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Nucleus-Targeting Domain of the Matrix Protein (M_1) of Influenza Virus

ZHIPING YE, DRUEN ROBINSON, AND ROBERT R. WAGNER*

Department of Microbiology and Cancer Center, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Received 7 September 1994/Accepted 14 December 1994

The matrix protein M_1 of influenza virus A/WSN/33 was shown by immunofluorescent staining to be transported into the nuclei of transfected cells without requiring other viral proteins. We postulated the existence of a potential signal sequence at amino acids 101 to 105 (RKLKR) that is required for nuclear localization of the M_1 protein. When CV1 cells were transfected with recombinant vectors expressing the entire M_1 protein (amino acids 1 to 252) or just the first 112 N-terminal amino acids, both the complete M_1 protein and the truncated M_1 protein were transported to the nucleus. In contrast, expression in CV1 cells of vectors coding for M_1 proteins with deletions from amino acids 77 to 202 or amino acids 1 to 134 resulted only in cytoplasmic immunofluorescent staining of these truncated M_1 proteins without protein being transported to the nucleus. Moreover, no nuclear membrane translocation occurred when CV1 cells were transfected with recombinant vectors expressing M_1 proteins with deletions of amino acids 101 to 105 or with substitution at amino acids 101 to 105 of SNLNS for RKLKR. Furthermore, a synthetic oligopeptide corresponding to M_1 protein amino acids 90 to 108 was also transported into isolated nuclei derived from CV1 cells, whereas oligopeptides corresponding to amino acid sequences 25 to 40, 67 to 81, and 135 to 164 were not transported into the isolated cell nuclei. These data suggest that the amino acid sequence $_{101}$RKLKR$_{105}$ is the nuclear localization signal of the M_1 protein.

All enveloped viruses with negative-sense RNA genomes appear to code for a matrix protein (M) which is membrane associated, binds to the genomic ribonucleoprotein (RNP) core and down-regulates viral transcription. Available evidence suggests that these matrix proteins are responsible for assembling the RNP core with that region of the infected cell membrane containing newly synthesized viral glycoproteins. There are many similarities among the matrix proteins of nonsegmented and segmented negative-strand viruses, to the extent that certain amino acids are conserved between matrix proteins of nonsegmented vesicular stomatitis virus and that of influenza virus (19). Of the eight segments of the influenza virus genome, segment 7 codes for the major matrix protein (1, 14), now designated M_1, consisting of 252 amino acids. A second matrix protein, designated M_2, is encoded in a separate reading frame of genome segment 7, migrates to the apical surface of polarized epithelial cells, and lines the inner surface of the virion membrane in proximity to the hemagglutinin protein trimer (12). The major known properties of the influenza virus M_1 protein are its binding to the cell membrane by means of stretches of hydrophobic amino acids (10) and down-regulation of viral transcription (23, 25), presumably secondary to binding of M_1 to RNP cores. Similar properties of RNP binding, down-regulation of viral transcription (4), and association with membrane bilayers (6, 17) had been assigned to the M protein of vesicular stomatitis virus.

After entry into the host cell, the incoming influenza virus RNP core is divested of its M_1 protein in the cytoplasm and is then transported to the nucleus, where viral transcription takes place (15). The newly transcribed mRNAs migrate back to the cytoplasm for translation. The M_1 protein is next transported into the nucleus, where it binds to the newly assembled progeny RNP cores for transport back to the cytoplasm (11, 13, 16, 18, 20).

The mechanism by which the matrix proteins of influenza virus, and perhaps matrix proteins of other viruses, are translocated from the cytoplasm to the nucleus and back to the cytoplasm is presumably similar to that for nuclear transport of endogenous cellular proteins (7). The experiments reported here were undertaken to determine the properties and the regions of the M_1 protein that promote its migration from the cytoplasm to the nucleus. The M_1 protein of influenza virus A/WSN/33 also has two RNA binding sites extending from amino acid 135 to 164 and 90 to 108 (21, 22); this latter RNA binding site also contains a sequence, Arg-Lys-Leu-Lys-Arg, extending from amino acid 101 to 105 similar to signal sequences for translocation across the nuclear membrane found in other peptides that are transported to the nucleus (5). On the basis of these fundamental premises, experiments were designed to determine which region of influenza virus M_1 protein is responsible for its transport from the cytoplasm to the nucleus.

