JOURNAL OF VIROLOGY, Jan. 1991, p. 245–253
0022-538X/91/010245-09$02.00/0

Vol. 65, No. 1

Two Signals Mediate Nuclear Localization of Influenza Virus (A/WSN/33) Polymerase Basic Protein 2

JUN MUKAIGAWA AND DEBI P. NAYAK*

Department of Microbiology and Immunology, UCLA School of Medicine, Jonsson Comprehensive Cancer Center, Los Angeles, California 90024-1747

Received 26 July 1990/Accepted 14 October 1990

Polymerase basic protein 2 (PB2), a component of the influenza virus polymerase complex, when expressed alone from cloned cDNA in the absence of other influenza virus proteins, is transported into the nucleus. In this study, we have examined the nuclear translocation signal of PB2 by making deletions and mutations in the PB2 sequence. Our studies showed that two distant regions in the polypeptide sequence were involved in the nuclear translocation of PB2. In one region, four basic residues (K-736 R K R) played a critical role in the nuclear translocation of PB2, since the deletion or mutation of these residues rendered the protein totally cytoplasmic. However, seven residues (M K R K R N S) of this region, including the four basic residues, failed to translocate a cytoplasmic reporter protein into the nucleus, suggesting that these sequences were necessary but not sufficient for nuclear translocation. Deletion of another region (amino acids 449 to 495) resulted in a mutant protein which was cytoplasmic with a perinuclear distribution. This novel phenotype suggests that a perinuclear binding step was involved prior to translocation of PB2 across the nuclear pore and that a signal might be involved in perinuclear binding. Possible involvement of these two signal sequences in the nuclear localization of PB2 is discussed.

Influenza virus, a negative-strand RNA virus, carries an RNA-dependent RNA polymerase (often referred to as transcriptase or replicase) in the virion particle. The viral polymerase functions as a heterocomplex (3P complex) and consists of three polymerase proteins, polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein, encoded by the three largest viral RNA segments (segment 1, 2, and 3). However, unlike most other negative- or positive-strand RNA viruses, influenza virus requires the host cell nucleus for both the transcription and the replication of the virus RNA (see reference 22). Therefore, the polymerase proteins, either individually or as a part of a complex, must be transported into the nucleus, the site of their function.

In virus-infected cells, at least six of the virus-encoded proteins, PB1, PB2, PA, NP, NS1, and NS2, are found in the cell nuclei. Furthermore, when these proteins are expressed independently from cloned cDNAs, they are transported to the nucleus in the absence of other viral proteins (1, 6, 15, 16, 23, 27, 37, 39), indicating that each protein is capable of nuclear localization independently and must carry one or more nuclear localization signal(s) in its polypeptide sequence. Of these, NP appears to possess a nuclear accumulation signal which is acidic in nature (6). NS1, on the other hand, contains two nuclear localization signals, both of which are basic in nature (15). Both of these NS1 nuclear localization signals possess a single stretch of contiguous amino acids and can function independently of each other in nuclear translocation. By using chimeric constructions of PB1 with chicken pyruvate kinase (PK), a cytoplasmic protein, we have recently shown that PB1 contains a novel bipartite nuclear localization signal separated by a spacer sequence (27). Both domains of the signal are required for nuclear localization of the PB1 chimeric protein, whereas the intervening spacer sequence could be deleted without affecting nuclear localization.

In this report, we have analyzed the signal requirement(s) for nuclear localization of PB2 and shown that two signals are required for its nuclear localization. However, the requirement, function, and structural features of these two signals appear to be different from those of the PB1 nuclear translocation signal, since only one of the PB2 signal domains possesses a stretch of basic residues and since deletion of the other yields a novel perinuclear phenotype.

MATERIALS AND METHODS

Viruses, cDNA clones, and antisera. Influenza virus A/WSN/33 (H1N1) was grown in MDCK cells as reported previously (28). Isolation and sequence analysis of cDNA clones of PB2 of A/WSN/33 have been described before (18). Recombinant vaccinia virus VTF7.3 expressing T7 RNA polymerase was obtained from B. Moss of the National Institutes of Health (14). Monospecific antisera against the fusion polymerase proteins, expressed in *Escherichia coli*, were prepared in rabbits (1). Standard procedures were followed for all DNA manipulations (24).

Construction of PB2 deletion mutants and chicken muscle PK chimeras. (i) Deletion 1 (del 1). The plasmid PB2/pGEM 4 was digested with *Bgl*II and *Sph*I. The large fragment was isolated, end-filled with DNA polymerase I (Klenow fragment), and ligated with *Cla*I linkers to insert the initiation codon (ATG).

(ii) del 2. The plasmid PB2/pGEM 4 was digested with *Bgl*II and treated with mung bean nuclease and then digested with *Hin*dIII and treated with Klenow fragment. The large fragment was isolated and recircularized by ligation.

(iii) del 3. The plasmid PB2/pGEM 4 was digested with *Hin*dIII and *Nco*I and treated with mung bean nuclease. The large fragment was isolated and recircularized.

(iv) del 4. The plasmid PB2/pGEM 4 was digested by *Nco*I

* Corresponding author.

