6, lanes 5 and 7), but become completely crosslinkable after phosphorylation by CK-II (Figure 6, lanes 6 and 8). The double mutant is completely crosslinked, like the phosphorylated wild type, but without treatment by CK-II (Figure 6, lane 9).

Table II. Activity and phosphorylation of P protein mutants

Mutant	Treatment with CK-II	Relative activity		
		1 μg P	10 μg P	Mol ^{32}P/mol protein
Wild-type	–	0	ND[a]	ND
	+	100[b]	ND	1.81
S60D	–	40	98	ND
	+	92	ND	0.88
T62D	–	42	101	ND
	+	90	ND	0.91
S60D/T62D	–	97	ND	ND
	+	ND	ND	0.01

[a] ND, not determined.
[b] 100 = 16.5 nmol UTP/mg protein/h.

Discussion

Perhaps the most striking conclusion to be drawn from these studies is that transcriptional activation of P by CK-II is simple and complete. While this might not be unexpected by analogy to other well-studied phosphorylated proteins, studies of VSV P protein have tended to emphasize the complexity of its phosphorylation pattern. As many as 21 different phosphorylation sites were inferred from proteolytic and chemical cleavages (Hsu *et al.*, 1982; Bell and Prevec, 1985), although the stoichiometry of phosphorylation was generally conceded to be much lower; hence, great heterogeneity was assumed. P protein from infected cells or purified virions was readily separated by gel electrophoresis or ion exchange chromatography into two fractions, which generally differed both in average degree of phosphorylation and in transcriptional activity. Differences were found between cell and viral P protein, and between fractions separated by several different techniques, adding further to the aura of complexity (Clinton *et al.*, 1979; Kingsford and Emerson, 1980; Barik and Banerjee, 1992a).

Our results suggest that most of that complexity may be irrelevant, at least for transcription. While viral P protein possessed four or five phosphates/P protein molecule (Table I), the activation and mutant studies described in this paper indicate that two or three of those phosphates were unnecessary for transcription activity. Specifically, phosphates added by kinases that are generally present (unless specifically removed) in the L- and N-RNA fractions of the transcription assay have no role in activation (Figure 1B), contrary to a recent report (Barik and Banerjee, 1992a).

On the other hand, the requirement for a single ubiquitously distributed kinase like CK-II (Tuazon and Traugh, 1991) ensures that activation of P protein for transcription will never be a limiting factor for VSV infectivity or host range. Our studies showed that transcriptional activation of P depends upon the addition of a single phosphate at either of two equivalent sites. This was demonstrated by comparing the kinetics of phosphorylation by CK-II with the kinetics of acquisition of transcriptional activity from CK-II: complete activation occurred by the time phosphorylation at the two CK-II reactive sites was only half complete (Figure 2). This conclusion was confirmed, and the specific sites identified, by showing that substitution of either or both of two phosphorylation sites, S60 or T62, by D residues conferred full transcriptional activity without phosphorylation. For the single mutants, however, larger amounts of protein were required for full activity (Table II).

The presence of two functionally important phosphorylation sites at closely neighboring positions is characteristic of many phosphorylated proteins, and has often confounded attempts to identify specific sites. These sites are generally assumed to be interdependent, with phosphorylation at one of the sites facilitating phosphorylation at the other, and at least one example of such behavior

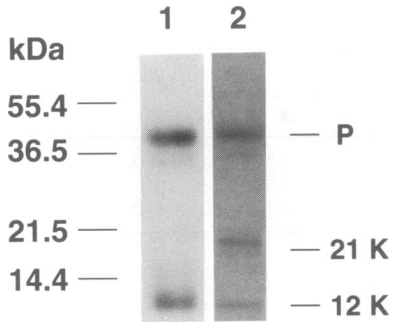

Fig. 3. SDS–polyacrylamide gel showing distribution of radiolabeled phosphate in wild-type ^{32}P-labeled P protein after hydrolysis in 10% acetic acid, 40°C, 48 h (Matsudaira, 1990). Lane 1, autoradiogram; lane 2, Coomassie blue staining.

