

Two of the Three Influenza Viral Polymerase Proteins Expressed by Using Baculovirus Vectors Form a Complex in Insect Cells

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Each of the influenza virus polymerase (P) genes PB1, PB2, and PA was inserted into a baculovirus vector under the control of the polyhedrin promoter. In insect (*Spodoptera frugiperda*) cells infected by each baculovirus recombinant containing a P gene insert, a large amount of the encoded P protein was synthesized. Gel electrophoretic analysis of the total proteins in infected cells revealed the presence of a new protein band corresponding to the encoded P protein that was abundant enough to be stained with Coomassie blue. In cells infected simultaneously with both the PB1 and PB2 baculovirus recombinants, a PB1-PB2 complex was formed that was immunoprecipitated with an antiserum specific for either PB1 or PB2. In cells infected simultaneously with all three P baculovirus recombinants, a PB1-PB2 complex lacking the PA protein was formed. Formation of this PB1-PB2 complex partially mimics events that occur in influenza virus-infected cells, where all three P proteins form a complex with each other (B. M. Detjen, C. St. Angelo, M. G. Katze, and R. M. Krug, *J. Virol.* 61:16-22, 1987). These results indicate that the ability of PB1 and PB2 to form a complex is an intrinsic property of these two proteins that does not require the participation of other influenza viral gene products. Possible reasons for the absence of the PA protein from the immunoprecipitable P protein complex in insect cells infected by the three P baculovirus recombinants are discussed.

Influenza viral mRNA synthesis is catalyzed by a complex of the three polymerase (P) proteins PB1, PB2, and PA associated with viral nucleocapsids (2, 8, 11, 14). Some of the roles of these proteins have been determined by analysis of the in vitro reaction catalyzed by virion nucleocapsids. Viral mRNA synthesis is initiated by heterologous capped RNA fragments generated by a viral cap-dependent endonuclease (11). The PB2 protein has been shown to be the viral protein that recognizes and binds to the cap of the heterologous RNA (1, 14, 15), but the protein(s) responsible for the endonucleolytic cleavage of the capped RNA has not been identified. The PB1 protein catalyzes the addition of nucleotides to the growing viral mRNA chains (2, 14). No role for the PA protein in viral mRNA synthesis has been found. The three proteins remain associated as a complex throughout viral mRNA synthesis (2).

Recently, it has been shown that infected cells contain a pool of P protein complexes that are not associated with viral nucleocapsids (3a). Almost all of the three P protein molecules in this nonnucleocapsid pool were apparently in complexes rather than free. Consequently, it is likely that it is these complexes, rather than a particular one of the P proteins, that recognizes and binds to the 3' ends of the virion RNAs to initiate viral mRNA synthesis. These complexes may also be involved in the reinitiation of viral mRNA synthesis. In addition, P protein complexes probably different from those involved in viral mRNA synthesis may be responsible for the initiation and reinitiation of the other two types of virus-specific RNA synthesis, i.e., template RNA and virion RNA syntheses. Finally, the nonnucleocapsid P protein complexes by themselves might possess some transcription-related activities like cap binding and cap-dependent endonucleolytic cleavage.

To address these issues, it is necessary to obtain a source of a large amount of the individual P proteins. This should allow reconstitution of P protein complexes, with the ultimate goal of determining the activity of such complexes both by themselves and in combination with viral nucleocapsid templates. Previous experiments in which the influenza viral P genes were expressed in eucaryotic cells employed bovine papillomavirus vectors (3, 9). The P proteins synthesized by using these vectors were functional in that they complemented the temperature-sensitive defects of viral mutants. However, the levels of production of the P proteins were insufficient for use in biochemical experiments (3). Here, we inserted the influenza viral P genes into baculovirus vectors under the control of the polyhedrin promoter, which is extremely active at late times of baculovirus infection (12, 13; M. D. Summers and G. E. Smith, *A manual of methods for baculovirus vectors and insect cell culture procedures*, in press). Using these vectors, we synthesized large amounts of each of the three influenza viral P proteins in insect cells. Further, we showed that two of the expressed proteins PB1 and PB2 form a complex with each other in insect cells, indicating that the ability to form this complex is an intrinsic property of these two influenza viral proteins.

