

Nucleocytoplasmic Traffic Disorder Induced by Cardioviruses

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Some picornaviruses, for example, poliovirus, increase bidirectional permeability of the nuclear envelope and suppress active nucleocytoplasmic transport. These activities require the viral protease 2A^{pro}. Here, we studied nucleocytoplasmic traffic in cells infected with encephalomyocarditis virus (EMCV; a cardiovirus), which lacks the poliovirus 2A^{pro}-related protein. EMCV similarly enhanced bidirectional nucleocytoplasmic traffic. By using the fluorescent “Timer” protein, which contains a nuclear localization signal, we showed that the cytoplasmic accumulation of nuclear proteins in infected cells was largely due to the nuclear efflux of “old” proteins rather than impaired active nuclear import of newly synthesized molecules. The nuclear envelope of digitonin-treated EMCV-infected cells permitted rapid efflux of a nuclear marker protein. Inhibitors of poliovirus 2A^{pro} did not prevent the EMCV-induced efflux. Extracts from EMCV-infected cells and products of *in vitro* translation of viral RNAs contained an activity increasing permeability of the nuclear envelope of uninfected cells. This activity depended on the expression of the viral leader protein. Mutations disrupting the zinc finger motif of this protein abolished its efflux-inducing ability. Inactivation of the L protein phosphorylation site (Thr47→Ala) resulted in a delayed efflux, while a phosphorylation-mimicking (Thr47→Asp) replacement did not significantly impair the efflux-inducing ability. Such activity of extracts from EMCV-infected cells was suppressed by the protein kinase inhibitor staurosporine. As evidenced by electron microscopy, cardiovirus infection resulted in alteration of the nuclear pores, but it did not trigger degradation of the nucleoporins known to be degraded in the poliovirus-infected cells. Thus, two groups of picornaviruses, enteroviruses and cardioviruses, similarly alter the nucleocytoplasmic traffic but achieve this by strikingly different mechanisms.

Picornaviruses, small nonenveloped icosahedral animal viruses with a single-stranded RNA genome of positive (mRNA) polarity, encompass the *Enterovirus*, *Rhinoviruses*, *Cardiovirus*, *Aphthovirus*, *Parechovirus*, and some other genera (64). All essential steps of their reproduction, such as translation, RNA synthesis, and encapsidation, take place in the cytoplasm of infected cells. The nonessential role of the nucleus for their reproduction follows from their ability to fulfill the complete infectious cycle in nuclei-free cytoplasts (31, 60) or cytoplasmic extracts (7, 52, 71). This fact, however, does not mean that the nuclei are not involved in the infectious process. Indeed, virus-specific proteins have been detected in the nuclei of poliovirus-infected (11, 29) and encephalomyocarditis virus (EMCV)-infected (5, 6) cells. Poliovirus proteases 2A and 3C are known to target a variety of nuclear transcription factors and histones (66, 78, 79, 80). The EMCV 2A protein enters the nucleoli and interacts there with a ribosome precursor, contributing thereby to alterations in the translation control of the virus-infected cells (5, 49). Also, nuclear changes occurring during the apop-

otic response to infection with poliovirus (3, 9, 73), coxsackievirus B3 (38), Theiler's murine encephalomyelitis virus (TMEV) (41), and foot-and-mouth disease virus (56) imply that the relevant cytoplasmic host proteins, e.g., caspases and DNases, also find their way into nuclei of the infected cells. On the other hand, picornavirus infection triggers translocation of a number of nuclear host proteins into the cytoplasm where they may stimulate translation (12, 37, 40, 50, 70) and replication (48, 74) of the viral genome. Thus, the macromolecular nucleocytoplasmic exchange plays a significant part in the outcome of the picornavirus infection.

Such exchange, in uninfected cells, is a tightly regulated process. The central role in the nucleocytoplasmic transport is played by a 125-MDa structure called nuclear pore complex (NPC), which forms an aqueous channel in the nuclear envelope. It is composed of about 30 different proteins called nucleoporins (Nup), forming an eightfold symmetrical core, which surrounds the channel and carries filamentous extensions directed to both the cytoplasm and nucleus. Small molecules such as ions, metabolites, and even proteins with a molecular mass below 40 kDa can cross the NPC channel in the nuclear envelope by simple diffusion (25). Generally, the proteins that need to be transported into the nucleus must contain nuclear localization signals (NLS), which are most commonly represented by arginine-lysine-rich motifs (55).

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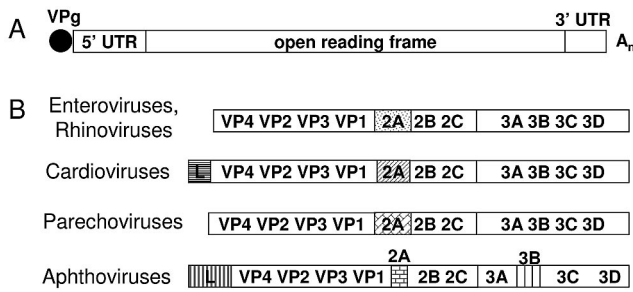


FIG. 1. A schematic representation of picornavirus genomes. (A) The general structure of the genome, which harbors a single open reading frame, flanked with 5' untranslated (5'UTR) and 3' untranslated (3'UTR) regions terminated with a genome-linked viral protein VPg and poly(A) tail, respectively. (B) Differences in the organization of coding regions of some picornaviruses. Nonhomologous regions are differently hatched. Aphthoviruses contain three very similar, though not identical, copies of 3B (VPg)-coding sequences.

These "classical" NLS are recognized in the cytoplasm by their soluble receptor, importin- α/β . The resulting complex docks at a nuclear pore receptor binding site and is transferred into the nucleus, where the cargo dissociates and the receptor is reexported back to the cytoplasm. The directionality of transport is achieved with the aid of a small GTPase Ran, which differently affects the stability of importin-cargo complexes depending on whether it is in a GTP-bound or GDP-bound form (32). The nuclear export machinery works similarly and usually exploits a variety of nuclear export signals and carriers (55). Thus, the NPC, in collaboration with a set of carriers and their regulators, is responsible for the recognition and translocation of specific cargoes in and out of the nucleus, thereby controlling appropriate compartmentation of high-molecular-mass soluble compounds.

Some picornaviruses are known to affect the normal control of nucleocytoplasmic traffic. Two relevant mechanisms have been described. Poliovirus infection induces an increase in the bidirectional permeability of the nuclear envelope, apparently due to the destruction of nuclear pores, as visualized by electron microscopy (8, 10). In addition, active nuclear import via different pathways is also impaired in cells infected with poliovirus and rhinovirus (34, 35). Both these events seem to be caused by the proteolysis of some components on the NPC, such as nucleoporins p62, Nup153, and Nup98 (34), accomplished directly or indirectly by the viral 2A^{pro} protease activity (10; K. Gustin, T. Skern, N. Park, and M. Halver, Abstr. Meeting of the European Study Group on the Molecular Biology of Picornaviruses, Luntenen, The Netherlands, abstr. G08, 2005).

