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Intracellular Localization of the Viral Polymerase Proteins in Cells Infected with Influenza Virus and Cells Expressing PB_1 Protein from Cloned cDNA

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The biosynthesis, nuclear transport, and formation of a complex among the influenza polymerase proteins were studied in influenza virus-infected MDBK cells by using monospecific antisera. To obtain these monospecific antisera, portions of cloned cDNAs encoding the individual polymerase proteins (PB_1, PB_2, or PA) of A/WSN/33 influenza virus were expressed as fusion proteins in *Escherichia coli*, and the purified fusion proteins were injected into rabbits. Studies using indirect immunofluorescence showed that early in the infectious cycle (4 h postinfection) of influenza virus, PB_1 and PB_2 are present mainly in the nucleus, whereas PA is predominantly present in the cytoplasm of the virus-infected cells. Later, at 6 to 8 h postinfection, all three polymerase proteins are apparent both in the cytoplasm as well as the nucleus. Radiolabeling and immunoprecipitation analyses showed that the three polymerase proteins remain physically associated as a complex in either the presence or the absence of ribonucleoproteins. In the cytoplasm, the majority of the polymerase proteins remain unassociated, whereas in the nucleus they are present as a complex of three polymerase proteins. To determine whether a polymerase protein is transported into the nucleus individually, PB_1 was expressed from the cloned cDNA by using the simian virus 40 late promoter expression vector. PB_1 alone, in the absence of the other polymerase proteins or the nucleoprotein, accumulates in the nucleus. This suggests that the formation of a complex with other viral protein(s) is not required for either nuclear transport or nuclear accumulation of PB_1 protein and that the PB_1 protein may contain an intrinsic signal(s) for nuclear transport.

Influenza virus, a negative-stranded RNA virus, has been shown to contain a functional RNA transcriptase activity in mature virions (3, 22). After infection of a permissive cell, the ribonucleoprotein (RNP)-polymerase complex is uncoated and migrates into the nucleus, where influenza virus-specific mRNAs are transcribed. Genetic and biochemical studies have demonstrated that three polymerase proteins (PB_1, PB_2, and PA) play a crucial role in both transcription and replication of the viral genome (see references 13 and 16). Krug and his colleagues (1) have demonstrated that PB_2 is involved in initiation of mRNA transcription and that PB_1 is involved in mRNA chain elongation. However, except for the role of these two proteins in primary transcription of viral mRNAs, little information is available about the structure of these proteins or their active sites. The three polymerase proteins are found as an RNP-polymerase complex in the virion and, therefore, are believed to function as a complex. Little is known as to how and where the polymerase complex is formed or how the newly synthesized polymerase proteins are transported into the nucleus. Because only minute amounts of the polymerase proteins are formed in the virus-infected cells and only a few molecules are present in virions, it has not been possible to obtain enough polymerase protein for producing monospecific antibodies or for use in studying the function of individual components of the transcriptase complex.

To elucidate the structure-function relationship of these polymerase proteins, we have undertaken a detailed analysis of the polymerase genes and their products. In this report we show that we have expressed portions of each polymerase protein as a fusion protein in *Escherichia coli* and prepared monospecific antibodies against each of these three polymerase proteins. Using these antibodies, we have studied the biosynthesis, intracellular localization, and complex formation of polymerase proteins in influenza virus-infected cells. Furthermore, we have expressed PB_1 protein by using a simian virus 40 (SV40) late promoter expression vector in CV1 cells and shown that the expressed protein migrates to the nucleus in the absence of other viral proteins.

MATERIALS AND METHODS

Virus, plasmids, and cells. Influenza virus, A/WSN/33 (WSN), was plaque purified and grown in MDBK cells as described (21). All experiments were carried out using standard virus in MDBK cells at a multiplicity of infection of 3 to 5 PFU per cell. The cDNA cloning of polymerase genes using pBR322 in *E. coli* 294 has been reported previously (25). Expression plasmids used for expressing portions of polymerase cDNAs in bacteria as a fusion polypeptide were constructed from pNCV as described previously (6, 19). Plasmids containing the SV40 late promoter expression vector (pA11SVL3) and the SV40 helper DNA (pSVSal · 32) used for the expression of PB_1 cDNA have been described previously (5). All manipulations with restriction enzymes,

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ligases, DNA polymerases, etc., were carried out according to the suppliers' specifications.

Production of polymerase-specific antisera. Bacterially expressed fusion proteins containing parts of influenza virus polymerase proteins were used as immunogens for antibody production. Bacterial extracts containing the fusion proteins were solubilized, fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and electroeluted as described (6). Approximately 250 μg of fusion protein was mixed with complete Freund adjuvant and injected subcutaneously into female New Zealand white rabbits. Booster injections using 250 μg of each protein in incomplete Freund adjuvant were given at 4- to 6-week intervals. Rabbits were bled from the ears 10 days after each booster injection.

Indirect immunofluorescence The procedure for indirect immunofluorescence has been described previously (16a). Briefly, cell cultures grown on cover slips were infected with virus and fixed at different times postinfection (p.i.) with methanol-acetone (1:1) at −20°C. Fixed cells were overlaid with rabbit antipolymerase antibodies (1:20) or with mouse monoclonal antihemagglutinin (HA) antibodies (1:12) and incubated in a humidified chamber for 30 min at 37°C. The cells were then washed, treated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (1:50) or goat anti-mouse immunoglobulin G (1:30) for 30 min at 37°C, and examined in a Leitz photomicroscope by epifluorescence.

