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Nuclear Trafficking of Influenza Virus Ribonucleoproteins in Heterokaryons

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The influenza virus nucleoprotein (NP), matrix protein (M1), and ribonucleoproteins (vRNPs) undergo regulated nuclear import and export during infection. Their trafficking was analyzed by using interspecies heterokaryons containing nuclei from infected and uninfected cells. Under normal conditions, it was demonstrated that the vRNPs which were assembled in the nucleus and transported to the cytosol were prevented from reimport into the nucleus. To be import competent, they must first assemble into virions and enter by the endosomal entry pathway. In influenza virus mutant *ts*51, in which M1 is defective, direct reimport took place but was inhibited by heterologous expression of wild-type M1. These data confirm M1's role as the inhibitor of premature nuclear import and as the main regulator of nuclear transport of vRNPs. In addition to this vRNP shuttling, M1 also shuttled between the nucleus and the cytoplasm in *ts*51-infected cells. When NP was expressed in the absence of virus infection, it was also found to be a shuttling protein.

When viruses that replicate in the nucleus enter their host cells, the viral genome and accessory proteins must be delivered first to the cytosol and then into the nucleus. Cellular macromolecules transit across the nuclear membrane via the nuclear pore complex (NPC), a large supramolecular structure spanning the nuclear envelope (48). However, viruses and viral nucleocapsids are usually larger than the 23-nm size limit for passage through the NPC (11). Viruses have therefore evolved a variety of strategies to overcome the nuclear membrane barrier. DNA viruses such as adenoviruses and herpesviruses have evolved injection mechanisms, whereby the capsids dock onto the NPC and deliver their DNA into the nucleus via the nuclear pore (reviewed in references 13 and 64). Most retroviruses, with the exception of human immunodeficiency virus type 1 and the lentiviruses, must wait until the nuclear membrane is disrupted during mitosis (36, 66). Infection is therefore limited to dividing cells. Human immunodeficiency virus type 1 can, however, infect nondividing cells, and nuclear import of the viral preintegration complex is controlled by either the virion matrix or Vpr protein (14, 21). Influenza viruses, the subject of this study, have a segmented genome with individual ribonucleoprotein complexes (vRNPs) small enough to pass through the NPCs (44).

The influenza virus vRNP contains one of the eight single-stranded, negative-sense RNA segments. The protein components are the viral nucleoprotein (NP), of which there is one for about every 20 nucleotides, and a single copy of the trimeric polymerase enzyme (34, 47). The vRNPs are helical and rod-like, with a width of approximately 10 to 20 nm (7). Within the intact virus particle, the vRNPs are tightly associated with the matrix protein (M1), and M1 is thought to form a shell between the vRNPs and the viral membrane (60).

During entry, influenza virus first binds to sialic acid-containing receptors on the cell surface and is rapidly internalized by receptor-mediated endocytosis (45). In the late endosomes, a low-pH-triggered membrane fusion step releases the vRNPs into the cytoplasm (41, 69). M1 then dissociates, and the vRNPs are imported into the nucleus by an active mechanism through the NPCs (44). RNA replication and transcription take place in the nucleus (32).

The nuclear trafficking events during influenza virus infection are interesting because the vRNPs undergo bidirectional transport; the incoming vRNPs enter the nucleus while progeny vRNPs are transported from the nucleus to the cytosol (70). Altogether, the nuclear transport events include (i) import of incoming vRNPs, (ii) export of viral mRNAs, (iii) import of newly synthesized structural and nonstructural proteins, and (iv) export of assembled progeny vRNPs. Each of these events is highly regulated and occurs at specific phases of the replication cycle. M1, a protein synthesized late in infection, is known to be essential for the export of the newly assembled vRNPs from the nucleus and into the cytoplasm (43).

In this study, we have analyzed the nuclear transport of NP, M1, and vRNPs in heterokaryons containing nuclei from infected and uninfected cells. We have defined the role of M1 in preventing premature uptake of vRNPs and have analyzed the shuttling of M1 and NP between the nucleus and the cytosol. The results revealed M1 as the master regulator during nuclear trafficking of viral components.

MATERIALS AND METHODS

Cells and virus. L929 cells were passaged twice weekly and grown in alpha minimal essential medium (αMEM) containing 10% fetal calf serum (FCS), 1% glutamine, 100 U of penicillin per ml, 10 μg of streptomycin per ml, 0.8 g of sodium bicarbonate per liter, 10 mM *N*-2-hydroxypiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.3), and nonessential amino acids. L929 cells have been reported to give abortive infections for some influenza strains, but synthesis of virus proteins was normal and infectious virus was produced for the WSN strain used here (71). HeLa cells were also passaged twice weekly and grown in αMEM as for L929 cells except that medium contained 7% FCS. The 3PNP-4 cell line (37) was a gift from Mark Krystal (Bristol Myers Squibb, Wallingford, Conn.) and was derived from the mouse cell line C127. 3PNP-4 cells constitutively express influenza virus (strain WSN) NP and the three polymerase proteins and were passaged twice weekly and grown in αMEM containing 10% FCS, 1% glutamine, 100 U of penicillin per ml, 10 mg of streptomycin per ml, 0.8 g of sodium bicarbonate per liter, and 400 μg of geneticin (Gibco) per ml.

Influenza A virus (strain WSN) and the temperature-sensitive mutant *ts*51 (59, 65) were a gift from Robert Krug (Rutgers University, New Brunswick, N.J.). *ts*51 was plaque purified three times on MDBK cells at 33°C prior to use. Stocks of virus were prepared as described previously (44) and grown at 37°C for WSN and 33°C for *ts*51 in MDBK or MDCK cells, and titers were determined by plaque assay on MDBK or MDCK cells. Recombinant Semliki Forest virus

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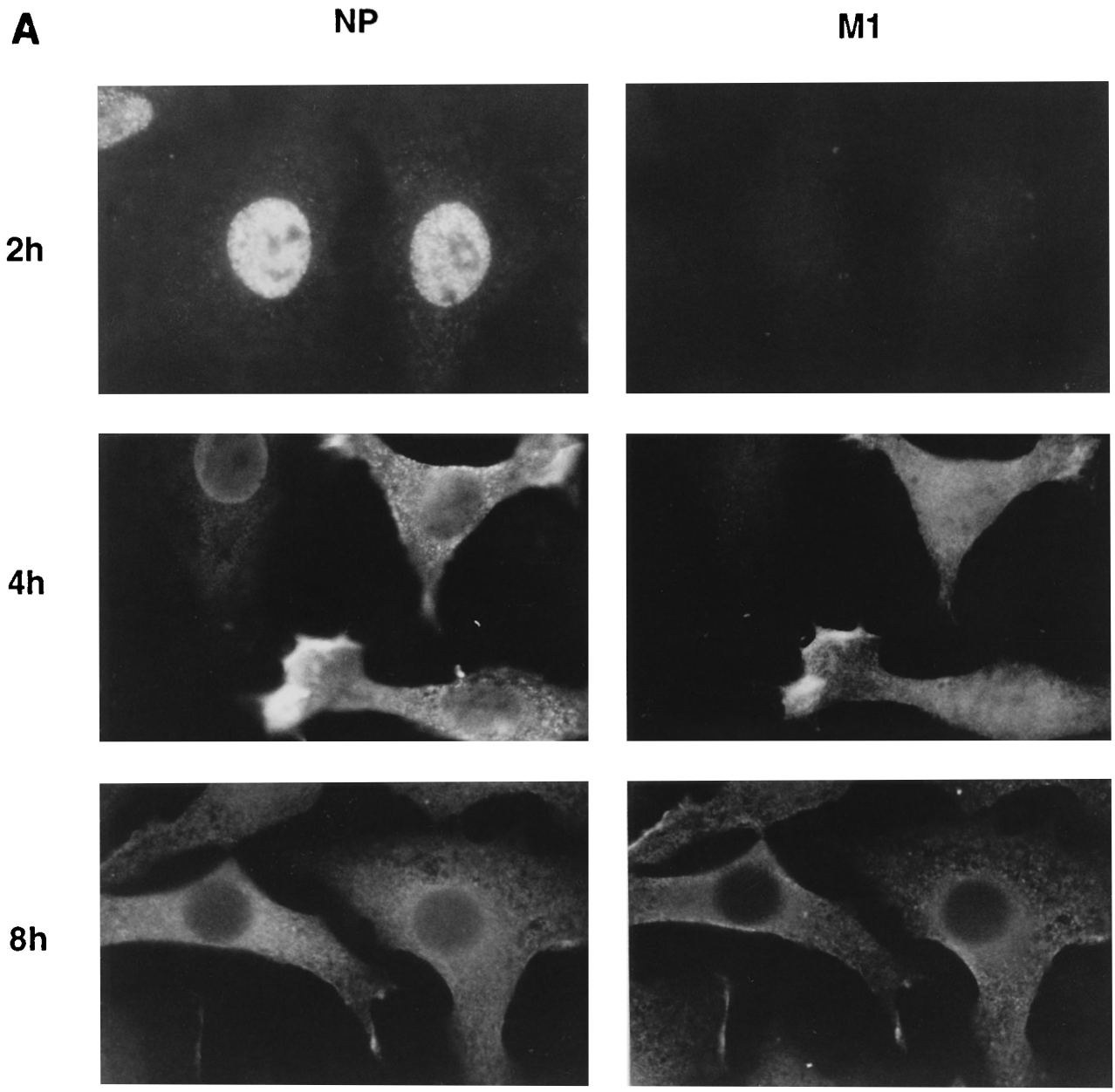

FIG. 1. Immunofluorescence of infected L929 and HeLa cells at various times after infection. (A) L929 cells were infected with influenza virus and analyzed by immunofluorescence microscopy at 2, 4, and 8 h postinfection, using anti-NP and anti-M1 antibodies. At early time points, NP is entirely nuclear and M1 is not synthesized. At later time points, both NP and M1 are synthesized and are distributed predominantly in the cytoplasm. (B) HeLa cells were infected with influenza virus and analyzed by immunofluorescence microscopy at 2, 4, and 8 h postinfection, using anti-NP and anti-M1 antibodies. At early time points, NP is entirely nuclear and M1 is not synthesized. At later time points, both NP and M1 are synthesized and are distributed in both the cytoplasm and the nucleus.