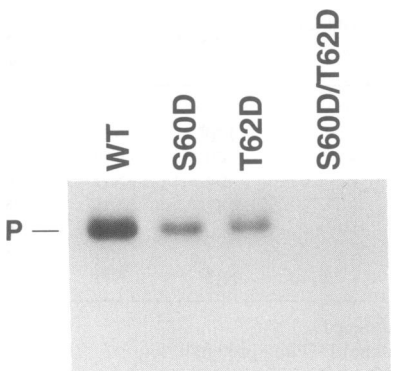

Fig. 4. Amount of radiolabeled phosphate incorporated from [γ-^{32}P]ATP into identical amounts of wild-type and mutant P proteins by CK-II. From left to right: wild-type; S60D; T62D; S60D/T62D.

is known (Redpath *et al.*, 1993). A protein possessing two closely located sites that are phosphorylated independently with identical functional effect has not, to our knowledge, been found previously. Both sites seem to be advantageous, since they are rigorously conserved through extensive evolutionary change (Bilsel *et al.*, 1990; Nichol *et al.*, 1993), raising the question of what function this apparent duplication might serve. Two separate answers may be suggested. First, double phosphorylation might enhance multimerization, and hence activation, compared with single phosphorylation. This is suggested by the properties of the singly substituted D mutants (Figures 5 and 6; Table II). The kinetics of Figure 2, however, suggest that the singly phosphorylated species may be more active than the corresponding D substituted mutants, perhaps even identical to the doubly phosphorylated form. Second, double phosphorylation might provide protection from cellular phosphatases, which now must remove two phosphates instead of one in order to inactivate the protein. Continuing action by the ubiquitous CK-II might make simultaneous removal of both phosphates difficult, once they become attached.

Although phosphorylation at the two sites is independent, the sites themselves may be capable of some interaction, since substitution of either site by an A residue completely abolished transcriptional activity (Takacs *et al.*, 1992). In contrast, substitution of these sites by D residues produced a protein that was not only inherently active, but that also permitted unimpeded phosphorylation by

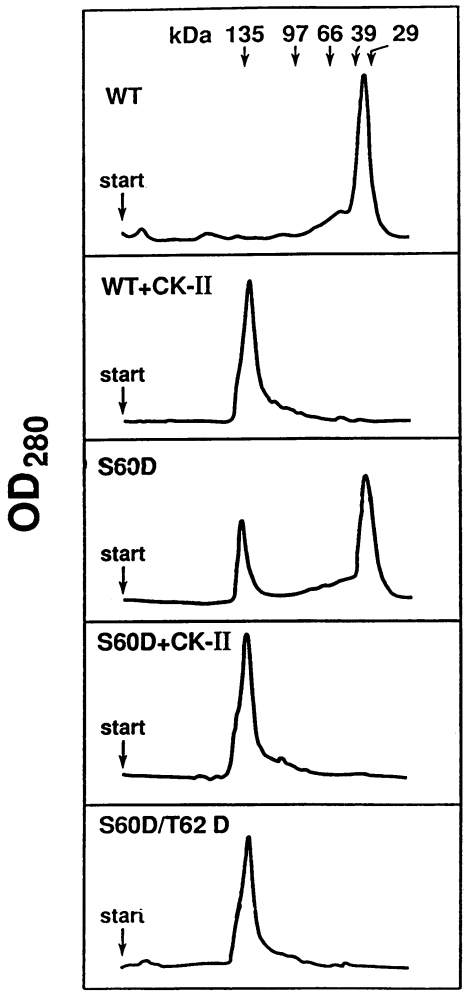

Fig. 5. Superose-12 elution profiles of P proteins in transcription buffer. From top to bottom: wild-type P, untreated; wild-type P, treated with CK-II; S60D, untreated; S60D, treated with CK-II; S60D/T62D, untreated.