Radiolabeling, fractionation of cells, and immunoprecipitation. At different times p.i., WSN-infected MDBK cells were labeled with [^{35}S] methionine (50 to 100 μCi/ml) for various lengths of time. The cells were lysed in cold lysis buffer (10 mM Tris hydrochloride, pH 7.4, 2 mM EDTA, 100 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1% aprotinin [Trasylol; Boehringer Mannheim Biochemicals, Indianapolis, Ind.]), and the lysate was clarified for 30 min in an Eppendorf centrifuge. Immunoprecipitation of viral polypeptides was performed as described previously (5, 16a).

Cytoplasmic and nuclear fractionations were carried out as described by Briedis et al. (2) with minor modification. Briefly, cells were lysed by vortexing for 4 s in a buffer containing Nonidet P-40 (0.5%), Triton X-100 (0.5%), sodium deoxycholate (0.2%), Tris hydrochloride (pH 7.2) (10 mM), NaCl (140 mM), and $MgCl_2$ (1.5 mM). After the lysate was incubated at 0°C for 30 min, nuclear and cytoplasmic fractions were separated by centrifugation through 24% sucrose at 10,000 × *g* for 20 min. The nuclear pellet was further washed twice in the same buffer and lysed by sonication. Both the nuclear and cytoplasmic fractions were clarified for 30 min in an Eppendorf centrifuge and used for immunoprecipitation. The nuclear fraction isolated by this procedure contained histone proteins but lacked the viral HA, whereas the cytoplasmic fraction contained the viral HA but little or no histone protein. Viral polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (8% SDS; gels containing 4 M urea) (14).

Isolation of RNP-polymerase complexes. At 4 h p.i., infected cells were labeled with [^{35}S]methionine for 2 h and disrupted in the lysis buffer containing RNasin (1,000 U/ml; Promega Biotec, Madison, Wis.). To fractionate the RNP on a sucrose gradient, the clarified cell lysate was layered on a discontinuous sucrose gradient containing 75 mM NaCl, 2 mM EDTA, and 10 mM potassium phosphate (pH 7.4). The gradient was made sequentially in an SW40 tube using 2 ml of 65% sucrose, 2 ml of 35% sucrose, and 7 ml of 15 to 30% sucrose gradient. The cell lysate was laid on the top of the gradient. After centrifugation for 16 h at 39,000 rpm at 4°C, 1-ml fractions were collected from the bottom, and portions of these were analyzed by SDS-PAGE and pooled for immunoprecipitation.

Construction of plasmid pSVd · PB_1 for eucaryotic expression of PB_1. A cDNA clone containing the complete WSN PB_1 sequence was constructed by recombining the plasmid 1-72b (25), containing PB_1 nucleotides 1 to 2260, with the plasmids D1-3 (20), which contains the sequence of WSN defective interfering RNA L2b including the complete 3′ end of PB_1 (PB_1 nucleotides 1929 to 2341). The insert of the resulting plasmid (p1-72 · L2b) was subsequently modified by removal of G-C tails and addition of *Eco*RI linkers at nucleotide 17 of PB_1. The modified PB_1 insert possessed the entire coding sequence of the complete PB_1 polypeptide (757 amino acids [aa]) together with its initiation and termination codons. This insert was cloned into the *Eco*RI site of the SV40 expression vector pA11SVL3 (5, 22), substituting for the late region of SV40 (*Hpa*II to *Bam*HI), to yield pSVd · PB_1. For transfection, the pBR322 sequences of pSVd · PB_1 were removed by *Xba*I treatment and the SV40 portion was circularized using T4 DNA ligase. CV1 cells were cotransfected with SVd · PB_1 DNA and a helper DNA, SVSal · 32, using the DEAE-dextran technique (16a). SVSal · 32 contains a complete SV40 late region but a nonfunctional early region (5, 16a). From the transfected cells, virus stocks of SVd · PB_1 were prepared.

RESULTS

Expression of polymerase fusion proteins in *E. coli*. To produce large quantities of influenza virus polymerase polypeptides (PB_1, PB_2, PA) for use as immunogens, cDNA fragments corresponding to parts of the influenza virus WSN strain PB_1, PB_2, or PA coding sequences were joined in phase via the *Eco*RI site to a bacterial promoter-operator and leader DNA of the deletion plasmid (17) containing *ΔtrpLE1413* derived from the *E. coli* tryptophan operon. Figure 1 shows a diagram of the expression plasmid and the cDNA fragments used for expressing the fusion proteins. Each fusion protein contains the 190 aa of *trpLE* sequence, including the translation initiation codon (AUG) at the NH_2 terminus followed by a portion of the coding region of PB_1, PB_2, or PA. The construction strategy for placing portions of DNA of PB_1, PB_2, and PA in the same reading frame as the DNA encoding the 190 aa of the NH_2 terminus of the *trpLE* polypeptide is noted in the legend of Fig. 1. In pHDP1-1, 190 aa of the *trpLE* polypeptide is joined via 3 linking aa (provided by the *Eco*RI linker), followed by aa 1 to 377 of the WSN PB_1 protein (WSN PB_1 contains 757 aa). At the COOH terminus of this fusion protein, 2 aa and the translation termination codon are contributed by pBR322. This construction codes for a fusion protein of M_r 65,000.