(SFV) expressing the WSN wild-type M1 protein was produced in BHK cells by cotransfecting SP6-derived transcripts of helper and pSFV-M1 plasmids as described previously (39). The SFV plasmids were a gift from Henrik Garoff (Karolinska Institute, Stockholm, Sweden). Details of the M1 expression system will be described elsewhere (5).

Virus infection. L929 cells were seeded onto round 12-mm-diameter no. 1 coverslips coated with poly-L-lysine and incubated for 2 days to a density of approximately 2×10^5 cells per coverslip. Cells were infected with virus at a multiplicity of infection of 1 to 2 PFU per cell in RPMI 1640 medium containing 0.2% bovine serum albumin and buffered to pH 6.8 with 20 mM HEPES. Cells were incubated at 37°C for 60 min to allow virus binding and internalization, or at 4°C for 90 min to allow virus binding only, then washed twice with RPMI, transferred to αMEM containing 2% FCS, and maintained at 37°C. The start of infection (0 h) was the time from which cells were incubated at 37°C. HeLa cells (10^5) were then seeded onto the same coverslip at 1 h postinfection, and cells were incubated at 37°C in αMEM–2% FCS containing 20 mM NH_4Cl. NH_4Cl raises the pH of endosomes (51) and therefore blocks the penetration of influenza virus and other viruses (23). The addition of NH_4Cl thus effectively blocks any further infection of cells by unbound virus remaining in the medium.

For experiments involving *ts*51, virus was bound initially in RPMI at 4°C for 90 min and then incubated at the nonpermissive temperature (39.5°C) or the permissive temperature (33°C), again in the presence of NH_4Cl. Cycloheximide (0.5 mM) was added to cells 30 min prior to fusion, and cells were fused at room temperature at 10 h postinfection. Cells were maintained at 39.5 or 33°C until 11 h postinfection and then fixed. The phenotype of *ts*51 is known to be irreversible in the presence of cycloheximide at late times after infection (56).

For analysis of incoming vRNPs, L929 cells were grown on coverslips as for virus infections, and then HeLa cells (10^5) were added and allowed to attach to the coverslip for 90 min at 37°C in growth medium. Cells were fused as described below, infected for 60 min after fusion with 100 to 200 PFU of WSN per cell in

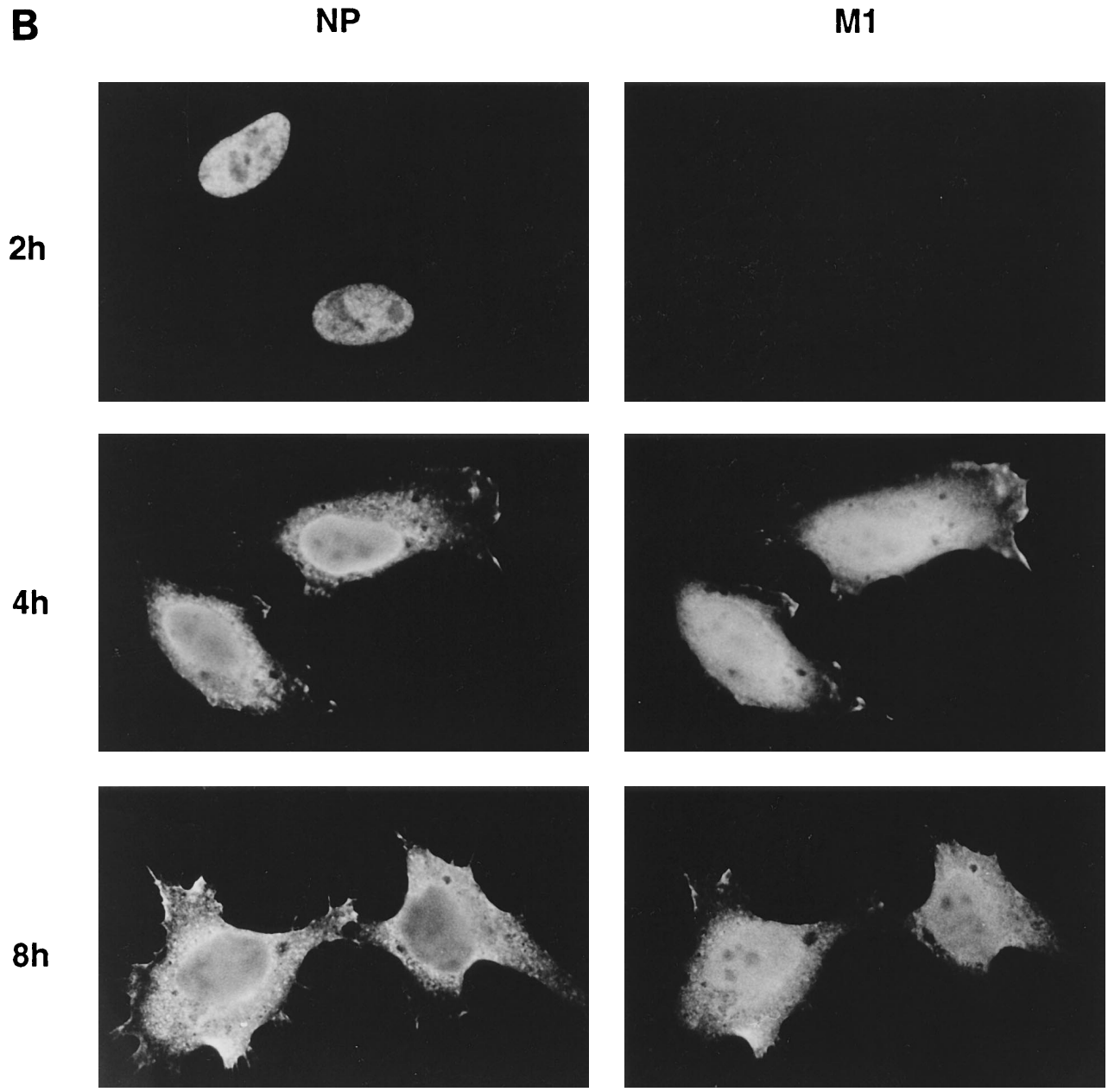

FIG. 1—*Continued.*

RPMI, and incubated for 90 min at 4°C. Cells were then washed and incubated at 37°C for 60 min in medium containing 1 mM cycloheximide.

Cell fusion. Cells were fused by using polyethylene glycol (PEG) (10). Coverslips were washed with calcium and magnesium-free phosphate-buffered saline (PBS) containing 1 g of glucose per liter and then inverted onto a drop of 50% PEG 8000 (Sigma) made up in PBS-glucose. The PEG solution was prepared by autoclaving 10 g solid PEG, which was then mixed with 10 ml of PBS-glucose. After 2 min at room temperature, the coverslip was washed three times with PBS-glucose and returned to αMEM without NH_4Cl but containing 1 mM cycloheximide (to prevent further protein synthesis) or to αMEM without cycloheximide. Cells were incubated at 37°C for 45 to 60 min, fixed, and processed for indirect immunofluorescence. In other experiments, HeLa cells were also infected and then fused with L929 cells; in this case, all experimental conditions were identical except for the reversal of the cell lines.

For fusion of 3PNP-4 cells with HeLa cells, 3PNP-4 cells were grown on coverslips for approximately 24 h to a density of approximately 10^5 cells per coverslip and then transferred to serum-free medium for 12 to 14 h. HeLa cells (10^5) were then added, and cells were maintained at 37°C for 3 to 4 h in serum-free 3PNP-4 growth medium. Cells were then fused as described above, fixed at various times after fusion, and analyzed by immunofluorescence microscopy.

Microinjection. For microinjection, cells were seeded 2 days prior to use onto etched coverslips (Bellco Biotechnology) coated with poly-L-lysine. Before injection, cells were transferred to αMEM containing 20% FCS and 20 mM HEPES (pH 7.3). Cells were injected at room temperature (44) and then incubated at 37°C and 5% CO_2 in cell growth medium for 45 min before being fixed and analyzed by immunofluorescence. Trimethyl rhodamine isothiocyanate (TRITC)-protein A (Sigma) was dissolved in microinjection buffer (120 mM KCl, 10 mM Tris [pH 7.4]) to a concentration of 1 mg/ml. We estimated that the volume of material injected was 1/10 of the cell volume.

