Cloning and Expression of the Vesicular Stomatitis Virus Phosphoprotein Gene in *Escherichia coli*: Analysis of Phosphorylation Status versus Transcriptional Activity

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The phosphoprotein (P, previously known as NS) genes of vesicular stomatitis virus serotypes New Jersey and Indiana have been cloned in the *Escherichia coli* expression vector pET-3a. Transcription of P genes in these clones initiated from a phage T7 RNA polymerase promoter, whereas translation was driven by the Shine-Dalgarno sequence and the initiator AUG codon of the T7 gene 10 message. The clones were introduced into an appropriate *E. coli* strain in which T7 RNA polymerase was expressed under the control of the *lac* promoter. Under optimal conditions of induction with isopropylthiogalactopyranoside, P protein made in these bacterial strains constituted 5 to 20% of total cellular protein. P protein expressed in bacteria was unphosphorylated and transcriptionally active in an in vitro reconstitution assay with viral L protein and an N-RNA template. However, the P protein was phosphorylated in vitro by the kinase activities associated with L and the N-RNA template.

The virion-associated RNA-dependent RNA polymerase of vesicular stomatitis virus (VSV), a prototype rhabdovirus, is composed of a large polypeptide (L) and a phosphoprotein (P). Within the virion, these two proteins are associated with the negative-strand (anti-message sense) genomic RNA which is tightly wrapped with the nucleocapsid protein (N). This transcription complex synthesizes, both in vivo and in vitro, five viral mRNAs and a 47-nucleotide-long leader RNA (1, 2). Genetic and biochemical studies have suggested that the L protein contains all the basic transcriptional activities: RNA polymerase activity, 5'-capping activity, and poly(A) polymerase activity (4). This is also consistent with the finding that L protein is required in catalytic amounts for optimal RNA synthesis, whereas the requirement for P is stoichiometric (11).

Because of their essential role as transcription factors, the P proteins of VSV and other nonsegmented negative-strand RNA viruses have been studied in some detail in recent years. P proteins made in eukaryotic environments, including those present in mature virions and in VSV-infected cells, exist in a variety of phosphorylated forms. Proteolytic and chemical cleavage studies have mapped the "constitutively" phosphorylated residues in the amino-terminal region of the polypeptide (domain I), between residues 35 and 106 (6, 17, 24). The exact role of constitutive phosphorylation in P function is not known. From several lines of evidence, it has been suggested that phosphorylation might be important for the transcriptional activity of P; for example, (i) the more highly phosphorylated forms have higher transcriptional activity (21) and (ii) inhibition of phosphorylation of P by various compounds or extensive dephosphorylation of P also inhibits VSV transcription in vitro (8, 18, 20, 21, 29–32). In addition, phosphorylation of two specific serine residues in P protein domain II, at positions 236 and 242, catalyzed by L protein-associated protein kinase, seems to be required for transcription (7). Site-directed mutagenesis

of these serines to alanine significantly reduced the transcriptional activity of the P protein as well as its phosphorylation by L and its binding to L and the N protein-RNA complex (N-RNA) in vitro.

However, a number of important questions regarding P phosphorylation still remain unanswered. Is constitutive phosphorylation in domain I essential for P function? Is phosphorylation of a few critical residues more important than that of others? Is there a specific pathway of phosphorylation leading from one phosphorylated species to another? Does P possess an autophosphorylation activity that might be responsible for constitutive phosphorylation? Can L kinase also phosphorylate domain I, at least in part? Is phosphorylation in domain I an essential prerequisite for L-mediated phosphorylation in domain II? And lastly, what is the nature of the protein kinase?

In order to obtain a definitive answer to many of these questions, it is essential to obtain a P protein totally devoid of phosphorylation. Thus, our attempt to express P protein in bacteria was based on the following assumptions: (i) since bacterial protein kinases are generally specific for their natural substrates and are not as prevalent as in eukaryotes, P protein synthesized in bacteria might be completely free of phosphates, and (ii) bacterial expression would provide a quick and inexpensive source of large quantities of P protein amenable to detailed biochemical and physical studies, including crystallography. In this communication, we describe the subcloning of the P genes of two VSV serotypes, New Jersey (NJ) and Indiana (IND) and their expression in the gram-negative bacterium *Escherichia coli*. We also show that in spite of the lack of phosphorylation, the P protein synthesized in *E. coli* is transcriptionally active in vitro. However, it can also be phosphorylated in vitro by protein kinase activities associated with viral L protein and the N-RNA template. These results provide the first report of bacterial synthesis of a functional VSV protein. Throughout the text, we have referred to the P protein synthesized in bacteria as bacterial P protein for the sake of brevity.