MATERIALS AND METHODS

Construction of recombinant baculoviruses. The plasmids pAPR206, pAPR101, and pAPR303 containing the PB1, PB2, and PA genes, respectively, of the PR8 strain of influenza virus were provided by Peter Palese (16). The PB2 gene was excised with *Bam*HI and ligated into the unique *Bam*HI site of the baculovirus transfer plasmid pAc373 (Summers and Smith, in press). The PB1 and PA genes were cut out of their parent plasmids with *Hind*III and *Eco*RI, respectively, blunt ended with T7 DNA polymerase, and then ligated into the *Hind*III site of pAc373, which had also been blunt ended with T7 DNA polymerase. The recombinant plasmids were used to generate recombinant baculoviruses as described

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previously (12; Summers and Smith, in press). Briefly, 3 μg of each of the recombinant plasmids containing a P gene insert was cotransfected into *Spodoptera frugiperda* cells with 1 μg of the DNA of wild-type (wt) *Autographa californica* nuclear polyhedrosis virus (AcNPV). After 4 days at 27°C, the cell medium was collected and analyzed by plaque assay in *Spodoptera* cells. AcNPV (wt) forms plaques containing occlusions of polyhedrin protein. Plaques lacking these occlusions were identified with a dissecting microscope. These plaques were picked and plaque purified until the plaque assays were devoid of all wt plaques. Stocks of the recombinant plaques were then produced in *Spodoptera* cells.

Analysis of proteins in infected cells. Monolayer *Spodoptera* cells in a 75-cm² flask were infected with 3 to 5 PFU per cell of wt AcNPV or of a P recombinant virus or were mock infected (Summers and Smith, in press). After a 1-h adsorption, the inoculum was removed, and 10 ml of TnMFH medium (6) was added. Where indicated, the cells were labeled with [³⁵S]methionine. Labeling was for 1 or 2 h in Grace medium (5) lacking methionine at the times indicated in the text. Cells were disrupted in Laemmli gel sample buffer (10), and the proteins were resolved by electrophoresis on 7 or 8% polyacrylamide gels containing 4 M urea. Where indicated, proteins in the gel were detected by staining with Coomassie blue. Gels containing [³⁵S]methionine-labeled proteins were fluorographed.

Preparation of cell extracts and immunoprecipitation. At the times indicated in the text, infected cells labeled with [³⁵S]methionine were collected in RSB (10 mM Tris hydrochloride [pH 7.4], 10 mM NaCl, 5 mM MgCl₂). After swelling in ice for 10 min, the cells were disrupted by vortexing. The extract was centrifuged for 5 min at 500 \times g to yield a nuclear pellet and a cytoplasmic extract. Nuclear extracts were prepared by treating the nuclear pellet with Dignam buffer (4) lacking glycerol. This nuclear extract was combined with the cytoplasmic extract. The combined extract was adjusted to 20 mM Tris hydrochloride [pH 7.4]–100 mM NaCl–0.5% sodium deoxycholate–1% Triton X-100–0.1% sodium dodecyl sulfate–1 mM phenylmethylsulfonyl fluoride (RIPA buffer). Immunoprecipitations were then carried out as described previously (3a) with an anti-PB1 (3a) or an anti-PB2 antiserum. The latter, prepared in collaboration with Jonathan Yewdell, consisted of a pool of three monoclonal antibodies directed against a 152-amino-acid fragment of the PB2 protein expressed in *Escherichia coli* under the control of the T7 bacteriophage promoter (A. Rosenberg and T. W. Studier, manuscript submitted for publication).

RESULTS

Preparation of three recombinant baculoviruses expressing each of the influenza virus P proteins. Our goal was to use baculovirus vectors to synthesize large amounts of each of the three influenza virus P proteins. The DNA copies of the PB1, PB2, and PA virion RNAs were each inserted into the unique *Bam*HI site of the baculovirus transfer vector pAc373 (Summers and Smith, in press), which contains the polyhedrin promoter and most of the rest of the polyhedrin gene. The unique *Bam*HI site, which occurs 42 nucleotides downstream from the polyhedrin cap site, was generated by BAL 31 nuclease digestion of the polyhedrin gene. This removed sequences extending from 8 nucleotides upstream of the polyhedrin ATG initiator codon to the *Bam*HI site 175 nucleotides downstream of this ATG. Thus, the ATG translation initiation codon for the polyhedrin protein was de-