Immunofluorescence. Immunofluorescence was carried out essentially as described previously (43). Briefly, cells were fixed with 3% paraformaldehyde in PBS for 15 min, quenched with 50 mM NH_4Cl-PBS, and permeabilized for 2 min with 0.1% Triton X-100–PBS. After blocking 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,

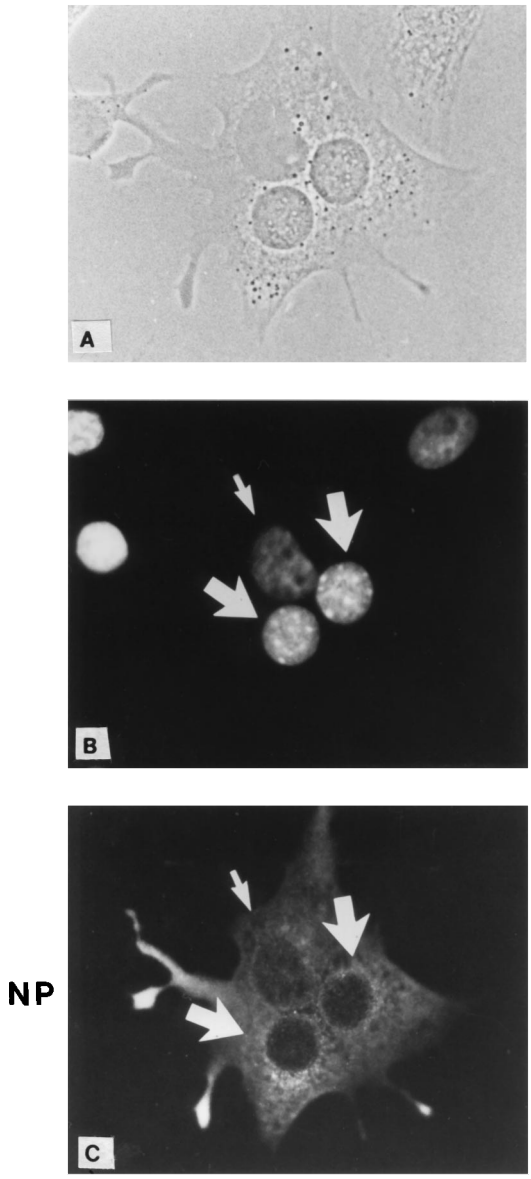

FIG. 2. vRNPs do not shuttle when infected L929 cells are fused with uninfected HeLa cells at 8 h postinfection. L929 cells (large arrows) were infected with influenza virus and fused with uninfected HeLa cells (small arrows) at 8 h postinfection in the presence of cycloheximide. Cells were analyzed by phase-contrast microscopy (A), by fluorescence microscopy using the DNA stain Hoechst 33258 (B), or after indirect immunofluorescence with anti-NP antibodies (C). Trafficking of vRNPs does not occur from the cytoplasm into the newly provided HeLa cell nucleus.

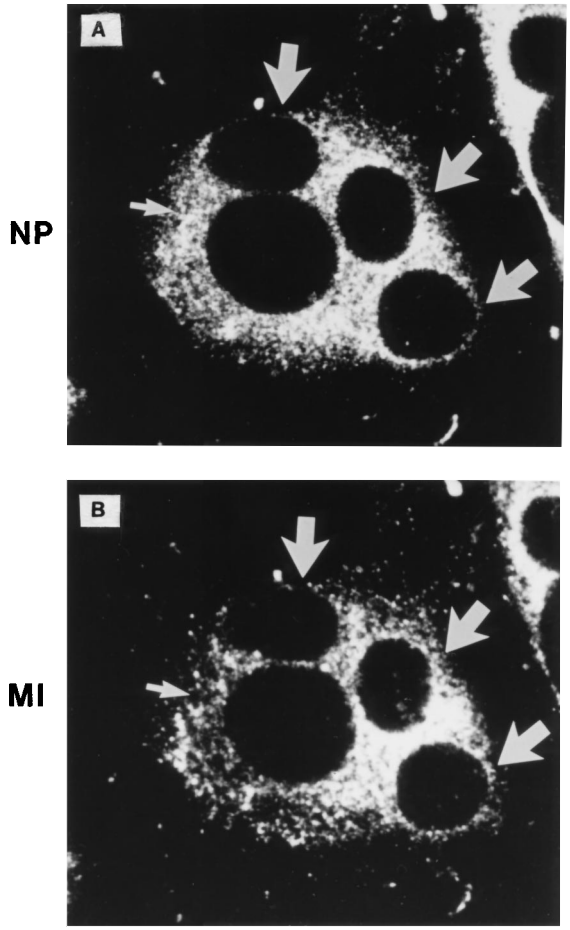

FIG. 3. Confocal microscopy of L929-HeLa heterokaryons. L929 cells (large arrows) were infected with influenza virus and fused with uninfected HeLa cells (small arrows) at 8 h postinfection in the presence of cycloheximide. Cells were analyzed by confocal microscopy using anti-NP antibodies (A) or the anti-M1 antibody Mprtn (B). Trafficking of neither vRNPs nor M1 takes place from the cytoplasm into the newly provided HeLa cell nucleus.

5/1, 150/4, and 469/6 (provided by Robert Webster, St. Jude's Children's Research Hospital, Memphis, Tenn.) or monospecific anti-M1 polyclonal antibodies Mprtn (provided by Mark Krystal, Bristol Myers Squibb) and 7648 (71). As secondary antibodies, we used Texas red-labeled goat anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG and fluorescein isothiocyanate-labeled goat anti-mouse IgG (Zymed). Cells were viewed with a Zeiss Axiophot microscope fitted with a 40× objective lens, and images were photographed with TMAX 400 film (Kodak).

To visualize nuclei, cells were incubated with 1 μg of bisbenzimide (Hoechst 33258; Sigma) per ml for 1 min. This dye gave differential staining of mouse cell nuclei and human cell nuclei, allowing easy distinction between the infected and uninfected nuclei. L929 cell nuclei were relatively small and stained brightly, with many very bright speckles. HeLa cell nuclei, however, tended to be larger and stained less brightly overall, with some dark areas. Other DNA stains, such as Syto 16 (Molecular Probes) and propidium iodide (Sigma), were also used for confocal microscopy, but the different nuclei were less easily distinguished, and therefore most experiments used epifluorescence microscopy, which allows detection at the short wavelengths emitted by Hoechst 33258.

Scanning laser confocal microscopy. Cells were viewed on a Bio-Rad MRC-600 confocal imaging system mounted on a Zeiss Axiovert 10 and using a 63× objective lens with a 1.4 numerical aperture. The dual-channel mode was used, and images of cells were collected through the center of the cell. Images were photographed directly from the computer screen by using TMAX 100 film (Kodak).

RESULTS

The distribution of NP and M1 in infected L929 and HeLa cells. The intracellular distribution of NP and M1 proteins in infected, unfused cells was first analyzed by immunofluorescence microscopy at different times after influenza virus infection (Fig. 1). A human cell line (HeLa) and a mouse cell line (L929) were chosen for this study because their nuclei are easily distinguished from each other in heterokaryons. Although HeLa cells have been shown to have some defects in virus entry (17), the synthesis of virus proteins and the intracellular trafficking events are normal in both HeLa cells and L929 cells (17, 71).

NP synthesis began within the first 2 h postinfection, and the newly synthesized NP was localized in the nucleus. Since no NP

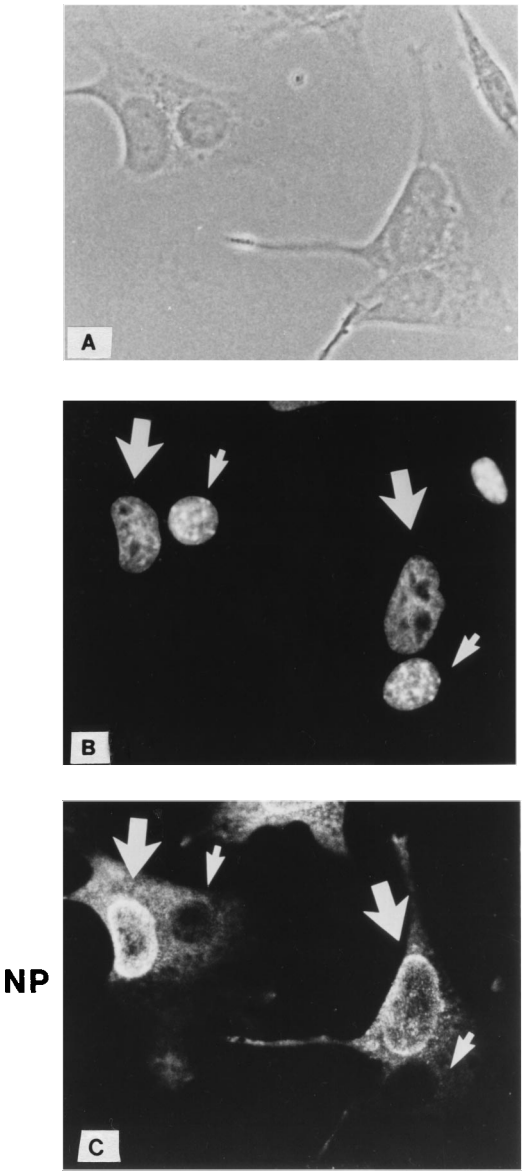

FIG. 4. Fusion of infected HeLa cells with uninfected L929 cells at 8 h postinfection. HeLa cells (large arrows) were infected with influenza virus and fused with uninfected L929 cells (small arrows) at 8 h postinfection in the presence of cycloheximide. Cells were analyzed by phase-contrast microscopy (A), by fluorescence microscopy with the DNA stain Hoechst 33258 (B), or with anti-NP antibodies (C). Trafficking of vRNPs does not occur into the newly provided nucleus.