In order to determine which region of the influenza virus A/WSN/33 M_1 protein is essential for its translocation into the nucleus, we constructed deletion and site-directed mutations in the M_1 gene of the vaccinia virus-based expression vector designated pTFM21(3) driven by the bacteriophage T7 polymerase (2). Current and previous studies provided some insight into potentially important regions of the M_1 protein that were candidates for mutations resulting in deficiencies in nuclear transport (21, 22). A computer search of the M_1 protein amino acid sequence revealed a potential pentapeptide (Arg-Lys-Leu-Lys-Arg) at amino acids 101 to 105 as a consensus candidate for a nuclear transport signal sequence (5).

We had previously constructed plasmid pTFM21(3) which contains the entire M_1 gene cDNA of influenza virus A/WSN/33 inserted into the *Bam*HI site of the vaccinia virus-

* Corresponding author. Mailing address: Department of Microbiology, School of Medicine, Box 441, University of Virginia, Charlottesville, VA 22908. Phone: (804) 924-1948 or (804) 924-5111. Fax: (804) 982-1071. Electronic mail address: rrw@virginia.edu.

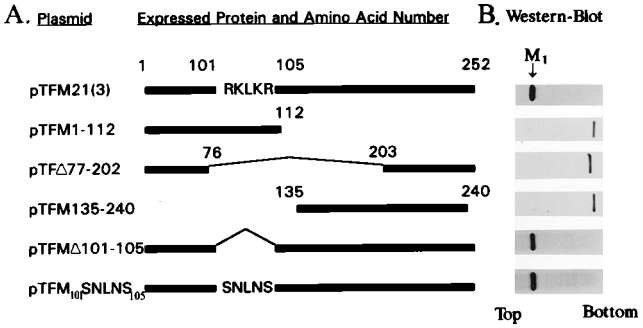


FIG. 1. Stick models of vaccinia virus-based expression vectors of the wt, deletion mutants, and a site-directed mutant of the M_1 gene of influenza virus A/WSN/33 (A) and their expressed products identified by SDS-PAGE and immunoblotting (B). As described previously (2), the plasmid carrying the entire coding region of the M_1 gene is designated pTFM21(3); also displayed are the wt amino acid sequences designating a putative nuclear localization signal sequence as well as deletions and substitutions at amino acids 101 to 105. To validate the capacity of these plasmids to express either the entire M_1 protein, truncated proteins, or the site-directed mutant, plasmids were transfected into CV1 cells after infection with T7 polymerase-expressing vaccinia virus recombinant vTF1-6,2. Transfected CV1 cells incubated for 16 h were extracted, subjected to SDS-PAGE, and electroblotted onto nitrocellulose membranes. Expressed wt and mutant M_1 proteins were identified by reactivity with a mixture of anti-M_1 MAbs prior to exposure to ^{125}I-labeled protein A and autoradiography. Gel migration of expressed proteins is shown in panel B from left (top) to right (bottom), and the arrow designated M_1 shows the migration position of the virion M_1 protein.

based plasmid pTF7-IHB-1 obtained from Bernard Moss (2, 9). This insert is flanked by the promoter and terminator sequences of the bacteriophage T7 RNA polymerase and is expressed by coinfection of cells with vaccinia virus recombinant vTF1-6,2 that expresses the phage T7 polymerase. The deletion mutants used in these experiments were constructed by PCR or by deleting segments of the M_1 gene in pTFM21(3) with restriction enzymes and reannealing with DNA ligases. The M_1 gene and its mutated constructs were expressed by transfecting CV1 cells coinfected with vTF1-6,2, as described previously (24). The expressed proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with a mixture of three monoclonal antibodies (MAbs) directed to the M_1 protein of influenza virus (22, 23).

Figure 1 depicts a stick model and Western immunoblot of the previously constructed plasmid pTFM21(3) coding for the entire M_1 protein (2). Also illustrated in Fig. 1A are stick models of C-terminal truncation of M_1 protein with pTFM1-112 (expression of amino acids 1 to 112) and N-terminal truncation with pTFM135-240 (expression of amino acids 135 to 240), a deletion of amino acids 77 to 202, a deletion of amino acids 101 to 105, and a complete protein with substitutions at amino acids 101, 102, 104, and 105.

Figure 1B shows the relative migration by SDS-PAGE and immunoblotting with a mixture of MAbs of wild-type (wt), truncated, and mutant M_1 proteins expressed by the various cDNA constructs. The truncated, rapidly migrating proteins corresponding to the first 112 amino acids and to amino acids 135 to 240 of the M_1 protein were well expressed by pTFM1-112 and pTFM135-240, respectively. The deletion mutant pTFMΔ77-202 expressing amino acids 1 to 76 and 203 to 252 of the M_1 protein gave rise to a protein of 126 amino acids that is devoid of the putative nuclear translocation signal, as is truncated protein M135-240. The deletion mutant pTFMΔ101-105, also missing the potential nuclear translocation signal at amino acids 101 to 105, and mutant vector $pTFM_{101}SNLNS_{105}$ with substitutions at amino acids 101 to 105 both gave rise to M_1 protein with SDS-PAGE migration similar to that of wt M_1 protein.