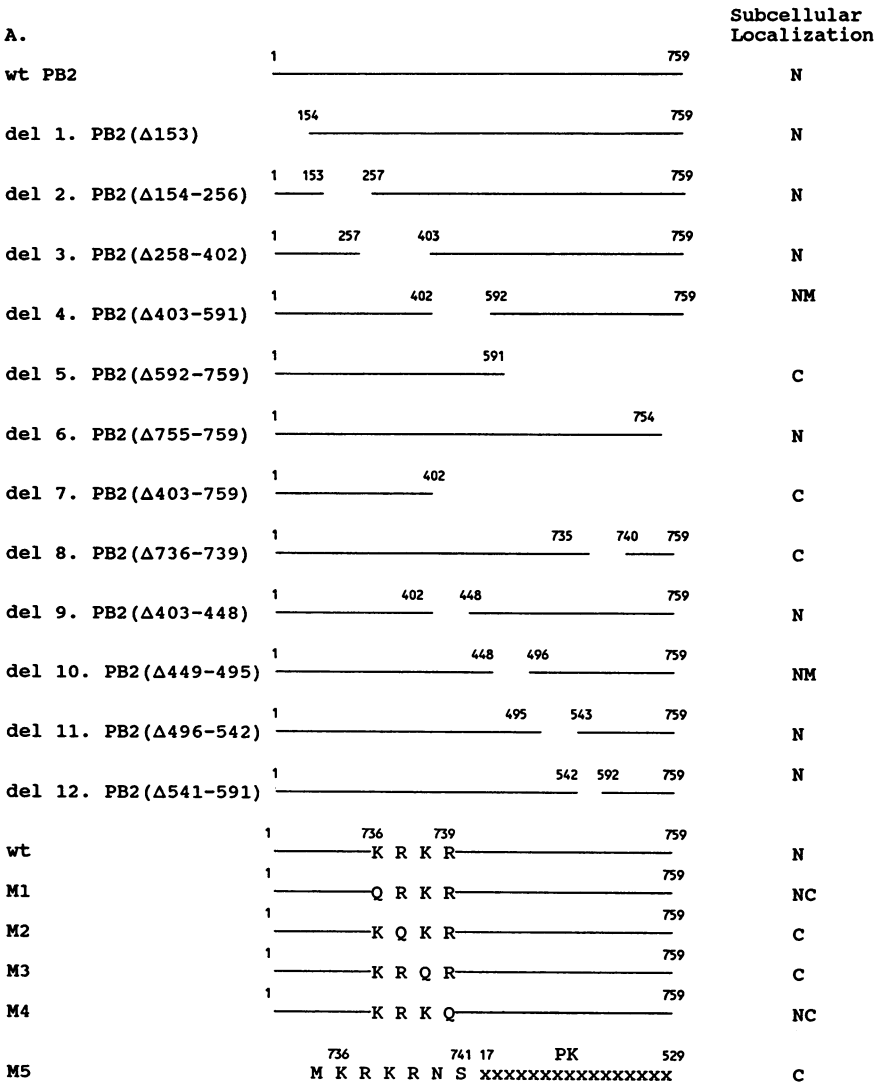

FIG. 1. Schematic representation of deletion mutants of PB2 of A/WSN/33 virus and chimeric construction with PK. Numbers on the top of the lines denote the specific amino acid numbers of the polypeptide. N, Nuclear; NM, nuclear membrane; C, cytoplasmic; x, PK amino acids from aa 17 to 529 (32).

and *Bst*XI and treated with mung bean nuclease. The large fragment was isolated and recircularized.

(v) del 5. The plasmid PB2/pGEM 4 was digested by *Bst*XI and *Sma*I and treated with Klenow fragment. The large fragment was isolated and ligated with a *Hpa*I linker to generate a stop codon.

(vi) del 6. The plasmid PB2/pGEM 4 was digested by *Eco*RI and treated with Klenow fragment. The large fragment was isolated and ligated with *Hpa*I linker to generate a stop codon.

(vii) del 8 to 12. To remove the residues specific for nuclear localization, the PB2 cDNA fragment was first inserted into the *Pst*I site of M13mp18. Oligonucleotides ranging from 34 to 40 bases were used to create specific deletions of DNA encoding the desired amino acids by using an Amersham in vitro mutagenesis system.

To construct the chicken muscle PK chimera (Fig. 1, M5), the following two complementary DNA primers with an *Eco*RI site on each end were made and inserted into the *Eco*RI site at the 5′ end of the PK DNA (17):

```
AA TTC ATG AAA CGG AAA CGG AAC TCT AGC G
    . ... ... ... ... ... ... ... ... .
    G TAC TTT GCC TTT GCC TTG GGA TCG C TTAA
```

Transfection, labeling, and lysis of cells. The vaccinia virus-T7 expression system (14) was used for the transient expression of PB2 proteins in baby hamster kidney (BHK) cells. Procedures for the transfection of plasmid DNA under the control of the T7 promoter and infection by vaccinia virus have been described previously (14, 27). Usually, 30 μg of DNA was used for lipofectin-mediated transfection of cells in a 60-mm dish (12). At the appropriate time, the transfected BHK cells were labeled with [^{35}S]methionine (100 μCi/ml) and [^{35}S]cysteine (100 μCi/ml) for 15 min at 39°C. Cells were washed twice with phosphate-buffered saline (PBS) and then chased for various times in a medium containing an excess of unlabeled amino acids (20 mM). Cells were then scraped from the plates, centrifuged, and lysed in the appropriate lysis buffer. For preparing total cell lysates, cells were suspended in 1 ml of lysis buffer (50 mM

Tris hydrochloride [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 2 mM EDTA, 1 μM phenylmethylsulfonyl fluoride, and 10 μg of aprotinin per ml) and sheared through a 25-gauge needle. The lysate was clarified by centrifugation in an Eppendorff microcentrifuge for 20 min at 4°C.

Subcellular fractionation. The procedure of Ramsay et al. (32) was used for isolation of nuclear and cytoplasmic fractions. Briefly, cells were resuspended in hypotonic TMK buffer (25 mM Tris hydrochloride [pH 7.4], 1 mM $MgCl_2$, and 5 mM KCl). After incubation on ice for 15 min, an equal volume of hypotonic TMK buffer containing 1% Nonidet P-40 was added, making the final Nonidet P-40 concentration 0.5%. After an additional 5 min on ice, nuclei were pelleted at 1,000 × *g* for 5 min at 4°C. The nuclei were washed twice with the hypotonic TMK buffer containing 0.5% Nonidet P-40, then resuspended in lysis buffer, sheared through a 25-gauge needle, and clarified by centrifugation. For fractionation of the nuclear envelope, cells were treated as described above to obtain the cytoplasmic and nuclear fractions. The nuclear fraction was then treated further with a buffer containing a higher detergent concentration, which has been shown to remove the nuclear membrane (1, 3). Briefly, the nuclear fraction obtained above was mixed with ice-cold buffer containing 10 mM Tris hydrochloride (pH 7.4), 2 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40, 0.5% Triton X-100, 0.2% sodium deoxycholate, and 1 mM $MgCl_2$ and kept for 20 min on ice. The nuclear and nuclear membrane fractions were separated by centrifugation. The salt and detergent concentrations of the cytoplasmic and nuclear membrane fractions and of nuclear fractions were adjusted to those of lysis buffer. Each fraction was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Immunofluorescence, immunoprecipitation, and PAGE analysis. Procedures for indirect immunofluorescence, immunoprecipitation, sample preparation, and PAGE analysis have been described previously (1, 27).