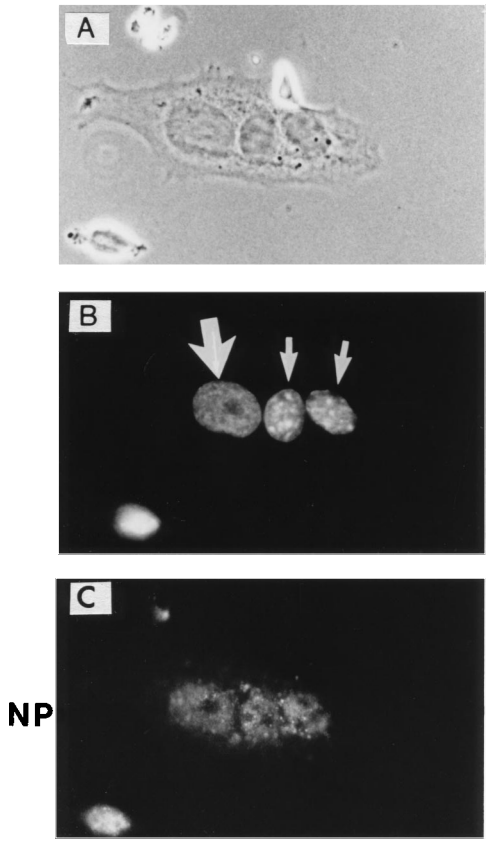

FIG. 5. Incoming vRNPs distribute into both nuclei in heterokaryons. L929 cells (small arrows) were fused with HeLa cells (large arrows) and then infected with a high multiplicity of infection of WSN in the presence of cycloheximide. Cells were fixed 60 min after infection and analyzed by phase-contrast microscopy (A), by fluorescence microscopy with the DNA stain Hoechst 33258 (B), or with anti-NP antibodies (C). Import of incoming vRNPs occurs equally into both the L929 and HeLa cell nuclei.

was detectable in the cytosol, where it had been translated, rapid import into the nucleus was taking place. M1 synthesis did not occur at this early time. By 4 to 5 h of infection, M1 synthesis had started in most of the cells; the protein was detectable in the nucleus, in the cytoplasm and along the plasma membrane (Fig. 1). In the cells that expressed M1, NP staining was now seen in the cytoplasm and along the periphery of the cell. Staining in the nuclei was weaker because of M1-dependent nuclear export of the assembled vRNPs (43).

By 8 h, all cells expressed M1 (Fig. 1). In L929 cells, both NP and M1 were now exclusively cytoplasmic, indicating that the vRNPs, assembled in the nucleus, had been exported and that newly synthesized NP and M1 no longer entered the nucleus or accumulated there. The nuclear depletion of NP and M1 late in infection was more complete in L929 cells than in HeLa cells, in which some NP and M1 fluorescence remained nuclear even late in infection.

Nuclear trafficking of vRNPs and NP in heterokaryons. The loss of nuclear NP staining with advancing infection suggested that once exported from the nucleus to the cytosol, the vRNPs were somehow prevented from reentering the nucleus. To test whether this was the case, we fused infected L929 cells with uninfected HeLa cells at 8 h postinfection and determined whether the vRNPs in the cytosol could enter the uninfected nuclei. NH_4Cl was present to block the entry of incoming viruses (45), and cycloheximide was added to inhibit the synthesis of new NP and M1.

Fusion was induced by the addition of PEG (10). One hour after fusion, the cells were analyzed by phase-contrast and fluorescence microscopy, using anti-NP antibodies, and stained with Hoechst 33258 (Fig. 2). The L929 nuclei were readily distinguished as smaller and having a speckled Hoechst staining pattern, while the HeLa cell nuclei were larger and more evenly stained. The infected heterokaryons could be observed for about 2.5 h, but longer incubations were prevented by the relatively poor survival of cells.

As shown in Fig. 2C, the distribution of NP in the heterokaryons remained cytoplasmic. Although the experimental system uses anti-NP antibodies, all cytoplasmic NP protein is

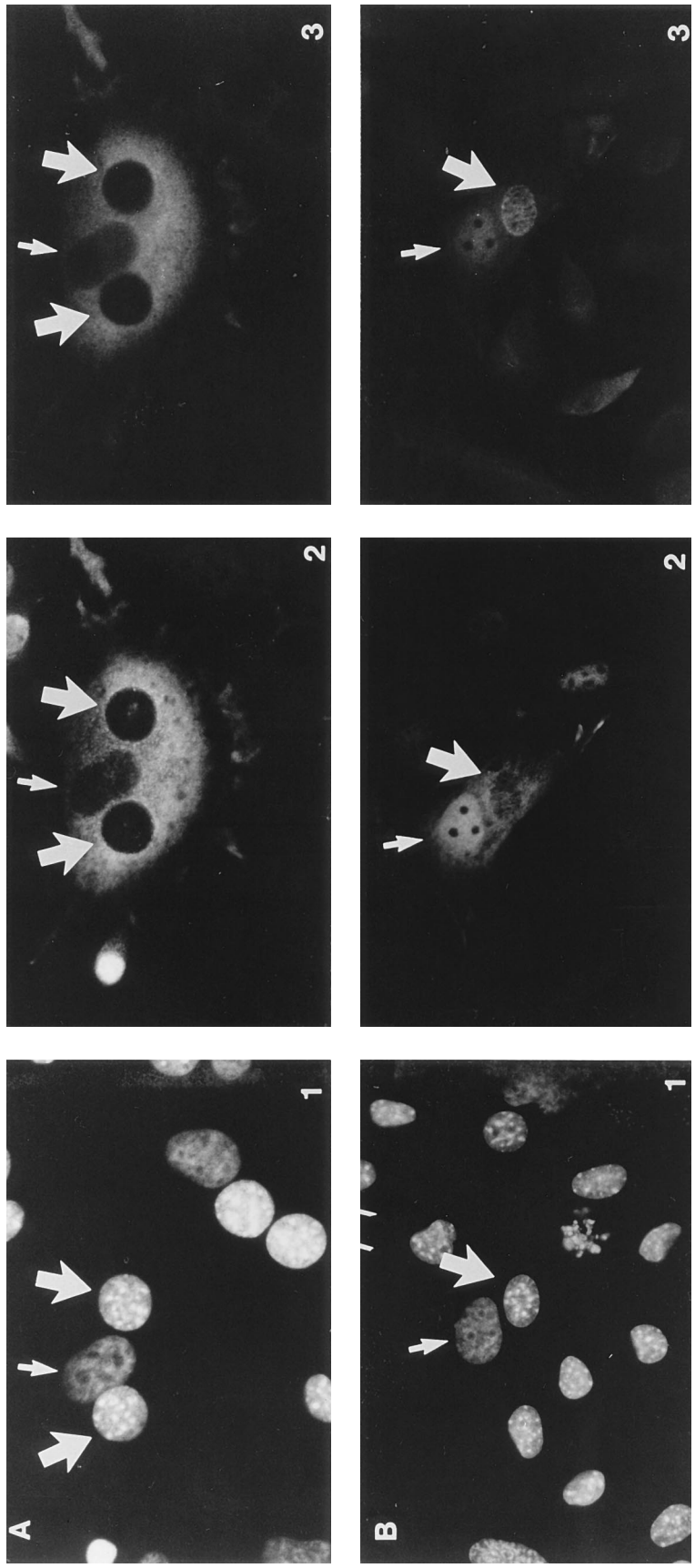

FIG. 6. vRNPs and M1 shuttle in *ts*51-infected heterokaryons. L929 cells (large arrows) were infected with *ts*51 at 32°C, the permissive temperature (A), or 39.5°C, the nonpermissive temperature (B), and fused with uninfected HeLa cells (small arrows) at 10 h postinfection in the presence of cycloheximide. Cells were analyzed by fluorescence microscopy with the DNA stain Hoechst 33258 (panels 1), with a pool of anti-NP antibodies (panels 2), or with the anti-M1 antibody 7648 (panels 3). Trafficking of both vRNPs and M1 takes place into the newly provided nucleus at the nonpermissive temperature, implying that both species are shuttling into and out of the nucleus.