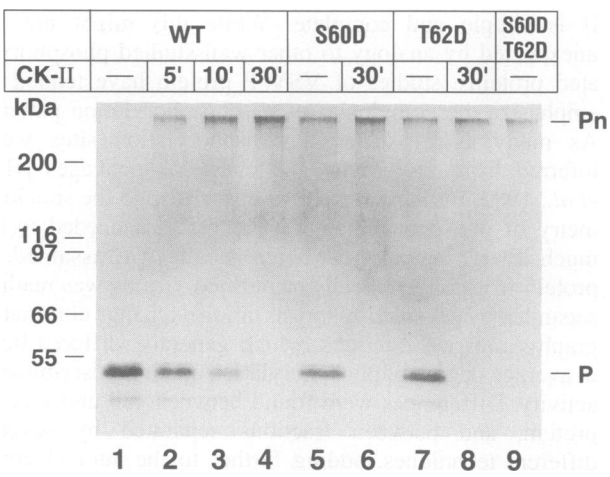

Fig. 6. SDS–polyacrylamide gels of P proteins after crosslinking. Lanes 1–4, wild-type P: untreated (lane 1); treated with CK-II for 5 min (lane 2); 10 min (lane 3); or 30 min (lane 4). Lanes 5–6, mutant S60D: untreated (lane 5) or treated with CK-II for 30 min (lane 6). Lanes 7–8, mutant T62D: untreated (lane 7); or treated with CK-II for 30 min (lane 8). Lane 9, mutant S60D/T62D.

CK-II at the remaining unmodified site (Figure 4), with concomitant increase in activity (Table II). The simplest interpretation of these results considered together is that an A residue at either position prevents substrate recognition of the other by CK-II, whereas D residues, or phosphoserines, do not have this effect. If this is so, then the phosphorylation that was observed when the A substituted mutants were expressed in COS cells (Takacs *et al.*, 1992) would have arisen from the action of other cellular kinases at different sites in the molecule.

It is noteworthy that no sites close to the C-terminus were phosphorylated by CK-II (Figure 3), or needed to be modified for activity. Earlier work had shown that substitution of either of two closely spaced C-terminal S residues by A reduced transcriptional activity by 75%, while the double substitution inhibited transcription by >90% (Chattopadhyay and Banerjee, 1987). Since the present work shows that phosphorylation at these sites is not required for activity, another explanation for the data must be sought, presumably in a conformational change or in the direct interaction between P and another component of the transcription machinery (Emerson and Schubert, 1987). Phosphorylation by LAK was also completely blocked by either of these substitutions (Chattopadhyay and Banerjee, 1987), but, as shown in Figure 1, this has no bearing on transcriptional activity.

The second major finding from these studies is that active forms of P protein self-associate into discrete multimers, probably tetramers. The evidence that this multimer is the active form of P is correlative, but striking: fully active forms are completely multimeric in transcription buffer, while the single D mutants, which require more P protein for full activity were only partially multimeric; unphosphorylated, hence inactive, wild-type P is negligibly multimeric. We suggest that the function of phosphorylation may be to permit the protein to adopt the active, multimeric form. Thus a third interaction of P, in addition to its necessary association with N-RNA template and L protein (Emerson and Schubert, 1987), must be postulated for transcriptional activity: self-association into the active multimeric form.

The finding that the transcriptionally active form of P is multimeric has a bearing on another function of P as well: complex formation with N protein. This association is thought to prevent aggregation of N and to provide the substrate for encapsidation of the replicative forms of genomic RNA. The functional complex is apparently a 1:1 dimer of N and P, consistent with its sedimentation velocity and its migration on gels (Davis *et al.*, 1986; Peluso, 1988; Masters and Banerjee, 1988). Thus, the transcriptionally active multimer of P does not participate in this other important function. It is interesting that recent studies have suggested that phosphorylation of P may not be required for complex formation with N (Takacs *et al.*, 1993), raising the intriguing possibility that phosphorylation and complexation with N might be competing reactions.

Materials and methods

Growth and purification of VSV

Monolayers of BHK-21F cells grown in roller bottles were infected with VSV_{IND} (Mudd–Summers strain; a gift of Dr J.Perrault) at m.o.i. = 0.01–0.1 p.f.u./cell for 16–18 h. Progeny virions were harvested and purified on a sucrose gradient as described (Rigaut *et al.*, 1993).