RESULTS

Construction and expression of PB2 deletion mutants. It has been previously shown that the influenza virus PB2, expressed alone from cDNA, becomes translocated into the nucleus (16, 37). Since a computer search failed to identify any obvious nuclear localization signal, we made a series of deletions (Fig. 1, del 1 through 7) covering the entire coding region of PB2, as described in Materials and Methods. These deleted DNAs were expressed in BHK cells by using a T7 vaccinia virus expression system (14). Intracellular localization of the wild-type (wt) and mutant PB2 proteins was examined by indirect immunofluorescence with monospecific rabbit antiserum against PB2 (1). Results showed that the wt PB2, as expected, was localized into the nucleus (Fig. 2A). Analysis of deletion mutants showed that the nuclear signal was not present in either the extreme amino terminus (Fig. 2B, del 1) or the carboxy terminus (Fig. 2G, del 6). Furthermore, since del 1 (Fig. 2B), del 2 (Fig. 2C), and del 3 (Fig. 2D) were also present in the nucleus, whereas del 7 (Fig. 2H) was present in the cytoplasm and not in the nucleus, the nuclear signal was not present in the amino acids (aa) 1 to 402 of PB2. del 4, lacking 190 amino acids (aa 403 to 591), presented a different picture: its distribution was not intranuclear, but rather perinuclear (Fig. 2E). However, its distribution was also different from that of the cytoplasmic PB2 proteins such as del 5 (Fig. 2F), del 7 (Fig. 2H), and del 8 (Fig. 2I), which presented a diffuse cytoplasmic distribution. Since del 6 (Fig. 2G, aa 1 to 754) was positive for nuclear translocation but del 5 (Fig. 2F, aa 1 to 591) was totally cytoplasmic, the nuclear localization signal(s) was likely to be present in the region encompassing aa 592 to 754. Upon further examination for a possible nuclear localization signal, a stretch of four basic residues K-736 R K R was found within this region. Since putative nuclear localization signals often consist of a stretch of basic residues, we examined the function of these residues by making specific deletion of them (Fig. 1, del 8). del 8, when expressed in BHK cells, was totally cytoplasmic (Fig. 2I), indicating that these four basic residues (K R K R) formed a critical part of the nuclear localization signal of PB2.

Effect of single-amino-acid changes in the nuclear localization signal of PB2. Since basic residues have been found to be critical in providing the nuclear localization signal and since the positions of some residues in the nuclear localization signal appear to be more critical than others (36), we changed each of these four residues separately to glutamine, a nonbasic residue (M1 to M4, Fig. 1), and determined the effect of each replacement in the nuclear transport of PB2. Results show that the replacement of K-736→Q (Fig. 3B) or R-739→Q (Fig. 3E) reduced the efficiency of nuclear translocation of PB2, since both mutant proteins were present in the nucleus as well as in the cytoplasm. However, the replacement of R-737→Q (Fig. 3C) or K-738→Q (Fig. 3D) made the protein completely cytoplasmic. These results showed that all four basic amino acids were important for efficient nuclear localization of PB2, but R-737 and K-738 played the most critical roles in nuclear translocation of PB2 since replacement of either of these basic amino acids with a nonbasic amino acid rendered the protein totally cytoplasmic. Mutation of the second lysine (K-128) to a nonbasic residue in the simian virus 40 large T nuclear signal also rendered the mutant protein totally cytoplasmic (36).

Role of the PB2 nuclear localization signal in translocating a heterologous protein into the nucleus. Since the residues K-736 R K R formed a critical part of the nuclear localization signal, we wanted to determine whether this sequence was sufficient to transport a heterologous cytoplasmic protein into the nucleus. Since 5 to 7 residues have been shown to be a minimum requirement for a nuclear translocation signal and in some cases were shown to translocate a heterologous cytoplasmic protein into the nucleus (17), seven residues including a methionine (M K R K R N S) were tethered at the NH_2 terminus of PK, a cytoplasmic protein (26) which has been extensively used as a reporter protein for nuclear translocation (36). The results showed that the chimeric PK was cytoplasmic (Fig. 3F) and was not transported into the nucleus, indicating that these basic residues alone were not sufficient for nuclear translocation of a foreign cytoplasmic protein.

Deletion analysis of the sequence required for binding to the perinuclear region. Since the intracellular distribution of del 4 [Δ(403 to 591)] was different from either the cytoplasmic or the nuclear distribution of PB2 and appeared to be bound to the perinuclear region, we wanted to further delineate sequences responsible for this phenotype. We therefore made four smaller deletions within this 190-aa region, each ranging from 46 to 50 residues (Fig. 1; del 9, del 10, del 11, and del 12), and determined the phenotype of these proteins upon expression in BHK cells. The results show that del 9 [Fig. 4A, Δ(403 to 448)], del 11 [Fig. 4C, Δ(496 to 542)], and del 12 [Fig. 4D, Δ(543 to 591)] were transported to the nucleus and exhibited the same phenotype as the wt, whereas del 10 [Fig.