Although different picornaviruses share many aspects of genome organization, they can differ from one another in important features (Fig. 1) (2). Thus, a protein structurally and functionally similar to the enterovirus and rhinovirus 2A^{pro} is absent from cardioviruses, aphthoviruses, and parechoviruses. In fact, the 2A proteins of these latter three virus groups share with 2A proteins of enteroviruses and rhinoviruses nothing except the name and position in the viral polyprotein. On the other hand, several picornaviruses, cardioviruses included, have various types of leader (L) proteins marking the beginnings of their reading frames. In some instances, e.g., in aphthoviruses, the L proteins possess protease activity (22, 68),

whereas in others, e.g., cardioviruses, they do not exhibit proteolytic or any other known enzymatic activity. The actual function of L in the cardiovirus life cycle is yet to be defined, although it was reported that it might affect host translation (81), interferon response (19, 82; S. Hato et al., submitted for publication), and, in the case of TMEV, distribution of the proteins between the nucleus and cytoplasm (19).

Taking into account the essential role of poliovirus 2A^{pro} in triggering alterations of the nucleocytoplasmic traffic, on the one hand, and the absence of a homologous protein in cardioviruses, on the other hand, we decided to study whether EMCV infection is accompanied by similar alterations of the cellular infrastructure, and if so, what viral protein(s) may be involved. As reported here, two closely related EMCV strains do facilitate the bidirectional relocation of proteins between the nucleus and cytoplasm, and it is the L protein which is largely, if not entirely, responsible for this phenomenon.

MATERIALS AND METHODS

Cells and viruses. Cells were grown on petri dishes in Dulbecco's modified Eagle's medium with 10% bovine serum for HeLa-B cells (a subline of HeLa cells) (73) and HeLa-3E cells (constitutively expressing 3 \times EGFP-NLS, i.e., three copies of the enhanced green fluorescent protein fused to the simian virus 40 [SV40] NLS) (10) or with 10% fetal bovine serum for BHK-21 cells. The cells were washed with serum-free medium and infected with cardioviruses at a multiplicity of infection (MOI) of 30 PFU/cell, if not indicated otherwise. After 30 min of adsorption with agitation at 18°C, the cells were washed again and incubated with 5% CO₂ in serum-free medium at 37°C for various time intervals, with or without inhibitors. Two strains of EMCV were used: a strain obtained from Yuri Drygin (Moscow State University) and named here EMCV and strain mengo (mengovirus). Derivates of mengovirus were described previously (81, 82; S. Hato et al., submitted).

Plasmids. The plasmid pEGFP-nuc was generated by fusing three copies of the SV40 NLS to the C terminus of EGFP. The cyclin B-EGFP-encoding vector was a gift from Dmitry Bulavin (National Cancer Institute). The plasmids pTimer-NLS encoding the Timer protein (72) fused to the SV40 NLS (10), pLg encoding firefly luciferase under the control of the TMEV internal ribosome entry site (59), and pE-luc, an EMCV-based replicon containing the firefly luciferase gene in place of a portion of the region encoding the VP3 and VP1 capsid proteins (5), were described previously. Derivative EMCV replicons, pE-luc- Δ 2A and pE-luc- Δ 3D, harbored deletions in the 2A- or 3D-coding sequences, respectively (5). Another replicon with a deletion in the leader protein, pE-luc- Δ L, had a similar organization, but the leader sequence was almost completely excised. This was achieved by the removal of a fragment between a natural EMCV BssHI site (within a 5' part of the L-coding sequence) and an artificial BssHI site, engineered immediately upstream of the VP4-coding sequence. The remaining 5' terminal portion of the L-coding RNA segment (21 bases encoding 7 amino acids) is known to be important for efficient translation (42). The modified region of the replicon was verified by sequencing. Plasmids encoding the full-length mengovirus genome or its mutants harboring alterations in the Zn finger motif or a phosphorylation site (T47) of the leader protein have been described previously (81, 82; S. Hato et al., submitted).

Transient transfection. For transfection, Lipofectamine 2000 (Invitrogen) or Fugene (Roche) was used essentially according to the manufacturers' recommendations. Briefly, the reagent was diluted in Dulbecco's modified Eagle's medium, mixed with 1 to 2 μ g of DNA or 3 μ g of RNA, incubated for 20 min at room temperature, and added to monolayers of HeLa or BHK-21 cells.

Preparation of cell extracts. HeLa-B cells were grown in 1.5-liter roller bottles until near confluence and then mock-infected or infected with EMCV at an input MOI of \sim 30 PFU/cell. After incubation for 4.5 h at 37°C, cells were collected by treating with EDTA, placed into a hypotonic buffer (50 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol [DTT], 10 μ g/ml cytochalasin B, pH 7), and subjected to three cycles of freezing-thawing, with subsequent centrifugation at 100,000 \times g, as previously described (10).

Cell permeabilization. Monolayers of HeLa-3E cells were treated with digitonin (1), as previously described (10). Briefly, the cells were washed consecutively with phosphate-buffered saline and the permeabilization buffer (50 mM PIPES, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, pH 7), exposed to

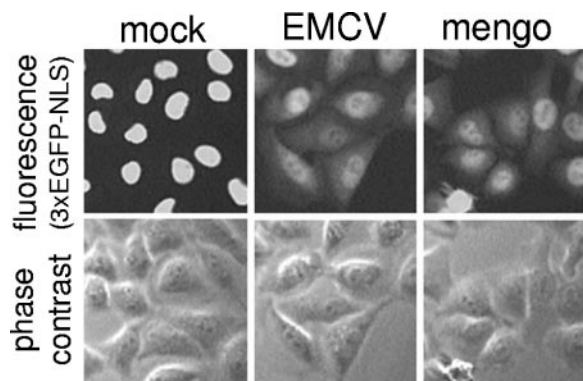


FIG. 2. Exit of a nuclear protein to the cytoplasm upon cardiovirus infection. Mock-infected and cardiovirus-infected HeLa-3E cells. Images were captured at 3 h p.i.

the detergent (40 μ g/ml for 5 min), and then carefully washed again with digitonin-free permeabilization buffer. The digitonin solution was supplemented with Hoechst 33258 (Sigma) stain, which penetrates through the plasma membrane relatively slowly. Under the conditions used, the permeabilized cells were stained well, whereas the cells with intact plasma membranes were stained poorly, if at all.

