The Three Influenza Virus Polymerase (P) Proteins Not Associated with Viral Nucleocapsids in the Infected Cell Are in the Form of a Complex

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Received 15 July 1986/Accepted 24 September 1986

The three influenza virus polymerase, or P, proteins (PB1, PB2, and PA) that are associated with viral nucleocapsids and are responsible for viral mRNA synthesis are in the form of a complex that moves down the template in association with the growing mRNAs during transcription (J. Braam, I. Ulmanen, and R. M. Krug, *Cell* 34:609-618, 1983). We determined whether infected cells contained a pool of P proteins not associated with viral nucleocapsids and, if so, whether the P proteins in this pool were in the form of a complex with each other. The cytoplasmic and nuclear extracts from infected cells were depleted of nucleocapsids by centrifugation, and the resulting supernatants were subjected to immunoprecipitation with an antiserum specific for either the PB1 protein or the PB2 protein. Both antisera precipitated all three P proteins, indicating that the P proteins were in a complex that was largely resistant to disruption by the detergents present in the immunoprecipitation buffer. Sucrose density gradient analysis showed that the P protein complexes ranged from about 11S to 22S and that almost all of the PB1 and PB2 protein molecules synthesized during a 1-h period (2.5 to 3.5 h postinfection) were in these complexes. Little or no free PB1 or PB2 protein was detected. The possible role of these nonnucleocapsid P protein complexes in the initiation and reinitiation of virus-specific RNA synthesis is discussed.

Influenza virus mRNA synthesis is initiated by capped RNA fragments generated by a viral cap-dependent endonuclease (26, 31). This synthesis is catalyzed by virion nucleocapsids, which consist of the individual negative-stranded virion RNAs (vRNAs) associated with four viral proteins, the nucleocapsid (NP) protein and the three P proteins (PB1, PB2, and PA). The P proteins are responsible for viral mRNA synthesis, and some of their roles have been determined by analyses of the *in vitro* reaction catalyzed by virion nucleocapsids (4, 30, 31). The PB2 protein recognizes and binds to the cap of the primer RNA, and the PB1 protein catalyzes the addition of nucleotides to the growing viral mRNA chains. The three P proteins are in the form of a complex that starts at the 3' ends of the vRNA templates and moves down the template in association with the elongating mRNAs during transcription (4).

Many important questions remain about the mechanism of viral mRNA synthesis and the role of the P proteins. For example, it is not known how the complex of the three P proteins is assembled at the 3' ends of the vRNA templates, where transcription initiates. One possibility is that one of the P proteins recognizes and binds to the 3' ends of the vRNAs and then the other two P proteins bind to form the P protein complex. Alternatively, the P protein complex may form first, because only the formed complex may have the ability to bind to the 3' ends of the vRNAs. Another important question concerns the reinitiation of viral mRNA synthesis. With virion nucleocapsids, reinitiation is extremely limited or does not occur at all (4). If reinitiation does occur in the infected cell, it is conceivable that it is mediated by a pool of P proteins not associated with viral nucleocapsids; these P proteins might be in the form of a complex. Similar questions about the assembly of P protein complexes and the reinitiation of transcription apply to the

other two types of virus-specific RNA synthesis in the infected cell, the synthesis of template RNAs (i.e., full-length copies of the vRNAs) and the synthesis of vRNAs. Template RNA synthesis differs from viral mRNA synthesis in two respects: (i) initiation without a primer and (ii) antitermination at the polyadenylate addition site 17 to 22 nucleotides before the 5' ends of the vRNAs (12, 14, 27). The synthesis of vRNAs also almost certainly occurs without a primer, since the vRNAs contain a triphosphorylated 5' end (34). Thus, the P protein complexes involved in template RNA and vRNA synthesis may differ from those involved in viral mRNA synthesis.

As an initial approach to these questions, we investigated whether there was a pool of P proteins in the infected cell that were not associated with viral nucleocapsids and, if so, whether this pool consisted of complexes of the three P proteins. Two earlier reports indicated that some of the P proteins in the infected cell were not associated with viral nucleocapsids, but these P proteins were not characterized (13, 22). Here, using two antibodies specific for individual P proteins, we show that infected cells contain a large pool of P proteins that are not associated with viral nucleocapsids and that these proteins are predominantly, if not entirely, in the form of complexes of the three P proteins.