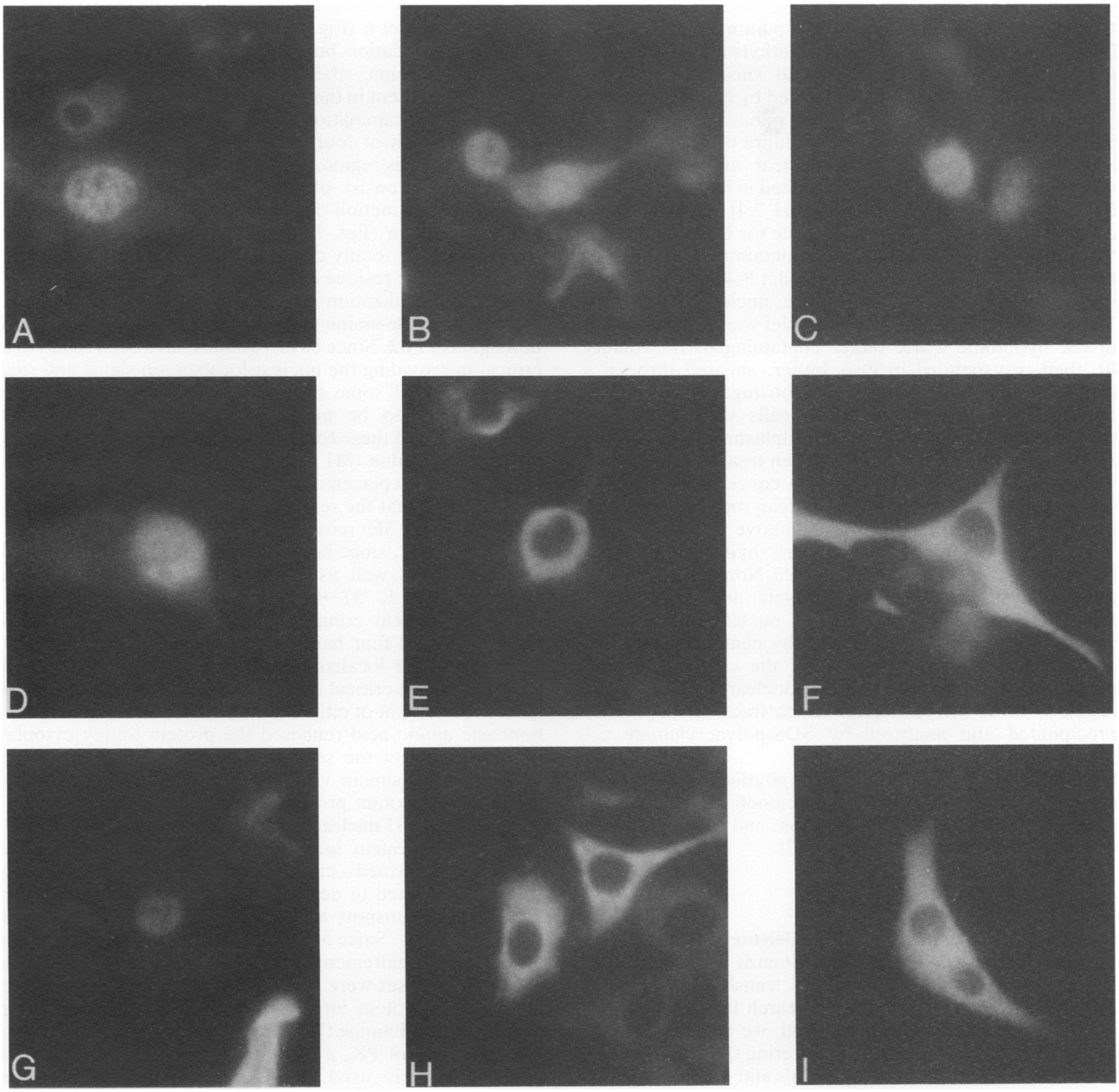

FIG. 2. Localization of PB2 protein in BHK cells transfected with wt or mutant PB2 cDNAs. At 16 h after transfection and T7 vaccinia virus infection, cells were fixed and stained with anti-PB2 antiserum by indirect immunofluorescence as described in Materials and Methods. BHK cells were transfected with wt PB2 DNA (A) and with del 1 (B), del 2 (C), del 3 (D), del 4 (E), del 5 (F), del 6 (G), del 7 (H), and del 8 (I) mutants of PB2 DNA.

4B, Δ(449 to 495)] exhibited the nuclear membrane phenotype of del 4. These results indicated that the region (aa 449 to 495) was required for nuclear translocation, since the protein lacking this region remained bound to the nuclear membrane and was not translocated into the nucleus.

Subcellular localization of the wt PB2 and mutant proteins by cell fractionation. To further determine the subcellular localization of the wt and mutant proteins, relative distribution of the wt and the two mutants del 8 and del 4 were determined. Accordingly, transfected cells were pulse-labeled for 15 min and chased for different times in the presence of unlabeled amino acids in excess. Cytoplasmic and nuclear fractions were isolated as described in Materials and Methods. Cells were fractionated into either the cytoplasmic and total nuclear fractions (Fig. 5A) or the cytoplasmic, nuclear, and nuclear membrane fractions (Fig. 5B). The results showed that after a 15-min pulse, the wt PB2 (Fig. 5A, upper panel) was present both in the cytoplasm and in the nucleus, indicating that a fraction of PB2 was already transported into the nucleus. During the chase, PB2 disappeared from the cytoplasm with a half-life ($t_{1/2}$) of 30 to 40 min. However, there was no corresponding increase in the nucleus, where PB2 was also unstable ($t_{1/2} < 1$ h). It therefore appeared that the wt PB2 was being degraded with