In pHDP2-2, 190 aa of the *trpLE* polypeptide are linked by an additional amino acid (provided by the *Eco*RI linker sequence) to a 221-aa sequence (aa 159 to 379) of the WSN PA protein (WSN PA contains 716 aa), followed by 12 aa (provided by pBR322 sequence) and the termination codon contributed by pBR322. This construction codes for a fusion protein of M_r 45,000.

In pHDP3-1, the *trpLE* polypeptide is joined directly to a 242-aa sequence (aa 17 to 258) from the WSN PB_2 protein (759 aa), followed by 8 aa and the termination codon contributed by pBR322. This construction codes for a fusion protein of M_r 50,000.

Figure 2 demonstrated that the polymerase fusion proteins of the expected size encoded by pHDP1-1, pHDP2-2, and

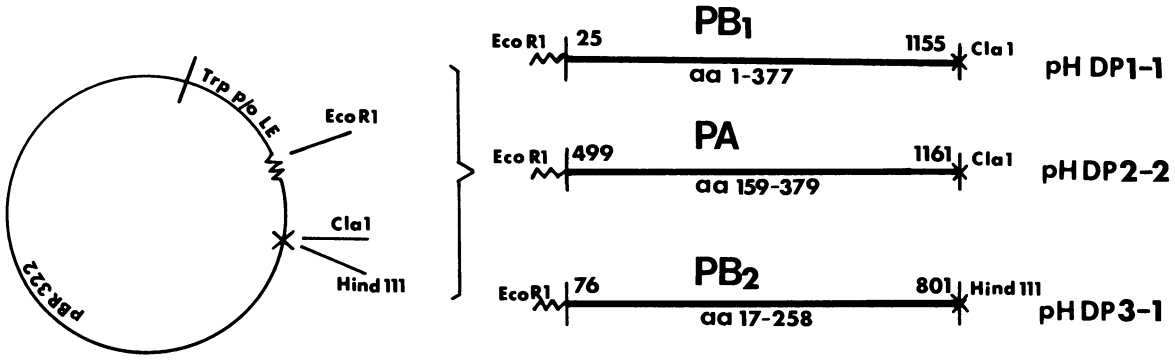

FIG. 1. Construction of expression plasmids containing insert cDNAs of PB_1, PB_2, and PA. To construct the expression plasmids, portions of the cDNA insert of PB_1, PB_2, and PA were joined in frame to the *trp* promoter-operator (p/o) containing a deletion in the *trpL* and *trpE* genes and pBR322. A DNA fragment spanning from the *Eco*RI site to either the *Cla*I or *Hin*dIII site was excised from the expression plasmid and replaced by the insert DNA as indicated below. For PB_1, the DNA fragment spanning from the *Bst*XI site (nucleotides 23 to 28) to the *Cla*I site (nucleotides 1150 to 1155; reference 25) was used. At the 5′ end, the *Bst*XI site was first blunt-ended using T4 DNA polymerase. A *Hin*dIII linker was added, the *Bst*XI site was blunt-ended again using DNA polymerase (Klenow fragment), and finally, an *Eco*RI linker was added. For PA, the DNA fragment spanning from the *Bgl*I site (nucleotides 489 to 500) to the *Acc*I site (nucleotides 1159 to 1164; D. Nayak, unpublished data) was used. At the 5′ end, the *Bgl*I site was filled in with T4 DNA polymerase and an *Eco*RI linker was added. At the 3′ end, the *Acc*I site was blunt-ended with DNA polymerase (Klenow fragment) and a *Cla*I linker was added. For PB_2, the DNA fragment spanning from the *Nru*I site (nucleotides 75 to 80) to the *Hin*dIII site (nucelotides 798 to 803; 9) was used. At the 5′ end an *Eco*RI linker was added. The zigzag line shows the EcoRI site; × indicates either the *Cla*I site (nucleotides 23 to 28) or the *Hin*dIII site (nucleotides 29 to 34) in pBR322.

pHDP3-1 are produced in large quantity when *E. coli* harboring the plasmid is grown in tryptophan-depleted medium. For immunization, each fusion protein was identified on SDS-polyacrylamide gels, excised, electroeluted, and injected with Freund adjuvant into rabbits as described in Materials and Methods.

Specificity of antisera against polymerase proteins. To determine the specificity of each antiserum made against the bacterially expressed polymerase proteins, [^{35}S]methionine-labeled infected-cell lysate was immunoprecipitated with each antiserum and analyzed by SDS-PAGE. The results show that the antibodies against each polymerase protein precipitated all three polymerase proteins. (Fig. 3, lanes 1, 2, and 3). These data suggest that either the three polymerase proteins remain physically associated as a complex, or they contain shared epitopes. To distinguish between these two possibilities and to determine the specificity of antisera against individual polymerase proteins, the cell lysate was treated first with SDS (0.8%) to dissociate the putative polymerase complex and then adjusted to 0.1% SDS before immunoprecipitation. The results show that the anti-pHDP1-1 serum (anti-PB_1), the anti-pHDP2-2 serum (anti-PA), and the anti-pHDP3-1 serum (anti-PB_2) immunoprecipitated exclusively PB_1, PA and PB_2, respectively (Fig. 3 lanes 4, 5, and 6). A small amount of nucleoprotein (NP) was also nonspecifically immunoprecipitated. The level of immunoprecipitated NP varied with sera from different rabbits, and NP was also precipitated by normal rabbit serum (data not shown). These data demonstrate that each antibody is, in fact, monospecific and reacts only with the corresponding antigen, with no cross-reactivity among the three polymerase proteins. Furthermore, the above results strongly suggest that some of the PB_1, PB_2, and PA polypeptides exist as a physical complex in the infected cells.