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MATERIALS AND METHODS

Cells, viruses, and viral proteins. VSV (IND), Mudd-Summers strain, and VSV (NJ), Ogden strain, were grown in roller bottles containing freshly confluent monolayers of baby hamster (Syrian) kidney cells (BHK-21; ATCC CCL 10). The cells were infected with plaque-purified VSV stock at a multiplicity of infection of 0.05 in complete Eagle's minimal essential medium (Eagle's 1× MEM [2 mM glutamine, penicillin, streptomycin, and nonessential amino acids] at 5 ml per roller bottle) without serum. Adsorption was allowed to proceed at 37°C for 2 h. Then 75 ml of fresh complete Eagle's medium containing 10% fetal calf serum was added to each bottle, and virus growth was continued for another 24 to 30 h. Cells were harvested when approximately 90% of the cells showed characteristic morphological change (spherical shape or lysis and detachment). All cell culture reagents and media were purchased from GIBCO.

Following lysis, medium containing virus was poured off and centrifuged in the cold (4°C) at $4,500 \times g$ for 10 min to remove unlysed cells and debris. The supernatant was centrifuged at $120,000 \times g$ for 1 h. The pellet, containing nearly pure virus, was rinsed with TE (20 mM Tris-Cl [pH 8.0], 1 mM EDTA), resuspended in 2 ml of TE (per roller bottle) by soaking overnight, then by pipetting, and finally by vigorous sonication in an ice-bath sonicator with a strong focus. The virus was further purified by centrifuging through glycerol-tartrate gradients as follows. Linear gradients (10 ml) were made with 5.5 ml of 30% glycerol and 4.5 ml of 50% potassium tartrate in TE, so that the concentration of potassium tartrate increased from the top to the bottom of the gradient while that of glycerol decreased in the same direction. Virus suspension (2 ml) was layered on each of the gradients, which were then centrifuged at $150,000 \times g$ in an SW40 rotor for 16 h (overnight) at 4°C. The opaque virus band near the middle of each gradient was collected, pooled, and diluted approximately fivefold with cold TE. Virus was again pelleted by high-speed centrifugation and subjected to a second cycle of glycerol-tartrate gradient purification as above. The final preparation was resuspended in TE to a protein concentration of ~5 mg/ml and stored in liquid nitrogen.

Viral L and P proteins were purified from virions by detergent disruption followed by phosphocellulose chromatography as described before (10), except that following elution from phosphocellulose with 1 M NaCl buffer, the L protein was directly concentrated by Amicon ultrafiltration without dialysis. The N-RNA template was purified exactly as described previously (26). All purified viral components were stored in small portions in liquid nitrogen.

The polyclonal mouse antibody against VSV (NJ) was kindly supplied by Robert E. Shope.

Subcloning of P genes in pET-3a and production of P proteins in *E. coli*. P genes of both serotypes (NJ and IND) cloned in pGEM-4 (15, 27) were amplified by polymerase chain reaction (PCR), with the reagents and DNA thermal cycler of Perkin-Elmer Cetus. PCR reaction mixes contained 50 mM KCl, 20 mM Tris-hydrochloride (pH 8.4), 2.5 mM MgCl₂, bovine serum albumin (100 µg/ml), 200 µM each of the four dNTPs, 2 U of Ampli-Taq DNA polymerase, 0.5 µg of supercoiled pGEM-4-P template, and 0.2 µg of each primer per 100-µl reaction mix. Thermal cycle parameters were 94°C, 2 min (denaturation); 40°C, 2 min (annealing); 72°C, 2 min (elongation); and a total of 30 cycles. A time delay of 94°C and 7 min (for initial denaturation of the supercoiled template) and 72°C and 4 min (final extension)

was incorporated at the beginning and end, respectively. The following oligonucleotides were synthesized in an Applied Biosystems DNA synthesizer model 380B with phosphoramidite chemistry. For the NJ serotype: upstream primer, 5'-TATATACATATGGACAGTGTGATAGGC-3'; downstream primer, 5'-TATATAGGATCCAATTAAGACGATATTTCATTCTTGCC-3'. For the IND serotype: upstream primer, 5'-TATATACATATGGATAATCTCACAAAAGTTCGTGAG-3'; downstream primer, 5'-TATATAGGATCCA GTCTACAGAGAATATTTGACTCTCGCC-3'. Note that the upstream primers contain an *Nde*I site (CATATG) and the downstream ones contain a *Bam*HI site (GGATCC) (underlined). All nucleotides 3' of these sites have homology with the P gene 5' (for upstream primer) and 3' (for downstream primer) ends (14). All primers also contain an A+T-rich hexanucleotide ("clamping") sequence at the 5' end for optimal restriction enzyme recognition.