Nuclear envelope permeability assay. Permeabilized HeLa-3E cells were overlaid with appropriate cell extracts, usually diluted 10-fold in permeabilization buffer. Samples were incubated for 1 h at 37°C and examined under an epifluorescence microscope. Effects of the following protease inhibitors were tested on the capacity of cellular extracts to trigger nuclear protein efflux: chymostatin, leupeptin, pepstatin A, antipain, phenylmethylsulfonyl fluoride, *N*-(methoxysuccinyl)-L-alanyl-L-alanyl-L-prolyl-L-valine chloromethylketone (MPCMK), *N*-benzyloxycarbonyl-L-valyl-L-alanyl-L-aspartyl-fluoromethylketone (zVAD.fmk) (all from Sigma), and *N*-benzyloxycarbonyl-L-valyl-L-alanyl-L-aspartyl-(*O*-methyl)-fluoromethylketone [zVAD(Ome).fmk] (Enzyme Systems or Sigma), as well as Zn^{++} and Cd^{++} ions. The extracts were preincubated with the inhibitors for 10 min at 4°C before being added to the permeabilized cells.

Assay for nucleoporin degradation by Western blotting. HeLa cells were seeded in a six-well plate, and the next day they were infected with the specified viruses at an MOI of 50 50% tissue culture infective doses for 6 or 7 h. After infection, cells were washed with phosphate-buffered saline and lysed in Laemmli buffer. Samples were separated on a gradient (4 to 12%) polyacrylamide gel, transferred onto a nitrocellulose membrane, and probed with monoclonal antibody (MAb) 414, a mouse ascites MAb raised against an NPC mixture and that recognizes the conserved domain FXFG repeats in nucleoporins like p62 and Nup153 (Covance, Berkeley, Calif.). As a loading control the blot was probed with an antibody against β -actin.

Fluorescence microscopy. Examination of the cells was performed with a Leica DMLS fluorescent microscope equipped with green I3 (for the Timer-protein and GFP-derivatives) and blue A (for Hoechst-stained nuclei) filter cubes. The images were captured with a Leica DC100 digital camera.

Electron microscopy. The cells were fixed in 2.5% glutaraldehyde (Sigma) prepared on Sorensen phosphate buffer (pH 7.4), postfixed in 1% OsO₄ (Sigma), dehydrated (70% ethanol containing 2% uranyl acetate), and embedded in Epon 812 (Fluka). Ultrathin sections were cut with an LKB Ultratome-III, stained with lead citrate, and examined with an HU-12 electron microscope (Hitachi).

Luciferase assay. The luciferase assay was performed with a Luciferase Assay System Kit (Promega) according to the manufacturer's recommendations, using a 1250 Luminometer (LKB Wallac).

In vitro transcription. The plasmids encoding pE-luc derivatives and mengovirus mutants were linearized with SalI and BamHI, respectively, and used for a T7 polymerase-driven transcription reaction with consequent purification of the transcripts in a sucrose density gradient, as previously described (58). The quality of RNA was checked by electrophoresis in agarose gel.

Isolation of viral RNA. Isolation of viral RNA was carried out by phenol extraction of BHK cell-grown mengovirus preparations obtained by centrifugation of virus-containing medium at 100,000 \times g through a 30% sucrose cushion.

In vitro translation. The RNA transcripts were translated in extracts from Krebs-2 cells essentially as previously described (71). Briefly, the lysate obtained by Dounce homogenization in a hypotonic buffer (25 mM HEPES-KOH, pH 7.3,

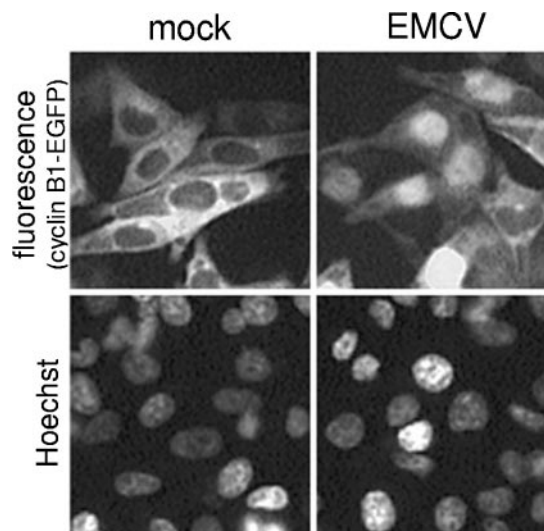


FIG. 3. Entry of a cytoplasmic protein into the nuclei upon cardiovirus infection. HeLa cells were transiently transfected with a vector encoding cyclin B1-EGFP and then infected with EMCV at 24 h posttransfection. Hoechst 33342 (a DNA dye that permeates plasma membrane) was added, and the images were captured at 3 h p.i.

50 mM KCl, 1.5 mM MgCl₂, 1 mM DTT) was supplemented with a one-ninth volume of a 10 \times translation buffer (25 mM HEPES-KOH, pH 7.3, 1 M KCH₃COO, 30 mM MgCl₂, 30 mM DTT) and centrifuged at 18,000 \times g for 20 min at 4°C. The supernatant was stored at -70°C as small aliquots. Before being used for translation, the lysates were treated with micrococcal nuclease. The translation mixtures (40 μ l) contained 20 μ l of the nuclease-treated lysate, 4 μ l of a master mix (10 mM ATP, a 2 mM concentration of each of the three other nucleoside triphosphates, 100 mM creatine phosphate, 1 mg/ml of rabbit muscle creatine phosphokinase, a 0.2 mM concentration of each of 19 unlabeled L-amino acids [without methionine], 125 mM HEPES-KOH, pH 7.3), 4 μ l of a salt solution (0.75 M KCH₃COO, 10 mM MgCl₂, 2.5 mM spermidine), 0.5 μ l of [³⁵S]methionine or 0.02 mM L-methionine, and RNA transcript (700 ng/sample). The samples were incubated for 3 h at 37°C. The radiolabeled probes were counted for isotope incorporation into the trichloroacetic acid-insoluble material and analyzed by a 12% polyacrylamide gel using a PageRuler Prestained Protein Ladder (Fermentas) as a marker with consequent radiography. Products of replicon translation (reactions lacking ³⁵S) were tested for luciferase activity and for their effects on the nuclei of permeabilized cells.