MATERIALS AND METHODS

Preparation of antisera. Peptides corresponding to the more hydrophilic terminus of each of the viral P proteins were chosen as antigens. The amino acid sequences were based on the nucleotide sequences of cloned DNA copies of the P genes (11, 17, 28, 33). The PB1-specific peptide, purchased from Bachem, Inc. (Torrance, Calif.), contained the 10 carboxy-terminal amino acids of the PB1 protein plus an amino-terminal tyrosine residue; the PB2-specific peptide, kindly provided by R. B. Merrifield, contained the 9 amino-terminal amino acids of the PB2 protein plus a

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carboxy-terminal tyrosine residue; and the PA-specific peptide, purchased from Bachem, Inc., contained the 7 amino-terminal amino acids of the PA protein plus a carboxy-terminal tyrosine residue. Each of these peptides was diazotized via its terminal tyrosine residue to keyhole limpet hemocyanin (32); the peptides were labeled with ^{125}I to monitor the efficiency of coupling (2, 3). For each of the three peptide-keyhole limpet hemocyanin conjugates, three rabbits were each injected subcutaneously with 0.45 mg of the conjugate in complete Freund adjuvant and intravenously with 0.15 mg of conjugate in phosphate-buffered saline. Boosters (with incomplete Freund adjuvant) were given 2, 4, and 8 weeks later. The sera were screened by an enzyme-linked immunosorbent assay (ELISA), in which purified virion nucleocapsids, prepared as described previously (26), were the test antigen. Positive sera were then tested in immunoblots against either virion nucleocapsids or cytoplasmic and nuclear extracts from infected cells prepared as described below. The proteins in virion nucleocapsids or in infected cell extracts were resolved by electrophoresis on 8% polyacrylamide gels containing 4 M urea (23), and the proteins were electrophoretically transferred to nitrocellulose paper (9). After being stained with Coomassie blue to localize proteins, the nitrocellulose blots were destained and incubated with the specific antiserum diluted 1:100 in 5% bovine serum albumin for 2 h. Bound immunoglobulin G (IgG) was detected with ^{125}I -protein A.

Anti-PB2 antiserum was prepared by immunizing rabbits with a 607-amino-acid-long carboxy-terminal fragment of the PB2 protein expressed in *Escherichia coli* under the control of the T7 RNA polymerase promoter (29). Details of this construction will be published elsewhere (A. Rosenberg and F. W. Studier, submitted for publication). The PB2-specific protein was isolated by preparative gel electrophoresis. The portion of the gel containing this protein was ground into a slurry in a small volume of phosphate-buffered saline and injected subcutaneously into two rabbits. The rabbits were injected again at 2 and 4 weeks and were bled at 6 weeks. The sera were screened by immunoblots against cytoplasmic and nuclear extracts from infected cells prepared as described below. Ascites fluid containing a mixture of monoclonal antibodies against the NP protein was kindly provided by Robert Webster.

Preparation of infected-cell extracts. MDCK and BHK-21 cells were infected with the WSN strain of influenza A virus as described previously (15, 20, 21). Where indicated, the cells were labeled from 2.5 to 3.5 h postinfection with 600 μCi of [^{35}S]methionine per ml in Dulbecco modified Eagle medium lacking methionine. At the indicated times postinfection, the cells were collected into reticulocyte standard buffer (10 mM Tris hydrochloride, pH 7.5, 10 mM KCl, 1.5 mM MgCl_2) and disrupted by Dounce homogenization. The extracts were centrifuged at $250 \times g$ for 5 min to yield a nuclear pellet and a cytoplasmic extract. The cytoplasmic extracts were clarified by centrifugation at $10,000 \times g$ for 10 min. Nuclear extracts were prepared by treating the nuclear pellet with the 0.42 M NaCl-containing buffer described by Dignam et al. (10), except that glycerol was omitted. After the extract was clarified by centrifugation at $10,000 \times g$, the NaCl concentration of the extract was reduced to 0.2 M by adding the appropriate volume of a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH, pH 7.6, 120 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). In many experiments, viral nucleocapsids were removed from the nuclear and cytoplasmic extracts by

centrifugation for 2.2 h at 45,000 rpm in the SW50.1 rotor in 0.8-ml centrifuge tubes ($234,000 \times g$), yielding what were designated high-speed supernatants.

