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Hyperphosphorylation of Mutant Influenza Virus Matrix Protein, M1, Causes Its Retention in the Nucleus

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The matrix (M1) protein of influenza virus is a major structural component, involved in regulation of viral ribonucleoprotein transport into and out of the nucleus. Early in infection, M1 is distributed in the nucleus, whereas later, it is localized predominantly in the cytoplasm. Using immunofluorescence microscopy and the influenza virus mutant *ts*51, we found that at the nonpermissive temperature M1 was retained in the nucleus, even at late times after infection. In contrast, the viral nucleoprotein (NP), after a temporary retention in the nucleus, was distributed in the cytoplasm. Therefore, mutant M1 supported the release of the viral ribonucleoproteins from the nucleus, but not the formation of infectious virions. The point mutation in the *ts*51 M1 gene was predicted to encode an additional phosphorylation site. We observed a substantial increase in the incorporation of $^{32}P_i$ into M1 at the nonpermissive temperature. The critical role of this phosphorylation site was demonstrated by using H89, a protein kinase inhibitor; it inhibited the expression of the mutant phenotype, as judged by M1 distribution in the cell. Immunofluorescence analysis of *ts*51-infected cells after treatment with H89 showed a wild-type phenotype. In summary, the data indicated that the *ts*51 M1 protein was hyperphosphorylated at the nonpermissive temperature and that this phosphorylation was responsible for its aberrant nuclear retention.

Influenza virus is a negative-sense RNA virus which replicates in the cell nucleus. Infection begins with the virus binding to the plasma membrane, followed by receptor-mediated endocytosis and a low-pH-dependent fusion step in the late endosome (20, 37). This releases the viral ribonucleoproteins (vRNPs), together with the matrix protein (M1), into the cytoplasm. We have previously shown that the dissociation of M1 from the vRNPs allows subsequent import of the vRNPs into the nucleus (11, 18).

The first viral proteins to be synthesized are the polypeptides of the polymerase complex (PA, PB1, and PB2), the nonstructural protein NS-1, and the nucleoprotein (NP), with synthesis commencing within 1 to 2 h of infection (17). These proteins are all imported into the nucleus shortly after their synthesis. Later in infection, the remainder of the virus proteins, hemagglutinin (HA) and neuraminidase (NA), the nonstructural protein NS-2, and the M1 and M2 proteins, are synthesized. HA, NA, and M2 are synthesized at the endoplasmic reticulum and transported to the plasma membrane, where they are available for virus budding. The M1 protein is distributed to the nucleus shortly after its synthesis (2, 24).

The newly synthesized NP associates with the negative-sense viral RNA in the nucleus, thereby forming the vRNPs, which remain in the nucleus until the onset of M1 synthesis. After its import into the nucleus, M1 protein acts in an, as yet, unknown manner to promote export of the vRNPs from the nucleus to the cytoplasm (18). The vRNPs are further transported to the plasma membrane, where additional M1 molecules are involved in the budding and release of virus particles (24). M1 colocalizes with the vRNP in the nucleus, in the cytoplasm, and at the plasma membrane (18, 19, 24).

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The M1 protein, therefore, plays a pivotal role in influenza virus assembly and budding. It has both nucleic acid- and membrane-binding properties (9, 35, 39, 40). In the virus particle, where it is the most abundant protein, it is thought to act as a bridge between the viral envelope and the capsid. In addition, it interacts with the NS-2 protein in the virus particle (38). With a molecular mass of 27 kDa, it is small enough to diffuse through the nuclear pore and gain access to the nuclear matrix. Most nuclear proteins are, however, imported into the nucleus by an active import process. Active import is brought about by the interaction of a basic nuclear localization signal (NLS) on the protein with cytosolic receptor molecules (5). Because of its basic nature, it is likely that the M1 protein contains an NLS, but specific NLSs have not yet been identified.

M1 occurs in two forms which differ slightly in apparent molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In the WSN strain used in our studies, both species are phosphorylated on serine and threonine residues (6–8). Other phosphorylated proteins of influenza virus are NP, NS-1, and NS-2 (1, 25, 27, 28, 30). NP phosphorylation has been studied extensively. The sites and overall levels of phosphorylation vary depending on the virus strain, the cell line in which the virus grows, and the time after infection (15, 16). M1 phosphorylation is, however, weak in comparison to that of NP and, to date, has been observed only in the WSN strain (8, 16).

Our previous studies have shown that M1 entry into the nucleus is required for vRNP export. To explain this phenomenon, we previously proposed a model in which vRNPs released from the nucleus are exported into the cytosol, complexed with the M1 protein (18). This model has been questioned by Rey and Nayak (29), who showed that in a temperature-sensitive mutant (*ts*51), the export of vRNPs out of the nucleus occurred normally whilst M1 was retained in the nucleus. A contradictory report from Enami et al. (4), however, showed nuclear retention of both NP and viral RNA (vRNA) for the *ts*51 mutant.

To resolve these contradictory results and to investigate the role of M1 in the nuclear transport of vRNPs, we analyzed the *ts*51 mutant in further detail. We found that its nuclear retention was caused by hyperphosphorylation of M1. Most likely this was induced by a single-amino-acid change in M1, which creates a new protein kinase recognition site. Unlike what occurs with wild-type virus, both NP and M1 were retained in the nucleus at intermediate time points. Late in infection, however, the vRNPs were exported whereas M1 was still retained. The hyperphosphorylated, mutant M1 was thus capable of inducing efficient, albeit somewhat delayed, vRNP export out of the nucleus but was not available for the budding of infectious virions at the plasma membrane.