RESULTS

Cardioviruses induce redistribution of nuclear and cytoplasmic proteins. To ascertain whether cardiovirus infection alters the nucleocytoplasmic traffic, HeLa-3E cells expressing a fluorescent nuclear protein marker (3xEGFP-NLS) were infected with EMCV or mengovirus and then examined under an epifluorescence microscope. In control cells, fluorescence was confined exclusively to the nuclei. However, at 3 h postinfection (p.i.), the nuclear marker was observed also in the cytoplasm of the majority of the cells (Fig. 2), indicating that the infection facilitated efflux of NLS-containing proteins from the nucleus. The infection also triggered the penetration of cytoplasmic proteins into the nucleus. Indeed, HeLa cells expressing a fluorescent cytoplasmic protein (cyclin B1 fused to EGFP), and consequently exhibiting primarily cytoplasmic fluorescence before infection, demonstrated the appearance of this protein in the nucleus after infection (Fig. 3).

To investigate whether the accumulation of nuclear protein in the cytoplasm was due to its exit from the nucleus or to the

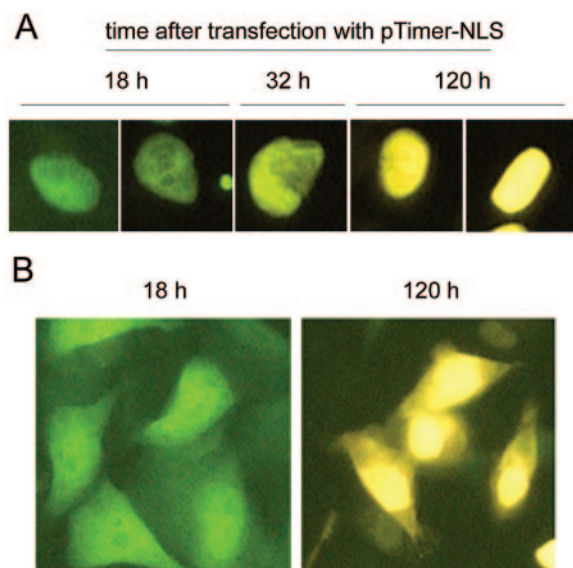


FIG. 4. Cytoplasmic accumulation of the nuclear protein in cardiovirus-infected cells is due to its efflux from the nucleus. (A) Uninfected HeLa cells were transiently transfected with the vector encoding Timer-NLS and observed for different time intervals. Nuclei of these cells change the color of fluorescence from green to yellow with time. (B) HeLa cells transiently expressing Timer-NLS were infected with EMCV at different times after transfection. Note that fluorescence is readily observed in the cytoplasm and that the fluorescence color is the same as that of nuclear fluorescence. At 120 h posttransfection, fluorescence corresponded to the “old” protein species present in the nucleus prior to the infection. Images were captured at 4 h p.i.

cytoplasmic retention of newly synthesized molecules as a result of impaired active nuclear import, experiments with cells expressing the NLS-fused “Timer” protein were performed. The *Anthozoa*-derived Timer protein contains a GFP-like fluorophore, which is autocatalytically modified in a time-dependent but concentration-independent manner, changing fluorescence toward the red spectral area (72). Because of its NLS, Timer was observed exclusively in the nuclei of uninfected cells. When observed with a green filter, this protein changes the color of its fluorescence from green to yellow, marking its age relative to synthesis. In pure preparations, the transition from “young” to “old” forms of the protein takes about 8 h (72). But in uninfected cells expressing Timer, a clear-cut color transition requires a few days (Fig. 4A), perhaps because of the continuous replenishment of the newly synthesized “young” form. Indeed, when such replenishment is prevented by inhibition of translation, the nuclei of Timer-NLS-expressing cells are reported to change their fluorescence to yellow in less than 12 h (10). When HeLa cells were transfected with pTimer-NLS and then infected with EMCV 18 h or 120 h later, there was readily observable movement of fluorescence into the cytoplasm by 4 h p.i. (Fig. 4B). Importantly, the color of the cytoplasmic fluorescence corresponded to that observed in the nuclei, indicating that it was due to the “old” material already present in the nuclei prior to the infection and translocated into the cytoplasm after the infection.

We concluded that cardioviruses, like enteroviruses, disturb the normal asymmetry of nucleocytoplasmic protein distribution by facilitating bidirectional traffic through the nuclear envelope.

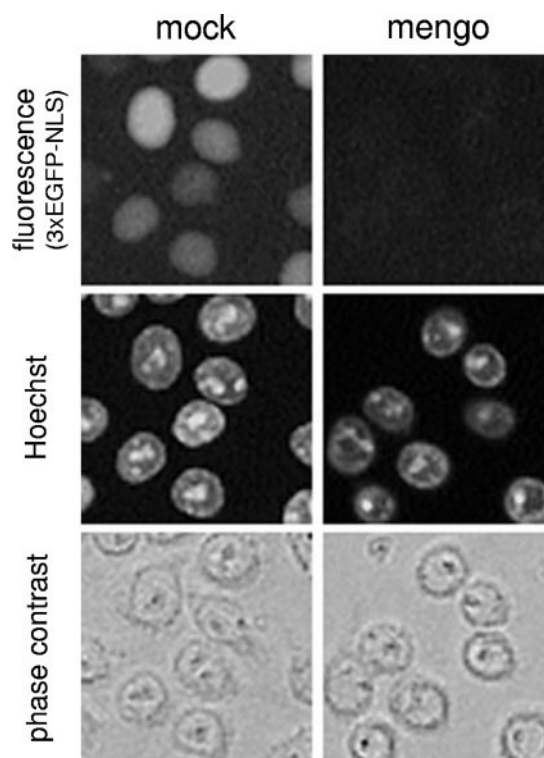


FIG. 5. EMCV-induced leakiness of the nuclear envelope. HeLa-3E cells were mock-infected or infected with EMCV, treated with digitonin at 3 h p.i., and examined for fluorescence within 3 to 5 min. The majority of nuclei in mock-infected cells exhibit fluorescence, whereas nuclei from infected cells lost their ability to retain the marker nuclear protein, pointing to an increase in nuclear envelope permeability. Staining with Hoechst 33258 allowed visualization of permeabilized cells because, under the conditions used, the cells with intact plasma membranes stained poorly, if at all, with this dye (data not shown).

The nuclear envelope becomes leaky in infected cells. To investigate whether cardiovirus-induced redistribution of proteins was caused by an increase in the leakiness of the nuclear envelope, experiments with permeabilized HeLa-3E cells (10) were performed. In digitonin-treated cells, the plasma membrane becomes damaged and permeable even for macromolecules, while the nuclear envelope essentially retains its barrier properties (1). EMCV-infected cells were treated with digitonin at 3 h p.i. and examined 3 to 5 min later. As evidenced by their staining with Hoechst 33258, the treatment resulted in the permeabilization of nearly all of the cells. While permeabilized uninfected cells mostly retained their nuclear fluorescence, the overwhelming majority of infected cells lost it completely, or nearly so (Fig. 5). This loss took place in the absence of an exogenous energy source, strongly suggesting that it was due to an increased leakiness of the nuclear envelope, and occurred by passive diffusion rather than active translocation.