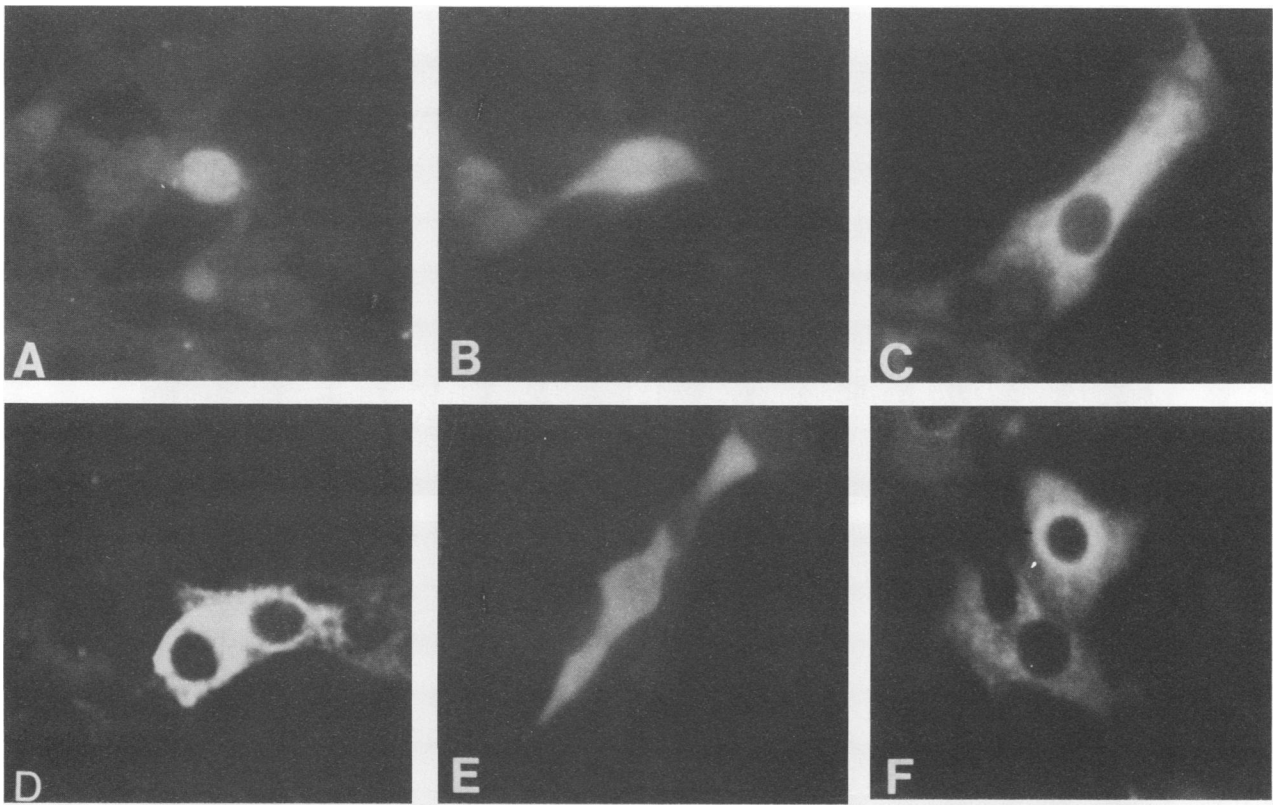

FIG. 3. Mutational analysis of the nuclear signal of PB2 protein. BHK cells were transfected with wt PB2 DNA (A), with K-736→Q (B), R-737→Q (C), K-738→Q (D), and R-739→Q (E) one-amino-acid exchange mutants, and with M K R K R N S-PK DNA (F). At 16 h posttransfection, the cells were fixed and stained with anti-PB2 (A to E) or anti-PK antisera (F) by using indirect immunofluorescence as described in Materials and Methods.

a relatively high turnover rate in PB2-expressing cells. del 8 (Fig. 5A, lower panel), on the other hand, was present only in the cytoplasm and not in the nucleus, and unlike the wt PB2, it did not exhibit a fast turnover rate ($t_{1/2} > 2$ h), suggesting that the degradation of the wt PB2 was probably taking place in the nucleus and not in the cytoplasm. These results concurred with the immunofluorescence data (Fig. 2A and I), which showed that the wt PB2 was transported into the nucleus, whereas the del 8 was transport defective and present only in the cytoplasm.

Since del 4 presented an intracellular distribution different from that of either the wt PB2 or del 8 and exhibited a perinuclear staining by immunofluorescence assay (Fig. 2E), we wanted to determine if del 4 would fractionate with the nuclear membrane. Accordingly, subcellular fractionations were carried out in two steps, as described in Materials and Methods. In the first step, cells were swelled in a hypotonic solution and treated with a mild detergent concentration (32). Such treatment removes the soluble cytoplasmic compartment and other membranous compartments, leaving the perinuclear membrane and perinuclear endoplasmic reticulum still attached to the nucleus. Subsequently, these nuclei were treated with a higher detergent concentration, which removes the perinuclear membrane components from the nuclear fraction (1, 3). The results show that during the 15-min pulse and subsequent chase of 2 h, the wt PB2 was present in the cytoplasm and nucleus and that very little, if any, PB2 cofractionated with the nuclear membrane (Fig. 5B, upper panel). On the other hand, during this period, del 4 was not found in the nucleus and a significant fraction cofractionated with the nuclear membrane (Fig. 5B, lower panel). These biochemical data support the immunofluorescence observations that del 4 was present in the perinuclear region and bound to the nuclear membrane.

Intranuclear PB2 was not degraded in influenza virus-infected cells. Since the wt PB2, expressed from the cDNA, exhibited a high turnover rate (Fig. 5A), we wanted to determine if PB2 has a similar turnover rate in influenza virus-infected cells. Accordingly, WSN virus-infected cells were pulse-labeled for 15 min and chased for different times. Nuclear fractions were obtained as described for Fig. 5A and analyzed by immunoprecipitation and SDS-PAGE. The results (Fig. 6) show that PB2 was present in the nucleus within a 15-min pulse. But unlike the PB2 expressed from cDNA, the level of PB2 in the nuclei of virus-infected cells essentially remained the same during the 2-h chase period. These results therefore indicate that the turnover rate of PB2 in virus-infected cells was different from that observed in transfected cells. In the nuclei of virus-infected cells, PB2 remained stable ($t_{1/2} > 2$ h), whereas in the nuclei of transfected cells, PB2 was unstable with a high turnover rate ($t_{1/2}$, 30 to 40 min). In another expression system using a bovine papillomavirus vector and NIH 3T3 cells, influenza virus PB2 was also found to be unstable (2), suggesting that PB2 expressed alone may be unstable and that complex formation with other viral proteins (e.g., 3P complex) and/or viral ribonucleoproteins may be required for the structural stability of PB2.