The availability of vectors expressing wt and mutant M_1 proteins allows us to pose this question: what region(s) of the M_1 protein, if any, is essential for its transport across the nuclear membrane? An attempt was made to answer this question by indirect fluorescence antibody staining with fluorescein isothiocyanate (FITC) for M_1 antigen present in the nuclei and/or cytoplasm of CV1 cells transfected with plasmids expressing wt or deletion mutant M_1 proteins. The vTF1-6,2-infected and plasmid-transfected cells were fixed in formaldehyde, permeabilized by exposure to 0.4% Triton X-100, used in reactions with pooled MAbs directed to various regions of the M_1 protein, and stained with FITC-labeled donkey anti-mouse immunoglobulin G (24).

Figure 2 shows representative fluorescence micrographs of CV1 cells 16 h after transfection with plasmids expressing the entire or truncated M_1 proteins. Cells transfected for 16 h with pTFM21(3) expressing the entire wt M_1 protein exhibited intense anti-M_1 antibody fluorescence in the nucleus and moderate, dispersed fluorescence in the cytoplasm (Fig. 2A). Cells transfected with pTFM21(3) for 3 or 6 h exhibited moderately intense fluorescence entirely confined to the nucleus (data not shown). These data indicate that newly synthesized wt M_1 protein rapidly migrates to the nucleus; these findings are consistent with those on transport of M_1 protein in cells infected with influenza virus (16, 18). Very similar nuclear and cytoplasmic distribution of truncated M_1 protein was found in CV1 cells 16 h after transfection with pTFM1-112 expressing the first 112 N-terminal amino acids of M_1 protein (Fig. 2B). Once again, the nucleus is intensely stained by anti-M_1 fluorescent antibody whereas more limited and dispersed fluorescence is found in the cytoplasm. The truncated M_1 protein expressed by pTFM1-112 retains the putative nucleus translocation signal sequence at amino acids 101 to 105 (Fig. 1).

Quite a different cellular distribution of M_1 protein was seen in CV1 cells 16 h after transfection with pTFMΔ77-202 (Fig. 2C) or pTFM135-240 (Fig. 2D). Clearly, CV1 cells transfected with these two deletion mutants exhibit intense cytoplasmic fluorescence and little if any nuclear fluorescence even after 16 h. A similar but less intense cytoplasmic localization of M_1 protein, but with no protein found in the nucleus, was found in CV1 cells 3 and 6 h after transfection with pTFMΔ77-202 or pTFM135-240 (data not shown). As depicted in Fig. 1, pTFMΔ77-202 expresses amino acids 1 to 76 and 203 to 252 of M_1 protein devoid of the putative nuclear transport signal sequence. Similarly, the deletion mutant pTFM135-240 expresses M_1 protein truncated to amino acid sequence 135 to 240 devoid of the putative nuclear transport signal sequence.

These experiments with wt and deletion mutant expression vectors of M_1 protein answer at least partially the questions about translocation of M_1 protein across the nuclear membrane of CV1 cells. First, wt M_1 protein synthesized in the cytoplasm is transported to the nucleus and, subsequently, back to the cytoplasm independently of any other influenza virus proteins. Second, studies with deletion mutants expressing truncated M_1 proteins indicate that the region from amino acid 76 to 112 appears to provide the signal for translocation of M_1 protein across the nuclear membrane.

The only reasonable lead available for identification of a nuclear transport signal in M_1 protein between amino acids 76 and 112 is the sequence Arg-Lys-Leu-Lys-Arg extending from residue 101 to 105 (Fig. 1). This sequence resembles certain nuclear transport consensus sequences collected by Chelsky et al. (5). A similar sequence of basic amino acids ($_{101}$Arg-Lys-