Extracts from EMCV-infected cells induce leakiness of the nuclear envelope. Cardioviruses translate and replicate in the cytoplasm. Therefore, a putative viral inductor of permeabilization of the viral envelope is expected to be generated in the cytoplasm. To ascertain whether this was the case, extracts from EMCV-infected cells were assayed for their capacity to disturb the nuclear envelope barrier function of uninfected,

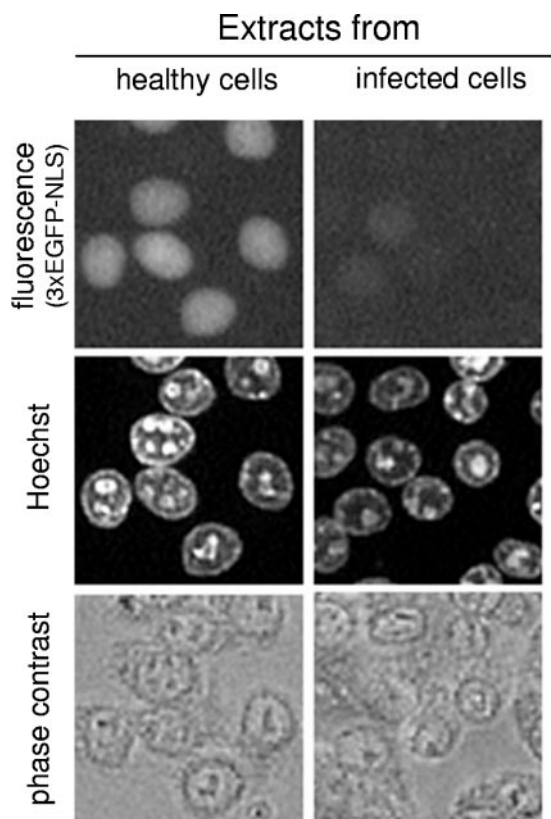


FIG. 6. Extracts from EMCV-infected cells induce nuclear envelope leakiness in permeabilized, uninfected cells. HeLa cells were mock-infected or infected with EMCV, and 4 h later, they were used to prepare S-100 extracts. The extracts, diluted 1:10 were added to monolayers of permeabilized Hoechst 33258-stained uninfected HeLa-3E cells for 1 h.

digitonin-permeabilized HeLa-3E cells. As demonstrated above, such permeabilized cells retained the fluorescent NLS-containing marker protein in their nuclei. However, after treatment with extracts from EMCV-infected HeLa cells, but not extracts from the mock-infected cells, the fluorescence of such preparations was lost (Fig. 6). Thus, the extracts from the infected cells contained an activity or activities capable of inducing permeabilization of the nuclear envelope of uninfected cells.

It should be noted that the extracts from EMCV-infected cells were markedly less active in this assay than equivalent extracts from poliovirus-infected cells. The latter usually retained their ability to induce the nuclear efflux after about a 1,000-fold dilution (10), while the extracts from EMCV-infected cells typically lost their relevant activity after being diluted more than 10-fold (data not shown). Thus, the EMCV-induced activity appeared to be some two orders of magnitude lower than that triggered by poliovirus infection. Nevertheless, the EMCV activity was sufficient to achieve essentially the same overall biological effect during a natural infection. The permeabilizing activity of the extracts from poliovirus-infected cells has been reported to be suppressed by inhibitors of the viral protease 2A^{Pro}, such as MPCMCK, antipain, chymostatin, zVAD(OMe).fmk, Zn⁺⁺, and Cd⁺⁺ (10). However, in similar assays, none of these inhibitors affected the relevant activity of extracts from EMCV-infected cells (Table 1). Nor did any

other tested protease inhibitors prove able to suppress the nuclear permeabilization ability of extracts from EMCV-infected cells. However, this ability was lost after incubation of the extracts at 65°C for 30 min, indicating that it was thermolabile and hence likely represented by a protein.

The viral leader protein triggers permeabilization of the nuclear envelope. To identify the viral product(s) responsible for the phenomena observed, we made use of a recombinant replicon expressing the EMCV genome, with a luciferase gene in place of a portion of the viral capsid sequence (5). Two series of experiments were performed with this replicon and its derivatives. First, the constructs were translated *in vitro*, and the protein products were added to permeabilized uninfected HeLa-3E cells. Since different replicon transcripts somewhat differed in their translational efficiencies, the relevant samples were appropriately diluted to equalize the expressed luciferase activities. With extracts templated by the parental replicon RNA (pE-luc) (Fig. 7, wt), the loss of fluorescence was readily observed (Fig. 7A), confirming that EMCV encodes a product(s) triggering permeabilization of the nuclear envelope. Three mutant derivatives of this replicon were tested under similar conditions. The products of *in vitro* translation of a construct lacking the EMCV protein 2A-coding sequence as well as a construct encoding an inactive 3D^{Pol} did not differ in their permeabilization-inducing activity from the original replicon. On the other hand, the replicon lacking the sequence for the leader protein was unable to induce efflux of the fluorescent protein from nuclei of permeabilized cells (Fig. 7A).

In a parallel set of experiments, the same replicon transcripts were transfected into HeLa-3E cells, and their capacity to trigger efflux of the nuclear fluorescent protein was monitored. As shown in Fig. 7B, transfection of any of the three leader-encoding RNA sequences (Fig. 7B, wt, Δ 2A, and Δ 3D) resulted in the appearance of a significant proportion of cells with cytoplasmic fluorescence (in assessing appropriate values given below the panels, one should take into consideration the relative inefficiency of transfection with the replicons). The L-lacking replicon was completely devoid of a similar level of activity.

Additional support for the involvement of the cardiovascular L protein in the alteration of intracellular trafficking was obtained with a mengovirus mutant engineered to lack the L-coding sequence. In contrast to wild-type mengovirus (Fig. 8a

TABLE 1. Protease inhibitors that failed to inhibit EMCV-induced efflux from the nuclear envelope

Inhibitor	Concn (mM)	Known targets (reference)
MPCMCK	0.5	Elastase, polio 2A ^{Pro} (53)
Antipain	0.7	Serine and cysteine proteases, polio 2A ^{Pro} (53)
Chymostatin	0.3	Serine and cysteine proteases, polio 2A ^{Pro} (53)
zVAD(OMe).fmk	0.1	Caspases, rhino 2A ^{Pro} (21)
zVAD.fmk	0.1	Caspases
Leupeptin	2.0	Serine and cysteine proteases
PMSF ^a	0.6	Serine and cysteine proteases
Pepstatin A	1.0	Acid proteases
Zn ⁺⁺	2.5	Proteases, polio 2A ^{Pro} (44)
Cd ⁺⁺	1.0	Proteases polio 2A ^{Pro} (44)

^a PMSF, phenylmethylsulfonyl fluoride.