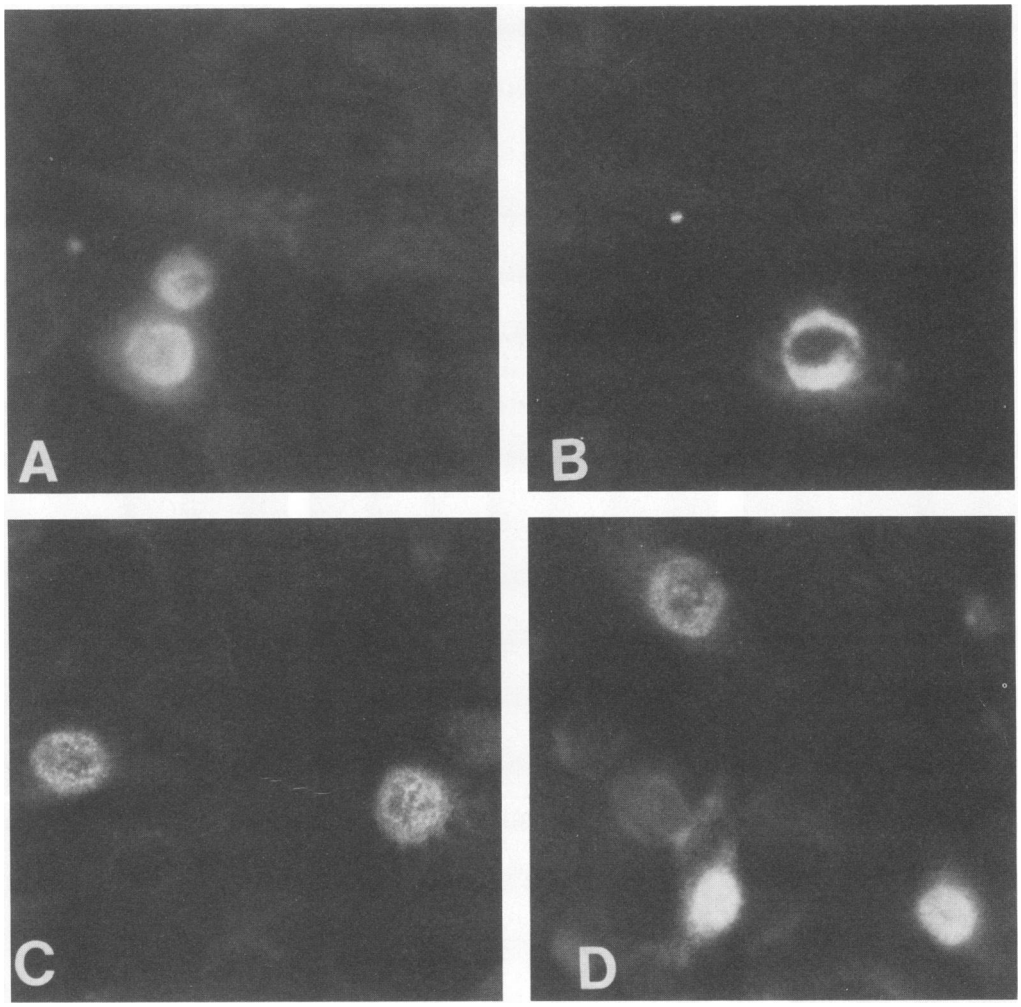

FIG. 4. Deletion analysis of the sequence required for binding to the perinuclear region. Small deletion mutants between aa 463 to 591 were used to determine the region required for binding to the perinuclear region. BHK cells were transfected with del 9 [Δ(403 to 448)] (A), del 10 [Δ(449-495)] (B), del 11 [Δ(496-542)] (C), and del 12 [Δ(543 -591)] (D) mutant DNA.

Subcellular fractionation data show that only a small fraction of the labeled PB2 cofractionated with the nucleus (Fig. 5), although most of the protein appeared to be present in the nucleus by immunofluorescence staining (Fig. 2A). This could be partly due to the fact that, unlike in the influenza virus-infected cells, the vaccinia virus-expressed PB2 was not present as a 3P-ribonucleoprotein complex and therefore was more prone to leach out from the nucleus into the cytoplasm during fractionation with detergent treatment. This could also explain the failure by another group to detect PB2 in the nuclei of PB2-expressing cells (2).

DISCUSSION

The data presented in this paper demonstrate that the function of two regions (aa 448 to 496 and aa 736 to 739) was required for the nuclear localization of influenza virus PB2. Deletion of either of these two regions abolished nuclear translocation of the protein. However, the phenotypes of these two mutant proteins were different. One, lacking a short stretch of basic amino acids (aa 736 to 739), exhibited a diffuse cytoplasmic distribution characteristic of a soluble cytoplasmic protein. However, the other, missing aa 448 to 496, exhibited a novel phenotype. Both the biochemical and immunofluorescence data showed that the mutant protein did not enter the nucleus but remained bound to the perinuclear membrane.

Although many nuclear proteins possess nuclear translocation signals consisting of a single stretch of contiguous basic amino acids, a number of nuclear proteins have recently been shown to possess a bipartite nuclear translocation signal consisting of two discontinuous regions separated by a spacer sequence (4, 9, 11, 20, 25, 31, 35). We have recently shown that the nuclear localization of influenza virus PB1 consisted of two discrete regions and that deletion of either one would result in cytoplasmic expression of the protein, whereas the removal of the intervening spacer sequence would not affect nuclear localization (27). Both nuclear localization signals of PB1 contained stretches of basic amino acid sequences. Unlike PB1, only one of the two PB2 signal regions contained a stretch of four basic residues (K-736 R K R), which upon deletion rendered the protein totally cytoplasmic. Furthermore, mutation of individual amino acids in this region showed that although each of the basic residues played an important role in nuclear localiza-

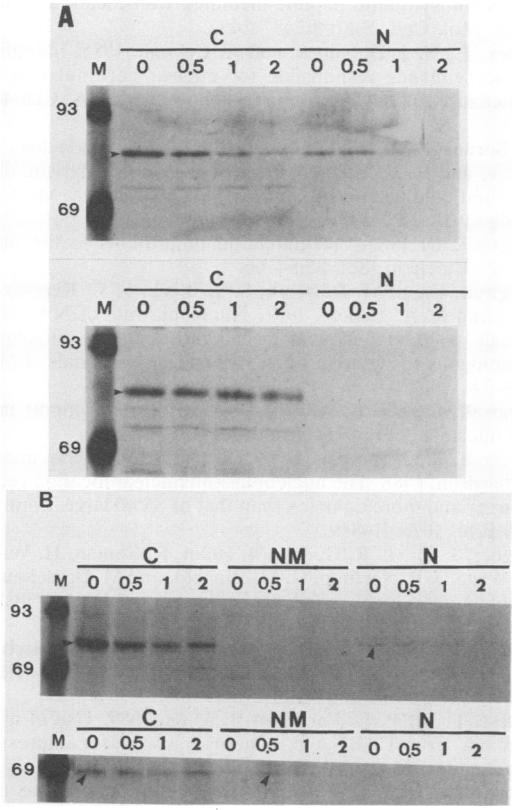

FIG. 5. Intracellular transport and processing of PB2 wt and mutant proteins. At 10 h posttransfection, BHK cells were labeled with [^{35}S]methionine (100 μCi/ml) and [^{35}S]cysteine (100 μCi/ml) for 15 min and chased in the presence of an excess of unlabeled methionine and cysteine for different time periods, as noted on the top of the gel (in hours). Cells were separated into cytoplasmic (C), nuclear (N) fractions and nuclear membrane (NM) fractions as described in Materials and Methods. Each fraction was immunoprecipitated with anti-PB2 antiserum. Panel A shows the wt PB2 (upper) and the del 8 mutant (lower). Panel B shows the wt (upper) and the del 4 mutant (lower). Precipitated proteins were analyzed on SDS-8% polyacrylamide gels containing 4 M urea. M, Molecular weight markers.

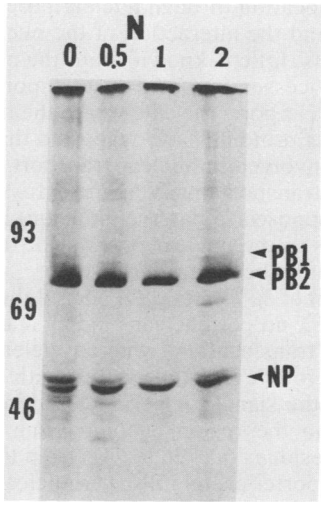

FIG. 6. Intracellular transport and processing of PB2 protein in the WSN virus-infected cells. BHK cells were pulse-labeled with [^{35}S]methionine (100 μCi/ml) and [^{35}S]cysteine (100 μCi/ml) for 15 min and chased in the presence of unlabeled methionine and cysteine for different time periods, as noted on the top of the gel (in hours). Nuclear (N) fractions were isolated as described in the legend to Fig. 5, adjusted to the detergent and salt concentrations of lysis buffer, immunoprecipitated with anti-PB2 antiserum, and analyzed by SDS-PAGE.

tion, residues R-737 and K-738 played the most critical roles. The conversion of either of these basic residues to nonbasic residues rendered the protein totally cytoplasmic (Fig. 3C and D).