leted. The pAc373 plasmid also contains a total of 6.0 kilobases of the baculovirus (AcNPV) sequences immediately surrounding the polyhedrin gene to serve as sites for in vivo recombination with wt AcNPV DNA. The structure of the recombinant plasmid (containing a P gene) pAc373P, where P stands for PB1, PB2, or PA, is shown in Fig. 1, which also indicates the predicted sequence from the polyhedrin cap site to the ATG translation initiation codon of the PB1, PB2, and PA proteins (Fig. 1B). The 5' untranslated region of the resulting PB1, PB2, and PA mRNAs should be 94, 93, and 87 nucleotides long, respectively. These mRNAs should contain both the P gene sequence and most of the polyhedrin protein-coding sequence and should terminate at the polyhedrin polyadenylation site. The P protein should be the only translation product directed by each of these mRNAs. Recombinant baculoviruses are formed by in vivo recombination between the appropriate pAc373P vector and wt baculovirus (AcNPV) DNA (12; Summers and Smith, in press). The recombinant viruses produced plaques that lack polyhedrin occlusions.

To determine whether cells infected by each of these recombinant viruses (AcPB1, AcPA, and AcPB2) synthesized the appropriate P protein, infected *Spodoptera* cells at 40 h postinfection were labeled with [³⁵S]methionine for 1 h, and the labeled proteins were resolved by gel electrophoresis (Fig. 2). As controls, proteins made in mock-infected cells and in cells infected by wt AcNPV were analyzed in the same way. Cells infected by AcPB1 (Fig. 2, lane 3), AcPA (lane 4), and AcPB2 (lane 5) synthesized large amounts of novel proteins of the sizes of PB1, PA, and PB2, respectively. These proteins were not synthesized in mock-infected cells (lane 1) or in cells infected by wt AcNPV (lane 2). The 33,000-molecular-weight polyhedrin protein, which was synthesized in extremely large amounts in cells infected by wt AcNPV (see bottom of lane 2), was not synthesized in detectable amounts in the cells infected by the recombinants. The synthesis of the PB1, PA, and PB2 proteins was first detected at about 24 h postinfection and increased until the cells began to die (about 44 h postinfection).

To estimate the amount of P proteins that were synthesized, we resolved the total proteins in 1.5×10^6 cells infected by each recombinant (40 h postinfection) by gel electrophoresis and detected the proteins in the gel by Coomassie blue staining (Fig. 3). Each influenza viral P protein was clearly visible as a new protein band (lanes 3 to 5). By comparing the intensity of staining of these bands with bovine serum albumin standards that were run on other lanes of the same gel, we estimated that the recombinant virus-infected cells produced 5 to 7 μg of PB1, PA, or PB2 protein per 1.5×10^6 cells.

Formation of a P protein complex in *Spodoptera* cells. In influenza virus-infected cells, the three P proteins are in the form of a complex with each other (2, 3a). A large proportion of these P protein complexes are not associated with viral nucleocapsids (3a). If the ability to form these complexes is an intrinsic property of the P proteins themselves, then the P proteins produced by the recombinant baculoviruses might form a complex with each other. To test this possibility, we infected *Spodoptera* cells simultaneously with two or all three P recombinants. In such multiple infections, all of the encoded P proteins were efficiently synthesized. For example, *Spodoptera* cells infected by AcPB1 and AcPB2 synthesized both the PB1 and PB2 proteins (Fig. 4A, lane 1), and cells infected by all three P baculovirus recombinants synthesized all three P proteins (lane 2). These cells were labeled with [³⁵S]methionine from 30 to 32 h postinfection,