Density gradient centrifugation. Cytoplasmic extracts were made 0.5% in lyssolecithin and layered onto 20 to 50% (wt/vol) linear glycerol gradients in 50 mM Tris hydrochloride (pH 7.8)–150 mM NaCl–1 mM DTT. The gradients were centrifuged for 3 h at 48,000 rpm in the SW50.1 rotor. The high-speed nuclear and cytoplasmic supernatants were analyzed on 5 to 20% (wt/wt) linear sucrose gradients in 20 mM Tris hydrochloride (pH 7.4)–1.5 mM magnesium acetate–1 mM DTT–1 mM PMSF. These gradients were centrifuged for 17.5 h at 27,300 rpm in the SW50.1 rotor, and fractions were collected from the top. Sedimentation values were estimated from the value for $\omega^2 t$ (Applications Data DS-528; Beckman Instruments, Inc., Palo Alto, Calif.).

Immunoprecipitation. [^{35}S]methionine-labeled samples were 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 (SDS), 1 mM PMSF, and 100 kallikrein inhibitory units of aprotinin per ml (RIPA buffer). All operations were carried out at 4°C. Each sample was first incubated overnight with 0.01 volume of normal rabbit serum. Subsequently, 0.05 volume of protein A-Sepharose beads (PAS; Sigma Chemical Co., St. Louis, Mo.) which had been swollen in RIPA buffer (0.1 g of beads per ml of suspension) was added, and the sample was incubated for an additional 30 min with shaking. After removal of the PAS by centrifugation, this precleared supernatant was incubated with a specific antiserum at the following dilutions: normal rabbit serum, 1:100; anti-PB1(C) antiserum, 1:200; anti-PB2 antiserum, 1:100; anti-NP ascites fluid, 1:1,000. After incubation for 2 h, PAS was added to bind antigen-antibody complexes. The PAS was spun out and washed five times with RIPA buffer. The immune complexes were solubilized by heating at 100°C in the SDS-containing gel loading buffer and resolved by electrophoresis on 8% polyacrylamide gels containing 4 M urea. After electrophoresis, the gels were fluorographed (16).

RESULTS

Antisera directed against influenza virus P proteins. Our goal was to characterize intracellular influenza virus polymerase (P) proteins that are not associated with viral nucleocapsids. For this purpose, we needed antisera directed against individual viral P proteins. As one approach, we prepared for each P protein an antiserum directed against a peptide that corresponded to a hydrophilic region of the P protein. The peptides chosen corresponded to the 10 carboxy-terminal amino acids of PB1 [PB1(C)], the 9 amino-terminal amino acids of PB2 [PB2(N)], and the 7 amino-terminal amino acids of PA [PA(N)]. Antisera raised against the PB1(C) and PB2(N) peptides were positive in the ELISA with purified nucleocapsids as the test antigen, while all antisera raised against the PA(N) peptide were negative in this ELISA (data not shown). The specificities of the anti-PB1(C) and anti-PB2(N) antisera were evaluated by immunoblots. As shown in Fig. 1A, the antiserum directed against PB1(C) specifically detected authentic PB1 protein and did not bind to any of the other proteins in purified virion nucleocapsids. The PB1 protein was the predominant protein recognized on immunoblots of infected-cell cytoplasmic proteins (Fig. 1B, lane 2). Little cross-reactivity with cellular proteins occurred; only a small amount of binding to a cellular protein of M_r 100,000 was detected (lanes 1 and 2).

FIG. 1. Specificities of the anti-PB1(C) and anti-PB2 antisera. (A) Immunoblot of the proteins in purified nucleocapsids probed with the anti-PB1(C) antiserum (lane 2). Lane 1 shows the Coomassie blue-staining pattern of the blot. The positions of the viral proteins are indicated on the left. (B) Immunoblot of the cytoplasmic proteins from uninfected (lane 1) and infected (lane 2) cells probed with the anti-PB1(C) antiserum. The positions of molecular weight markers (in thousands) are indicated. (C) Immunoblot of the total proteins from uninfected (lane 1) and infected (lane 2) cells probed with the anti-PB2 antiserum. Lane 3 shows the position of authentic PB2 protein synthesized in reticulocyte extracts with bacteriophage SP6 transcripts of PB2 DNA (5).

Similar results were obtained with nuclear extracts. In contrast to the high specificity of the anti-PB1(C) antiserum, the anti-PB2(N) antiserum bound not only to PB2 protein but also to NP protein when blots of nucleocapsids were used. This antiserum also bound to many of the major cellular proteins in uninfected and infected cells (data not shown). Thus, of the antipeptide antisera, only the anti-PB1(C) antiserum was judged suitable for the study of P proteins in infected-cell extracts.