The PCR-amplified products were gel purified, restricted with *Nde*I and *Bam*HI, and ligated with pET-3a restricted with these two enzymes. Initial transformation and screening were done in *E. coli* HB101; prospective clones were then introduced into *E. coli* BL21(DE3). For the production of the P protein, BL21(DE3) containing pET-3a-P was grown at 37°C in LB broth (Difco) containing glucose (0.3%) and ampicillin (100 µg/ml). Glucose improved the growth of the NJ strain and was absolutely necessary for the growth of the IND strain. We presume that P protein is toxic for *E. coli* and that catabolite repression mediated by glucose reduces a small, basal level of transcription that takes place from the *lac* promoter in DE3 (resulting in the synthesis of T7 RNA polymerase) even in the absence of isopropylthiogalactopyranoside (IPTG). When the culture reached an A_{590} of 0.3, IPTG was added to 1 mM, and growth was continued for another 2 h. The culture was then quick-chilled in ice-water, and cells were harvested by centrifugation. The cells were lysed with lysozyme-EDTA in the presence of Triton X-100; P protein was extracted from the insoluble fraction and purified through phosphocellulose and by two cycles of DE52 chromatography. The final preparation was at least 95% pure; approximately 15 mg of P protein of this purity was obtained from 1 liter of bacterial culture.

VSV transcription reconstitution in vitro. Reconstituted VSV transcription reaction mixes (20 µl) contained 50 mM Tris-hydrochloride (pH 8.0), 5 mM MgCl₂, 2 mM dithiothreitol (DTT), 50 µM CTP, 1 mM each ATP, GTP, and UTP, 2 µCi of [α -³²P]CTP (400 Ci/mmol; Amersham), 750 ng (with respect to N protein) of N-RNA, 300 ng (2 µl) of L protein, and the indicated amounts of P protein purified from either virus or bacteria. The L preparation contributed the optimum NaCl concentration (100 mM) for the reaction. Transcription reaction mixes were incubated at 30°C for 110 min and then chased with 1 mM CTP for 10 min; 100 ng of oligo(dT)₁₈ and 0.5 U of RNase H (Boehringer Mannheim) were then added to the reaction, and incubation was continued for another 10 min. In our experience, treatment of transcripts with oligo(dT) and RNase H in situ (i.e., in the transcription reaction) offered significant improvements over the existing practice of isolating the transcripts followed by a separate RNase H reaction. By minimizing handling of the RNA, the in situ RNase H treatment method produces more consistent results and saves time. The radiolabeled transcripts were deproteinized by phenol-chloroform, precipitated with ethanol, and resolved by electrophoresis in a 5% polyacrylamide-7 M urea gel as described by Barik et al. (3).

Gel electrophoresis and Western immunoblotting of proteins. Proteins were electrophoresed in 10% (total) discon-

tinuous polyacrylamide gels (acrylamide-bisacrylamide, 30:0.4) containing sodium dodecyl sulfate (SDS) as described by Laemmli (22). For autoradiography of ^{35}S -labeled protein bands, gels were treated with 1 M sodium salicylate for 30 min and then dried in a Bio-Rad gel dryer. Western blotting was performed with mouse polyclonal anti-VSV antibody as the first antibody and horseradish peroxidase-conjugated rabbit anti-mouse IgG as the second antibody, by the procedure described by Barik et al. (3).

Radiolabeling of bacterial cells. For the *in vivo* labeling experiments in Fig. 4, *E. coli* cells were grown in low-phosphate glucose-peptone medium of the following composition: Tris base, 1.2%; Bacto-peptone (Difco), 0.75%; NaCl, 0.3%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10^{-4} M; CaCl_2 , 10^{-5} M; glucose, 0.3% (23). The pH of the medium was adjusted to 7.1 before addition of Ca^{2+} and Mg^{2+} . Induction with IPTG was done as described above. Simultaneous with the addition of IPTG, either 15 μCi of [^{35}S]methionine (800 Ci/mmol; Amersham) or 50 μCi of carrier-free $^{32}\text{P}_i$ (Amersham) was added per ml of culture. Following harvest, cells were washed once with ice-cold normal saline, and total cell proteins were analyzed by electrophoresis and autoradiography, as described above.