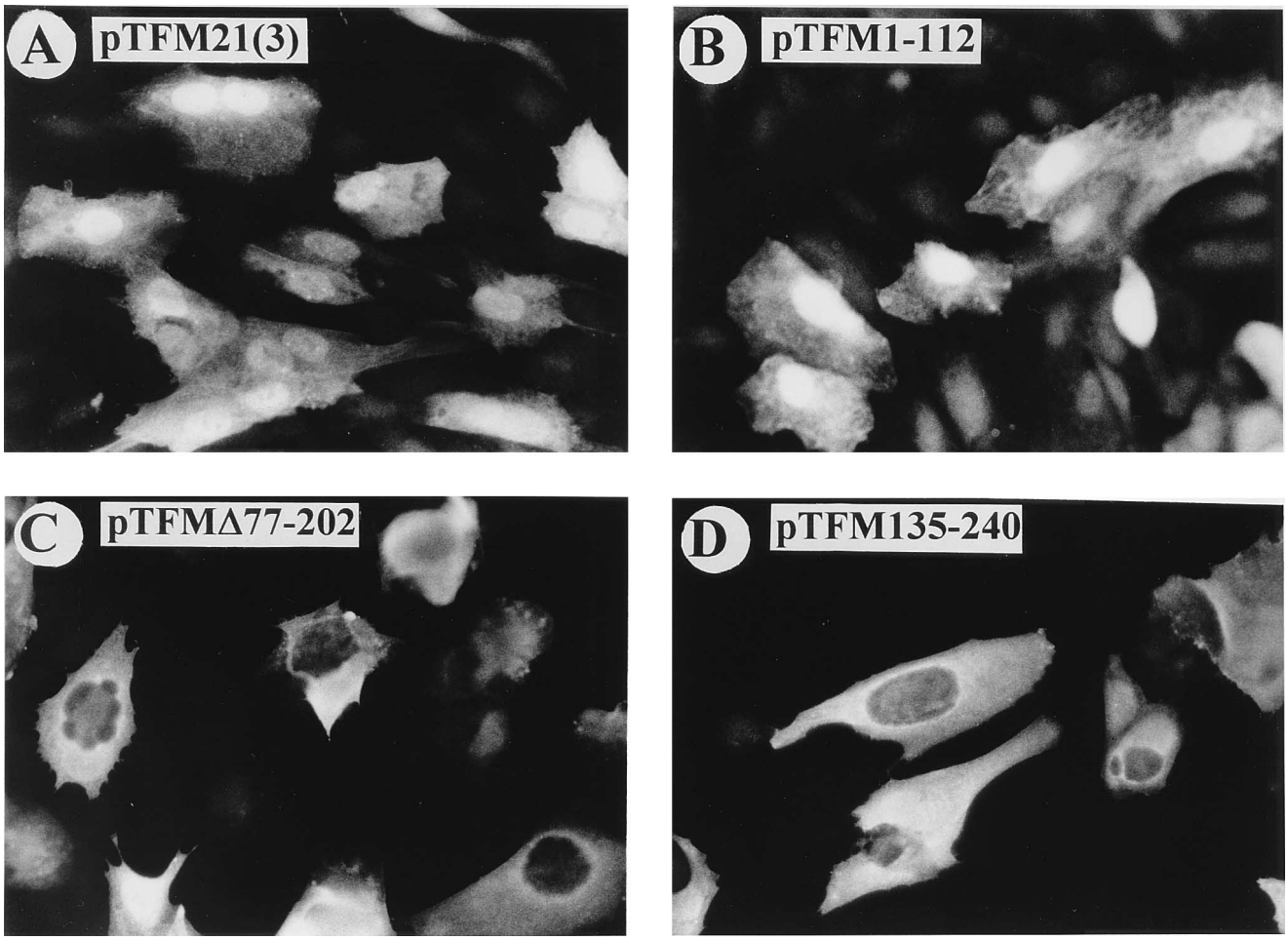


FIG. 2. Indirect immunofluorescence to determine the distribution of complete or truncated influenza virus M_1 proteins in the cytoplasm or nucleus of CV1 cells transfected with pTFM21(3) expressing the entire M_1 protein (A), pTFM1-112 expressing the first 112 N-terminal M_1 amino acids (B), pTFMΔ77-202 expressing M_1 amino acids 1 to 76 and 203 to 252 (C), and pTFM135-240 expressing M_1 amino acids 135 to 240 (D). As described previously (24), CV1 cells first infected with T7 polymerase-expressing vTF1-6,2 (multiplicity of infection, ~7.5) were transfected with M_1 gene recombinant plasmids assisted by Lipofectin and were incubated for 16 h at 37°C. Cells fixed with formaldehyde and permeabilized with 0.4% Triton X-100 were exposed to a mixture of anti-M_1 MAbs and then were stained with FITC-conjugated donkey anti-mouse immunoglobulin G. Cells on coverslips were examined by transmission microscopy under an epifluorescent UV light source, and representative cells were photographed at a magnification of ×627.

Met-Arg-Arg_{105}) is located at the same region of the M_1 protein of influenza B (Lee) virus (3). To determine whether the region from amino acid 101 to 105 of the influenza A virus M_1 protein is responsible for its nuclear translocation, we used the deletion mutant expression vector pTFMΔ101-105 (deletion of amino acids 101 to 105) and the site-specific mutant vector $pTFM_{101}SNLNS_{105}$ (substituting serines and asparagines for arginines and lysines at amino acids 101 to 105) as shown in Fig. 1. CV1 cells transfected with pTFMΔ101-105, $pTFM_{101}SNLNS_{105}$, and wt M_1 recombinant vector pTFM21(3) were examined by phase microscopy and by immunofluorescence microscopy for the presence of M_1 protein in the nucleus and cytoplasm.