This region of PB2 appears to be highly conserved among influenza A and B viruses. Six of the eight residues including the same four basic residues K-740 R K R were present in this region of influenza A and B viruses (7), suggesting that these residues might play an important role in nuclear transport in both influenza virus A and B PB2s. Furthermore, these four residues of PB2 would also fit the proposed consensus sequence, K R/K X R/K, predicted for the nuclear localization signal (5). However, in experiments reported here (M5, Fig. 1), the PB2 sequence was unable to translocate a heterologous cytoplasmic protein into the nucleus (Fig. 3F), indicating that other adjacent or distant sequences might also be required for nuclear localization. It should be noted that the proposed consensus sequence (K R/K X R/K) was predicted from experiments in which multiple synthetic peptides were randomly joined to a reporter protein and that it was not determined whether a single synthetic peptide was capable of nuclear translocation of a cytoplasmic protein (5).

The other region of PB2 (W-449 G I E S I D N V M G M I G I L P D M T P S T E M S M R G V R I S K M G V D E T S S A E K I V-495) involved in nuclear translocation neither contains stretches of basic amino acids nor exhibits any sequence homology to known nuclear signals. Finer deletion and mutation analyses of this region have to be done to define the functional requirement and boundaries of this domain. However, neither of these domains encompasses the sequence P-579 F Q S L, predicted by Jones et al. (16) to be the probable nuclear localization sequence of PB2 on the basis of its partial homology to A F E D L, the nuclear accumulation signal of influenza virus NP (6).

Deletion analyses reported in this paper exhibited two cytoplasmic phenotypes: (i) totally cytoplasmic or (ii) perinuclear. These results would suggest that perinuclear binding may be involved prior to the translocation of PB2 into the nucleus. However, since this phenotype was not observed with the wt protein, it is likely that the perinuclear binding was occurring only transiently just prior to nuclear translocation via nuclear pores and that the kinetics of the steps involved in nuclear transport subsequent to perinuclear binding was fast. Simian virus 40 VP1 exhibited a similar perinuclear phenotype, which became more pronounced in the absence of agnoprotein (19, 33, 38). However, the sequence requirement of simian virus 40 VP1 for its perinuclear binding remains to be determined. It should also be noted that like the PB2 basic residues, the nuclear localization signal of simian virus 40 VP1 could not translocate chicken serum albumin, a carrier protein, into the nucleus (5), indicating that additional sequences may be involved in nuclear translocation. These data would suggest that at least some nuclear proteins may undergo perinuclear binding prior to translocation into the nucleus through nuclear pores.

Although recent studies have elucidated the steps in-

volved in translocation through nuclear pores (8, 10, 13, 29, 30, 34–36, 39) and the interaction of the nuclear signal with the pore proteins, little is known about the posttranslational cytoplasmic processes involved in transporting these proteins to the nuclear pore, the gateway to the nucleus. Studies with herpesvirus proteins have suggested that the cytoskeleton might be involved in nuclear transport (21). However, whether active transport involving the cytoskeleton or other structures (as opposed to passive diffusion) is used in transporting proteins to the nuclear pores prior to nuclear translocation remains to be determined.

Since deletion of aa 736 to 739 rendered the protein totally cytoplasmic (i.e., no perinuclear binding and consequently, no intranuclear translocation), whereas deletion of aa 449 to 495 made the protein perinuclear (but with no intranuclear translocation), the signal for perinuclear binding was likely to be present in the region encompassing aa 736 to 739. However, six residues (aa 736 to 741) from this region failed to render the reporter protein PK, perinuclear (Fig. 3F, M5), suggesting that these residues may be necessary but not sufficient for perinuclear binding. However, an alternative explanation that the deletion (aa 449 to 495) may have altered the conformation of PB2 by partially masking the nuclear signal and thereby causing the protein to accumulate around the nucleus cannot be ruled out at present. A computer search did not reveal any obvious sequence homology within this region between influenza virus A and B PB2s. Further work is needed to determine whether there is a specific step involving perinuclear binding prior to the interaction with the nuclear pore and whether such a step requires a specific signal(s). This would involve making finer deletions and mutations and ultimately reconstructing the perinuclear binding signal with a reporter protein.

In conclusion, we have shown that influenza virus PB2, when expressed alone from cloned cDNA, is transported into the nucleus and that two discrete regions of the polypeptide are required for nuclear translocation. Deletion of one of the regions yields a novel phenotype protein with perinuclear distribution, suggesting the existence of a signal for binding the protein to the perinuclear region without translocating into the nucleus. Characterization of this signal should yield important information towards defining the steps in nuclear transport.