RESULTS

Cloning of the P gene and expression of P protein in *E. coli*. The pET-3a vector used in this study is a derivative of pBR322 and a member of the pET (expression T7) series of vectors, all of which contain the strong T7 RNA polymerase promoter preceding gene 10 in the T7 genome (28). pET-3a also contains, in the mRNA form, the highly efficient translation initiation signals—Shine-Dalgarno sequence and initiator AUG codon—of the gene 10 mRNA plus a portion of its N-terminal coding sequence. In addition, a strong transcriptional terminator of T7 RNA polymerase situated downstream of the cloning site prevents continuous circular transcription. We decided to use the unique *Nde*I and *Bam*HI sites of this vector to clone the P gene, since this would remove the gene 10 coding sequence and hence produce a P protein devoid of any extraneous amino acid residues (Fig. 1). We started with clones of P strain IND (P_{IND}) (27) and New Jersey (P_{NJ}) (15) serotypes in pGEM-4. By using these clones as templates and synthetic oligonucleotides with overhanging *Nde*I and *Bam*HI sequences as upstream and downstream primers, the P genes were amplified by PCR. The PCR products were inserted into pET-3a through restriction and ligation to produce the desired P clones (Fig. 1). The DNA sequence of both clones was confirmed by restriction analyses and dideoxy sequencing; relevant portions of the sequences common to both clones are shown at the bottom of Fig. 1. The pET-3a-P clones were then introduced into *E. coli* BL21(DE3), a lysogen containing lambda derivative prophage DE3, in which the T7 RNA polymerase gene is under the control of the *lacUV5* promoter (28). The transformants were induced with IPTG, and total cellular proteins were electrophoresed.

Figure 2 shows a Coomassie blue-stained protein profile of induced and control uninduced cultures. Clones of both serotypes expressed a major polypeptide upon induction: the P_{IND} clone produced a polypeptide of ~55 kDa, and P_{NJ} produced one of ~50 kDa. These polypeptides were absent in the strain containing vector pET-3a alone (lane H); in the uninduced P strains, these polypeptides were either barely detectable (NJ) or present in smaller amounts (IND).

The P protein of VSV is known to exhibit anomalous

FIG. 1. Cloning of P gene in pET-3a. The 4.5-kb vector pET-3a with relevant restriction sites is shown at the top (28). The T7 terminator (tT7) and the transcriptional and translational start regions (pT7-s10) are indicated. Unique restriction sites for *Nde*I and *Bam*HI (shown in boldface) were used for cloning of the P gene. PCR amplification of the P gene cloned in pGEM-4 with *Nde*I and *Bam*HI primers and subsequent insertion into pET-3a are described in the text. Sequences of transcriptional and translational control regions are shown at the bottom. The *Nde*I site (CATATG) overlaps the initiator methionine codon ATG of the P genes of both serotypes in the final clone. SD, Shine-Dalgarno sequence.

(slow) mobility in SDS-polyacrylamide gel electrophoresis (PAGE). While the calculated molecular masses of the IND and NJ polypeptides are 29 and 31 kDa, respectively, their mobilities relative to protein standards are in the range of ~49 to 55 and ~47 to 50 kDa, respectively, the actual value depending on the gel composition used. It is quite apparent in Fig. 2 that bacterial P proteins also showed this anomalous mobility, which is identical to that of their viral counterparts of the corresponding serotype.