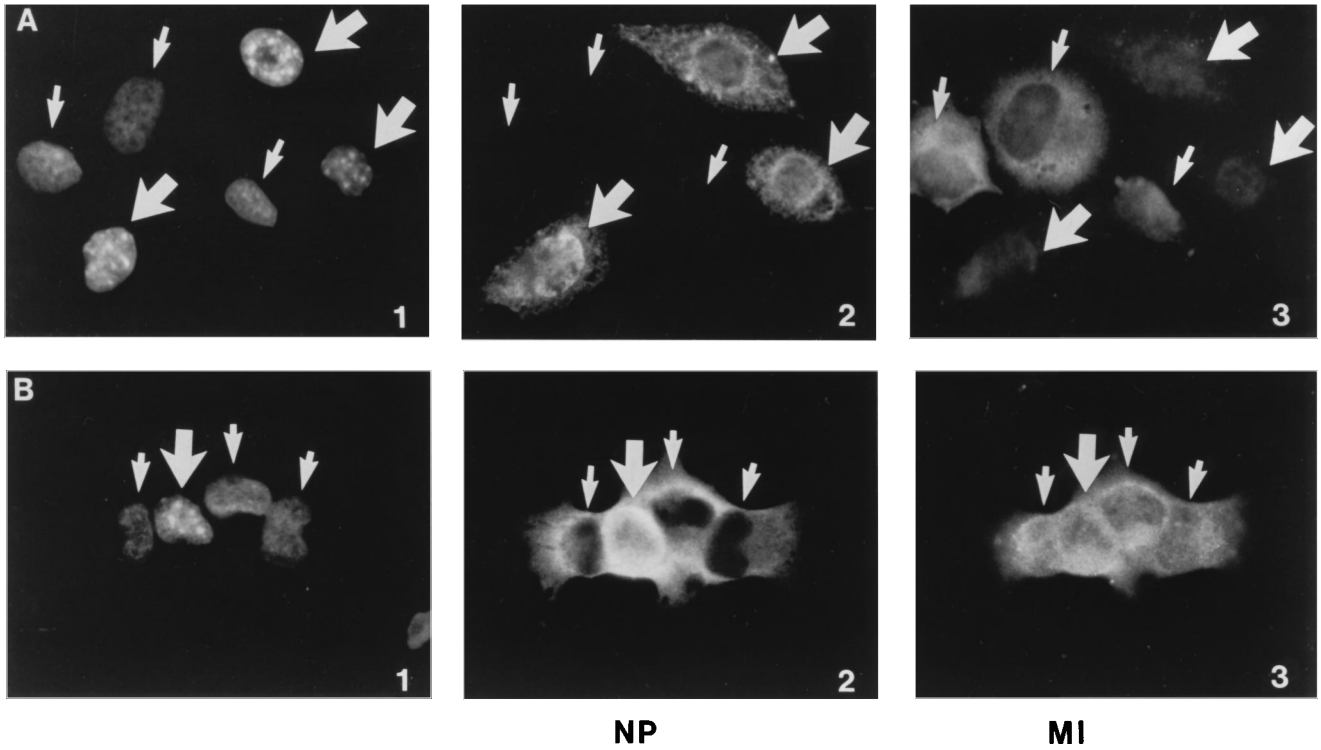

FIG. 7. vRNP shuttling in *ts*51-infected heterokaryons is reversed by heterologous expression of M1. L929 cells (large arrows) were infected with *ts*51 at 39.5°C, the nonpermissive temperature, and HeLa cells (small arrows), infected with recombinant SFV expressing M1, were seeded alongside at 10 h postinfection. At 14 h postinfection, cells were fused (B) or remained unfused (A) in the presence of cycloheximide. Cells were analyzed by fluorescence microscopy with the DNA stain Hoechst 33258 (panels 1), with a pool of anti-NP antibodies (panels 2), or with the anti-M1 antibody 7648 (panels 3). Trafficking of vRNPs does not take place into the newly provided nucleus, implying that recombinant M1 can substitute for the mutant viral M1 and prevent reimport of vRNPs into the nucleus.

believed to be assembled into vRNPs at late times after infection (55). Thus, the vRNPs that had been exported from the infected L929 cell nuclei and were present in the cytosol of the heterokaryon did not enter the uninfected HeLa cell nucleus within the period for which the cells were monitored. The lack of nuclear labeling by both NP and M1 antibodies was particularly clear when cells were analyzed by confocal microscope (Fig. 3). This technique allowed us to visualize a horizontal section of the nucleus, excluding any signal from the cytoplasm overlying the nucleus. The same results were obtained when the cells lines reversed, i.e., when the HeLa cells were infected and the L929 cells uninfected (Fig. 4C). No entry of vRNPs took place into the uninfected nucleus. These results demonstrated that newly formed vRNPs and M1 present in the cytosol of infected cells either were unable to enter a nucleus or, if they did enter, were rapidly recycled back to the cytosol. Whether a nucleus was previously infected or not seemed not to make a difference; the vRNPs and M1 did not accumulate in any of the nuclei at this late time of infection.

Nuclear import of incoming vRNPs. To determine whether nuclear import would occur when vRNPs entered heterokaryons via the normal virus entry pathway, uninfected HeLa cells were fused with uninfected L929 cells. The heterokaryons were then infected with a high multiplicity of virus in the absence of the entry blockers. Cycloheximide was included to inhibit synthesis of new NP and M1.

The results in Fig. 5C showed that the vRNPs of incoming viruses were able to enter the nuclei of both cell types with equal efficiency. This finding indicated that the block in nuclear import seen above for endogenous vRNPs did not apply for vRNPs entering via the endosomal entry route. Therefore, incoming vRNPs are different from exported vRNPs with respect to their nuclear import properties. This difference appears to depend on whether M1 is bound to vRNPs, since incoming vRNPs must dissociate from M1 for nuclear import and newly synthesized vRNPs require M1 for nuclear export (44).

Shuttling of vRNPs and M1 in *ts*51-infected heterokaryons. To test whether it is the association with M1 that prevents newly exported vRNPs from reentering the nucleus, we made use of a mutant influenza virus, *ts*51, which has a temperature-sensitive M1 protein. At the nonpermissive temperature of 39.5°C, the mutant M1 is hyperphosphorylated and is no longer seen to colocalize with vRNPs in the cytosol (56, 71). Like wild-type M1, it enters the nucleus and is capable of triggering the export of vRNPs to the cytosol. However, no virus particles are formed, and the vRNPs accumulate in the cytosol in a virtually M1-free form. Since the cytosol remains essentially M1 free, the mutant virus provided us with a tool to evaluate M1's role in preventing reimport of vRNPs into the nucleus.

L929 cells were infected with influenza virus mutant *ts*51 either at the permissive temperature of 33°C or at the nonpermissive temperature of 39.5°C and fused with uninfected HeLa cells at 10 h postinfection in the presence of cycloheximide (Fig. 6). When the heterokaryons were analyzed for NP at the permissive temperature, the NP was seen in the cytosol and excluded from the nuclei (Fig. 6A, panel 2). At the nonpermissive temperature, however, the NP was seen to enter the uninfected HeLa cell nucleus (Fig. 6B, panel 2). Since new protein synthesis had been blocked with cycloheximide, this could only mean that vRNPs were imported into the unin-