Figure 3 shows phase and immunofluorescent photomicrographs of representative fields of CV1 cells 16 h after transfection with recombinant plasmids expressing wt M_1 protein compared with fields for mutant M_1 proteins with deletion or substitution at amino acids 101 to 105. Comparison of the phase (Fig. 3A, C, and E) and fluorescent (Fig. 3B, D, and F) micrographs reveals high rates of successful transfection and expression of all three M_1 proteins. CV1 cells transfected with pTFM21(3) exhibited large concentrations of wt M_1 protein present in nuclei of cells exposed to pooled MAbs and stained with FITC-labeled donkey anti-mouse immunoglobulin G (Fig. 3B). In contrast, only the cytoplasm of CV1 cells contained immunofluorescently labeled M_1 proteins 16 h after transfection with pTFMΔ101-105 expressing M_1 protein deleted of amino acids 101 to 105 (Fig. 3D). Similarly, only cytoplasmic localization of M_1 protein was found in CV1 cells transfected with $pTFM_{101}SNLNS_{105}$ expressing M_1 protein with serines substituted at amino acids 101 and 105 and asparagines substituted at amino acids 102 and 104 (Fig. 3F). These data strongly suggest that the four basic amino acids located at amino acids 101 to 105 are required for translocation of M_1 protein across the nuclear membrane of CV1 cells.

Seeking confirmation that the sequence $_{101}RKLKR_{105}$ is responsible for nuclear membrane transport of influenza virus M_1 protein, we tested some previously prepared synthetic oligopeptides corresponding to M_1 protein sequences (22) for their uptake by isolated nuclei. For this purpose, we adapted the technique of Dean and Kasamatsu (8) who successfully imported into isolated nuclei a synthetic peptide analog of the