To ascertain that the overproduced polypeptide represented authentic P protein, we electrophoresed whole-cell extracts of IPTG-induced and uninduced strains in SDS-PAGE as before and probed the proteins with polyclonal anti-P antibody by Western blot. The results presented in Fig. 3B show that the antibody reacted specifically with the overproduced P polypeptides and did not react with host bacterial proteins which were constitutively expressed. A control extract of IPTG-induced *E. coli* BL21(DE3) containing the pET-3a vector did not cross-react with the anti-P antibody (Fig. 3B, lane 0). Finally, P proteins of both serotypes were purified to homogeneity as described briefly

FIG. 2. Overproduction of recombinant P protein in *E. coli*. IPTG-induced (+ lanes) and uninduced (– lanes) *E. coli* BL21(DE3) containing the pET-3a vector (lane H) or pET-3a-P clones of the IND and NJ serotypes were lysed by boiling in Laemmli sample buffer (22). Total cellular proteins (50 µg) were electrophoresed in polyacrylamide gels containing SDS (Bio-Rad) by the method of Laemmli (22). Lane M represents standard protein markers of indicated molecular mass (in kilodaltons). Lanes marked VSV IND and VSV NJ represent whole viral proteins (as indicated) of the respective serotypes. Following electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (Bio-Rad).

in Materials and Methods. A silver-stained profile of the pure preparations (used in transcription reconstitution and phosphorylation studies described later) is presented in Fig. 3C; corresponding pooled DE52 fraction obtained from

FIG. 3. Western blot of bacterial P proteins. (A) Coomassie blue-stained protein standards. Lane M, size markers (from the top: 97, 68, 46, and 30 kDa). The next two lanes show whole viral proteins of IND and NJ serotypes, as indicated. (B) Western blot. Total protein (50 µg) from IPTG-induced *E. coli* BL21(DE3) containing the pET-3a vector (lane 0) or pET-3a-P clone of the NJ and IND serotypes was resolved in SDS-polyacrylamide gels as in Fig. 2. The proteins were electroblotted to a nitrocellulose membrane and subjected to Western blot analysis with anti-VSV antibody as described in Materials and Methods. (C) Silver-stained pure P proteins (5 µg) of the two serotypes (as indicated) and the corresponding pooled DE52 fraction obtained from control BL21(DE3) containing the pET-3a vector (lane 0).

FIG. 4. Phosphorylation status of bacterial P proteins. (A) Coomassie blue-stained protein pattern of various bacterial cell extracts (50 µg of protein in each lane) and whole viruses. (B) Autoradiograph of the same gel. The symbols + and – indicate IPTG-induced and uninduced cell extracts, respectively. Host lanes represent extracts of BL21(DE3)(pET-3a). ^{35}S and ^{32}P indicate that the cell culture was labeled with either [^{35}S]methionine or $^{32}\text{P}_i$, as detailed in Materials and Methods. R, Rabbit reticulocyte lysates in which P proteins of indicated serotypes have been translated; V, whole viral proteins of the indicated serotype; M, size markers (in kilodaltons). The arrow shows an unidentified phosphorylated protein (see text).

BL21(DE3)(pET-3a) did not contain any detectable protein (Fig. 3C, lane 0).

Bacterial P protein is not phosphorylated. In order to test whether P protein expressed in *E. coli* is phosphorylated, we grew P_{IND} and P_{NJ} strains in a low-phosphate medium. Carrier-free $^{32}\text{P}_i$ was added to the cultures at the time of addition of IPTG. Parallel cultures were labeled with [^{35}S]methionine to confirm the synthesis of P protein during ^{32}P labeling. Figure 4 shows a Coomassie blue-stained total protein pattern of such cultures and an autoradiograph of the same gel. Whole viral proteins of both serotypes were also included (lanes marked V) for comparison of mobility. In addition, P mRNA synthesized *in vitro* by transcription of pGEM-P clones with phage SP6 polymerase was translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine (15); the labeled proteins were then electrophoresed in parallel lanes (marked R). Like the viral P protein, these *in vitro*-synthesized P proteins have been shown to be transcriptionally active and probably also phosphorylated (7). P proteins of both serotypes were seen to be induced by IPTG in the low-phosphate medium, as judged by staining as well as [^{35}S]methionine labeling. However, in cultures labeled with ^{32}P , the P protein bands did not contain any detectable radioactivity in spite of the considerable amounts of these proteins seen in the corresponding stained lanes. We therefore conclude that P proteins synthesized in *E. coli* are not phosphorylated. (Based on the total phosphate content of

FIG. 5. In vitro reconstitution of VSV transcription. Transcription of both serotypes of VSV was reconstituted in vitro with purified viral L protein (300 ng) and N-RNA template (750 ng) and P proteins purified from either bacteria or virus, as described in Materials and Methods; autoradiographs of ^{32}P -labeled transcripts of different genes (as indicated on the left and right for the NJ and IND serotypes, respectively) are shown. Lanes: 0, no P protein; R, an aliquot of a control transcription reaction with viral nucleocapsids (after treatment with low salt to remove M and G proteins [10]); v1 and v2, 50 and 150 ng of viral P protein, respectively; b1 and b2, 50 and 150 ng of bacterial P protein used in reconstituted transcription reactions, respectively.