Therefore, a second approach was used to prepare a PB2-specific antiserum. A 607-amino-acid-long carboxy-terminal fragment of the PB2 protein was expressed in *E. coli* under the control of the T7 RNA polymerase promoter (29; Rosenberg and Studier, submitted for publication). The antiserum raised against this protein detected a single protein in immunoblots of total infected-cell proteins (Fig. 1C, lane 2), and this protein comigrated with authentic PB2 protein (lane 3). No cross-reacting protein was detected in uninfected cells (lane 1).

Much of the PB1 protein in the infected cell is not associated with viral nucleocapsids. To determine the relative amount of the PB1 protein in the infected cell that was not associated with viral nucleocapsids, a cytoplasmic extract from infected MDCK cells was subjected to centrifugation on a glycerol gradient, and the proteins in each fraction were analyzed in immunoblots with the anti-PB1(C) antiserum (Fig. 2). Viral nucleocapsids sedimented in gradient fractions 3 through 6, as shown by the presence of a large amount of the nucleocapsid (NP) protein detected by Coomassie blue staining of the blot and also by the sedimentation of virion nucleocapsids in the same fractions in a parallel gradient. PB1 protein was, as expected, associated with the viral nucleocapsids. However, a much greater amount of the PB1 protein was found near the top of the gradient, predominantly in fractions 11 through 13. These data confirm that a large amount

of at least one P protein which is not associated with viral nucleocapsids accumulates in infected cells (13, 22).

Non-nucleocapsid-associated P proteins are in the form of a complex with each other. The three P proteins that are associated with virion nucleocapsids are in the form of a complex that moves down the template in association with the growing viral mRNA chain transcription (4). We determined whether the large amount of the PB1 protein that was not associated with nucleocapsids in infected cells was also in the form of a complex with the other two P proteins. Infected BHK-21 cells were labeled with [35 S]methionine from 2.5 to 3.5 h postinfection, and the cytoplasmic and nuclear extracts from these cells were depleted of nucleocapsids by centrifuging the extracts to remove structures of ≥ 30 S. The resulting high-speed supernatants were then subjected to immunoprecipitation in RIPA buffer with either the anti-PB1(C) antiserum or the anti-PB2 antiserum. If each of these antisera immunoprecipitated not only the P protein against which it is directed but also the other two P proteins, this would indicate that the three P proteins were a complex that was resistant to disruption by the 0.1% SDS and other detergents present in the RIPA buffer.

The anti-PB1(C) antiserum precipitated not only the PB1 protein but also two other proteins which comigrated with the marker PA and PB2 proteins (Fig. 3, lanes 3 and 8). Much larger amounts of these three proteins were precipitated from the nuclear supernatant (lane 8) than from the cytoplasmic supernatant (lane 3). Similarly, the anti-PB2 antiserum precipitated from the nuclear supernatant not only the PB2 protein, but also two proteins which comigrated with authentic PA and PB1 proteins (lane 9). Lower amounts of these three proteins were also precipitated from the cytoplasmic supernatant, as was seen with a longer exposure of lane 4. These results indicate that PB1 and PB2 formed a stable complex with each other and that PA was also probably in this complex.

Two other protein bands were also observed in various amounts in the immunoprecipitates. When the anti-PB1(C)

FIG. 2. Much of the PB1 protein in infected cells is not associated with viral nucleocapsids. A cytoplasmic extract from infected MDCK cells was sedimented through a 20 to 50% glycerol gradient, and the gradient fractions (1 through 15) were analyzed in immunoblots probed with the anti-PB1(C) antiserum. Fractions 1 through 6 (the bottom of the gradient) were run on a separate gel from fractions 7 through 15. Both gels included the indicated molecular weight markers (in thousands). The Coomassie stain of the blot of the second gel indicated that the gel had sagged during transfer, distorting the position of the PB1 protein in fractions 10 through 14. The proteins in purified virion nucleocapsids (NC) were also electrophoresed on the second gel, and the positions of the PB1 and NP proteins determined by Coomassie staining of the blot are indicated.

FIG. 3. Detection by immunoprecipitation of P protein complexes that are not associated with viral nucleocapsids. BHK-21 cells were labeled with [35 S]methionine from 2.5 to 3.5 h after infection. The high-speed supernatant from the cytoplasm (lanes 2 through 5) and nucleus (lanes 7 through 10) was immunoprecipitated with normal rabbit serum (lanes 2 and 7), anti-PB1(C) antiserum (lanes 3 and 8), anti-PB2 antiserum (lanes 4 and 9), or pooled anti-NP monoclonal antibodies (lanes 5 and 10). Each immunoprecipitation reaction contained equal amounts of radioactivity (10^6 cpm), which corresponded to cell-equivalent amounts of the cytoplasmic and nuclear samples. The immunoprecipitated proteins were analyzed on 8% polyacrylamide gels containing 4 M urea. Lanes 1 and 6 show the pattern of labeled proteins in the high-speed supernatant from the cytoplasm and nucleus, respectively (3×10^4 cpm), without immunoprecipitation. Lane 11 shows the results of immunoprecipitating glycerol gradient-purified intracellular nucleocapsids (7.5×10^4 cpm) with the anti-PB1(C) antiserum. The positions of the nucleocapsid-associated proteins are indicated.