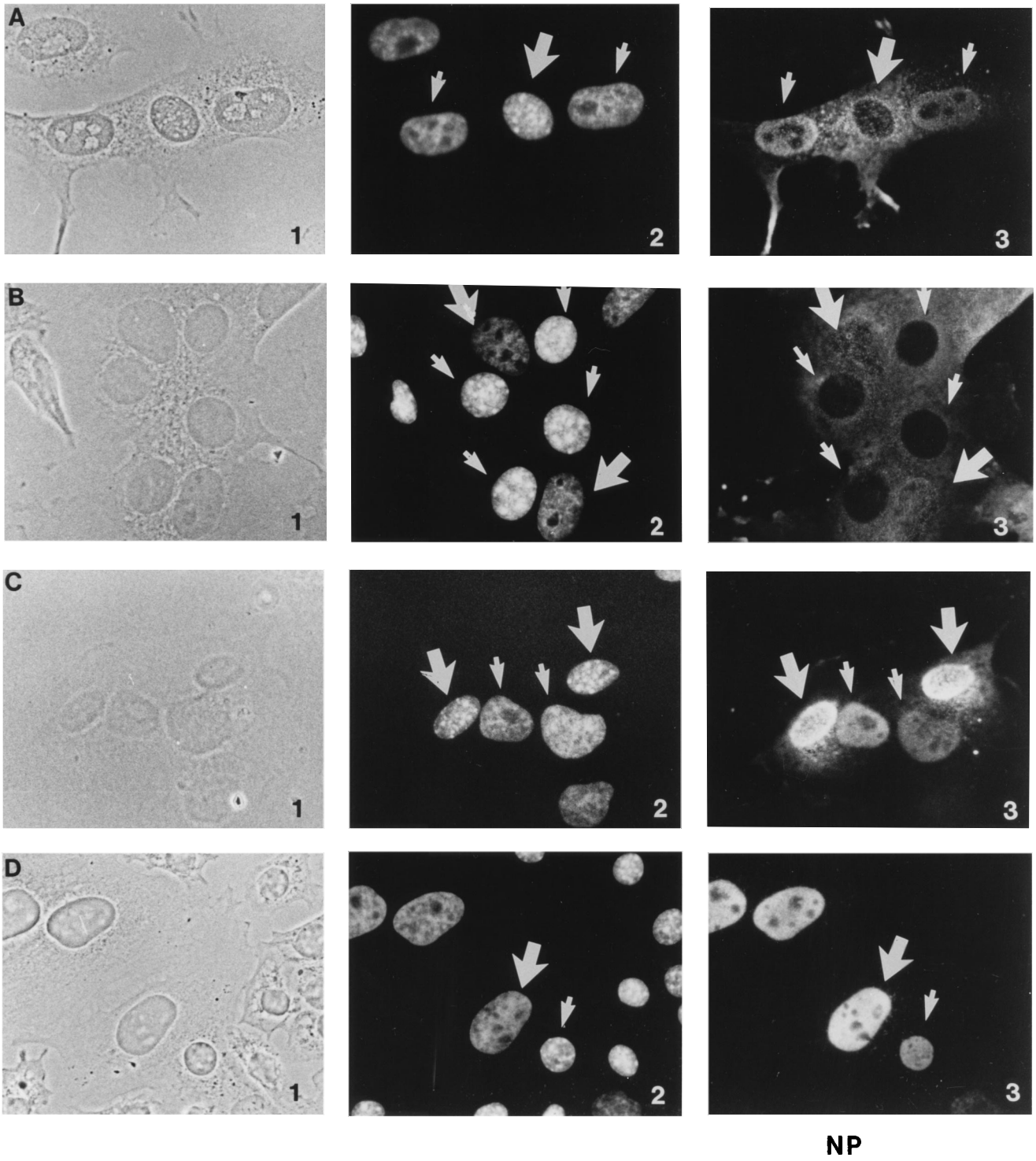

FIG. 8. Trafficking of NP in heterokaryons in the absence of cycloheximide. (A) L929 cells (large arrows) were infected and fused with uninfected HeLa cells (small arrows) at 8 h postinfection and analyzed by phase-contrast microscopy (panel 1), by fluorescence microscopy with the DNA stain Hoechst 33258 (panel 2), or with anti-NP antibodies (panel 3). Trafficking of newly synthesized, free NP can occur into the HeLa cell nucleus but not, apparently, into the L929 cell nucleus. (B) HeLa cells (large arrows) were infected and fused with uninfected L929 cells (small arrows) at 8 h postinfection, and analyzed by phase-contrast microscopy (panel 1), by fluorescence microscopy with the DNA stain Hoechst 33258 (panel 2), or with anti-NP antibodies (panel 3). In this case, trafficking of newly synthesized, free NP cannot occur into the L929 cell nucleus. (C) L929 cells (large arrows) were infected and fused with uninfected HeLa cells (small arrows) at 2.5 h postinfection and analyzed by phase-contrast microscopy (panel 1), by fluorescence microscopy with the DNA stain Hoechst 33258 (panel 2), or with anti-NP antibodies (panel 3). Trafficking of newly synthesized, free NP can occur into the HeLa cell nucleus. (D) HeLa cells (large arrows) were fused with uninfected L929 cells (small arrows) at 2.5 h postinfection and analyzed by phase-contrast microscopy (panel 1), by fluorescence microscopy with the DNA stain Hoechst 33258 (panel 2), or with anti-NP antibodies (panel 3). In this case, trafficking of newly synthesized, free NP can occur into the L929 cell nucleus.

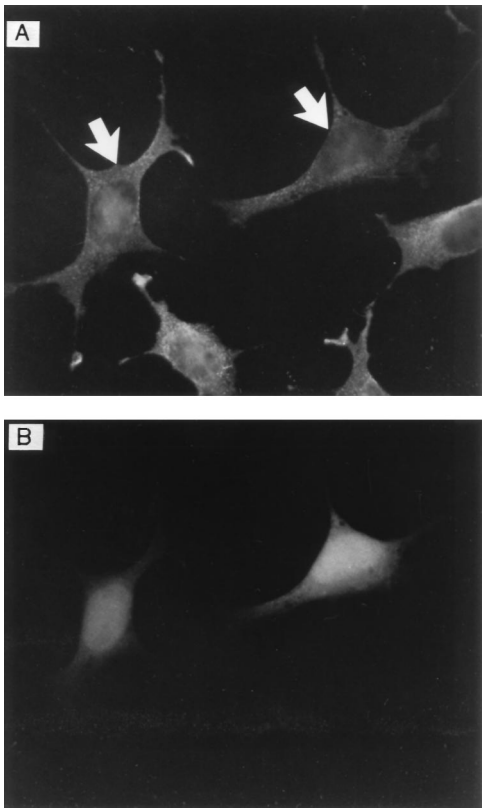

FIG. 9. Influenza virus-infected L929 cell nuclei are import competent. L929 cells were infected with influenza virus and microinjected with TRITC-protein A (1 mg/ml) at 7 to 8 h postinfection. Cells were analyzed by fluorescence microscopy using anti-NP antibodies (A) or directly for protein A (B). Injected cells are indicated with arrows. Influenza-infected cell nuclei are able to import nuclear localization signal-containing proteins late in infection.

fected nuclei and that the newly synthesized M1 is, indeed, a critical factor in preventing such reimport.

We reasoned that the reimport should be blocked if *ts*51-infected cells were fused with cells expressing wild-type M1. We expressed M1 in HeLa cells by using a recombinant SFV, which gave high transient levels of M1 expression. In contrast to *ts*51-infected cells, in which the M1 staining was predominantly nuclear, the distribution of M1 in the transfected cells was both nuclear and cytoplasmic (Fig. 7A, panel 3, small arrows). When *ts*51-infected L929 cells were fused with M1-expressing cells at the nonpermissive temperature, the vRNPs were found to stay in the cytosol (Fig. 7B, panel 2). Thus the wild-type M1 protein prevented the nuclear import of *ts*51 vRNPs, confirming the role of M1 as a blocker of premature nuclear import.

It is noteworthy that in heterokaryons held at the nonpermissive temperature, the mutant M1 was distributed between the *ts*51-infected L929 nuclei and uninfected HeLa nuclei (Fig. 6B, panel 3). Since it had been initially localized in the L929 cell nuclei only, and since cycloheximide was included to block new synthesis, some M1 must have moved from one nucleus to another. This finding indicated that the *ts*51 M1, unlike its wild-type counterpart, was able to shuttle between the nucleus and the cytosol in an infected cell and could therefore equilibrate between nuclei in a heterokaryon. The likely reason for the difference was the abnormal phosphorylation state of *ts*51 M1 (71), which prevented it not only from associating permanently with vRNPs but also from associating with other cytosolic and nuclear structures that normally limit its mobility within the cell. No such redistribution of M1 between nuclei was seen in heterokaryons infected with wild-type virus (Fig. 3) or with *ts*51 at the permissive temperature (Fig. 6A).

Nuclear import of free NP. In the experiments described above, cycloheximide was included after cell fusion to prevent continued synthesis of viral proteins. The signals were thus limited to NP and M1 synthesized prior to fusion. When heterokaryons were examined without added cycloheximide, the fate of newly synthesized, free NP and M1 could also be monitored. In this case, cell fusion experiments were performed both late and early in infection in the presence of NH_4Cl.

When cells were fused after only 2 to 3 h of infection, heterokaryons in which NP was actively synthesized but M1 was not yet expressed were obtained. As shown in Fig. 1 for single cells, all of the NP synthesized during this period accumulated in the nucleus. To determine whether it would also enter and accumulate in an uninfected nucleus, we inspected infected L929 cells that had been fused 2.5 h postinfection with uninfected HeLa cells (Fig. 8C) and vice versa (Fig. 8D). We found that both the L929 and HeLa nuclei were labeled with anti-NP antibodies. There was little or no labeling in the cytosol. These results indicated that early in infection, the newly synthesized NP entered both infected and uninfected cell nuclei. It did not make any difference whether the nuclei were from L929 or HeLa cells. We concluded that unlike assembled vRNPs, the newly synthesized, unassembled NP was able to enter uninfected nuclei in a heterokaryon.

When infected L929 cells were fused with uninfected HeLa cells 8 h postinfection, the starting situation was different; M1 was synthesized and the vRNPs were present in the cytosol. Unlike the vRNPs, the NP synthesized at this time, was found to enter the uninfected nucleus (Fig. 8A, panel 3). The block in nuclear import that prevented vRNP uptake thus did not apply to the free NP.

Surprisingly, a different result was observed in the reverse experiment in which the HeLa cell nuclei were from late-infected cells and the L929 cells were from uninfected cells. In this case, no NP labeling of the L929 cells could be observed (Fig. 8B, panel 3). This result implied that NP, synthesized late in infection, could not enter (or accumulate in) an uninfected L929 cell nucleus. That NP was actually being synthesized at this time was shown by pulse-labeling L929 cells with [^{35}S]methionine at 8 h postinfection (not shown).

To investigate whether the block in import into infected L929 cell nuclei reflected a generalized inhibition of nuclear import, we microinjected infected L929 cells with TRITC-labeled protein A. Protein A is a karyophilic molecule which enters nuclei via the NPC via an active uptake mechanism (38). Consistent with its entering through the nuclear pore, we have found that its import is inhibited by injected monoclonal antibodies against components of the NPC (70). TRITC-protein A was microinjected into L929 cells which had been infected with influenza virus for 7 to 8 h (Fig. 9). Nuclear import appeared to be identical to that seen in microinjected, uninfected cells. It was also independent of the cell line used; infected and uninfected HeLa cells showed identical distributions (not shown). Thus, the ability to import and accumulate molecules into the nucleus, via NPC-specific pathways was unaffected by influenza virus infection. This observation implied that the lack of NP accumulation in the infected L929 nuclei was due to selective exclusion of NP and M1 or rapid export of imported viral proteins back to the cytosol.