peptone used in the growth medium, the specific activity of ^{32}P calculated therefrom, and assuming that we should be able to detect 20 cpm of ^{32}P in our autoradiograph, we estimate that bacterial P proteins contain less than 1 phosphate residue per 20,000 polypeptides.) It is interesting that another polypeptide (migrating more slowly than the P protein) is strongly labeled by ^{32}P in the IPTG-induced cells. We presume that it is T7 RNA polymerase, since it has a very similar mobility (~ 99 kDa) and it is also induced in the host strain BL21(DE3) itself (data not shown). Whatever the nature of this polypeptide, it serves as a positive control in our phosphorylation studies, reinforcing the conclusion that bacterial P protein is not phosphorylated.

Bacterial P protein is transcriptionally active. Since bacterial P protein is free of phosphate, it is important to determine whether it is transcriptionally active. In vitro transcription reconstitution experiments were therefore performed with bacterial P proteins and viral L proteins and N-RNA templates of the homologous serotype. Transcripts were treated with oligo(dT) and RNase H in situ as described in Materials and Methods and resolved in a 5% polyacrylamide-7 M urea gel, an autoradiograph of which is shown in Fig. 5. The bacterial P protein of any given serotype is transcriptionally as active as its viral counterpart of the same serotype: (i) the sizes and relative ratio of different viral messages (G, N, P, and M) produced by viral and bacterial P proteins are indistinguishable; (ii) the specific activities of the two P proteins are very similar; with excess N-RNA template and L protein, 50 ng of P protein from either bacteria or virions produced comparable amounts of message; and finally, (iii) with a 10-fold molar excess (over L), both viral and bacterial P proteins produced similar amounts of mRNAs. The corresponding DE52 fraction (containing no detectable protein, as shown in Fig. 3C, lane 0) obtained from BL21(DE3)(pET-3a) was devoid of transcriptional activity when reconstituted with L and N-RNA in parallel transcription reactions (data not shown).

Phosphorylation of bacterial P protein by L protein- and N-RNA-associated kinases. Highly purified viral L and N-RNA preparations have been shown to contain potent protein kinase activities that phosphorylate viral P protein in

FIG. 6. Phosphorylation of P protein by L and N-RNA. In vitro phosphorylation reactions (20 μl) with 100 ng of viral or bacterial P protein of either serotype as the substrate and 150 ng of L and/or 300 ng of N-RNA of the homologous serotype as the source of kinase were performed at 30°C for 2 h in VSV transcription buffer (see Materials and Methods) containing 100 μM [$\gamma\text{-}^{32}\text{P}$]ATP (30 μCi) and 1 mM each of GTP, CTP, and UTP (to simulate transcription conditions). ^{32}P -labeled proteins were then resolved in SDS-urea-polyacrylamide gels and autoradiographed exactly as described previously (26). Where indicated, 2 μg of bacterial (*E. coli*) alkaline phosphatase (BAP; US Biochemicals) and ATP (to 1 mM) were added to a parallel reaction following phosphorylation, and incubation was continued for another 15 min at 30°C. In the control reaction, ATP alone was added. RNP indicates that viral nucleocapsid (as in Fig. 4) was incubated under identical conditions. P(B) and P(V), bacterial and viral P proteins, respectively. The two phosphorylated species, P1 and P2, of the P protein of Indiana serotype were previously called NS1 and NS2 (16, 26). The extreme right lanes show the mobility of the nonphosphorylated bacterial P proteins (^{35}S labeled).

vitro (26, 29). The various phosphorylated forms of P_{IND} can be resolved into two subsets of protein bands, called P1 and P2 (previously called NS1 and NS2) in polyacrylamide gels containing SDS and urea (9, 16); phosphorylated P_{NJ} , however, migrates as a single band under the same conditions (26). Since bacterial P protein was free of phosphorylation and yet transcriptionally active like phosphorylated viral P protein, we wanted to test whether L- and N-RNA-associated kinases could potentially phosphorylate bacterial P in vitro. L protein and N-RNA were purified from virions of both serotypes, and bacterial P proteins of a given serotype were then incubated with an L and/or N-RNA template of the same serotype in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP. The ^{32}P -labeled proteins were resolved in an SDS-urea gel; the autoradiograph of the gel is shown in Fig. 6.