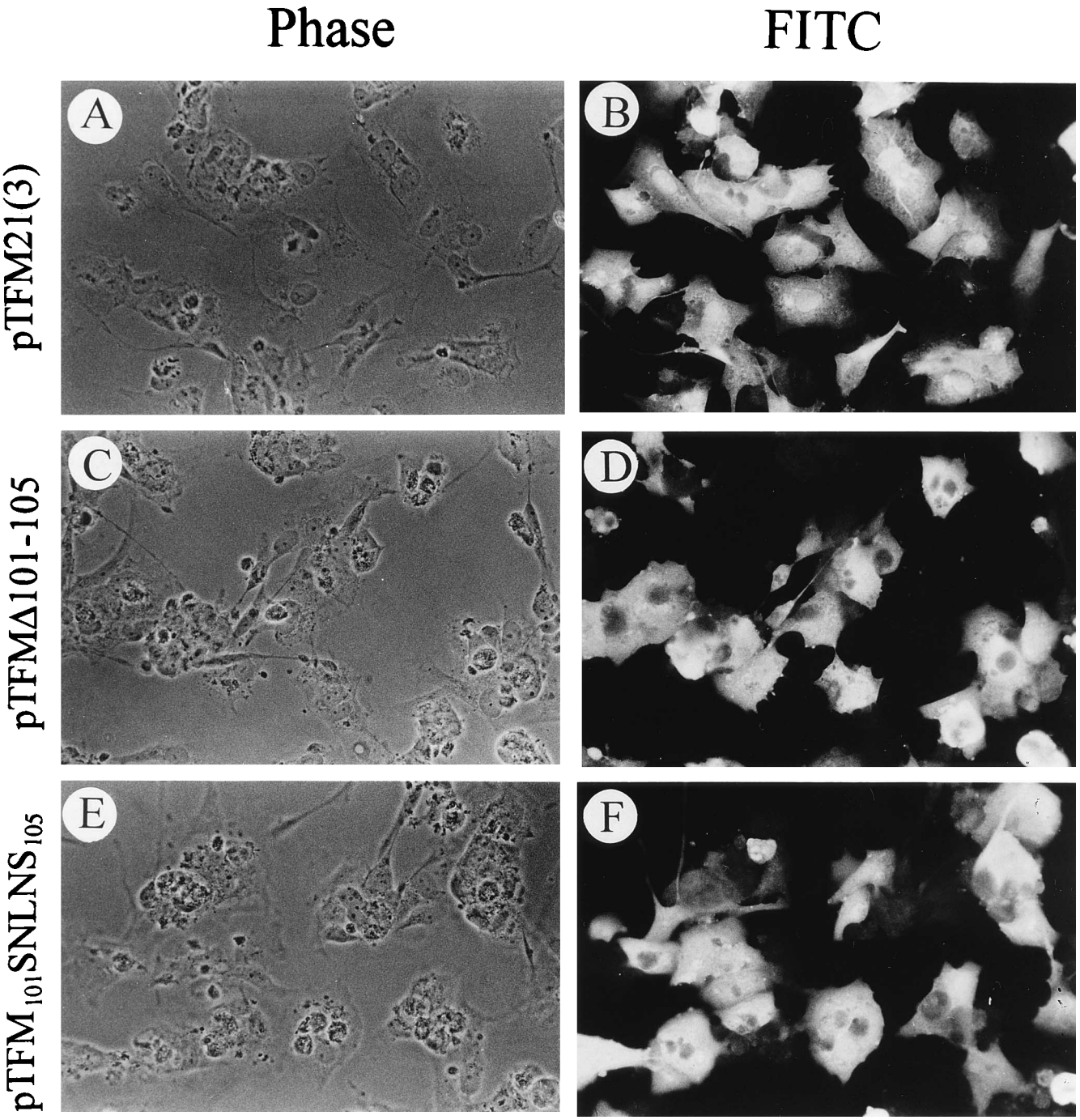
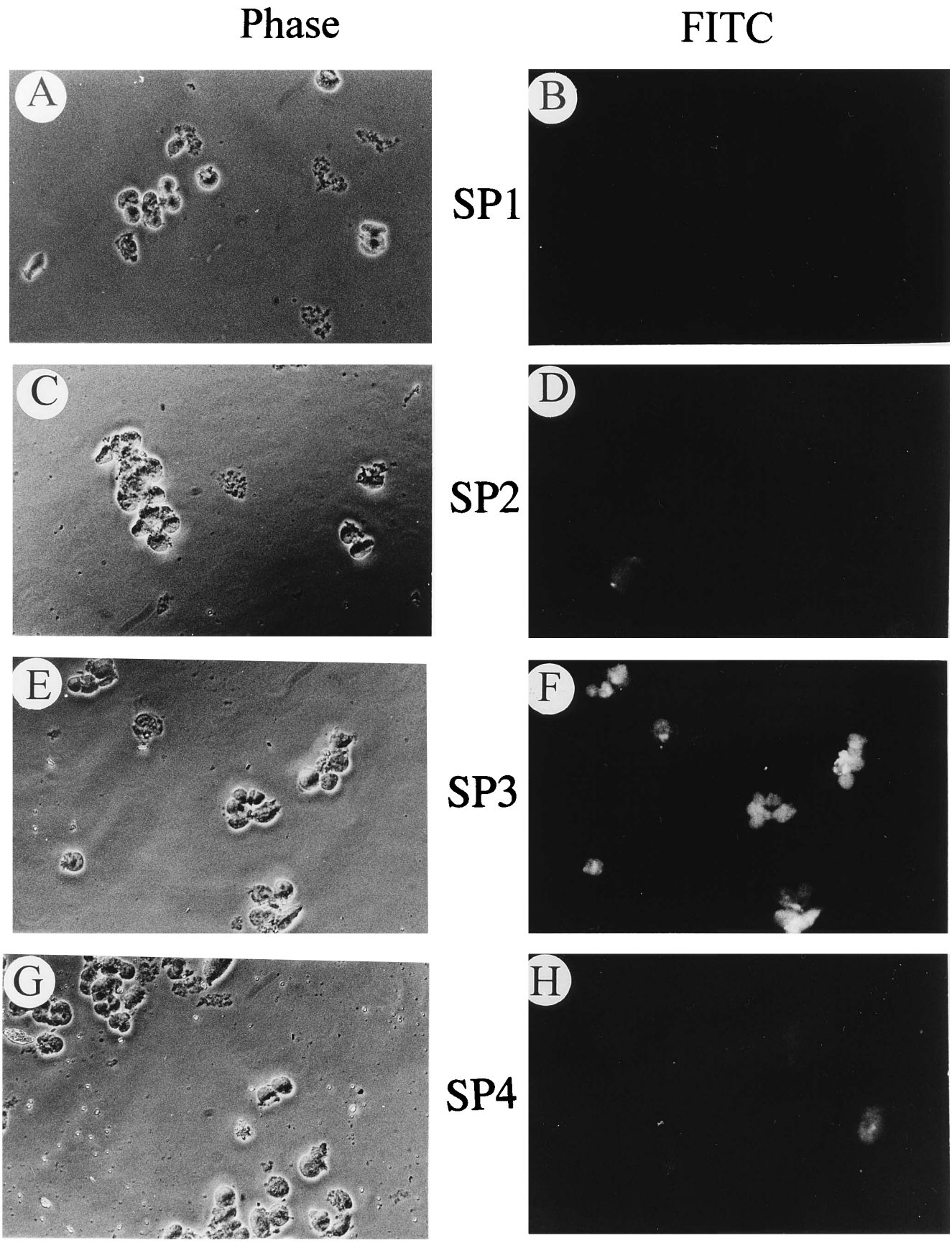