Do vRNPs and NP shuttle between nuclei before M1 is synthesized? To determine whether NP, early in infection, was confined to an infected cell nucleus or could shuttle in and out,

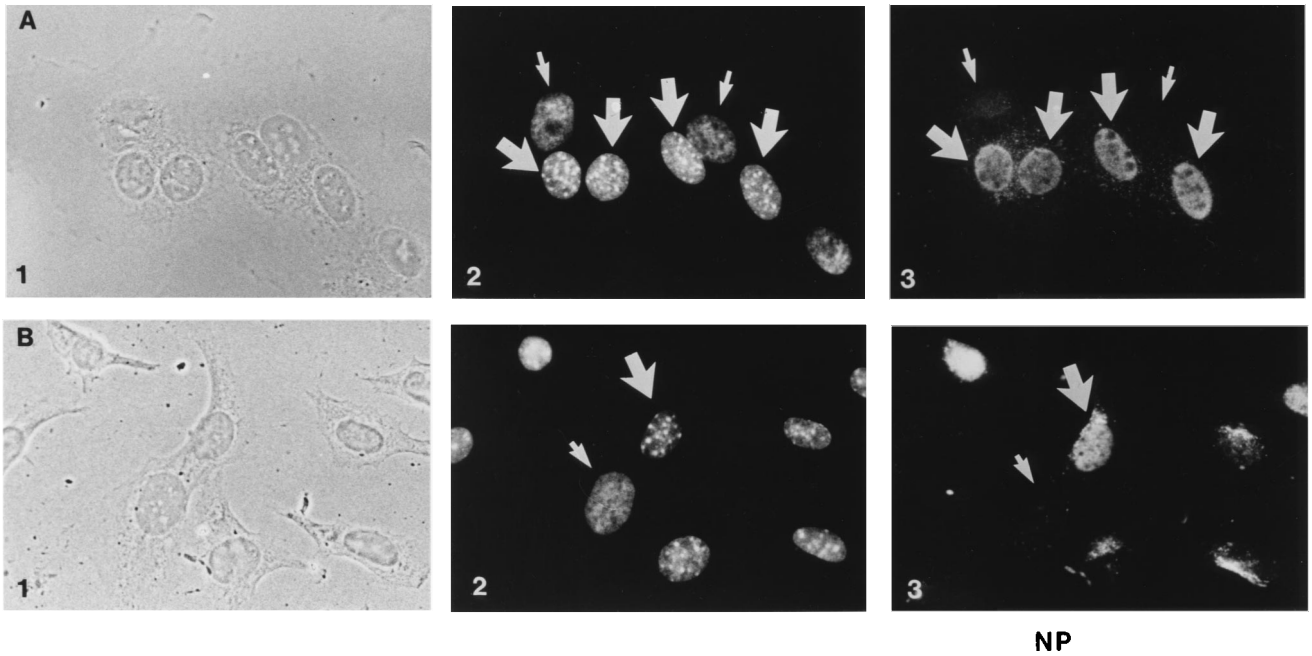

FIG. 10. Shuttling of vRNPs does not occur in the absence of M1. (A) L929 cells (large arrows) were infected with influenza virus and fused with uninfected HeLa cells (small arrows) at 2.5 h postinfection in the presence of cycloheximide. Cells were analyzed by phase-contrast microscopy (panel 1), by fluorescence microscopy with the DNA stain Hoechst 33258 (panel 2), or with anti-NP antibodies (panel 3). Trafficking of vRNPs present in the L929 cell nucleus does not take place into the newly provided HeLa cell nucleus. (B) L929 cells (large arrows) were infected with influenza virus and treated with 75 μM H7 to trap vRNPs in the nucleus. They were fused with uninfected HeLa cells (small arrows) at 8 h postinfection in the absence of cycloheximide. Cells were analyzed by phase-contrast microscopy (panel 1), by fluorescence microscopy with the DNA stain Hoechst 33258 (panel 2), or with anti-NP antibodies (panel 3). Trafficking of vRNPs does not take place into the newly provided nucleus.

uninfected HeLa cells were fused with infected L929 cells 2.5 h after infection. Cycloheximide was added to prevent further synthesis of NP. We found that NP remained confined to the infected L929 nuclei (Fig. 10A). This result showed that once synthesized and imported into the infected nucleus, NP remained there until it was time to undergo M1-mediated export late in infection. Apparently, it did not shuttle between the nucleus and the cytoplasm, because if shuttling had taken place, some of the NP would have been redistributed into the HeLa nucleus.

The protein kinase inhibitor H7 offered an opportunity to monitor, during a prolonged period of time, the fate of NP in the absence of M1 expression. This drug inhibits the synthesis of the late viral proteins (33, 43, 67). Negative-sense vRNA synthesis, although delayed, does take place, and NP is synthesized and transported to the nucleus. We found that when M1 synthesis was inhibited with H7, NP remained confined to the nucleus, even late in infection (41). When H7-treated, infected L929 cells were fused with uninfected HeLa cells (Fig. 10B), NP remained confined to the L929 cell nucleus, with no redistribution to the HeLa cell nucleus. This result confirmed that no NP export or shuttling took place prior to the activation of late protein synthesis. Either the NP itself was unable to exit the nucleus or it was bound to viral RNA or other components in the nucleoplasm.

To analyze if NP could shuttle into and out of the nucleus if the nucleus was uninfected, i.e., devoid of vRNA, we examined the distribution of NP in 3PNP-4 cells, a cell line permanently transfected with NP (37). The expressed NP was localized in most cells to the nucleus (Fig. 11A, panel 3, large arrows). We fused these 3PNP-4 cells with HeLa cells in the presence of cycloheximide. Transfer of NP from the 3PNP-4 cell nuclei to the HeLa cell nuclei could, in this case, be observed over a 4-h period (Fig. 11B, panel 3). This import was, however, relatively slow and incomplete. The NP signal in the HeLa cell nucleus remained weaker than that in the same cells analyzed in the absence of cycloheximide (Fig. 11C), in which case nuclear import of newly synthesized NP occurred. When cells were fixed immediately after fusion, no signal was present in the HeLa cell nuclei, confirming that some shuttling of NP does take place (not shown).

We concluded that in the absence of virus infection, NP can shuttle between the cytoplasm and the nucleus, but that the transfer of NP from one nucleus to another is slower than the import of newly synthesized NP.

DISCUSSION

Since influenza virus replicates in the nucleus and buds from the plasma membrane, its replication cycle involves a complex series of nuclear import and export events. The virally encoded components moving across the nuclear envelope in different phases of the infectious cycle include structural and nonstructural proteins, mRNAs, and inward- and outward-bound vRNPs. Together with previous experiments, the results of this study provide an increasingly detailed picture of this bidirectional traffic and its inherent selectivity. In particular, they highlight the mechanisms that allow directional regulation of the import of incoming vRNPs and export of newly synthesized vRNPs. Specifically, our results show that vRNPs, to be import competent, must assemble into virions and enter a cell via the normal endosomal route. The vRNPs cannot traffic directly from one nucleus to another or reenter the nucleus that they have just left.

The first nuclear transport event in the infectious cycle is the import of incoming vRNPs from the cytosol into the nucleo-