As reported previously (26), L and N-RNA preparations of both serotypes are seen to possess protein kinase activity. Upon phosphorylation, the IND serotype of bacterial P protein produced the same two electrophoretic classes, P1 (NS1) and P2 (NS2), referred to above, irrespective of

whether L or N-RNA was the source of kinase. However, the ratio of radioactivity in the two bands varied with the kinase source; whereas L-associated kinase labeled P1 and P2 to a comparable extent, the N-RNA-associated kinase labeled P2 more intensely than P1, suggesting that the two kinase activities are probably distinct and different. When incubated in vitro with [γ - 32 P]ATP alone, bacterial P of neither serotype was labeled. These results along with our earlier finding that P protein synthesized in vivo (in *E. coli*) fails to incorporate 32 P (Fig. 4) demonstrate that P protein lacks any autophosphorylation activity. The NJ serotype of bacterial P protein was also phosphorylated by L and N-RNA of the NJ serotype in a similar manner except that the phosphorylated forms of this serotype of P protein migrated as a single band in an SDS-urea gel. The mobility of this species seemed to be identical to that of P2 of the IND serotype. Interestingly, phosphorylation-free bacterial P itself migrated like one of its phosphorylated forms; bacterial P_{IND} migrated like P1, whereas bacterial P_{NJ} had the same mobility as its phosphorylated form. Thus, some of the phosphate groups of P do not affect the mobility of the protein in the SDS-urea gel system.

Using viral P protein as substrate, Kingsbury and coworkers (18) and Masters and Banerjee (26) showed earlier that the phosphate groups of P2 are highly susceptible to treatment with phosphatase whereas those of P1 are relatively resistant. We observed essentially similar properties of the P1 and P2 species obtained by phosphorylating bacterial P with L and/or N-RNA. As shown in Fig. 6, the P2 band completely lost its 32 P label upon phosphatase treatment, while that of P1 remained virtually unaffected. Similarly, a portion of the 32 P label of the single phosphorylated form of bacterial P_{NJ} was lost upon phosphatase treatment, while the rest of the label was resistant. The differential sensitivity to phosphatase strongly suggests that the two classes of phosphorylated bacterial P protein represent conformationally different states of P protein, those of P_{IND} only being resolved by SDS-urea gel electrophoresis.

DISCUSSION

We have been able to express the P phosphoprotein gene of VSV in *E. coli*. The resultant protein was shown to be transcriptionally active upon reconstitution with viral L protein and N-RNA template. Unlike its viral counterpart, P protein synthesized in *E. coli* is not phosphorylated. The L and N-RNA preparations, however, possess protein kinase activity, which can phosphorylate P in reconstituted transcription reactions. Thus, we cannot conclude whether unphosphorylated P itself is transcriptionally active or requires phosphorylation for activation. Massey et al. (25) recently claimed that the protein kinase activity present in L preparations could be separated from the transcriptase activity of the L protein by gel filtration chromatography. They were also able to remove about half of the casein kinase activity from the N-RNA template. The resultant L and N-RNA preparations were shown to be nearly free of viral P-phosphorylating activity. However, they appeared to retain transcriptional activity when reconstituted with viral P protein, suggesting that further phosphorylation of the viral P protein did not occur during transcription. Thus, it remains to be seen whether such "kinase-free" L and N-RNA preparations also fail to phosphorylate bacterial P protein and whether ribonucleoprotein reconstituted with bacterial P protein is transcriptionally active. Such studies should be decisive in establishing the role of constitutive phosphory-

lation in P function. In a different approach, we are also studying the effect of mutating specific serine and threonine residues to alanine in domain I of the NJ serotype P protein on the phosphorylation status and transcriptional activity of the protein.

A number of proteins with highly acidic domains exhibit slower mobility in SDS-PAGE. Although experimental evidence is lacking, the negative charge of the protein is believed to exclude binding of SDS, resulting in incomplete denaturation of the protein and hence its slower mobility. It has been suggested that the phosphate groups of the P protein may contribute to its aberrant mobility either by increasing the negative charge of the protein (8, 9) or through the formation of phosphodiester bonds, which leads to dimers of the protein (13). However, we have shown (Fig. 4) that unphosphorylated bacterial P has the same mobility as phosphorylated viral P; the phosphate groups, therefore, must not play any role in the anomalous mobility. We presume that the acidic amino acid residues (Asp and Glu) in domain I as well as some other structural features of this domain may be responsible for the anomalous mobility of P protein (30a).