MATERIALS AND METHODS

Cells and virus. MDBK and MDCK cells were grown as described previously (19). L929 cells (from the laboratory of Sandra Wolin, Yale University) were passaged twice weekly and grown in α-minimal essential medium (αMEM) containing 10% fetal calf serum; 1% glutamine; 100 U of penicillin per ml; 10 μg of streptomycin per ml; 0.8 g of sodium bicarbonate per liter; 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.3; and nonessential amino acids.

Influenza A virus (strain WSN) was a gift from Robert Krug (Rutgers University), and the temperature-sensitive mutant *ts*51 (32, 33) was a gift from Peter Palese (Mount Sinai Hospital, New York, N.Y.). *ts*51 was plaque purified three times on MDBK cells at 32°C prior to use. Stocks of virus were prepared as described previously (19) and grown at 37°C (WSN) or 32°C (*ts*51) in MDBK or MDCK cells.

For virus infections, cells were washed with RPMI 1640 medium containing 20 mM HEPES and 0.2% bovine serum albumin (BSA), pH 6.8. Virus was adsorbed in this medium for 45 to 60 min at 37°C or for 90 min at 4°C. The virus inoculum was then removed, and the cells were washed with αMEM containing 2% fetal calf serum before being incubated at various temperatures. Experiments with the mutant virus were carried out at 32°C in a 5% CO_2 incubator or by using water baths maintained at 32 or 39.5°C. When incubations were carried out in water baths, 20 mM HEPES, pH 7.4, substituted for sodium bicarbonate as a buffer in cell culture medium.

Plaque titers of viruses were determined on confluent monolayers of MDBK or MDCK cells. Virus was diluted in RPMI medium and adsorbed onto cells at 37°C for 60 min. Cells were washed and overlaid with Dulbecco's MEM containing 0.2% BSA, 2 μg of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma) per ml, and 1% SeaPlaque agarose (FMC BioProducts) and incubated at 37°C for 24 to 48 h in a 5% CO_2 incubator. Monolayers were fixed with formalin and stained with crystal violet.

Pulse labeling of cells with [^{35}S]methionine. L929 or MDBK cells were grown for 2 days to approximately 90% confluency; infected with 1 PFU of influenza virus (WSN or *ts*51) per cell at 37°C for 60 min in RPMI, pH 6.8; and incubated in αMEM containing 2% fetal calf serum. Cells were transferred to methionine- and cysteine-free αMEM (without serum) for 15 min prior to labeling. Cells were then labeled with 100 μCi of ^{35}S Trans label (Amersham) in the same medium for 15 min. The cell monolayer was lysed with 0.05% Triton X-100–0.1% SDS–0.3 M NaCl–10 mM Tris-HCl, pH 8.6, containing 10 mM each the protease inhibitors chymostatin, leupeptin, antipain, and pepstatin. Lysates were stored at −20°C and sonicated for 30 s, with a probe sonicator, before analysis.

Labeling of infected cells with $^{32}P_i$. For P_i labeling, MDBK cells were utilized, as these gave good incorporation of ^{32}P label. Cells were grown for 2 days to approximately 90% confluency, transferred to phosphate-free MEM containing 2% fetal calf serum, and maintained at 37°C overnight. Cells were then infected with 1 PFU of influenza virus (WSN or *ts*51) per cell at 37°C for 60 min in serum-free, phosphate-free MEM. Cells were washed and incubated in a water bath at 32 or 39.5°C in phosphate-free MEM containing 2% serum. Cells were labeled at various times with serum-free, phosphate-free MEM containing 0.5 to 1 mCi of $^{32}P_i$ (Amersham) per ml or 50 to 100 μCi of ^{35}S Trans label per ml. At the end of the labeling period, cells were washed twice in phosphate-buffered saline (PBS) and lysed as for ^{35}S pulse labeling, except for the addition of the following phosphatase inhibitors: 10 mM sodium fluoride, 25 mM sodium glycerophosphate, and 50 μM sodium vanadate. Lysates were stored at −20°C and sonicated for 30 s, with a probe sonicator, before analysis. Cells labeled with ^{35}S Trans label were treated in a manner identical to that used for those labeled with $^{32}P_i$, except for the substitution of label.

Immunoprecipitation. Immunoprecipitation was carried out with a polyclonal antibody, IBO, raised against purified influenza virus WSN, which recognizes NP, M1, and HA (18). A 50-μl volume of a 10% suspension of protein A-Sepharose was preincubated with 10 μl of antibody at 4°C for 30 min in MNT buffer [30 mM Tris, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 150 mM NaCl, pH 7.5] containing 0.1% Triton X-100. The beads were washed twice with MNT-Triton, solubilized cell lysate was added, and the mixture was incubated at 4°C for 1 to 2 h. Beads were then washed three times with immunoprecipitation wash buffer (0.05% Triton X-100, 0.1% SDS, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.6). Samples were heated to 95°C for 3 min, and the supernatant was loaded onto an SDS-PAGE gel containing 10 or 12.5% acrylamide. Gels were stained, dried, and subjected to autoradiography at −70°C by using Kodak X-ray film and intensifying screens.