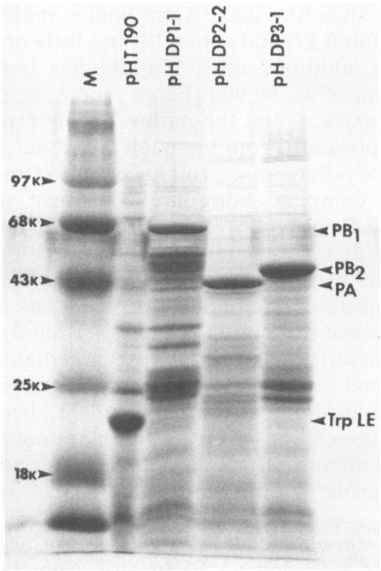

FIG. 2. Expression of bacterial polymerase-fusion proteins. *E. coli* harboring plasmids which contained portions of cDNA of PB_1, PB_2, or PA fused to the *trpLE* gene were expressed (6) in tryptophan-deficient medium. Proteins were separated by SDS-PAGE and stained with Coomassie blue. pHDP1-1 (PB_1), pHDP2-2 (PA), and pHDP3-1 (PB_2) expressed polypeptides of 65, 45, and 59 kilodaltons respectively. The positions of the trpLE (190 aa) protein (pHT190) and the polymerase fusion proteins are indicated by arrows.

Subcellular localization of polymerase proteins. To determine subcellular localization of the polymerase proteins, virus-infected MDBK cells at various times p.i. were analyzed by indirect immuno-fluorescence using monospecific antipolymerase antibodies. Monoclonal anti-HA antibodies were used for comparing the cytoplasmic localization of viral protein. As expected, HA was found to be accumulated in the rough endoplasmic reticulum and the Golgi complex at 4 h p.i. and later transported to the cell surface at 6 and 8 h p.i. (Fig. 4 b through d). Cellular nuclei remained free of HA throughout the infectious cycle. In contrast to the HA protein, three polymerase proteins were distributed differently in the infected cells. All three polymerase proteins were barely detectable over background at 2 h p.i. (Fig. 4e,

i, and m) but clearly visible at 4 h p.i. Both PB_1 (f) and PB_2 (n) were predominantly present in the nucleus, whereas PA (j) was mainly present in the cytoplasm. At 6 and 8 h p.i. all three polymerase proteins were present in the nucleus and cytoplasm (Fig. 4g, h, k, l, o, and p). The observed cytoplasmic fluorescence was diffuse, as expected for a soluble protein, and there was no staining of either the cell surface, the Golgi complex, or the perinuclear region. The nuclear staining was restricted to the nucleoplasm and absent in the nucleoli.

Formation of the polymerase complex. All three polymerase proteins are known to be associated with RNA and NP as the RNP-polymerase complex (10). Therefore, we wanted to determine whether the RNP-polymerase complex can be immunoprecipitated using antipolymerase antibodies. Accordingly, the labeled infected-cell lysate was clarified and fractionated on a discontinuous sucrose gradient as described in Materials and Methods. Portions of each fraction were analyzed by SDS-PAGE and pooled for immunoprecipitation. The RNP complexes were seen to sediment heterogenously (Fig. 5A). Large RNP complexes (fractions 1 and 2) contained an increased NP/polymerase ratio compared to the smaller RNP sedimenting in the middle of the gradient (fractions 3 through 6), whereas the fractions at the top of the gradient (fractions 9 through 11) contained more of the HA and M_1/NS_1 proteins and less NP and thus consisted of soluble proteins rather than RNP. To determine whether polymerase proteins are associated with RNP and also whether the RNP-free polymerase complex is present, every four consecutive fractions were pooled and immunoprecipitated, using the anti-PB_1 antibodies. Analysis of the immunoprecipitates showed that polymerase proteins are present both as an RNP-polymerase complex (pools 1 and 2) and as an RNP-free polymerase complex (pool 3) (Fig. 5B, lanes 1, 2, and 3). Essentially similar results were obtained when RNP was isolated by pelleting through a 30% sucrose cushion (40,000 rpm, 2 h at 4°C; SW60 rotor) and RNP-free supernatant was obtained from the top of the cushion. Polymerase complex could be immunoprecipitated from both the RNP pellet and the RNP-free supernatant (data not shown). These results demonstrated that the RNP-polymerase complexes can be immunoprecipitated with antipolymerase antibodies. In addition, these studies suggest that a fraction of the polymerase proteins may exist as a complex without being physically associated with the RNP and possibly forms the complex in absence of the RNP. However, it is not clear whether these RNP-free polymerase complexes are also associated with the NP. Because of its sticky nature, NP often is nonspecifically immunoprecipitated (8). Finally, the RNP immunoprecipitated by the antipolymerase antibodies was also shown to contain all eight viral RNA segments (data not shown). Next we attempted to define where the polymerase complex is formed. Accordingly, infected cells at 5 h p.i. were pulse-labeled for either 10 min or 1 h; nuclear and cytoplasmic extracts were prepared and immunoprecipitated with antipolymerase antibodies. These results were essentially similar for both 10 min (Fig. 6) and 1 h of pulse-labeling (data not shown). The cytoplasmic extract, when immunoprecipitated with anti-PB_2 antibodies, contained all three polymerase proteins, although the homologous protein was predominant (Fig. 6, lane 1). Similar results were obtained with anti-PB_1 antibodies (data not shown). Anti-PA antibodies preferentially immunoprecipitated PA and some PB_1 but little or no PB_2 (Fig. 6 lane 2). In addition, anti-PA antibodies immunoprecipitated a protein of M_r 60,000 (Fig 6, lane 2, arrow) from the cytoplasmic extract, and the nature of this protein remains unknown at present. From the nuclear extract, on the other hand, all three polymerase proteins could be immunoprecipitated as a complex using any of these monospecific antipolymerase antibodies (Fig. 6, lanes 3 and 4). These results show that polymerase proteins were present in the nucleus within a 10-min pulse and that essentially all of the polymerase proteins in the nucleus existed as a complex of three polymerase proteins. On the other hand, in the cytoplasm, the majority of the proteins immediately after synthesis remained unassociated and only a small fraction was found in the form of a complex. Again, because of the nonspecific association of NP in the immunoprecipitate we could not unambiguously determine the presence of NP-free polymerase proteins either in the cytoplasm or in the nucleus.