antiserum was used, variable amounts of a protein band migrating more slowly than PB1 were found in the immunoprecipitate (lane 8). In some experiments (see below), this band was also seen when the anti-PB2 antiserum was used. Analysis of this band by partial proteolysis with *Staphylococcus aureus* V8 protease (9) showed that it was indistinguishable from the immunoprecipitated protein band comigrating with authentic PB1 (data not shown). In immunoprecipitation experiments in which larger amounts of the slower-migrating band were present, there were decreased amounts of the protein band comigrating with authentic PB1 (see below). These results indicate that this slower-migrating band was the PB1 protein whose electrophoretic mobility had been decreased by an artifact of the procedure, e.g., anomalous detergent binding. We tried to eliminate this artifact without success.

The other band that was found in variable amounts in the immunoprecipitates was the NP protein. It was also found to various degrees after immunoprecipitation with normal rabbit serum (lanes 2 and 7). In fact, in most experiments (e.g., see Fig. 5), as much or more NP protein was immunoprecipitated by normal rabbit serum as by an anti-P antiserum. Consequently, the variable appearance of the NP protein after these immunoprecipitations is most likely due to nonspecific binding of the NP to immunoprecipitates. The amount of the NP protein in the immunoprecipitates obtained with the anti-PB1(C) or anti-PB2 antiserum (lanes 8 and 9) represented an extremely small fraction of the total NP protein in the high-speed supernatant, as determined by immunoprecipitation with pooled NP monoclonal antibodies (lanes 5 and 10). In addition, the amount of the NP protein relative to the three P proteins seen after immunoprecipitating the high-speed supernatant with the anti-PB1(C) anti-

serum (lane 8) was almost negligible compared to the amount of the NP protein relative to the three P proteins seen after immunoprecipitating intracellular viral nucleocapsids with the anti-PB1(C) antiserum (lane 11). We conclude that there was little or no NP protein associated with the P complexes in the high-speed supernatant fractions from infected cells.

As shown above, the P protein complex was stable after incubation (at 4°C) in RIPA buffer containing 0.1% SDS and other detergents. However, as expected, the complex was disrupted by boiling in 1% SDS. When the nuclear supernatant was boiled in 1% SDS prior to immunoprecipitation with either the anti-PB1(C) or the anti-PB2 antiserum, the PB1 protein or the PB2 protein, respectively, was the only P protein precipitated (Fig. 4). This result verifies that the other two P proteins were precipitated under nondisruptive conditions (RIPA buffer) because they were in a complex with the particular P protein (PB1 or PB2) against which the antiserum is directed. This result also indicates that the protein in the complex that comigrated with authentic PA was not related to PB1 or PB2 and was therefore most likely PA.

Densitometry of appropriate exposures of Fig. 3 and 4 indicated that the relative amounts of the individual P proteins that were precipitated under nondisruptive conditions depended on the antiserum used, i.e., the greatest relative amount of PB1 protein was detected with the anti-PB1(C) antiserum, and PB2 was the predominant P protein detected with anti-PB2 antiserum. One possible explanation for these results is that free P proteins as well as P protein complexes were present in the high-speed supernatant.

To investigate this possibility and to determine the size distribution of the P protein complexes, the [35 S]methionine-labeled high-speed supernatant from the nucleus was subjected to sucrose density gradient centrifugation (Fig. 5). The majority of the labeled proteins sedimented near the top of the gradient, indicating that most of the proteins in the nuclear supernatant existed as monomers. The gradient

FIG. 4. Disruption of non-nucleocapsid-associated P protein complexes. A sample of a [35 S]methionine-labeled, high-speed nuclear supernatant of virus-infected BHK-21 cells was adjusted to 1% SDS and boiled. Both the treated (lanes +) and untreated (lanes -) samples were immunoprecipitated with the anti-PB1(C) or anti-PB2 antiserum after dilution in excess RIPA buffer. The immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The positions of marker nucleocapsid-associated proteins are indicated.