ACKNOWLEDGMENTS

This work was partially supported by Public Health Service grants R01 AI 12749 and R01 AI 16348 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Akkina, R. K., T. M. Chambers, D. R. Londo, and D. P. Nayak.** 1987. Intracellular localization of the viral polymerase proteins in cells infected with influenza virus and cells expressing PB1 protein from cloned cDNA. J. Virol. **61**:2217–2224.
2. **Braam-Markson, J., C. Jaudon, and R. M. Krug.** 1985. Expression of a functional influenza viral cap-recognizing protein using a bovine papilloma virus vector. Proc. Natl. Acad. Sci. USA **82**:4326–4330.
3. **Briedis, D. J., G. Conti, E. A. Munn, and B. W. J. Mahy.** 1981. Migration of influenza virus-specific polypeptides from cytoplasm to nucleus of infected cells. Virology **111**:154–164.
4. **Burglin, T. R., and E. M. De Robertis.** 1987. The nuclear migration signal of *Xenopus laevis* nucleoplasmin. EMBO J. **6**:2617–2625.
5. **Chelsky, D., R. Ralph, and G. Jonak.** 1989. Sequence requirements for synthetic peptide-mediated translocation to the nucleus. Mol. Cell. Biol. **9**:2487–2492.
6. **Davey, J., N. J. Dimmock, and A. Colman.** 1985. Identification of the sequence responsible for nuclear accumulation of the influenza virus nucleoprotein in xenopus oocytes. Cell **40**:667–675.
7. **De Borde, D. C., A. M. Donabedian, M. L. Herlocher, C. W. Naeve, and H. F. Massab.** 1988. Sequence comparison of wild-type and cold-adapted B/Ann Arbor/1/66 influenza virus genes. Virology **163**:429–443.
8. **Dingwall, C.** 1985. Accumulation of proteins in the nucleus. Trends Biochem. Sci. **85**:64–66.
9. **Dingwall, C., S. M. Dilworth, S. J. Black, S. E. Kearsey, L. S. Cox, and R. A. Laskey.** 1987. Nucleoplasmin cDNA sequence reveals polyglutamic acid tracts and a cluster of sequences homologous to putative nuclear localization signals. EMBO J. **6**:69–74.
10. **Dingwall, C., and R. A. Laskey.** 1986. Protein import into the cell nucleus. Annu. Rev. Cell Biol. **2**:367–390.
11. **Dingwall, C., J. Robbins, S. M. Dilworth, B. Roberts, and W. D. Richardson.** 1988. The nucleoplasmin nuclear location sequence is larger and more complex than that of SV40 large T antigen. J. Cell Biol. **107**:841–849.
12. **Flegner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northop, G. M. Ringold, and M. Danielsen.** 1987. Lipofection: a highly efficient, lipid mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA **84**:7413–7417.
13. **Franke, W. W., U. Scheer, G. Krohne, and E. D. Jarasch.** 1981. The nuclear envelope and the architecture of the nuclear periphery. J. Cell Biol. **91**(3,Pt 2):39s–50s.
14. **Fuerst, T. R., P. L. Earl, and B. Moss.** 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol. Cell. Biol. **7**:2538–2544.
15. **Greenspan, D., P. Palese, and M. Krystal.** 1988. Two nuclear location signals in the influenza virus NS1 nonstructural protein. J. Virol. **62**:3020–3026.
16. **Jones, I. M., P. A. Reay, and K. L. Philpott.** 1986. Nuclear localization of all three polymerase proteins and a nuclear signal in polymerase PB2. EMBO J. **5**:2371–2376.
17. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39**:499–509.
18. **Kaptein, J. S., and D. P. Nayak.** 1982. Complete nucleotide sequence of the polymerase 3 gene of human influenza virus A/WSN/33. J. Virol. **43**:55–63.
19. **Kasamatsu, H., and A. Nehorayan.** 1979. Nuclear localization of viral polypeptides during simian virus 40 infection. J. Virol. **32**:648–660.
20. **Kleinschmidt, J. A., and A. Seiter.** 1988. Identification of domains involved in nuclear uptake and histone binding of protein N1 of *Xenopus laevis*. EMBO J. **7**:1605–1614.
21. **Knipe, D., and J. L. Smith.** 1986. A mutant herpes virus protein leads to a block in nuclear localization of other viral proteins. Mol. Cell. Biol. **6**:2371–2381.
22. **Krug, R. M., F. V. Alonso-Caplen, I. Julkunen, and M. G. Katze.** 1989. Expression and replication of influenza virus genome, *In* R. M. Krug (ed.), Influenza viruses. Plenum Press, New York.
23. **Lamb, R. A., and C.-J. Lai.** 1984. Expression of unspliced NS1 mRNA, spliced NS_2 mRNA and a spliced chimera mRNA from cloned influenza virus NS DNA in an SV40 vector. Virology **135**:139–147.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Morin, N., C. Delsert, and D. F. Klessig.** 1989. Nuclear localization of adenovirus DNA binding protein: requirements of two signals and complementation during viral infection. Mol. Cell. Biol. **9**:4372–4380.
26. **Muirhead, H., D. A. Clayden, D. Barford, C. G. Lorimer, L. A. Forthergill-Gilmore, E. Shiltz, and W. Schmitt.** 1986. The structure of cat muscle pyruvate kinase. EMBO J. **5**:475–481.
27. **Nath, S. T., and D. P. Nayak.** 1990. Function of two discrete

regions is required for nuclear localization of polymerase basic protein 1 of A/WSN/33 influenza virus (H1 N1). Mol. Cell. Biol. **10**:4139–4145.

28. **Nayak, D. P., K. Tobita, J. M. Janda, A. R. Davis, and B. K. De.** 1978. Homologous interference mediated by defective interfering influenza virus derived from a temperature-sensitive mutant of influenza virus. J. Virol. **28**:375–386.
29. **Newmeyer, D. D., and D. J. Forbes.** 1988. Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. Cell **52**:641–653.
30. **Newmeyer, D. D., J. M. Lucocq, T. R. Bürglin, and E. M. De Robertis.** 1986. Assembly in vitro of nuclei active in nuclear protein transport: ATP is required for nucleoplasmin accumulation. EMBO J. **5**:501–510.
31. **Picard, D., and K. Yamamoto.** 1987. Two signals mediate hormone dependent nuclear localization of the glucocorticoid receptor. EMBO J. **6**:3333–3340.
32. **Ramsay, G., L. Stanton, M. Schwab, and J. M. Bishop.** 1986. Human proto-oncogene N-*myc* encodes nuclear proteins that bind DNA. Mol. Cell. Biol. **6**:4450–4457.
33. **Resnick, J., and T. Shenk.** 1986. Simian virus 40 agnoprotein facilitates normal nuclear location of the major capsid polypeptide and cell-to-cell spread of virus. J. Virol. **60**:1098–1106.
34. **Richardson, W. D., A. D. Mills, S. M. Dilworth, R. A. Laskey, and C. Dingwall.** 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell **52**:655–664.
35. **Richardson, W. D., B. L. Roberts, and A. E. Smith.** 1986. Nuclear location signals in polyoma virus large-T. Cell **44**:75–85.
36. **Smith, A. E., D. Kalderon, B. L. Roberts, W. H. Colledge, M. Edge, P. Gillet, A. Markham, E. Paucha, and W. D. Richardson.** 1985. The nuclear location signal. Proc. R. Soc. Lond. B Biol. Sci. **226**:43–58.
37. **Smith, G. L., J. Z. Levin, P. Palese, and B. Moss.** 1987. Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses. Virology **160**:336–345.
38. **Wychowski, C., D. Benichou, and M. Girard.** 1986. Domain of SV-40 capsid polypeptide VP1 that specifies migration into the cell nucleus. EMBO J. **5**:2569–2576.
39. **Young, J. F., U. Desselberger, P. Palese, B. Ferguson, A. R. Shatzman, and M. Rosenberg.** 1983. Efficient expression of influenza virus NS1 nonstructural proteins in *E. coli*. Proc. Natl. Acad. Sci. USA **80**:6105–6109.