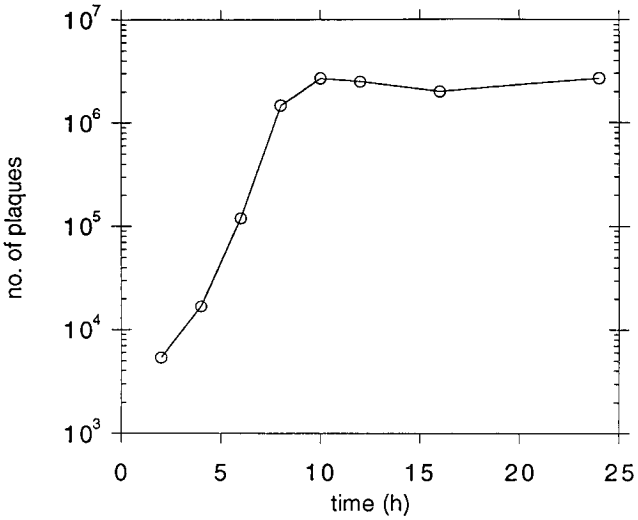

FIG. 1. Growth of influenza virus (WSN) in mouse L929 cells. L929 cells (10^6) were infected with 1 PFU of WSN per cell, and supernatants were harvested at various times. Supernatants were centrifuged at 500 × *g* for 5 min, and plaque titers of this supernatant were determined by using MDBK cells. Each datum point represents the mean for two assays.

Immunofluorescence. Immunofluorescence analysis was carried out with L929 cells, as these gave very clear patterns of M1 and NP distribution. The replication cycle of WSN in L929 cells was, however, slightly faster than in other cell lines typically used for influenza virus infection. Some of the time points utilized to study virus proteins during immunofluorescence experiments with L929 cells were therefore earlier than labeling studies with MDBK cells would predict. Some strains of influenza virus have been shown to give an abortive infection in L cells (13), so, to confirm that L929 cells were permissive for influenza virus (WSN) replication, we performed a single-cycle growth assay of virus production (Fig. 1). The curve shows a typical lag phase, followed by maximal yields of infectious virus at approximately 12 h and a subsequent plateau phase. This confirms that mouse L929 cells are permissive for influenza virus (WSN) growth.

For immunofluorescence studies, cells were grown to approximately 50% confluency on 12-mm-diameter, number 1 coverslips for 2 days prior to infection and then infected with 1 PFU of influenza virus WSN or *ts*51 per cell. Coverslips were pretreated with 10 μg of poly-L-lysine per μl to aid cell attachment and spreading. Immunofluorescence was carried out essentially as described previously (19). Briefly, cells were fixed with 3% paraformaldehyde in PBS (pH 7.4) for 15 min, quenched with 50 mM NH_4Cl, and permeabilized with 0.1% Triton X-100. After being blocked in 10% goat serum, cells were incubated with primary and secondary antibodies for 30 min each and mounted in mowiol. Antibodies used were a pool of anti-NP monoclonal antibodies, 3/1, 5/1, 150/4, and 469/6 (provided by Robert Webster, St. Jude's Childrens Research Hospital) (18), or a monospecific anti-M1 polyclonal antibody, 7648, raised against M1 purified from SDS-PAGE gels. Antibody 7648 specifically recognizes M1 by immunoprecipitation and Western blotting (immunoblotting). As secondary antibodies, we used Texas red (TR)-labeled goat anti-rabbit immunoglobulin G and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin G (Zymed). Cells were viewed and photographed on a Zeiss Axiophot microscope with Kodak T-Max 400 film.

RESULTS

Distribution of NP and M1 by immunofluorescence. Since there are apparent discrepancies in the published literature concerning the distribution of the M1 and NP proteins with the influenza virus mutant, *ts*51 (4, 29), we used immunofluorescence to monitor the distribution of newly synthesized M1 and NP proteins at various times after infection, at both the permissive temperature (32°C) and the nonpermissive temperature (39.5°C) (Fig. 2). At early times after infection (2 h) with