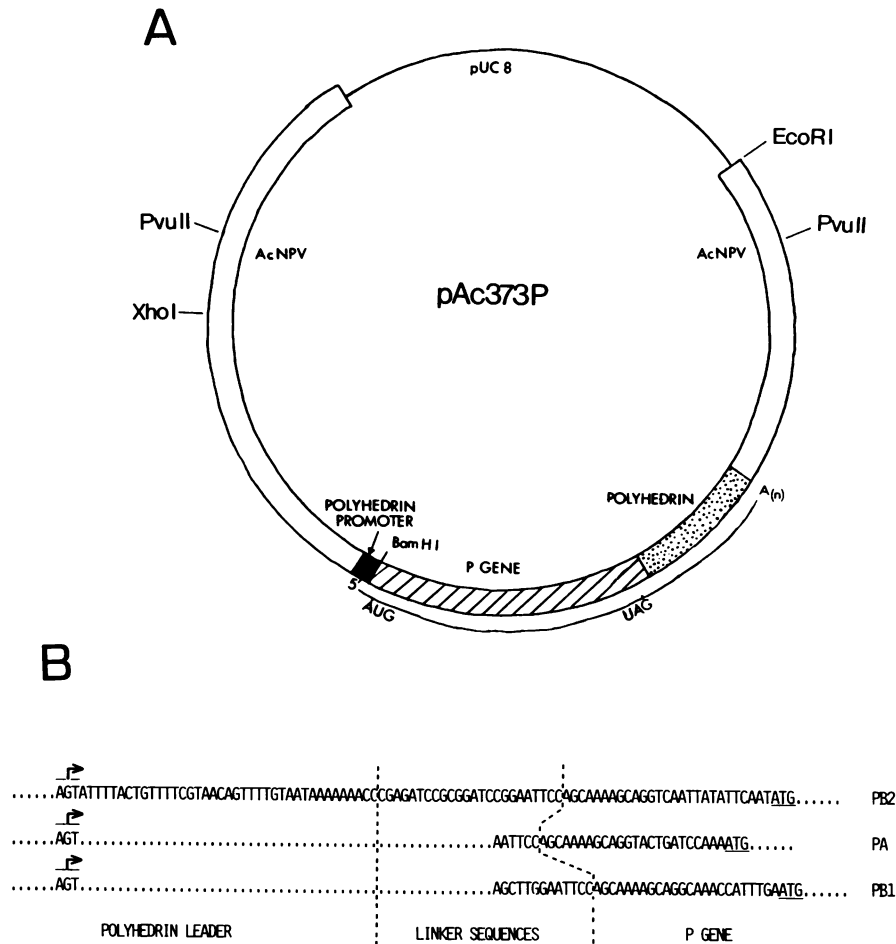


FIG. 1. (A) Structure of the recombinant baculovirus transfer plasmid pAc373P, where P stands for PB1, PB2, or PA. This plasmid contains the complete pUC8 sequence, 4.0 kilobases of AcNPV sequences immediately upstream from the polyhedrin promoter (corresponding to the left-hand terminal sequences of AcNPV), the polyhedrin gene (lacking the sequence described in the text), and 2.0 kilobases of AcNPV sequences immediately downstream from the polyhedrin gene. Several representative restriction sites are shown. The influenza viral P gene was inserted into the unique *Bam*HI site that occurs 42 nucleotides downstream from the polyhedrin cap site. The presumed structure of the chimeric P-polyhedrin chimeric RNA is shown outside the plasmid circle with translation initiation and termination codons indicated. (B) Predicted sequence from the polyhedrin cap site (7) to the ATG translation initiation codon of the PB1, PB2, and PA proteins. The linker sequences represent the fusion of the engineered polyhedrin *Bam*HI site (Verne Luckow, personal communication) and the linker at the end of the P gene (16).

so that the amount of synthesis of the P proteins relative to that of host proteins was lower than that shown in Fig. 1, for which the labeling was carried out at for 1 h at 40 h postinfection.

Complex formation between the expressed P proteins was monitored by immunoprecipitation with either an anti-PB1 or an anti-PB2 antiserum. The anti-PB1 antiserum was directed against a peptide corresponding to the 10 carboxy-terminal amino acids of PB1 (3a), and the anti-PB2 antiserum consisted of a pool of three monoclonal antibodies directed against a 152-amino acid fragment of the PB2 protein expressed in *E. coli*. If each of these antisera immunoprecipitated not only the P protein against which it was directed but also one or both of the other P proteins, this would indicate that the immunoprecipitated P proteins were in a complex that was resistant to disruption by 0.1% sodium dodecyl sulfate and other detergents in the RIPA buffer used in the immunoprecipitations.