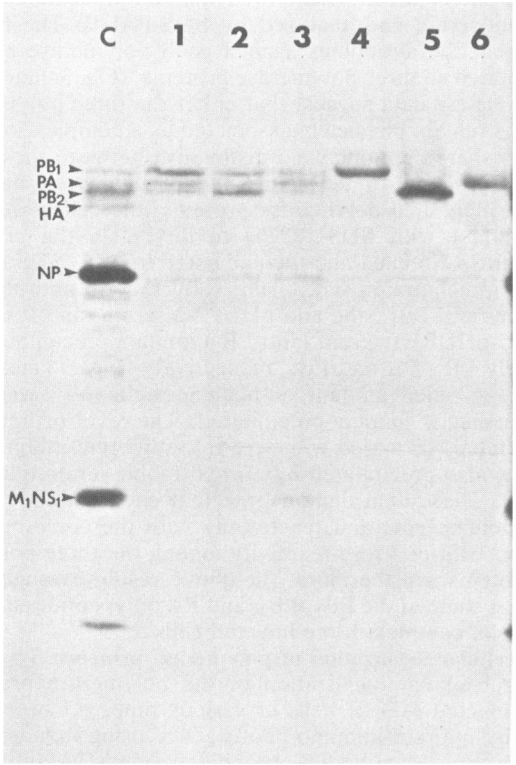

FIG. 3. Specificity of polymerase antisera. Virus-infected MDBK cells were pulse-labeled for 1 h with [^{35}S]methionine beginning at 5 h p.i. Labeled cells were disrupted in lysis buffer, and the clarified lysates were immunoprecipitated with each of the polymerase antisera (lanes 1 to 3). To determine the monospecificity of individual antisera, cell lysates were treated with 0.8% SDS at 37°C for 30 min and adjusted to 0.1% SDS with cold lysis buffer before immunoprecipitation (lanes 4 to 6). Lane C WSN-infected cell lysate. Immunoprecipitates: lanes 1 and 4, PB_1 antisera; lanes 2 and 5, PB_2 antisera; lanes 3 and 6, PA antisera.

Nuclear accumulation of PB_1 polymerase expressed from cloned cDNAs by using an SV40 late promoter expression vector. The above results suggest that either the individual polymerase proteins, after their synthesis, form a complex in the cytoplasm and are transported into the nucleus as a complex or alternatively, they are transported individually into the nucleus, where polymerase protein complexes and RNP-polymerase complexes are formed. To determine whether the individual polymerase proteins possess an intrinsic property of migrating to and accumulating in the