FIG. 5. Sucrose density gradient analysis of non-nucleocapsid-associated P protein complexes. (A) A [35 S]methionine-labeled high-speed nuclear supernatant of virus-infected BHK-21 cells was sedimented through a 5 to 20% linear sucrose gradient as described in Materials and Methods, and the amount of total acid-insoluble radioactivity was determined (6). Sedimentation was from right to left. The fractions were combined into five pools, A through E, as indicated. Sedimentation value ranges for each pool were: A, 30S to 22S; B, 22S to 16S; C, 16S to 11S; D, 11S to 6S; E, less than 6S. (B) Each gradient pool was divided into three equal parts and immunoprecipitated with normal rabbit serum (NRS), anti-PB1(C) antiserum, or anti-PB2 antiserum, and the immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The electrophoretic pattern shown is a composite of two exposures: detection of the proteins precipitated by the anti-PB1(C) antiserum required an exposure time approximately half that required for anti-PB2-precipitated proteins. The lanes showing normal rabbit serum control precipitations were exposed for the same length of time as the anti-PB2-precipitated proteins. Densitometry of appropriately exposed films yielded plots of the molar ratio of PB1 protein to PB2 protein for the anti-PB1(C) precipitations and the molar ratio of PB2 protein to PB1 protein for the anti-PB2 precipitations.

fractions were combined into five pools (A through E) prior to immunoprecipitation with normal rabbit serum, the anti-PB1(C) antiserum, or the anti-PB2 antiserum (Fig. 5B). The P protein complexes detected with either P antiserum sedimented considerably faster than protein monomers, as the majority of these complexes were found in pools B and C (sedimentation values of 11S to 22S). Similar analyses of the cytoplasmic complexes revealed identical sedimentation behavior. Few PB1 or PB2 protein molecules existed in free form, as shown by the absence of these proteins in gradient pool E; a small amount of free PB1 and PB2 might be present in pool D.

The P protein complexes in the gradient pools, like those in the unfractionated nuclear supernatant, varied in composition depending on the antiserum used for immunoprecipitation. The smaller P protein complexes (those in pool C) were found to contain a higher proportion of PB1 protein after immunoprecipitation with the anti-PB1(C) antiserum and a higher proportion of PB2 protein after immunoprecipitation with the anti-PB2 antiserum, suggesting that some of the P protein complexes in this gradient pool were deficient in the PB1 protein and some were deficient in the PB2 protein. With the large P protein complexes (predominantly in pool B and a small amount in pool A), a different relative content of the PB1 and PB2 proteins was observed depending on which of the two antisera was used. There are several possible explanations for this result. First, there might be several types of P protein complexes containing different PB1/PB2 ratios, and each of the two antisera might preferentially detect complexes enriched in the P protein against

which the particular antiserum was directed. Alternatively, some breakdown of the P protein complexes might occur in the RIPA buffer during the immunoprecipitation procedure, leading to an artifactual enrichment of the target P protein.

DISCUSSION

Previous studies have established that the three influenza virus P proteins that are associated with viral nucleocapsids and are responsible for viral mRNA synthesis are in the form of a complex that moves down the template during transcription (4). In the present study, we demonstrated that infected cells contain a large pool of P proteins that are not associated with nucleocapsids and that the P proteins in this pool are also in the form of a complex with each other. Using infected cell extracts that were depleted of their nucleocapsids by centrifugation, we showed that an anti-PB1 and an anti-PB2 antiserum both immunoprecipitated all three P proteins, indicating that the P proteins were in a complex that was largely resistant to disruption by the detergents present in the immunoprecipitation buffer. Sucrose density gradient analysis indicated that the P protein complexes ranged from about 11S to 22S and that almost all of the PB1 and PB2 protein molecules labeled during a 1-h period were in these complexes. Little or no free PB1 or PB2 protein was seen. These results suggest that the P proteins associate with each other soon after their synthesis.

The relative amounts of the individual P proteins in these nonnucleocapsid P complexes varied depending on which anti-P antiserum was used. We found that the immuno-

precipitated complexes were enriched in the P protein against which the antiserum was directed. These results indicate that (i) several types of P protein complexes containing different ratios of the individual P proteins were present in the infected cell or (ii) breakdown of the P protein complexes occurred to various degrees during the immunoprecipitation procedure. To distinguish between these alternatives, it will be necessary to use a method of analysis that does not involve immunoprecipitation.