The results obtained when cell extracts were precipitated with the anti-PB1 antiserum are shown in Fig. 4B. This

antiserum precipitated the PB1 protein synthesized in cells infected by AcPB1 (lane 1) but did not precipitate any proteins in cells infected by either AcPA or AcPB2 (lanes 2 and 3), verifying that the anti-PB1 antiserum recognized only the PB1 protein and did not cross-react with PA, PB2, or any AcNPV or *Spodoptera* cell proteins. In cells infected by both AcPB1 and AcPB2 (lane 4), the anti-PB1 antiserum precipitated not only the PB1 protein but also the PB2 protein. This indicates that the PB1 and PB2 proteins expressed in the *Spodoptera* cells formed a stable complex with each other. In contrast, in cells infected by both AcPB1 and AcPA (lane 5), only the PB1 protein, and not the PA protein, was precipitated with the anti-PB1 antiserum, indicating the absence of a stable PB1-PA complex. Finally, in cells infected by all three P virus recombinants (lane 6), the anti-PB1 antiserum precipitated both PB1 and PB2, but not PA, indicating that the PA protein was not in the stable PB1-PB2 complex.

These results were confirmed with the anti-PB2 antiserum (Fig. 4C). As shown by the single infections (lanes 1 through

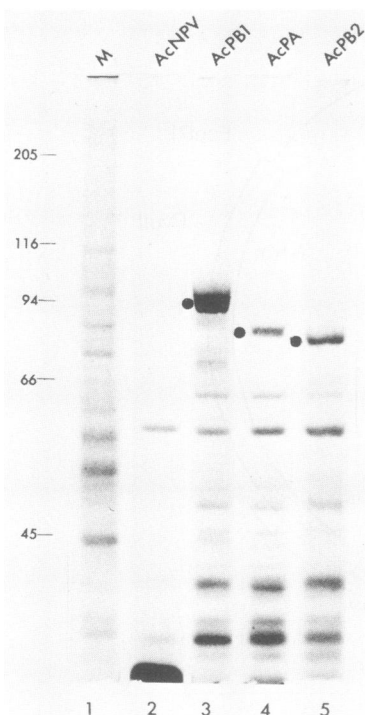


FIG. 2. Synthesis of the P proteins in *Spodoptera* cells infected by the P recombinant baculoviruses. Cells were mock infected (M) or infected with the indicated baculovirus. The cells were labeled at 40 h postinfection with [35 S]methionine for 1 h, and the proteins were analyzed on an 8% polyacrylamide gel. The dots denote the PB1, PA, and PB2 proteins, which comigrated with marker P proteins that were run on other lanes of the same gel. Molecular weight markers are indicated on the left.

3), this antiserum recognized only the PB2 protein and did not crossreact with PA, PB1, or any AcNPV or *Spodoptera* cell proteins. In cells infected by both AcPB1 and AcPB2 (lane 4), the anti-PB2 antiserum precipitated not only PB2 but also PB1, thereby verifying that the two proteins in this complex were PB1 and PB2. In contrast, no stable complex with PA was detected. Thus, in cells infected by both AcPA and AcPB2 (lane 5), only PB2, and not PA, was immunoprecipitated; in cells infected by all three P virus recombinants (lane 6), PB1 and PB2, but not PA, were immunoprecipitated. Consequently, a stable PB1-PB2 complex that lacked PA was formed in the *Spodoptera* cells.

DISCUSSION

Using baculovirus vectors, we succeeded in synthesizing large amounts of each of the three influenza viral P proteins in insect cells. On the basis of the amounts produced in 1.5×10^6 cells, we should be able to obtain about 3 to 5 mg of an individual P protein from 1 liter of a suspension culture. This should be sufficient for use in biochemical experiments.

The expressed PB1 and PB2 proteins form a complex with each other in insect cells infected with both the AcPB1 and AcPB2 baculovirus recombinants. This complex was detected by immunoprecipitation with an anti-PB1 or an anti-PB2 antiserum in a buffer containing detergents, including 0.1% sodium dodecyl sulfate. Formation of this complex partially mimics events that occur in influenza virus-infected cells, where all three P proteins form a complex with each

other (3a). Consequently, the ability of PB1 and PB2 to form a complex is an intrinsic property of these two proteins that does not require the participation of any other influenza viral gene products. Further, this result suggests that the P proteins expressed with baculovirus vectors possess at least some of the normal functions of the P proteins synthesized in influenza virus-infected cells.