Purification of transcriptionally active nucleocapsids

A modification of standard protocols (De and Banerjee, 1984; Hunt *et al.*, 1984) was used. Transcriptionally active nucleocapsids were prepared from purified virions (1 mg/ml) disrupted in 10% glycerol, 0.25% (v/v) Triton N-101, 10 mM Tris, 0.25 M NaCl, 0.65 mM dithiothreitol (DTT), pH 8.0. The disrupted virus mixture was sedimented at 45 000 r.p.m. for 1.5 h through a 30–50–100% glycerol step gradient, and nucleocapsids were collected from the 50–100% glycerol interface.

Dissociation of nucleocapsids into L, P and N-RNA template components

Purified nucleocapsids were diluted 5-fold to a final buffer concentration of 0.8 M NaCl, 0.6 mM DTT, 0.5 mM EDTA, 10 mM Tris, pH 8.0, 25 U/ml RNAsin (Promega). After incubating on ice for 1 h, the released L and P proteins were separated from the N-RNA template on a 30–50–100% glycerol step gradient containing 0.8 M NaCl. L and P proteins were recovered from the top of the gradient, and N-RNA template from the 50–100% interface.

The released L and P proteins were dialyzed into column buffer (10% glycerol, 0.2 mM DTT, 33 mM Tris, pH 8.0) containing 0.1 M NaCl and loaded onto a 5–8 ml phosphocellulose P11 column (Whatman) equilibrated with the same buffer. The unbound fraction, containing P protein, was collected and concentrated on Amicon concentrator 10. The column was then washed with two volumes of column buffer containing 0.2 M NaCl, and L protein finally eluted with column buffer containing 1 M NaCl, plus 0.056 mM magnesium acetate. The L protein was diluted with an equal volume of column buffer lacking NaCl and stored at −70°C.

Further purification of L protein to remove L-associated kinases (LAKs) was effected using FPLC as previously described (Massey *et al.*, 1990). L protein eluted from the phosphocellulose column was concentrated to a volume of 200 µl, applied to a Superose-6 column (1×30 cm) equilibrated with column buffer containing 1 M NaCl and eluted at a flow rate of 0.5 ml/min. Fractions containing L protein were diluted 2-fold, concentrated and stored at −70°C.

The N-RNA template collected from the 50–100% glycerol interface was re-suspended in buffer containing 1 M NaCl, re-sedimented on a glycerol gradient, and stored at −70°C. It was heated at 60°C for 30 s before use in order to inactivate associated protein kinases (Massey *et al.*, 1990; Barik and Banerjee, 1992a).

Construction of P protein expression plasmids

The pGNS1 plasmid containing the *P* gene was kindly provided by Dr Manfred Schubert. Procedures are outlined in Figure 7. The main steps are as follows. First, pGEM-3zf(−) from Promega was modified so as to replace its *Xba*I site with an *Xho*I site by the method of Kunkel *et al.* (1987) using the synthetic oligonucleotide 5′-GGTCGACTCGAGAGG-ATCCCC-3′. The modified plasmid was confirmed by sequencing and designated pGEM-3zfx(−). Second, the *P* gene was excised from pGNS1 with *Xho*I and ligated into *Xho*I-treated pGEM-3zfx(−). The product containing the P reading frame downstream of the T7 promoter was identified by sequencing and designated pGEMx-P. Four residues were removed to correct the reading frame, using as primer the synthetic oligonucleotide 5′-GTGAGATTATCCATCTCGAGAGGATCCCC-3′. The resulting plasmid, designated pGEMx-PΔ4, was used as template to prepare the three mutant P proteins used in this study, S60D, T62D and S60D/T62D. The synthetic oligonucleotides used in mutagenesis were, respectively, 5′-GATTCTGTGTCGTCATCATCTGC-3′, 5′-GTT-CAGATTCGTCGTCAGAATCATC-3′ and 5′-GTTCAGATTCGTCGT-CGTCATCATCTGC-3′. Third, expression plasmids were constructed by excising the *wt* or mutated *P* genes from their respective pGEM plasmids with *Xho*I and ligating them into *Xho*I-digested pET-19b expression vector to produce plasmid pET-P, or pET-PS60D, etc.