We wish to emphasize that bacterial P protein is naturally free of phosphorylation yet contains a wild-type primary sequence. This provided us with the opportunity to test whether constitutive phosphorylation of P is due to an autophosphorylating activity of the P polypeptide. As mentioned before, the very occurrence of unphosphorylated bacterial P and the absence of its self-phosphorylation in vitro demonstrate the lack of any autophosphorylation activity. Other evidence suggests that constitutive phosphorylation of viral P protein is catalyzed by a eukaryotic cellular kinase(s): recombinant P protein expressed in eukaryotic (COS) cells was found to be highly phosphorylated, probably at the constitutive sites, in the absence of any other VSV protein (19); and P protein translated from P mRNA in rabbit reticulocyte lysates in vitro appeared to be fully phosphorylated in domain I (7). Interestingly, our experiments on in vitro phosphorylation of bacterial P by L and N-RNA apparently produced the same phosphorylated species that are obtained by phosphorylating viral P which is already constitutively phosphorylated. It is possible that the microstates of the various phosphorylated forms of bacterial P protein comigrate as one band, P1, for example, which are different from those of the viral P proteins. However, the following similarities between viral and bacterial phosphorylated forms argue against such a possibility: (i) identical mobilities of viral and bacterial P1 (or P2), (ii) similar ratio of radioactivity in P1 and P2, and lastly (iii) both bacterial and viral P2 are totally sensitive to phosphatase, whereas the P1's of both are resistant. Thus, it seems that the majority of P species present in bacterial P1 and P2, like their viral counterparts, are most likely constitutively phosphorylated. Since in these experiments, phosphate-free bacterial P protein substrate was acted upon by the L or N-RNA preparation as the sole source of kinase, it follows that these preparations perhaps contain a cellular kinase or cellular kinase-like activity. Alternatively, the protein kinase activities associated with the L and the N-RNA complex can also phosphorylate at the constitutive phosphorylation sites of the bacterial P protein.

The nature and origin of the kinase activities present in L and N-RNA have not been studied in detail. As to the origin of the kinase, two models have been entertained: it is an inherent property of the L polypeptide (29) and the N-RNA complex; and the host kinase(s) is tightly bound to or

copurifies with L and N-RNA (25). We have argued above that at least part of the kinase activity associated with L and N-RNA might be of cellular origin; the other activity could be an intrinsic property of these macromolecules. In the case of L protein, this latter activity is believed to specifically phosphorylate two Ser residues in domain II of the P protein (7). More recently, Einberger et al. (12) have provided strong evidence that the L protein of Sendai virus, a member of the *Paramyxoviridae* family and closely related to VSV (4), also possesses protein kinase activity. These authors have renatured the L protein from SDS-polyacrylamide gels and then immunoprecipitated it by monoclonal antibodies against a synthetic peptide corresponding to the carboxyl terminus of the protein. This highly purified L protein preparation was shown to phosphorylate the viral nucleocapsid protein (NP) and phosphoprotein (P) in vitro. Based on sequence differences at the phosphate acceptor sites of M and P proteins, Perrault and coworkers have recently proposed the existence of at least two serine kinase activities in VSV virions (5).

Thus, it appears that both models (host kinase and intrinsic kinase) are correct and that the actual nature and number of host kinases copurified with L (and N-RNA) depend on the method of purification and the cell lines used. Using two serotypes of P, we have shown that *E. coli* is naturally deficient in P kinase activity. This raises the interesting possibility that cloning and expression of functional L protein in *E. coli* might help elucidate the origin of L-associated kinase and its requirement in VSV transcription. Lastly, we have shown that starting from either a totally unphosphorylated (bacterial) or hemiphosphorylated (viral) P protein, one can arrive at the same conformational state of P upon further phosphorylation. We postulate that the subset of phosphorylated P represented by P1 and P2 and by the single band of P_{NJ} are stable intermediates in the P protein phosphorylation pathway. Evidently, a complete dissection of the biochemistry of P phosphorylation will require purification of the various phosphorylated forms in preparative amounts, mapping of the sites of phosphorylation, and correlation of them with transcriptional activity. The availability of large quantities of unphosphorylated P protein synthesized in bacteria would provide the necessary substrate for such studies.

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