Because essentially all of the nonnucleocapsid P proteins were in the form of a complex, it is most likely that such a complex, rather than a particular one of the P proteins, recognizes and binds to the 3' ends of the vRNAs to initiate mRNA and template RNA synthesis and binds to the 3' ends of the template RNAs to initiate vRNA synthesis. The P protein complex would be expected to bind to the 3' ends of vRNAs or template RNAs that have already associated with NP protein, because, as is the case with other negative-strand RNA viruses (1, 8, 24, 25), NP protein almost certainly associates with template RNAs and vRNAs during their synthesis (2a; G. Shapiro and R. M. Krug, unpublished data). Binding of the P protein complexes to nucleocapsids need not be confined to newly synthesized nucleocapsids, so that the P protein complexes could also be responsible for reinitiation of mRNA, template RNA, and vRNA synthesis. Our finding that the nonnucleocapsid P protein complexes were predominantly in nuclear fractions from infected cells is consistent with their putative role in the initiation and reinitiation of virus-specific RNA synthesis, as the synthesis of all virus-specific RNAs occurs in the nucleus (15; G. Shapiro, T. Gurney, and R. M. Krug, *J. Virol.*, in press). To establish whether the P protein complexes have these roles, it will be necessary to use nuclear extracts from infected cells that catalyze virus-specific RNA synthesis *in vitro*. Recently, using such extracts, we showed that NP molecules not associated with nucleocapsids are required for the antitermination step that occurs as part of the switch from viral mRNA to template RNA synthesis (2a). These experiments did not address the requirements for initiation of template RNA synthesis. One goal of future experiments will be to determine whether similar or different types of P protein complexes are involved in the initiation of the three different types of virus-specific RNA synthesis, i.e., viral mRNA, template RNA, and vRNA synthesis.

It is conceivable that the nonnucleocapsid P protein complexes by themselves possess transcription-related activities, specifically cap-binding and cap-dependent endonuclease activities. It has been reported that a complex of the three P proteins associated with vRNA (lacking NP protein) isolated from virions possesses these activities (18, 19). We do not yet know whether the P protein complexes we have identified in infected cells are associated with vRNA or any other RNA. As one approach to determine the activities of P protein complexes free of vRNA or any other virus-specific components, workers in this laboratory have expressed each of the P proteins in baculoviruses under the control of the very strong polyhedrin promoter (C. St. Angelo, G. Smith, M. Summers, and R. M. Krug, *J. Virol.*, in press). In cells infected simultaneously by three recombinant baculoviruses encoding each of the P proteins, a PB1-PB2 complex was formed, indicating that the ability to form this complex is an intrinsic property of these two P proteins; PA, however, was not in the complex. It will be of great interest to examine the activities of this PB1-PB2 complex and to determine the factor(s) that is involved in the inclusion of the PA protein in P protein complexes.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants CA-08747 and AI-11772 from the National Institutes of Health. C.S.A. is a Ph.D. student in the Graduate Program of Molecular Biology.

We thank Elizabeth DeBeus for carrying out some of the initial experiments in this study, R. B. Merrifield for PB2(N) peptide, and Alan H. Rosenberg and F. William Studier for providing us with the gel slices containing the PB2 protein expressed in *E. coli*. We also thank Barbara Broni for expert technical assistance and Eveyon Farmer for typing the manuscript.

LITERATURE CITED

1. Arnheiter, H., N. L. Davis, G. Wertz, M. Schubert, and R. A. Lazzarini. 1985. Role of the nucleocapsid protein in regulating vesicular stomatitis virus RNA synthesis. *Cell* 41:259-267.
2. Baron, M. H., and D. Baltimore. 1982. Antibodies against the chemically synthesized genome-linked protein of poliovirus react with native virus-specific proteins. *Cell* 28:395-404.
- 2a. Beaton, A. R., and R. M. Krug. 1986. Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5' capped end. *Proc. Natl. Acad. Sci. USA* 83:6282-6286.
3. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ¹²⁵I-containing acylating agent. *Biochem. J.* 133:529-539.
4. Braam, J., I. Ulmanen, and R. M. Krug. 1983. Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. *Cell* 34:609-618.
5. 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.
6. Brendler, T., T. Godefroy-Colburn, R. D. Carlill, and R. E. Thach. 1981. The role of mRNA competition in regulating translation. II. *J. Biol. Chem.* 256:11747-11754.
7. Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.
8. Carlsen, S. R., R. W. Peluso, and S. A. Moyer. 1985. *In vitro* replication of Sendai virus wild-type and defective interfering particle genome RNAs. *J. Virol.* 54:493-500.
9. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102-1106.
10. 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.
11. Fields, S., and G. Winter. 1982. Nucleotide sequences of influenza virus segments 1 and 3 reveal mosaic structure of a small viral RNA segment. *Cell* 28:303-313.
12. Hay, A. J., G. Abraham, J. J. Skehel, J. C. Smith, and P. Fellner. 1977. Influenza virus messenger RNAs are incomplete transcripts of the genome RNAs. *Nucleic Acids Res.* 4:4197-4209.
13. Hay, A. J., and J. J. Skehel. 1975. Studies on the synthesis of influenza virus proteins, p. 635-655. *In* B. W. J. Mahy, and R. D. Barry (ed.), *Negative strand viruses*, vol. 2. Academic Press, Ltd., London.
14. Hay, A. J., J. J. Skehel, and J. McCauley. 1982. Characterization of influenza virus RNA complete transcripts. *Virology* 116:517-522.
15. Herz, C., E. Stavnezer, R. M. Krug, and T. Gurney, Jr. 1981. Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. *Cell* 26:391-400.
16. Jen, G., and R. E. Thach. 1982. Inhibition of host translation in encephalomyocarditis virus-infected L cells: a novel mechanism.