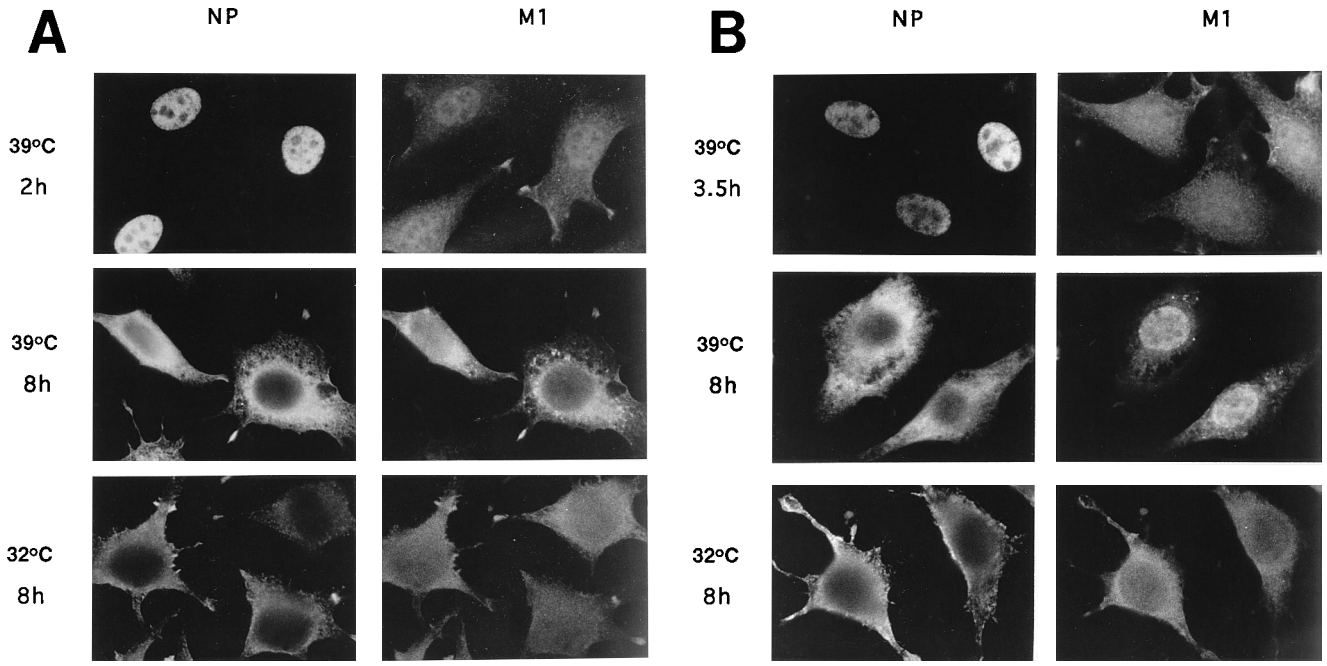

FIG. 2. *ts*51 M1 is retained in the nucleus at the nonpermissive temperature. L929 cells were infected with WSN (A) or *ts*51 (B) at 32 or 39.5°C. Cells were fixed at early time points (2 h for WSN and 3.5 h for *ts*51) or at a late time point (8 h) and labeled with mouse anti-NP (FITC labeled) and rabbit anti-M1 (TR labeled).

the wild-type strain, NP labeling was entirely nuclear. In the same cells, M1 was localized in both the nucleus and the cytosol (Fig. 2A). At later times postinfection (8 h), the distribution of NP and M1 was cytoplasmic, with no labeling in the nucleus, indicating export of NP and M1 to the cytosol. Merging the two fields of view revealed overlap of NP and M1 within the cytoplasm, with a predominance of NP towards the cell surface (data not shown). The distributions of the two proteins were similar whether the cells were incubated at 39.5 or at 32°C, except that less of the M1 was along the cell surface at 39.5°C. The reasons for its perinuclear distribution at this temperature are unclear.

For *ts*51 at the permissive temperature (32°C), both NP and M1 showed distributions identical to those seen for the wild-type virus (Fig. 2B). At the nonpermissive temperature (39.5°C) at early times (3.5 h), the distributions of NP and M1 were also essentially the same for WSN and *ts*51. However, late in infection (8 h), M1 was found in the nucleus while the distribution of NP was entirely cytoplasmic. Merging the two fields showed no overlap of NP and M1 inside the nucleus and a strong predominance of NP in the cytoplasm (data not shown). The data were consistent with the findings of Rey and Nayak (29), who found that *ts*51 M1 protein accumulates in the nucleus at the nonpermissive temperature but export of vRNPs proceeds normally.

Synthesis and export of NP are delayed in *ts*51-infected cells. The immunofluorescence experiments indicated that the kinetics of viral replication might not be identical for *ts*51 and WSN. To determine the overall rate of viral protein synthesis at the nonpermissive temperature, we infected MDBK cells and pulse labeled them with [^{35}S]methionine prior to cell lysis at various time points postinfection. Viral protein synthesis was then analyzed by SDS-PAGE and autoradiography (Fig. 3a). At the nonpermissive temperature, the synthesis of both NP and M1 was indeed delayed in the *ts*51 mutant compared with the wild type: whereas synthesis of WSN virus proteins commenced at approximately 2 h, synthesis of *ts*51 virus proteins occurred later, commencing at approximately 3 h postinfection.

We also analyzed L929 cells, infected with WSN and *ts*51 at the nonpermissive temperature, at an intermediate time point, i.e., 5 to 6 h postinfection, by immunofluorescence microscopy (Fig. 3b). Nuclear labeling of NP was apparent in *ts*51-infected cells, in contrast to findings for WSN-infected cells, which showed cytoplasmic labeling. The distribution of M1 was entirely cytoplasmic in WSN-infected cells but both nuclear and cytoplasmic in *ts*51-infected cells. Taken together, these experiments showed a delay in the synthesis of *ts*51 proteins, which caused an apparent retention of NP in the nucleus at intermediate time points (5 to 6 h). This difference in kinetics of infection and vRNP export may explain the results obtained by Enami et al. (4), who observed a retention of *ts*51 vRNA at the nonpermissive temperature.

***ts*51 M1 contains an additional phosphorylation site.** We next examined the published amino acid sequences of the wild-type and mutant M1 proteins (4, 29) to find an explanation for the mutant phenotype. First, we looked for potential phosphorylation sites in wild-type M1, using the consensus sequences described by Kemp and Pearson (14). We identified two putative phosphorylation sites at positions 54 and 194. These were consensus sites for proline kinase, X-Ser-Pro-X, and casein mammary gland kinase, X-Ser-X-Glu-X, respectively (26, 34) (Fig. 4).