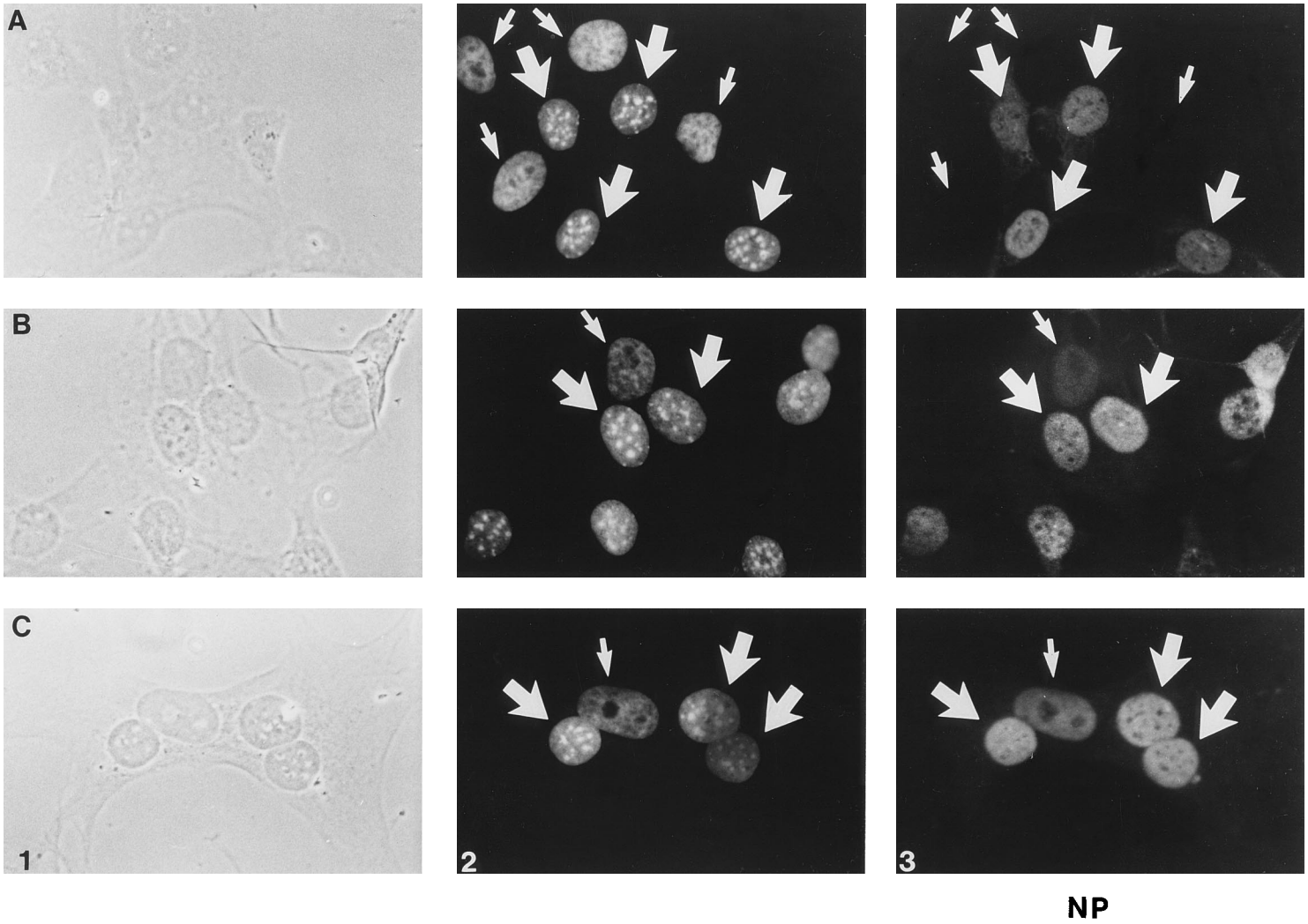

FIG. 11. NP does shuttle between the nucleus and the cytoplasm in the absence of virus replication. 3PNP-4 cells (large arrows) were analyzed directly by immunofluorescence (A) or were fused with HeLa cells (small arrows) and then analyzed 4 h after fusion in the presence (B) or absence (C) of cycloheximide. Cells were analyzed by phase-contrast microscopy (panels 1), by fluorescence microscopy with the DNA stain Hoechst 33258 (panels 2), or with anti-NP antibodies (panels 3). NP shuttles from the 3PNP-4 cell nuclei into the HeLa cell nuclei, but this process is slower than import of newly synthesized protein.

plasm. Our previous studies (43, 44) have shown that the vRNPs enter the nucleus through the nuclear pore. Import can occur only if the M1 protein associated with the vRNPs in the incoming virus particle has been dissociated. M1 dissociation is thought to be triggered by exposure of the nucleocapsid to low pH in the endosomes, and it occurs via an acid-activated proton channel in the viral membrane (22, 43). This channel is made up of the M2 protein (54) and is the target for amantadine (20), an antiviral agent that blocks M1-vRNP dissociation and nuclear import (6, 43).

After transcription in the nucleus, the mRNAs for translation of the early viral proteins are exported from the nucleus and translated in the cytosol. NP and other proteins carry nuclear import and/or retention signals and utilize cellular mechanisms for uptake into the nucleus (2, 9, 26, 50, 75). Our results show that it makes no difference at this stage whether the nuclei are infected or not. It is well known that NP in the infected nucleus associates with vRNA (31) and that replication is, in fact, dependent on the availability of newly synthesized NP (3). Free NP does enter an uninfected nucleus, but we found that it also shuttles between the cytoplasm and the nucleus.

In an infected cell, the uptake of NP into the nucleus continues for many hours despite the fact that assembled vRNPs begin to be exported out of the nucleus around 3 to 4 h postinfection. In the L929 cell line, we observed a cell-type-specific anomaly; at late times postinfection, neither free NP nor M1 entered the nucleus, resulting in a situation in which the nucleus became entirely devoid of M1 and NP labeling. The same block also occurred for M1-free vRNPs in *ts*51-infected cells. Import of other karyophilic proteins was not inhibited. The reasons for this virus protein specific block remain unclear but may be related to a cell-specific change in posttranslational modification of NP or M1, e.g., phosphorylation or dephosphorylation, as both of these proteins are phosphorylated (16, 29, 30, 71). There are many examples of nuclear transport being controlled by phosphorylation, such as import of lamin B and simian virus 40 T antigen (24, 58). Regulation of NP and M1 import and export by some form of phosphorylation event is a promising possibility.

Export of newly assembled vRNPs begins when the synthesis of late proteins is turned on. Of these, M1 is crucial for initiating the export process (43). M1 enters the nucleus and somehow releases the vRNPs for export. Whether it does this directly, by associating with the vRNPs, or indirectly, by supporting some late step in vRNP assembly or inducing vRNP dissociation from the nuclear matrix, is unclear. We previously reported that the hyperphosphorylated M1 protein of *ts*51 is able to perform the release function, but it does not undergo permanent association with the vRNPs (71). This is why the vRNPs transported to the cytosol fail to associate with M1, and virus particles are therefore not formed (56). It is also noteworthy that the vRNPs fail to move to the plasma membrane. Normally, M1 is intimately associated with cytosolic vRNPs being packaged in viral particles.

Our results strongly suggest that one of the functions of M1's association with cytosolic vRNPs is to prevent reimport into the nucleus. The M1-free vRNPs generated in *ts*51-infected cells at the nonpermissive temperature were efficiently imported into nuclei, bypassing the need for assembly into viral particles and entry via the normal endosomal pathway. This finding was consistent with our recent observation that isolated M1-free vRNPs are able to enter the nucleus of cells after microinjection into the cytoplasm, whether pretreated with low pH or not (27). The reimport of *ts*51 vRNPs was effectively abrogated by expression of wild-type M1. In this case, the vRNPs exported from the nuclei of *ts*51-infected cells remained in the cytosol. In addition, recent work from our laboratory has shown that expression of M1 in cells prevents nuclear import of incoming virus and microinjected vRNPs (5).

The most significant difference between import-competent and -incompetent vRNPs seems, therefore, to be the absence or presence of bound M1. To the many functions of M1, we can thus add its role in preventing premature nuclear uptake of newly synthesized vRNPs. This is an important role in a virus that replicates in the nucleus, assembles in the cytosol, and buds from the plasma membrane. It should be remembered that such a role is not automatically present, however, and in the case of hepatitis B virus, it has been suggested that newly assembled capsids do reenter the nucleus and that this leads to continuous reinfection of hepatocytes and chronic disease (49).

The actual mechanisms by which M1 achieves its import-inhibitory effects are not yet clear, but there are several possibilities. It may cover import signals present on the vRNPs and thus prevent their association with receptor proteins for nuclear import. Such a receptor may be NPI-1, a 60-kDa protein which interacts with NP (52) and is a member of the importin family of receptors necessary for nuclear import of nuclear localization signal-containing proteins (1). Alternatively, M1 may mediate interaction between the vRNPs and cytoskeletal elements needed for anchoring and perhaps for transport in the cytosol. Finally, it may be possible that M1 orchestrates the assembly of vRNP multimers which makes the particles too large to enter via the NPC.

It is also possible that M1 acts like other proteins involved in RNA export from the nucleus. One of the best characterized is the Rev protein of human immunodeficiency virus, which binds to both spliced and unspliced viral RNA and promotes its nuclear export (12). Cellular proteins involved in RNA export such as the eukaryotic initiation factor 4E (57) and the cap-binding protein responsible for export of small nuclear RNAs (19, 25) bind to the caps of RNA polymerase II-derived transcripts. While such proteins are likely to be involved in influenza virus mRNA export, they are unlikely to have a role in export of the uncapped vRNAs. While M1 is known to bind to RNA (68, 73), the details of the interaction are poorly understood. The precise conformation of the translocating vRNPs is unknown but appears to be analogous to the nuclear export of specific pre-mRNPs, which are partially unfolded and oriented with the 5′ end of the RNA in the lead, prior to export from the nucleus (46).

As NP import into either infected or uninfected nuclei did not take place in the presence of cycloheximide, there appeared to be no pool of free NP in the cytoplasm that was not part of a vRNP complex (Fig. 4). This finding was in agreement with previous observations by Rees and Dimmock (55), who found that at 4 h postinfection, all cytoplasmic NP was complexed into RNP structures. This observation also indicated that NP and M1 are not free in the cytosol but are bound to other viral or cellular structures. NP synthesized late in infection either rapidly entered the nucleus after synthesis, where it associated with vRNA and was then quickly exported, or interacted directly with previously exported vRNPs in the cytoplasm. M1 was also not retained in the nucleus late in infection, possibly because it was associated either with the cytoplasmic vRNPs (43) or with cellular membranes (74).

In the absence of vRNA, we found that NP shuttled between the nucleus and the cytoplasm. NP, free of vRNA, has been studied quite extensively, by expression of NP often by using virus vectors or in the presence of amino acid analogs (18, 28, 40, 42, 61, 63). In all cases, NP was shown to be a nuclear protein, but the shuttling capacity of NP has not been studied. Interestingly, expressed NP was often found to be in a soluble

fraction of the nucleus, (8, 18), in contrast to influenza virus infection, in which NP was assembled into vRNPs and was insoluble. This difference in nuclear binding may well account for the NP shuttling seen in the 3PNP-4 cells. The 3PNP-4 cell line also expresses the three polymerase proteins (PA, PB1, and PB2), and a role for the viral polymerases in the shuttling of NP cannot be excluded. This is unlikely, however, as no direct association is thought to exist between the viral polymerase complex and NP.

Heterokaryons have previously been used to study nuclear trafficking of nucleolin (4) and heterogeneous nuclear RNPs (53). In both cases, the molecules studied were found to shuttle between the cytoplasm and the nucleus. It has been concluded that nucleocytoplasmic shuttling is a general mechanism for signal transfer between the nucleus and the cytoplasm (35) and that shuttling proteins need not necessarily contain positive export signals (62). Not all nuclear proteins undergo shuttling, however (15). We show here that shuttling of newly synthesized NP, M1, and vRNPs does not take place in the infected cell. The only instances in which we observed shuttling were with *ts*51, when the M1 molecules were defective and both vRNPs and M1 shuttled or when NP was expressed in the absence of viral infection, when it was also a shuttling protein.

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