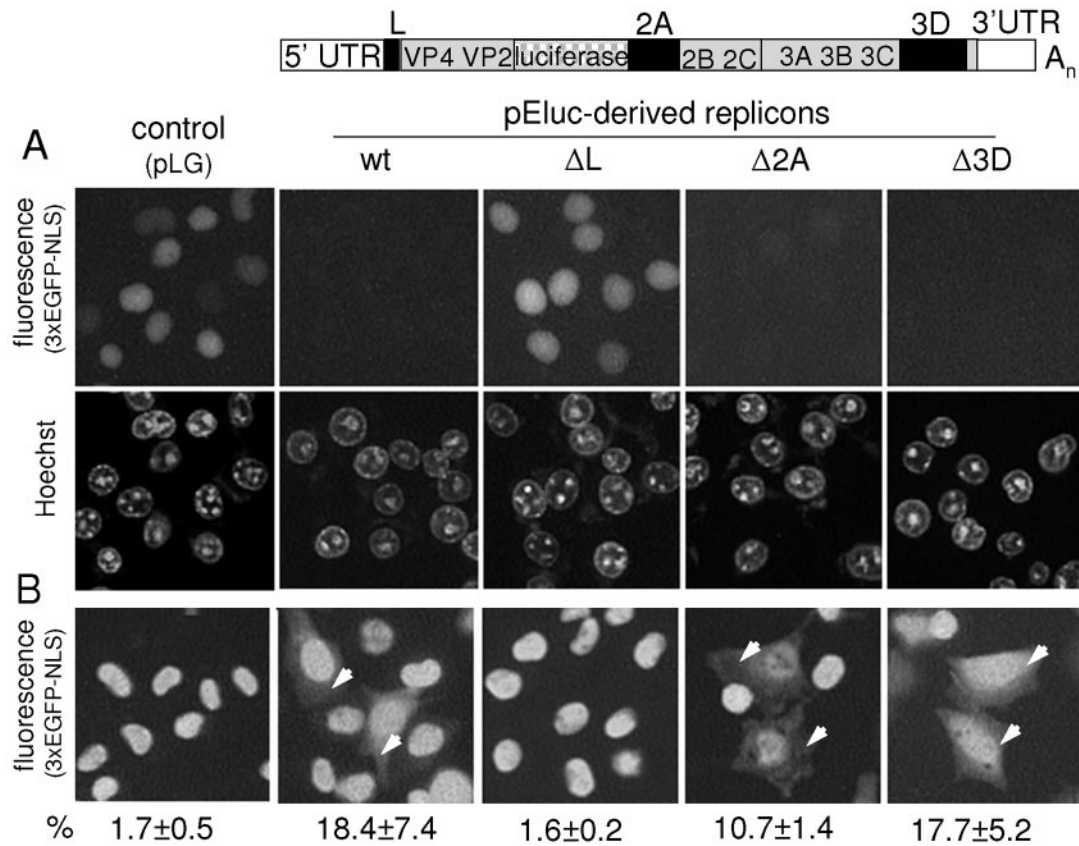


FIG. 7. Leader protein triggers permeabilization of the nuclear envelope. (A) RNA transcripts from EMCV-derived replicons harboring a firefly luciferase gene in place of a portion of the capsid-coding sequence were translated *in vitro*. The indicated replicons contained engineered deletions in the leader, 2A, or 3D sequences. Samples of the translation reactions were added to digitonin-permeabilized Hoechst 33258-stained HeLa-3E cells and incubated for 1 h. A transcript of pLG, encoding only the luciferase under the control of the TMEV internal ribosome entry site (58), was translated and assayed in parallel. Before being added to the cells, the translation products were assayed for luciferase activity and, based on the results of this assay, diluted to a normalized concentration. (B) RNA transcripts from the same replicons were transfected into HeLa-3E cells, and the samples were examined by microscope 6 h later. Cytoplasmic fluorescence in transfected cells is indicated by arrows. The percentage of cells with cytoplasmic fluorescence is indicated below each panel. wt, wild-type.

and f), this mutant failed to trigger redistribution of the nuclear marker protein in HeLa-3E cells (Fig. 8b and g).

We concluded that an intact cardiovascular leader protein is required to observe permeabilization of the nuclear envelope.

Functional motifs of the cardiovascular L protein involved in induction of nuclear envelope leakiness. To elucidate which structural elements of the L protein might be required to make the nuclear envelope permeable, several additional mengovirus mutants were created and tested. The L protein contains a predicted Zn finger near its N terminus (16, 24) and is phosphorylated at Thr47 (82). A mutant with the Zn finger domain of L disturbed by two engineered substitutions (Cys19→Ala and Cys22→Ala) was unable to induce relocation of the nuclear marker (Fig. 8c and h). Another virus with a mutation in a phosphorylation site (Thr47→Ala) did induce the efflux, but the phenotype showed a significant delay. The protein relocation was clearly evident by 5 h p.i. (Fig. 8i) but not at 3 h p.i. (Fig. 8d). Supporting a potential functional role for phosphorylation at this site, introduction of the phosphate-mimicking glutamate mutation, in place of threonine (Thr47→Glu), gave a pseudorevertant virus that was again efficient at inducing nuclear efflux at 3 h p.i. (Fig. 8e and j).

The functional significance of both the Zn finger and the phosphorylation site of the leader protein was confirmed also in experiments in which products of the *in vitro* translation of appropriate viral RNAs were assayed for their efflux-promoting activity. RNAs isolated from partially purified virions of different mutants were translated *in vitro*, and products of this translation were investigated by polyacrylamide gel electrophoresis. The products directed by all RNAs were shown to be equally efficiently processed (Fig. 9B). Then these products were added to permeabilized HeLa-3E cells and were investigated for their capacity to trigger nuclear efflux of the fluorescent marker NLS-containing protein. The templates containing L-damaging mutations (C19A/C22A or T47A) failed to generate a product promoting the efflux, whereas the RNA encoding the phosphorylation-mimicking mutation (T47E) in the leader protein proved to be fully, or nearly so, active in this respect (Fig. 9A). Similar conclusions could be also drawn from the experiments in which wild-type and mutant RNAs used as translation templates were generated by *in vitro* transcription of appropriate plasmids (data not shown).