The sequence of the M gene in *ts*51, reported by two groups (4, 28), contains a single-base-pair change resulting in an amino acid substitution of phenylalanine to serine at position 79. We found that this generated an additional serine/threonine kinase recognition site, namely, Phe-Arg-Arg-Arg-Ser-Val. This sequence fits exactly with the proposed consensus recognition site for cyclic AMP (cAMP)-dependent protein kinase, which is given as X-Arg-Arg-X-Ser-X, with more distal arginine residues on the N-terminal side of the phosphorylated

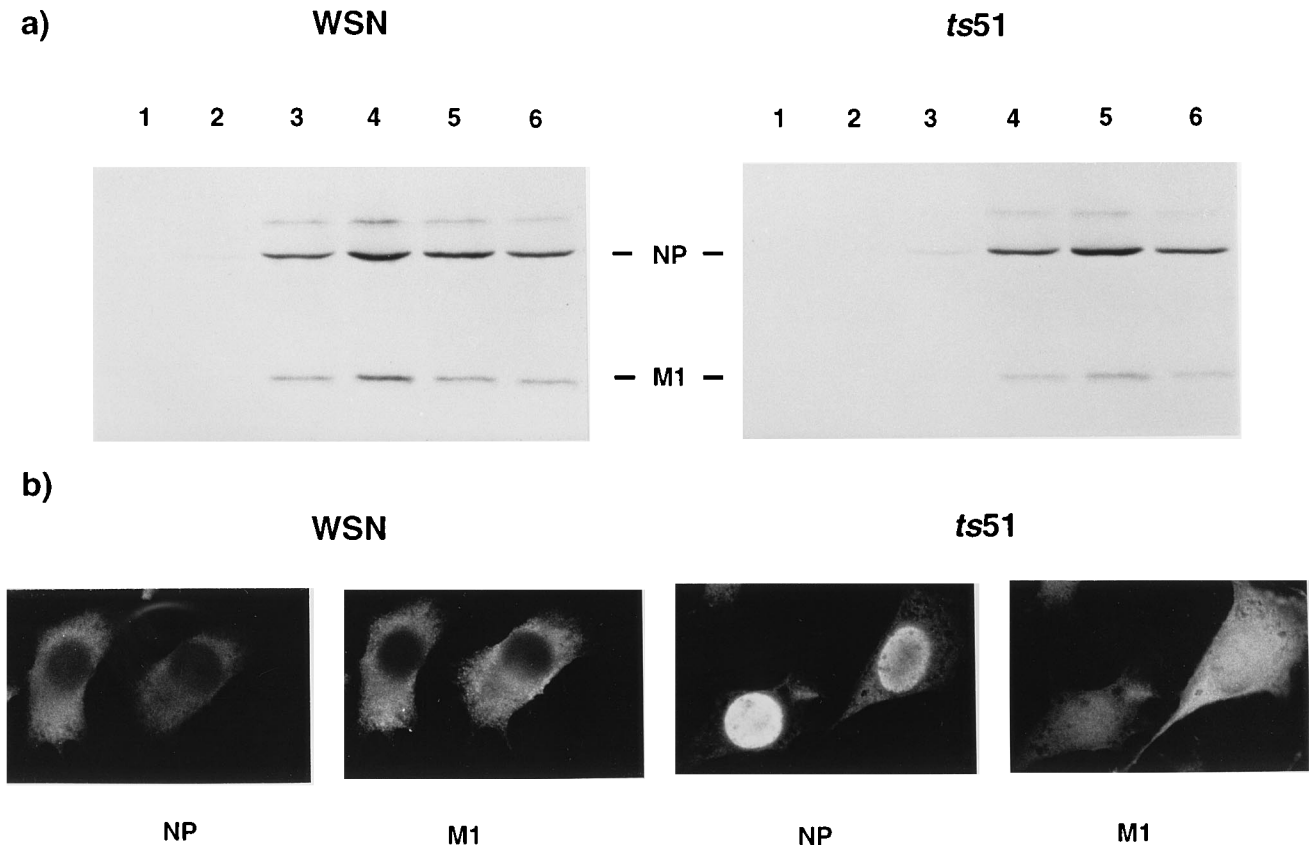

FIG. 3. Synthesis of virus proteins and export of NP are delayed in *ts*51 at the nonpermissive temperature. (a) Time course of viral protein synthesis. MDBK cells were infected with WSN or *ts*51 at 39.5°C, and samples were taken at various time points (1 to 6 h) after infection. Cell lysates were immunoprecipitated with the polyclonal antiserum IBO and analyzed on an SDS-PAGE gel containing 10% acrylamide. M1 and NP are indicated. (b) Indirect immunofluorescence of WSN- and *ts*51-infected cells at an intermediate time point. L929 cells were infected with WSN or *ts*51 at 39.5°C, fixed at 5 to 6 h postinfection, and labeled with mouse anti-NP (FITC labeled) and rabbit anti-M1 (TR labeled).

serine having a positive effect (41). A hydrophobic residue (such as valine) C terminal to the serine also promotes phosphorylation by cAMP-dependent protein kinase. Phosphorylation by other kinases, namely, by S6 kinase II and calmodulin-dependent protein kinase II, which have similar recognition sites, is also possible at this site. Ser-79 of *ts*51 M1 thus represents an ideal putative site for phosphorylation.