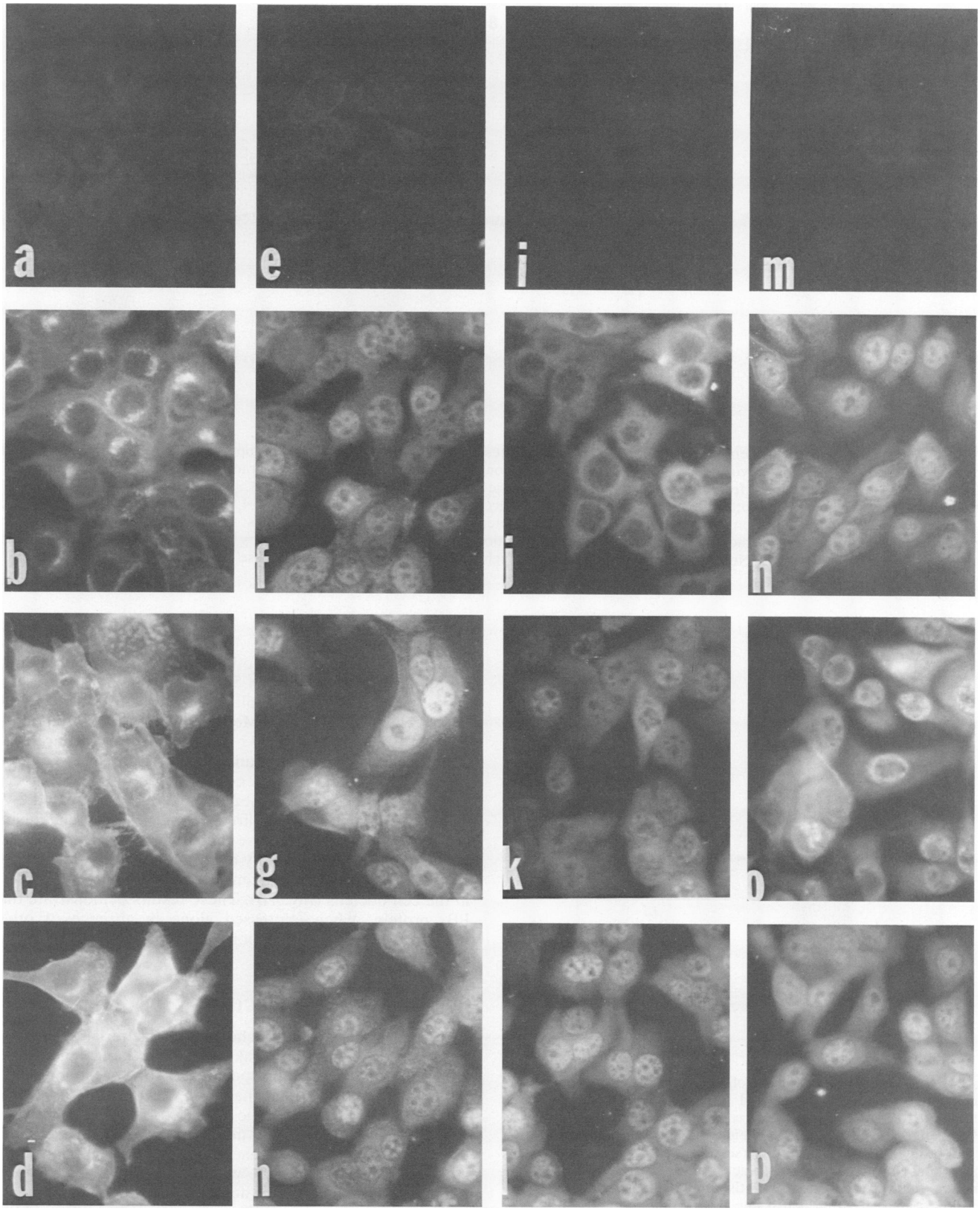

FIG. 4. Analysis of the synthesis and localization of individual polymerase proteins and hemagglutinin in WSN virus-infected MDBK cells by indirect immunofluorescence. Mock-infected or WSN-infected cells, at various times p.i. were fixed and stained with either monospecific antipolymerase antisera or anti-HA monoclonal antibodies, using indirect immunofluorescence as described in Materials and Methods. (a) Mock-infected cells with anti-PB_1. (b, c, and d) Anti-HA monoclonal antibodies at 4, 6, and 8 h p.i. respectively. (e, f, g, and h) Anti-PB_1 at 2, 4, 6, and 8 h p.i. respectively. (i, j, k, and l) Anti-PA at 2, 4, 6, and 8 h p.i. respectively. (m, n, o, and p) Anti-PB_2 at 2, 4, 6, and 8 h p.i. respectively.

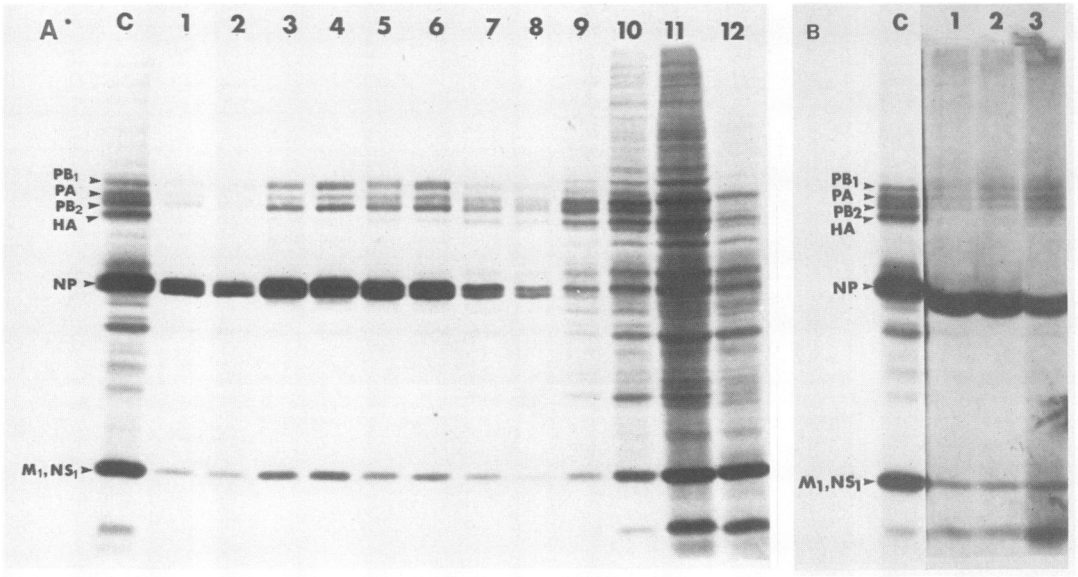

FIG. 5. Sucrose density gradient fractionation of RNP complexes from infected cells and immunoprecipitation of pooled RNP using anti-PB_1 antibodies. (A) Labeled virus-infected cell lysate was fractionated by a discontinous sucrose gradient (see Materials and Methods). Fractions of 1 ml were collected from the bottom, and samples were analyzed by SDS-PAGE. Lane C, Total infected cell lysate; lanes 1 through 12, fractions from the bottom of the gradient. (B) Four successive fractions were pooled (1–4, 5–8, 9–12) and diluted 10-fold to decrease the sucrose concentration, and each pool immunoprecipitated with anti-PB_1 antibodies. Lanes: C, total infected-cell lysate before immunoprecipitation; 1, pooled fractions 1 through 4; 2, pooled fractions 5 through 8; 3, pooled fractions 9 through 12; after immunoprecipitation with anti-PB_1 antibodies. Note that the NP band migrated slightly lower than the control because of the presence of a large amount of immunoglobulin heavy chains.