It should be noted, however, that the leader mutations tested above markedly affected the efficiency of mengovirus

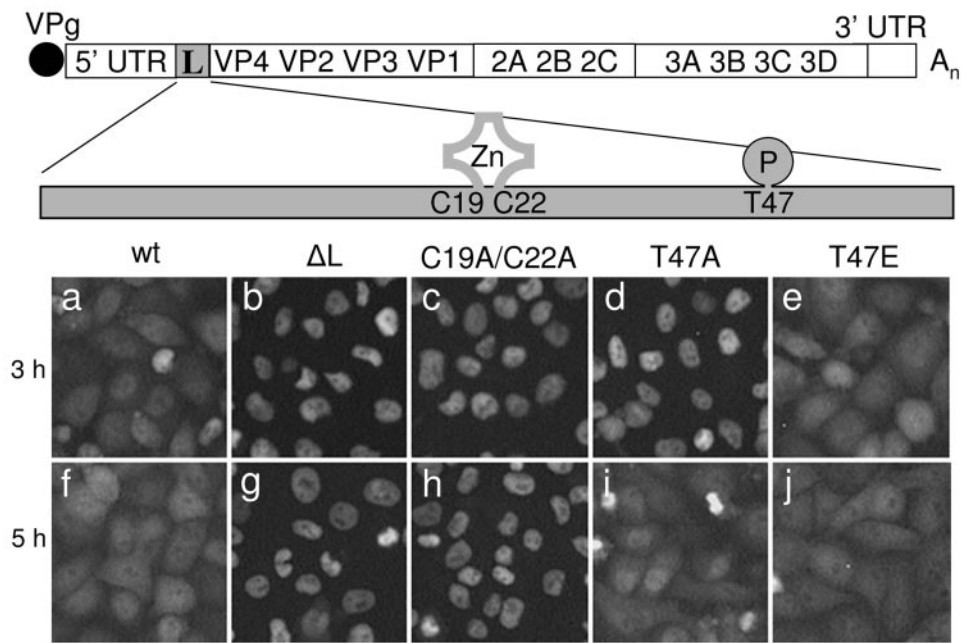


FIG. 8. Effects of mutations within the mengovirus leader protein on nucleocytoplasmic trafficking. HeLa-3E cells were infected with the indicated mengovirus variants at an MOI of 10 PFU and examined at different time intervals. The wild-type (wt) virus induced efflux of the marker nuclear protein into the cytoplasm as early as at 3 h p.i. The deletion within the leader (ΔL) or a double point mutation disturbing the zinc finger domain (C19A/C22A) resulted in a virus unable to induce the efflux. A T47A substitution, abolishing a phosphorylation site, resulted in a delay of nuclear efflux, while no such delay was seen with a pseudoreversion mutation mimicking phosphorylation at this position (T47E).

reproduction in HeLa cells (not shown). It might be argued, therefore, that the inability of these mutants to affect the nucleocytoplasmic traffic was a consequence of poor viral reproduction and low virus yield rather than a direct effect of altered L protein. To assess the validity of this argument, the mengovirus leader mutants were retested for their effects on the intracellular protein redistribution in BHK-21 cells, where dependence on L protein and differences among the above set of mutants with respect to their growth potential are not so marked (15, 81) (Fig. 10B). The results (Fig. 10A) fully confirmed the significance of both the Zn finger motif and the phosphorylation site as contributors to the phenotype of mengovirus L protein and its ability to influence the permeability of the nuclear envelope.

Modifications of the nuclear envelope upon cardiovirus infection. Increased bidirectional permeability in the poliovirus-infected cells was accompanied, and likely associated, with destruction of the nuclear pores readily detectable by electron microscopy (10). To investigate whether a similar alteration of the nuclear envelope took place upon cardiovirus infection, mengovirus-infected HeLa cells were subjected to the same analysis. The cross-sections and tangential sections of nuclear envelope were made. In uninfected cells (Fig. 11a to g), nuclear pores appeared as distinct substructures arranged in a complex with eightfold radial symmetry. The major visible components of the NPC were two coaxial annuli (facing the cytoplasm and nucleoplasm, respectively), a central body or transporter globule, and internal filaments connecting this globule with the pore periphery. The chromatin was condensed and aligned the internal nuclear membrane. In infected cells (Fig. 11h to n), the pore complexes did not appear to be totally destroyed, and their

diameters were comparable to those in the mock-infected cells, but their structural organization was markedly altered: the central bodies were missing from most of the NPCs and subunits of the annuli were not clearly visible, as seen particularly clearly in tangential sections (compare panels l to n and e to g in Fig. 11). Chromatin appeared to be more condensed with less dense areas adjoining the pores.

We conclude that mengovirus infection leads to a significant morphological alteration of the NPC, the phenomenon closely resembling that observed in poliovirus-infected cells.

The nucleoporins that are degraded upon enterovirus infection are stable in mengovirus-infected cells. As mentioned above, infection of cells with poliovirus and rhinovirus triggers degradation of nucleoporins p62 and Nup153 (34, 35), which may be a factor contributing to NPC destruction. Degradation of the nucleoporins has been demonstrated by Western blotting with MAb 414 from Covance that recognizes the nucleoporin's FXFG repeats. By using the same antibody and the same technique, we extended this observation also to another enterovirus, coxsackievirus B3 (Fig. 12). However, no appreciable degradation of these nucleoporins could be detected in the cells infected with either wild-type mengovirus or its C19A/C22A mutant (Fig. 12), providing another strong argument in favor of a fundamental difference between the mechanisms underlying damage to the nucleocytoplasmic traffic in cells infected with enteroviruses, on the one hand, and with cardioviruses, on the other.

Staurosporine modulates the efflux-triggering activity of the L protein. The modulating effect of changes in the phosphorylation site of the L protein on its ability to affect the nucleocytoplasmic traffic prompted us to investigate the effect of