To investigate whether the phosphorylation state of the *ts*51 M1 protein was changed from that of wild-type M1, we labeled influenza virus-infected MDBK cells with $^{32}P_i$ and [^{35}S]methionine between 2 to 5 h and 6 to 8 h postinfection, at both 32 and 39.5°C. Cell lysates were analyzed by immunoprecipitation, followed by SDS-PAGE and autoradiography (Fig. 5). In agreement with previous reports (1, 8, 25, 27), cells infected with WSN (Fig. 5, lanes 1 and 2) showed a major phosphorylated band corresponding to NP and a minor phosphorylated band corresponding to the M1 protein. Under the same conditions, *ts*51-infected cell lysates showed a significantly larger amount of ^{32}P incorporated into the M1 protein at a late time point (Fig. 5, lane 6). The increase in phosphorylation was accompanied by a slight shift in the mobility of the phosphorylated M1, i.e., the phosphorylated *ts*51 M1 showed slightly

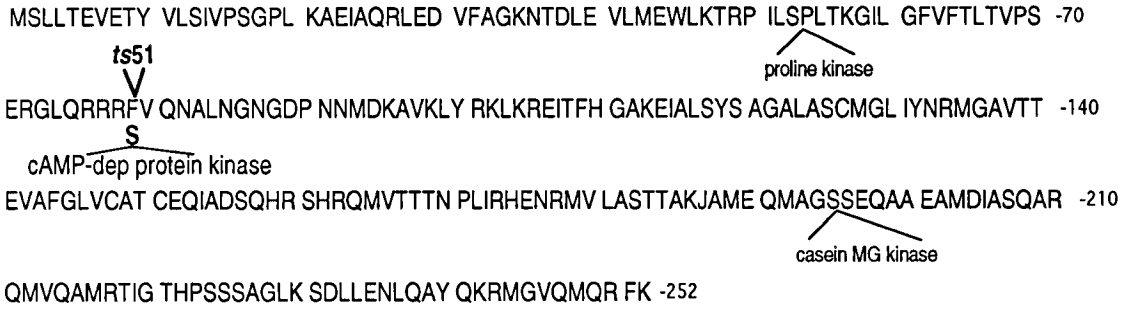

FIG. 4. *ts*51 M1 protein contains an additional phosphorylation site. The amino acid sequence (positions 1 to 252) of WSN M1 is shown, and the mutation in *ts*51 (F to S) is indicated in boldface type. The predicted phosphorylation sites of wild-type M1 (proline kinase and casein mammary gland [MG] kinase) are indicated, and the novel phosphorylation site (cAMP-dependent [dep] protein kinase) in *ts*51 is shown in boldface type.

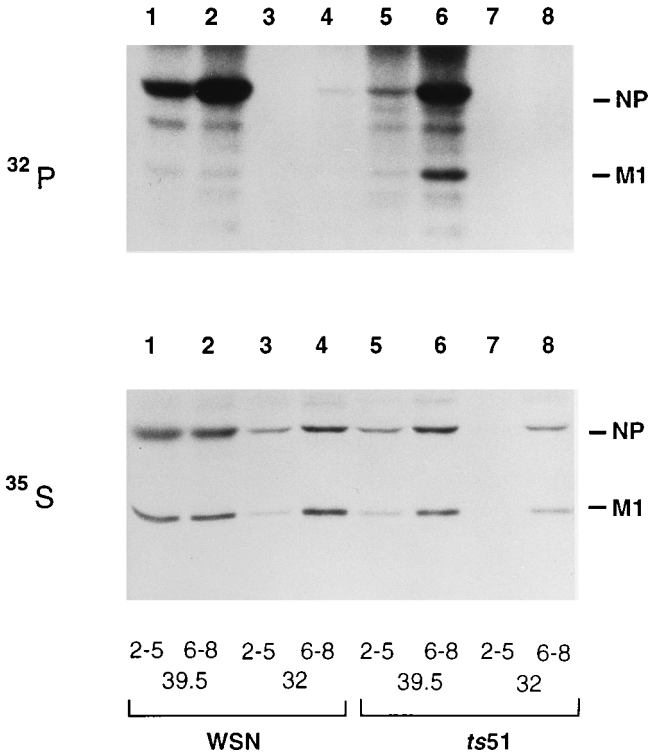

FIG. 5. *ts*51 M1 is hyperphosphorylated at late time points at the nonpermissive temperature. MDBK cells were infected with WSN or *ts*51 at 32°C (lanes 3, 4, 7, and 8) or at 39.5°C (lanes 1, 2, 5, and 6). Cells were labeled with $^{32}P_i$ or [^{35}S]methionine at early times (2 to 5 h [lanes 1, 3, 5, and 7]) or at late times (6 to 8 h [lanes 2, 4, 6, and 8]) and analyzed on an SDS-PAGE gel containing 12% acrylamide. M1 and NP are indicated.

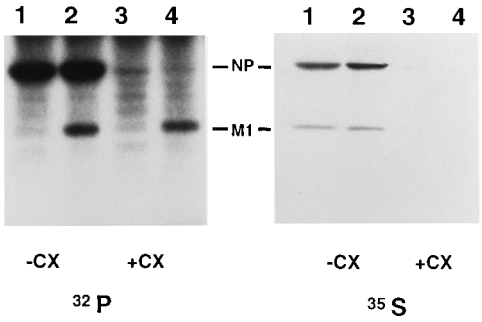

FIG. 6. *ts*51 hyperphosphorylation is independent of protein synthesis. MDBK cells were infected with WSN (lanes 1 and 3) or *ts*51 (lanes 2 and 4) at 39.5°C and treated with 0.5 mM cycloheximide from 5.5 to 8 h (lanes 1 and 2) or left untreated (lanes 3 and 4). Cells were labeled with $^{32}P_i$ or [^{35}S]methionine from 6 to 8 h postinfection. Cell lysates were immunoprecipitated with the polyclonal antiserum IBO and analyzed on an SDS-PAGE gel containing 12% acrylamide. M1 and NP are indicated.

slower migration than did the WSN M1. At an early time point (2 to 5 h) at the nonpermissive temperature, no significant difference was seen between WSN and *ts*51 M1 phosphorylation (Fig. 5, lanes 1 and 5).