FIG. 3. Phase and indirect immunofluorescence microscopy of CV1 cells transfected with plasmid pTFM21(3) expressing the entire M_1 protein of influenza A virus (panels A and B), pTFMΔ101-105 expressing M_1 protein deleted of amino acids 101 to 105 (panels C and D), and $pTFM_{101}SNLNS_{105}$ expressing M_1 protein with substitutions at amino acids 101, 102, 104, and 105 (panels E and F). As described in the legend for Fig. 2, vTF1-6,2-infected CV1 cells were transfected with each plasmid assisted by Lipofectin and were incubated for 16 h at 37°C. Fixed and permeabilized cells were then exposed to a mixture of anti-M_1 MAbs and were stained with FITC-conjugated donkey anti-mouse immunoglobulin G. Each representative field was examined by both phase microscopy (panels A, C, and E) and fluorescence microscopy (panels B, D, and F) for comparison of individual cells. The cells were photomicrographed at a magnification of ×275.

simian virus 40 Vp3 protein with a nucleus localization signal sequence (PPGPNKKKRKL).

Synthetic peptides (SP) with sequences corresponding to M_1 protein amino acids, SP1 ($_{25}$AQRLEDVFAGKNTDLF$_{40}$), SP2 ($_{67}$TVPSERGLQRRRFVQ$_{81}$), SP3 ($_{90}$PNNMDKAVKLYRKLKREIT$_{108}$), and SP4 ($_{135}$MGAVTTEVAFGLVCATCEQIADSQHRSHRQ$_{164}$) were previously prepared by the University of Virginia Biomedical Support Facility (22). These oligopeptides were labeled with FITC by adding 50 μg of FITC (in 0.25 M bicarbonate solution, pH 8.2) to 1 mg of each oligopeptide (in 0.5 M bicarbonate solution, pH 9.0) for 30 min with continuous stirring and were incubated overnight at room


Phase
FITC
A
B
SP1
C
D
SP2
E
F
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G
H
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FIG. 4. Phase and fluorescence microscopy of isolated nuclei incubated with FITC-labeled synthetic peptides (SP) corresponding to M_1 protein amino acid sequences $_{25}$AQRLEDVFAGKNTDLE$_{40}$ (SP1; panels A and B), $_{67}$TVPSERGLQRRRFVQ$_{81}$ (SP2; panels C and D), $_{90}$PNNMDKAVKLYRKLKREIT$_{108}$ (SP3; panels E and F), and $_{135}$MGAVTTEVAFGLVCATCEQIADSQHRSHRQ$_{164}$ (SP4; panels G and H). As described in the text, nuclei isolated from 2.5×10^7 CV1 cells (8) were suspended in sucrose-TKMC buffer and were incubated with 25 mg of FITC-labeled synthetic peptides per ml before being fixed with formaldehyde. Each representative field was examined both by phase microscopy (panels A, C, E, and G) and by fluorescence microscopy (panels B, D, F, and H). The cells were microphotographed at a magnification of ×275.

temperature. The free dye was removed by filtration on a Sephadex G-25 column. Nuclei were isolated by the method of Dean and Kasamatsu (8). Briefly, CV1 cells removed from subconfluent monolayers were disrupted in 3 ml of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–dithiothreitol buffer with 20 strokes of a Dounce homogenizer. The suspension was adjusted to 1.25 M sucrose in TKMC buffer (50 mM Tris [pH 7.6], 25 mM KCl, 2.5 mM $MgCl_2$, 15 mM $CaCl_2$, 10 mg of aprotinin per ml) and was incubated for 15 min on ice. The sucrose concentration was adjusted to 1.6 M. The nuclei were harvested by centrifugation onto a 2.3 M sucrose-TKMC cushion and resuspended in TKMC in a final volume of 200 μl (2.5×10^7 nuclei per ml). Cytoplasmic extract was also prepared from 10^8 disrupted CV1 cells and concentrated fivefold by Amicon filtration. Peptide uptake assays were carried out by the addition of nuclei to TKMC buffer containing 5 mM ATP, cytoplasmic extract derived from 5×10^6 cells, and 2 mg of bovine serum albumin per ml to a final concentration of 10^7 nuclei per ml. After equilibration to room temperature for 5 min, 25 mg of FITC-labeled peptides per ml was added to the mixture in a final volume of 20 μl before incubation at room temperature. After 15 min, 5 μl of the mixture was removed and the nuclei were fixed with 0.5 ml of 3% formaldehyde. The localization of FITC-labeled peptides in isolated nuclei was determined by direct fluorescence microscopy; comparisons were made by phase microscopy of nuclei in the same optical field.