The same immunoprecipitation method was used to identify both the PB1-PB2 complex in insect cells infected by the baculovirus recombinants and the complex of all three P proteins in the nonnucleocapsid fraction of influenza virus-infected cells (3a). In both cases, the relative amounts of the individual P proteins in the complexes varied, depending on which anti-P antiserum was used. Usually the immunoprecipitated complexes were enriched with the P protein against which the antiserum was directed. This result could be explained by the presence of free P proteins not in the complex. This possibility has been ruled out in influenza virus-infected cells (Detjen et al., in press) but has not been evaluated in the insect cells infected by the baculovirus recombinants. The other possibilities are that several types of P complexes containing different ratios of the two (or three) P proteins exist, that breakdown of the P protein complexes occurs to various degrees during immunoprecipitation in the detergent-containing buffer, or that both take place.

Using either the anti-PB1 or the anti-PB2 antiserum with the extracts from insect cells infected by all three P baculovirus recombinants, we detected no PA protein in the immunoprecipitates. In contrast, each of these antisera precipitates PA from nucleocapsid-depleted extracts from influenza virus-infected cells (3a). Thus, unlike the situation in influenza virus-infected cells, the PA protein expressed with the baculovirus vector either does not associate with the PB1-PB2 complex or is dissociated from this complex during immunoprecipitation in the presence of detergents. This suggests that the incorporation of PA into an im-

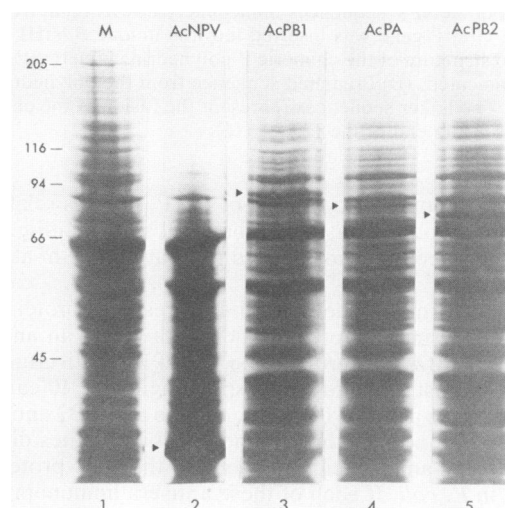


FIG. 3. Accumulation of the P proteins in *Spodoptera* cells infected by the P recombinant baculoviruses. Cells were mock infected (M) or infected with the indicated baculovirus. The cells were collected at 40 h postinfection, and the proteins were analyzed on an 8% polyacrylamide gel. The proteins in the gel were stained with Coomassie blue. The arrowheads denote the novel bands corresponding to the positions of PB1, PA, and PB2. Molecular weight markers are indicated on the left.