Bacterial expression and purification of wt and mutant P proteins

Proteins were produced in *Escherichia coli* strain BL21(DE3)plysS (Novagen). Cells containing the appropriate plasmid were grown in 1 l of LB medium/ampicillin (50 µg/ml)/chloramphenicol (35 µg/ml) at 30°C to OD_{600} = 0.3. Isopropylthiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and growth was continued for 5 h at 30°C. Cells were harvested by centrifugation and resuspended in 20 ml of binding buffer containing 5 mM imidazole, 0.5 M NaCl, 20 mM Tris, pH 7.9, supplemented with protease inhibitors: 2 mM phenylmethyl-

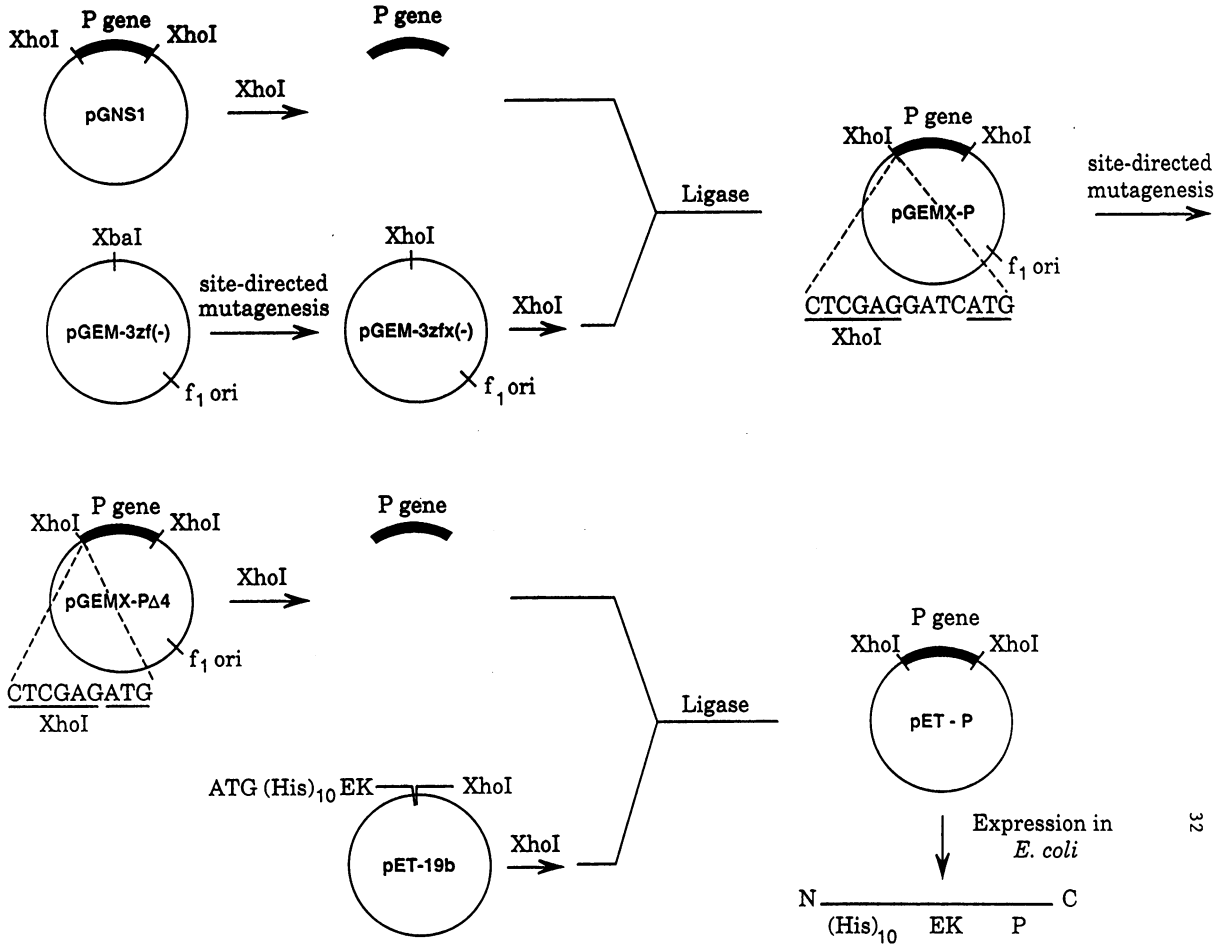

Fig. 7. Construction of plasmids used for mutating and expressing P proteins.

sulfonyl fluoride (PMSF), 20 μg/ml each of pepstatin A, leupeptin and aprotinin (all from Sigma). The cell suspension was sonicated until it was no longer viscous. The lysate was centrifuged at 20 000 *g* for 20 min to remove debris. The supernatant contained 15–20 mg P protein/l. Up to 90% of expressed protein was present in soluble form.