Figure 4 shows side-by-side representative phase and fluorescent photomicrographs of isolated CV1 cell nuclei after incubation with the four FITC-labeled synthetic peptides (SP1, SP2, SP3, and SP4) corresponding to amino acid sequences of M_1 protein. As noted from the phase micrographs, each field contained ample numbers of nuclei, which tended to aggregate (Fig. 4A, C, E, and G). Fluorescent microscopic examination of the same fields exposed to FITC-labeled peptides SP1, SP2, and SP4 revealed the complete absence of nuclear fluorescence (Fig. 4B, D, and H). In comparison, isolated nuclei exposed to FITC-labeled SP3 exhibited considerable fluorescence (Fig. 4F), involving most of the nuclei identifiable by phase microscopy in the same field (Fig. 4E).

These results indicate selective transport into isolated nuclei of synthetic peptide SP3 corresponding to amino acid sequence 90 to 108 of influenza virus M_1 protein, whereas synthetic peptides corresponding to three other M_1 protein sequences were not transported into isolated nuclei. The presence in synthetic peptide SP3 of the putative nuclear translocation signal sequence at amino acids 101 to 105 supports the hypothesis that this region of the M_1 protein is responsible, at least in part, for its transport from the cytoplasm to the nucleus of transfected cells.

In this study, we present evidence for a potential signal sequence ($_{101}$RKLKR$_{105}$) responsible for transport of the influenza virus M_1 protein from the cytoplasm to the nucleus. We are well aware that this sequence is not identical to those of endogenous nuclear transport proteins previously described (5), but the general grouping of positively charged amino acids fits with the scheme for a nuclear targeting sequence. An energy-dependent nuclear transport signal sequence (KKKRKL) recently described for the simian virus 40 Vp3 protein (8) is quite similar to our putative influenza virus M_1 protein nuclear localization sequence. With this lead in mind, we used an M_1 gene vaccinia virus-based, T7 polymerase-driven expression vector previously constructed in our laboratory (2), from which we produced deletion mutants. The deletion mutant pTFM135-240, which expresses M_1 protein truncated to amino acids 135 to 240, was incapable of being transported to the nucleus despite marked cytoplasmic expression. In sharp contrast, the deletion mutant pTFM1-112, which expresses a truncated M_1 protein limited to the first 112 N-terminal amino acids, rapidly and efficiently migrated to the nucleus; this M_1 truncated protein retains the putative nuclear translocation signal sequence at amino acids 101 to 105. In support of the concept that this amino acid sequence is essential for transport of M_1 protein across the nuclear membrane was the finding that deletion mutant pTFMΔ77-202, devoid of amino acids 101 to 105, was only minimally transported to the nucleus despite ample expression in the cytoplasm of CV1 cells.

Our hypothesis that a nuclear transport signal sequence in the region of amino acids 101 to 105 directs the M_1 protein through nuclear membrane pores required additional proof. When CV1 cells were transfected with recombinant vectors expressing the M_1 proteins deleted of only amino acids 101 to 105 or with SNLNS substituted for RKLKR, nuclear transport activity of the M_1 protein was essentially eliminated. Moreover, a synthetic oligopeptide corresponding to M_1 protein amino acids 90 to 108, which contains the RKLKR sequence of M_1 protein, was also readily transported into isolated nuclei obtained from CV1 cells. In contrast, oligopeptides corresponding to M_1 protein amino acids 25 to 40, 67 to 81, and 135 to 164 could not be transported into isolated nuclei. These data strongly suggest that amino acid sequence RKLKR from amino acids 101 to 105 is the nuclear localization signal of the M_1 protein. It is of interest that the nuclear localization signal sequence is located within one of the previously reported (22) RNA-binding regions (amino acids 90 to 108). Perhaps this sequence is inactivated as a nuclear transport signal when it binds RNA, thereby conceivably allowing transport out of the nucleus to the cytoplasm. The presence of a nuclear translocation sequence capable of transporting influenza virus M_1 protein into the nucleus is presumably essential for its assembly with RNP cores in the nucleus and their subsequent migration into the cytoplasm of infected cells.

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