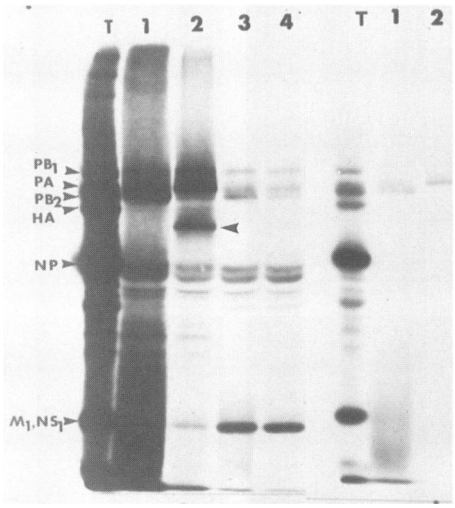

FIG. 6. Immunoprecipitation of pulse-labeled polymerase proteins present in the nuclear and cytoplasmic fractions of WSN virus-infected cells. Infected cells were pulse-labeled for 10 min with [^{35}S]methionine at 5 h p.i. Labeled cells were fractionated into cytoplasmic and nuclear extract (see Materials and Methods). The polymerase proteins in each extract were immunoprecipitated with monospecific antipolymerase antiserum and separated by SDS-PAGE. Lanes: T, total cell lysate before immunoprecipitation; 1, cytoplasmic fraction with anti-PB_2; 2, cytoplasmic fraction with anti-PA, (arrow shows the position of 60-kilodalton cellular protein); 3, nuclear fraction with anti-PB_2; 4, nuclear fraction with anti-PA. The lanes on the right-hand side are a shorter exposure of the lanes on the left-hand side.

nucleus in the absence of other viral or other polymerase proteins, we constructed an SV40 late promoter expression vector containing the complete PB_1 cDNA (SVd · PB_1) as described in Materials and Methods. Virus stocks containing SVd · PB_1 and SVSal · 32 viruses were prepared by cotransfection of CV1 cells and used for studying the expression of PB_1 protein in CV1 cells. To determine the subcellular location of the expressed PB_1, SVd · PB_1-infected CV1 cells were examined by indirect immunofluorescence using monospecific antipolymerase antisera. Positive immunofluorescence was detected only after staining with the anti-PB_1 antibodies, and the fluorescence was predominantly present in the cell nucleus (Fig. 7B). These results demonstrate that PB_1 protein, when expressed alone in absence of other influenza proteins, is transported to and accumulates in the cell nucleus.

DISCUSSION

In this report we have shown that bacterial expression vectors can be used to make relatively large amounts of fusion proteins containing specific portions of the individual polymerase proteins, and that these fusion proteins can be used to make monospecific antisera. Thus it should be possible in the future to make antisera against specific domains of each polymerase protein. Using monospecific antipolymerase antibodies, we have shown that all three polymerase proteins can be detected in infected MDBK cells by immunofluorescence (Fig. 4). At 4 h p.i. both PB_1 and PB_2 can be detected in the nucleus, whereas all three polymerase proteins including PA appear in the nucleus later in the infectious cycle. Recently, Jones et al. (8) have reported that all three polymerase proteins are present in the nucleus of

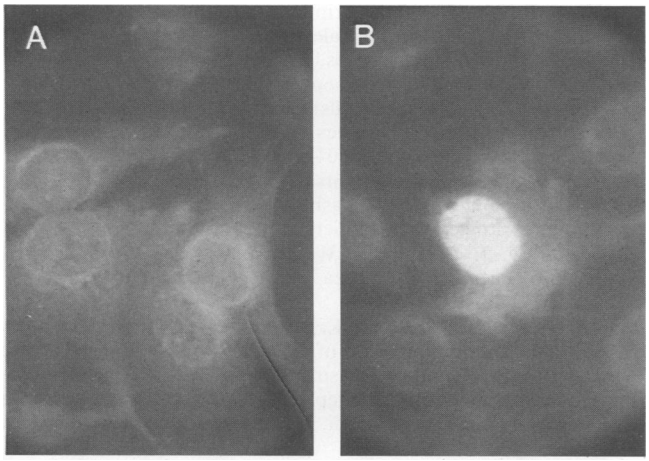

FIG. 7. Intracellular localization of PB_1 in SVd · PB_1-infected CV1 cells. CV1 cells were infected with SVd · PB_1 virus stocks (which also contain SVSal · 32 virus). At 48 h p.i. the infected cells were washed and fixed and then treated with anti-PB_1 antibodies and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G as described (5, 16a). (A) SNC-infected cells (5) reacted with anti-PB_1 (negative control). (B) SVd · PB_1-infected cells reacted with anti-PB_1 antibodies.