- nism. *J. Virol.* **43**:250–261.
17. **Kapteijn, J. S., and D. P. Nayak.** 1982. Complete nucleotide sequence of the polymerase 3 gene of human influenza virus A/WSN/33. *J. Virol.* **42**:55–63.
 18. **Kato, A., K. Mizumoto, and A. Ishihama.** 1985. Purification and enzymatic properties of an RNA polymerase-RNA complex from influenza virus. *Virus Res.* **3**:115–127.
 19. **Kawakami, K., and A. Ishihama.** 1983. RNA polymerase of influenza virus. III. Isolation of RNA polymerase RNA complexes from influenza virus PR8. *J. Biochem.* **93**:989–996.
 20. **Krug, R. M.** 1971. Influenza viral RNP'S newly synthesized during the latent period of viral growth in MDCK cells. *Virology* **44**:125–136.
 21. **Krug, R. M.** 1972. Cytoplasmic and nucleoplasmic viral RNPs in influenza virus-infected MDCK cells. *Virology* **50**:103–113.
 22. **Krug, R. M., and P. R. Etkind.** 1973. Cytoplasmic and nuclear virus-specific proteins in influenza virus-infected MDCK cells. *Virology* **56**:334–348.
 23. **Lamb, R. A., and P. W. Choppin.** 1976. Synthesis of influenza virus proteins in infected cells: translation of viral proteins, including three P polypeptides, from RNA produced by primary transcription. *Virology* **74**:504–519.
 24. **Patton, J., N. L. Davis, and G. W. Wertz.** 1984. N protein alone satisfies the requirement for protein synthesis during RNA replication of vesicular stomatitis virus. *J. Virol.* **49**:303–309.
 25. **Peluso, R. W., and S. A. Moyer.** 1984. Vesicular stomatitis virus proteins required for the *in vitro* replication of defective interfering particle genome RNA, p. 153–160. *In* D. H. L. Bishop and R. W. Compans (ed.), *Nonsegmented negative strand viruses*. Academic Press, Inc., Orlando, Fla.
 26. **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.
 27. **Robertson, J. S., M. Schubert, and R. A. Lazzarini.** 1981. Polyadenylation sites for influenza virus mRNA. *J. Virol.* **38**:157–163.
 28. **Sivasubramanian, N., and D. P. Nayak.** 1982. Sequence analysis of the polymerase 1 gene and the secondary structure prediction of polymerase 1 protein of human influenza virus A/WSN/33. *J. Virol.* **44**:321–329.
 29. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**:113–130.
 30. **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.
 31. **Ulmanen, I., B. A. Broni, and R. M. Krug.** 1983. Influenza virus temperature-sensitive cap (m⁷GpppNm)-dependent endonuclease. *J. Virol.* **45**:27–35.
 32. **Walter, G., K. Scheidtmann, A. Carbone, A. P. Laudano, and R. F. Doolittle.** 1980. Antibodies specific for the carboxy- and amino-terminal regions of simian virus 40 large tumor antigen. *Proc. Natl. Acad. Sci. USA* **77**:5197–5200.
 33. **Winter, G., and S. Fields.** 1982. Nucleotide sequence of human influenza A/PR/8/34 segment 2. *Nucleic Acids Res.* **10**:2135–2143.
 34. **Young, R. J., and J. Content.** 1971. 5'-terminus of influenza virus RNA. *Nature (London) New Biol.* **230**:140–142.