Purification was effected using the poly(His) tail present at the N-terminus of each protein (Figure 7). A His-Bind resin column (Novagen) of 2 ml volume was charged with 10 ml of 50 mM $NiSO_4$ solution. After equilibrating the charged column with binding buffer minus protease inhibitors, the extract was loaded onto the column at a flow rate of 0.3 ml/min. The column was then washed with 25 ml of binding buffer and 15 ml of binding buffer plus 60 mM imidazole, and the P protein then eluted using binding buffer plus 1 M imidazole. The protein was dialyzed against 10% glycerol, 0.1 M NaCl, 33 mM Tris, pH 8.0 to remove imidazole, diluted to a final concentration of 1 mg/ml with the same buffer, and stored at −70°C. The purified protein generally migrated as a single band on SDS gels, although a small band representing a proteolytic digestion product was sometimes present.

In vitro transcription assay

The standard reaction mixture (100 μl), in 0.1 M Na Cl, 5 mM $MgCl_2$, 50 mM Tris, pH 8.0, 4 mM DTT, 1 mM each ATP, GTP and CTP, 0.1 mM UTP, 5 μCi of [^{3}H]UTP, contained 1 μg L protein, 2.5 μg N-RNA template and 1 μg viral or recombinant P protein. Except where specifically indicated, L and N-RNA template had been freed of associated protein kinase activities as described above. Reactions were incubated at 32°C. At indicated times, 25 μl aliquots were taken, and the reaction terminated by addition of 50 μl yeast RNA (2 mg/ml in 0.067 M sodium pyrophosphate). Unincorporated label was removed from reaction products on a Bio-spin 30 spin column (Bio-Rad), and radioactivity of the products determined by scintillation counting.

Phosphorylation of P protein by CK-II

The standard reaction was carried out for 45 min at 32°C in 0.1 M NaCl, 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 1 mM each of DTT and ATP. Experiments in which the kinetics of phosphorylation and transcription were compared (Figure 2) contained 50 μg of P protein, 200 ng of CK-II (Sigma) and 40 μCi [γ-^{32}P]ATP (4500 Ci/mmol) in a total volume of 100 μl. Aliquots of 10 μl were taken at each time point, the reaction terminated by addition of SDS sample buffer, and P protein separated by SDS−PAGE. The labeled band was cut out of the gel and counted. Heparin (1 μg/ml) was added to stop the kinase reaction in aliquots that were to be used in transcription assays (performed as above).

Multimer analysis by FPLC

A Superose-12 column (Pharmacia, 1× 30 cm) was equilibrated with transcription buffer lacking the nucleoside triphosphates. An amount weighing 100 μg of each protein was applied to the column in 200 μl volume, and eluted at a flow rate of 0.5 ml/min.

Crosslinking

Experiments were carried out in 10 μl of transcription buffer containing 1 μg P protein and 0.09 mM disuccinimidyl suberate (DSS, Pierce) for 30 min at 4°C. Reactions were stopped by addition of SDS sample buffer and analyzed by SDS−PAGE.

Matrix-assisted laser desorption mass spectrometry (LDMS)

LDMS was performed on a Finnegan MAT Lasermat time-of-flight mass analyzer. Purified P proteins were dissolved in water at concentrations between 2×10^{-6} and 1×10^{-4} M. The matrix, alpha-cyano-4-hydroxy-cinnamic acid (Sigma), was dissolved in 70% (v/v) acetonitrile−30% H_2O containing 0.1% sequence grade trifluoroacetic acid (Sigma). Equal

aliquots (0.5 μl) of sample and matrix were loaded onto the gold spot of a standard slide for analysis.

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