MDCK cells infected with influenza virus A/NT/60/68 at 4 h p.i. Since these authors used only a single time point p.i. for immunofluorescence, they might have failed to detect the difference in the kinetics of nuclear accumulation of PA in virus-infected cells. Moreover, use of a different virus and host cells as well as a different multiplicity of infection may have caused nuclear accumulation of PA earlier than that observed in our studies. Previous genetic studies using temperature-sensitive mutants have indicated that in addition to PB_1 and PB_2, PA is involved in viral RNA replication (15). As the peak of viral RNA replication also appears to occur later in the infectious cycle than does the peak of mRNA transcription (24), the late nuclear accumulation of PA may modulate polymerase activity in favor of viral RNA synthesis (replication) over mRNA synthesis (transcription). PA is synthesized at the same rate as the other two polymerases throughout the infectious cycle, and all three polymerase proteins are equally stable in the infected cell (unpublished data). However, factors responsible for the delayed transport of PA into the nucleus remain unknown. It is possible that the kinetics of nuclear transport of PA could just be slower or that PA undergoes some posttranslation modification. However, we have been unable to detect any phosphorylation of PA (unpublished data).

Our immunoprecipitation data demonstrate that all three polymerase proteins can be precipitated as a complex by using any of the monospecific antisera. This provides direct evidence that the three polymerase proteins remain physically associated as a complex. Earlier studies using UV cross-linking have provided indirect evidence that polymerase proteins may remain associated as a complex with the viral RNA (1). Furthermore, antibodies against individual polymerase proteins also immunoprecipitated RNP-polymerase complex, confirming an earlier observation of the tight association of polymerase proteins, with RNA (10). In addition, polymerase proteins were immunoprecipitated as a complex from the RNP-free supernatant, indicating that polymerase complex can exist free from RNP. However, these results should be interpreted with caution because polymerase complexes may become dissociated from the RNP during the centrifugation procedure.

Influenza polymerase proteins, soon after synthesis, are found in the nucleus, where they are present as a complex of three polymerase proteins (Fig. 6). Two possibilities exist: either each polymerase protein is individually transported into the nucleus, where they immediately form complex with each other, or the three polymerase proteins form complex in the cytoplasm and are transported into the nucleus. To determine whether an individual polymerase protein possesses an intrinsic property for nuclear transport, we have expressed the PB_1 protein by using the SV40 late promoter expression vector. Our results demonstrate that PB_1 alone can be transported into the nucleus. Recently, Jones et al. (8) have shown that PB_2, when expressed alone, also accumulates in the nucleus. These data taken together show that two of the three polymerase proteins, PB_1 and PB_2, possess the intrinsic signal(s) for nuclear transport and accumulation. It remains to be seen whether PA alone possesses a nuclear transport signal. However, the nuclear transport of the individually expressed polymerase proteins does not rule out the possibility that in virus-infected cells, where all viral proteins are present, cytoplasmic complexes are formed among the three polymerase proteins and with NP and that such a complex may further regulate the transport of polymerase proteins into the nucleus. Recently, Knipe and Smith (12) have shown that two herpesvirus proteins (ICP8 and ICP0) when expressed alone become localized in the nucleus; however, in herpesvirus-infected cells the nuclear localization of these two proteins is modulated by another herpesvirus protein ICP4. Therefore, the kinetics of the nuclear transport of the influenza virus polymerase proteins, expressed alone or in combination with other viral proteins, have to be determined before the regulation of the nuclear transport of polymerase proteins can be elucidated.

Finally, the nature and the localization of nuclear transport signals of the polymerase proteins have yet to be identified. Two types of nucleophilic signals have been proposed. Proteins smaller than 70 kilodaltons may passively diffuse through the nuclear pores and may accumulate in the nucleus because of their nuclear "accumulation" signal (4). Ala-337–Phe–Glu–Asp–Leu-341 of influenza NP has been proposed as the nuclear accumulation signal (4). However, proteins larger than 70 kilodaltons, or proteins which form complex in the cytoplasm and are transported as a complex, may require nuclear "transport" signals for active transport. Nuclear transport signals have been identified for SV40 T antigen (7), polyomavirus T antigen (23), SV40 VP1 protein (26), and a yeast ribosomal L3 protein (18). The principal motif of the nuclear transport signal is a linear sequence of 4 or 5 aa containing a proline residue followed by a series of positively charged amino acids. Computer search of PB_1 sequence identifies a sequence Pro-668–Lys–Arg–Asn–Arg–Ser (25). Influenza B PB_1 (11) also contains sequence conservation in this region (Thr-Lys-Arg-Asn-Arg-Ser). In fact, 9 aa (668 to 676) in this region are completely conserved between A and B PB_1. In addition, the B PB_1 protein also contains another sequence, Pro-192–Ala–Lys–Asn–Arg–Lys–Gly, which closely resembles the putative nuclear transport signal. However, PB_2, although transported individually into the nucleus, does not appear to contain such a sequence (8, 9). Studies involving mutation, deletion, and chimeric construction with the putative nuclear signal should elucidate their role, if any, in nuclear transport of the PB_1 protein.

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ADDENDUM

Recently, D. M. Detjen, C. St. Angelo, M. G. Katze, and R. M. Krug (J. Virol. **61**:16–22, 1987) have also reported the presence of RNP-free polymerase complex in influenza virus-infected cells.

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