When cells were infected at the permissive temperature (32°C), less incorporation of ^{32}P into both NP and M1 was seen for both strains (Fig. 5, lanes 3, 4, 7, and 8). Upon prolonged exposure of ^{32}P-labeled gels, both NP and M1 were visible and showed no detectable difference between the wild-type and mutant viruses. The reasons for the low degree of overall phosphorylation of the virus proteins at lowered temperatures are unclear, but this is consistent with a previous report showing a decrease in the phosphorylation of fowl plague virus NP at 34°C in comparison with phosphorylation at 40.5°C (1).

Analysis of duplicate cell lysates after ^{35}S labeling showed that any differences in ^{32}P incorporation between WSN and *ts*51 seen at late times were not due to differences in protein production, as the labeling with ^{35}S was essentially identical between 6 and 8 h at 39.5°C (Fig. 5, lanes 2 and 6). An overall reduction in protein synthesis was seen at 32°C, and in some samples, e.g., *ts*51 at 2 to 5 h, synthesis of both NP and M1 was very much reduced. This is due to both the overall delay in viral protein synthesis in *ts*51 described above (cf Fig. 3) and a general reduction of the rate of protein synthesis at the lower temperature. In summary, these experiments showed that the M1 protein of *ts*51 was hyperphosphorylated at 39.5°C and that this only occurred late in infection.

L929 cells were also infected and labeled with $^{32}P_i$ from 5 to 8 h postinfection. Overall, the level of phosphorylation was very much lower in this cell line than in MDBK cells, despite similar levels of [^{35}S]methionine incorporation. A similar increase in *ts*51 M1 phosphorylation, compared with that for WSN, was apparent, however (data not shown).

We next investigated whether the phosphorylation of *ts*51 M1 at the nonpermissive temperature was dependent on protein synthesis. MDBK cells were infected with WSN or *ts*51 and incubated at 39.5°C. Protein synthesis was arrested at 5.5 h postinfection, and then cells were labeled with either $^{32}P_i$ or [^{35}S]methionine from 6 to 8 h, in the presence or absence of cycloheximide (Fig. 6). As expected, the cycloheximide treatment completely abolished protein synthesis, as shown by the lack of incorporation of [^{35}S]methionine into both NP and M1. The phosphorylation of *ts*51 M1 was apparently unaffected, but NP phosphorylation was reduced markedly for both WSN and *ts*51. These data showed that whereas for NP, synthesis and phosphorylation were linked, the hyperphosphorylation of *ts*51 M1 was independent of protein synthesis.

H89, a protein kinase inhibitor, abolishes nuclear retention of *ts*51 M1. To test whether the hyperphosphorylation of *ts*51 M1 was the cause of its nuclear retention at the nonpermissive temperature, we used H89, a competitive inhibitor of cAMP-dependent protein kinase (3). L929 cells were infected with influenza virus WSN and *ts*51, maintained at 39.5°C, and analyzed by immunofluorescence at 8 h postinfection (Fig. 7). In the absence of the drug, M1 was retained in the nucleus and NP was cytoplasmic. When H89 was added at 4 h postinfection, both NP and M1 were synthesized normally but M1 was now distributed in the cytoplasm. This experiment showed that a specific phosphorylation inhibitor, when added 4 h after *ts*51 infection, can modify the distribution of M1 from the mutant phenotype of nuclear retention to a wild-type phenotype of cytoplasmic labeling. We also added H89 to WSN- and *ts*51-infected cells 4 h after infection at 39.5°C and assayed virus production at 32°C, to determine whether the kinase inhibitor could rescue the production of infectious virus. The addition of the drug caused a general inhibition of virus production, both for *ts*51 and for WSN (data not shown). Thus, although we could not rescue virus production with H89, the abnormal distribution of *ts*51 M1 at the nonpermissive temperature was most likely a consequence of its hyperphosphorylation.

DISCUSSION

Our results show that the point mutation in the *ts*51 M1 protein creates a novel phosphorylation site which, at the nonpermissive temperature and late in infection, leads to its

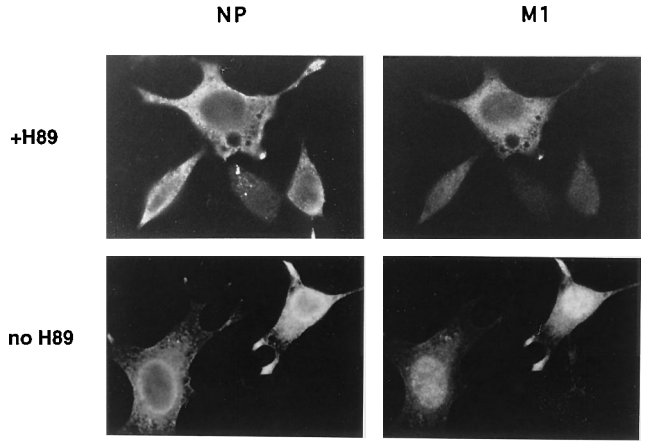

FIG. 7. Treatment of *ts*51-infected cells with the protein kinase inhibitor H89 results in normal export of M1. L929 cells were infected with *ts*51 at 39.5°C and incubated with 20 μM H89 from 4 to 8 h or left untreated. Cells were fixed at a late time point (8 h) and analyzed by indirect immunofluorescence. Cells were labeled with mouse anti-NP (FITC labeled) and rabbit anti-M1 (TR labeled).