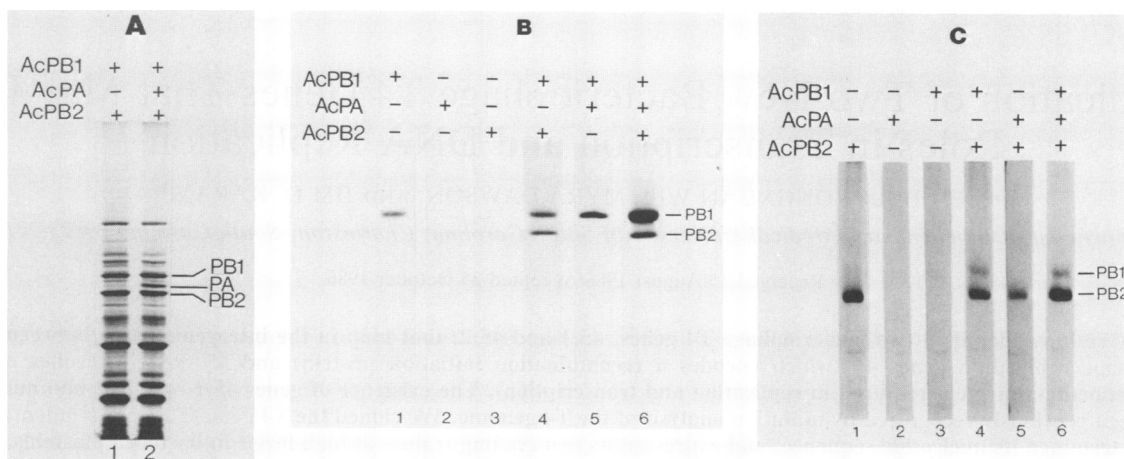


FIG. 4. Formation of a PB1-PB2 complex in infected *Spodoptera* cells. (A) [³⁵S]methionine-labeled proteins in cells infected (+) by both AcPB1 and AcPB2 (lane 1) or by all three P virus recombinants (lane 2), as indicated at the top of the gels. The cells were labeled from 30 to 32 h postinfection, and the proteins were resolved on a 7% polyacrylamide gel. (B) Immunoprecipitation with anti-PB1 antiserum of extracts from cells infected by AcPB1, AcPA, and AcPB2 alone or in combination as indicated. The cells were labeled from 30 to 32 h postinfection. (C) Immunoprecipitation with anti-PB2 antiserum of extracts from cells infected by AcPB2, AcPA, and AcPB1 alone or in combination as indicated.

munoprecipitable P complex in influenza virus-infected cells requires the participation of other influenza viral gene products. Perhaps the PA protein must be modified before it can stably associate with the other two P proteins, or perhaps this association requires or is stabilized by the presence of virus-specific RNA.

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LITERATURE CITED

- Blaas, D., E. Patzelt, and E. Keuchler. 1982. Identification of the cap binding protein of influenza virus. *Nucleic Acids Res.* **10**:4803-4812.
- Braam, J., I. Ulmanen, and R. M. Krug. 1983. Molecular model of a eukaryotic transcription complex: functions and movements for influenza P proteins during capped RNA-primed transcription. *Cell* **34**:609-618.
- Braam-Markson, J., C. Jaudon, and R. M. Krug. 1985. Expression of a functional influenza viral cap-recognizing protein by using a bovine papilloma virus vector. *Proc. Natl. Acad. Sci. USA* **82**:4326-4330.
- Detjen, B. M., C. St. Angelo, M. G. Katze, and R. M. Krug. 1987. The three influenza virus polymerase proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. *J. Virol.* **61**:16-22.
- Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475-1489.
- Grace, T. C. C. 1962. Establishment of four strains of cells from insect tissues grown *in vitro*. *Nature (London)* **195**:788-789.
- Hink, W. F. 1970. Established insect cell line from the cabbage looper, *Trichoplusia ni*. *Nature (London)* **226**:466-467.
- Howard, S. C., M. D. Ayres, and R. D. Possee. 1986. Mapping the 5' and 3' ends of *Autographa californica* nuclear polyhedrosis virus polyhedrin mRNA. *Virus Res.* **5**:109-119.
- Inglis, S. C., A. R. Carroll, R. A. Lamb, and B. W. J. Mahy. 1976. Polypeptides specified by the influenza virus genome. 1. Evidence for eight distinct gene products specified by fowl plague virus. *Virology* **74**:489-503.
- Krystal, M., L. Ruan, D. Lyles, G. Paulakis, and P. Palese. 1986. Expression of the three influenza virus polymerase proteins in a single cell allows growth complementation of viral mutants. *Proc. Natl. Acad. Sci. USA* **83**:2709-2713.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Plotch, S. J., M. Bouloy, I. Ulmanen, and R. M. Krug. 1981. A unique cap (m⁷GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell* **23**:847-858.
- Smith, G. E., M. D. Summers, and M. J. Fraser. 1983. Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* **3**:2156-2165.
- Smith, G. E., J. M. Vlak, and M. D. Summers. 1983. Physical analysis of *Autographa californica* nuclear polyhedrosis virus transcripts for polyhedrin and 10,000-molecular-weight protein. *J. Virol.* **45**:215-225.
- Ulmanen, I., B. A. Broni, and R. M. Krug. 1981. Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m⁷GpppNm) on RNAs and in initiating viral RNA transcription. *Proc. Natl. Acad. Sci. USA* **78**:7355-7359.
- Ulmanen, I., B. A. Broni, and R. M. Krug. 1983. Influenza virus temperature-sensitive cap (m⁷GpppNm)-dependent endonuclease. *J. Virol.* **45**:27-35.
- Young, J. F., U. Desselberger, P. Graves, P. Palese, A. Shatzman, and M. Rosenburg. 1983. Cloning and expression of influenza virus genes, p. 129-138. *In* W. G. Laver (ed.), *The origin of pandemic influenza viruses*. Elsevier Science Publishing, Inc., New York.