hyperphosphorylation. The additional phosphorylation results in the accumulation of the majority of M1 in the nucleus. The mutant protein is able to carry out its nuclear function; it mediates the release of NP from the nucleus to the cytosol. However, it is not capable of performing its cytosolic function, the formation of infectious virus at the plasma membrane. A kinase inhibitor, which prevented hyperphosphorylation, restored the intracellular distribution of M1 to normal, confirming that phosphorylation was directly responsible for M1's nuclear retention.

The abnormal distribution of *ts*51 M1 at the nonpermissive temperature has been described in two previous articles (4, 29), but these came to opposite conclusions about the fate of vRNPs. Rey and Nayak reported normal export of NP out of the nucleus, while Enami et al. found, by in situ hybridization, that the vRNAs were retained in the nucleus. While it remains theoretically possible that NP might be exported from the nucleus in *ts*51 without the vRNA molecules, our results suggest a simpler explanation for the apparent discrepancy. We found that *ts*51 infection is delayed by 1 to 2 h in comparison with wild-type, WSN, infection. Rey and Nayak (29) predominantly analyzed cells at late times of infection (when vRNPs are efficiently transported), while Enami et al. (4) analyzed cells at relatively early times, when, according to our data, the vRNPs are not yet transported. Overall, we found that for *ts*51, NP and, presumably, vRNPs are transported from the nucleus to the cytosol, albeit with somewhat delayed kinetics. In these experiments, we relied on immunofluorescence microscopy to study the distribution of NP and M1. Analysis of nuclear and cytoplasmic fractions produced from infected cells was also carried out, but this failed to give reproducible results (data not shown).

M1 has two known functions in virus assembly: it activates vRNP export from the nucleus (18), and it supports virus budding (24). As the most abundant protein component in the mature virions, it is likely to play a crucial functional role as a bridge between the envelope and the vRNAs. Under normal infection conditions, the two functions are likely to be carried out by different, though possibly overlapping, populations of M1 protein. Some M1 molecules enter the nucleus and promote the release of vRNPs. The other population remains in the cytosol and participates in the assembly of the nucleocapsid and budding of the virion at the plasma membrane. That the majority of M1 proteins in virus particles belong to the latter group is suggested by results obtained by Hay and Skehel (10). They found that M1 is incorporated into the virions almost immediately after synthesis. In contrast, NP, with its obligatory routing into and out of the nucleus, showed a lag of 15 min before it could be incorporated into virus particles. Thus, it is likely that M1 proteins that enter the nucleus do not constitute a large fraction of M1 in the final virion.

The proportion of M1 molecules that become phosphorylated during the course of a normal infection is not known. Since the increase in incorporation of ^{32}P between wild-type and *ts*51 M1 is very large, it seems unlikely that the difference would be due solely to a single, extra phosphorylation event on those M1 molecules which are already phosphorylated. Instead, it is likely that a larger fraction of M1 molecules become phosphorylated. It is thus possible that in the *ts*51 mutant, the population of M1 that would normally be resident in the cytoplasm and incorporated into virions is, instead, phosphorylated and imported into the nucleus, where it becomes sequestered. The finding that hyperphosphorylation of *ts*51 M1 took place in the absence of protein synthesis indicated either that a pool of previously synthesized M1 was phosphorylated late in infection or that phosphate was constantly cycled on and off the protein.

Recently, many reports concerning the role of phosphorylation in controlling the nucleocytoplasmic distribution of proteins have been published. For instance, the simian virus 40 T antigen shows increased uptake into the nucleus when casein kinase II sites adjacent to the NLS are phosphorylated (31). Phosphorylation of lamin B2 by protein kinase C, on the other hand, inhibits its nuclear import (12). Other examples are the cell cycle-regulated nuclear import of the Rel family of transcription factors (23); the altered nuclear affinity of retinoblastoma protein due to G1/S phosphorylation (22); and the selective nuclear transport of developmental factors such as *Xenopus* nuclear factor, xnf7 (21), and the *Drosophila* morphogen, *dorsal* (36). A previous report (1) has correlated the phosphorylation state of NP with its localization in the nucleus. It is interesting that the new phosphorylation site in *ts*51 M1 is close to a basic region in the sequence that could serve as a potential nuclear localization sequence.

In *ts*51-infected cells at the nonpermissive temperature, mistargeting prevents M1 from playing its role in virus assembly and no infectious viruses are produced. The vRNPs seemed to be normally transported from the nucleus to the cytosol, indicating that the mutant M1 is capable of performing its nuclear functions. That M1 is needed for vRNP export (18) is well documented, but contrary to our previous speculations, it may not have to associate permanently with the vRNPs. Perhaps its role is simply to dissociate the vRNPs from a bound form, or to promote a final vRNP assembly event which renders it transport competent. On the basis of our data we cannot, however, entirely dismiss the possibility that *ts*51 M1 does, indeed, accompany the vRNPs through the nuclear pore complexes to the cytosol. It could conceivably dissociate and return to the nucleus. More detailed studies are needed to elucidate the precise role that M1 plays in the nucleus.

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