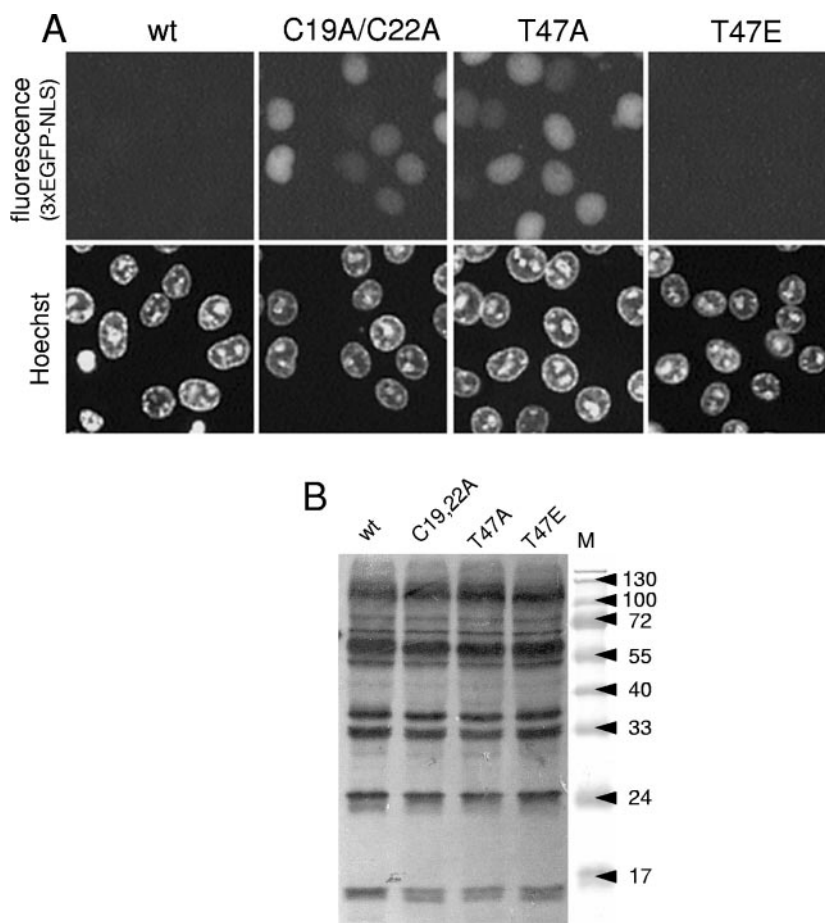


FIG. 9. Effects of preparations of mutated mengovirus leader proteins on the nuclear efflux in permeabilized HeLa-3E cells. (A) The unlabeled *in vitro* products of translation of mengoviral RNAs isolated from partially purified wild-type or mutant virions were normalized with respect to the incorporation of [S^{35}]methionine into the trichloroacetic acid-insoluble material in the labeled probes and were added to the digitonin-permeabilized HeLa-3E cells for 1 h. Hoechst 33258 was used to monitor the efficiency of the plasma membrane permeabilization. (B) Electrophoretic analysis showing that translation and processing of mengovirus RNAs was not affected by point mutations in the leader sequence. PageRuler Prestained Protein Ladder was used as a marker. wt, wild-type.

protein kinase inhibitors on this ability. To this end, inhibitors were added to the permeabilized uninfected HeLa-3E cells together with the extracts from EMCV-infected HeLa cells. As shown in Fig. 13A, the broad spectrum kinase inhibitor staurosporine (62) at a concentration of 1 μ M efficiently inhibited efflux of the nuclear marker protein triggered by the extract from infected cells (while exerting no detectable changes in protein distribution in the sample treated with the extract from mock-infected cells). Some other drugs, such as the tyrosine kinase inhibitor genistein (4) at a concentration of 50 μ M and the inhibitor of casein kinase 5,6-dichlorobenzimidazole riboside (51) at a concentration of 300 μ M, did not affect the nuclear efflux in this assay (not shown).

To investigate whether the effect of staurosporine was mediated through changes in the phosphorylation status of the leader protein, the drug was added together with the products of translation of mengovirus RNA harboring the T47E mutation in L. These products triggered nuclear efflux in a staurosporine-sensitive manner (Fig. 13B), suggesting that the drug affected an event downstream of the L protein rather than merely suppressing phosphorylation of this protein.

DISCUSSION

The overwhelming majority of DNA-containing and also several RNA-containing viruses exploit the nuclear machinery at different stages of their reproduction. To reach and/or leave the nucleus, these viruses not only utilize the canonical nuclear transport mechanisms but also change them for their own purposes. This problem has been studied rather extensively. We can give here only few, understandably arbitrarily selected, examples. Thus, many viral proteins possess specific signals targeting them into or out of the nucleus. In fact, the very first described NLS was identified in the T antigen of SV40 (76). DNA-containing parvoviruses (45) and hepadnaviruses (43, 61) possess NLS on their virions and use the importin-dependent mechanisms to deliver their genomes to the nucleus (reviewed in reference 33). The nuclear RNA viruses, such as influenza virus, harbor nuclear export signals and/or NLS on several proteins to accomplish ribonucleoprotein trafficking and, further, exploit several mechanisms to mask and unmask these signals to regulate RNP localization at different stages of infection (18, 77). Some viruses, e.g., retroviruses, encode

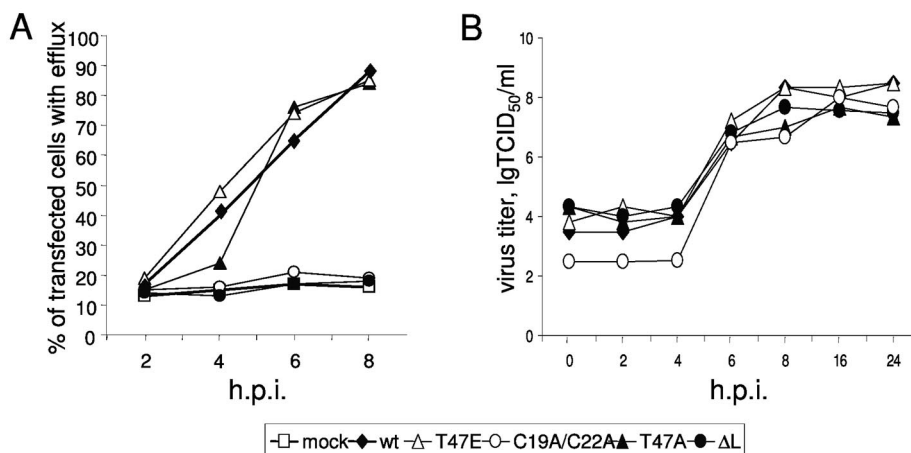


FIG. 10. Effect of mengovirus mutants on nucleocytoplasmic traffic in BHK-21 cells. (A) BHK-21 cells were infected with mengovirus mutants (MOI of 10 50% tissue culture infective doses) at 24 h after transient transfection with the EGFP-nuc-encoding vector. Cells were examined at appropriate time intervals p.i., and the proportion of cells with cytoplasmic fluorescence among the total number of fluorescent cells was counted. The overwhelming majority of cells infected with wild-type (wt) virus (diamond) exhibited efflux of the marker protein to the cytoplasm. Deletion of the leader (filled circle) or disrupting of the zinc finger (open circle) resulted in predominantly nuclear localization of the marker protein, typical of mock-infected cells (open square). Disruption of the T47 phosphorylation site (filled triangle) resulted in a delay of the efflux, while the virus harboring a phosphorylation-mimicking mutation (open triangle) efficiently induced the efflux. (B) Single-cycle growth curves of these mutants in BHK-21 cells.

shuttling proteins that allow them to export unspliced, virus-specific RNA from the nucleus to the cytoplasm, making use of the canonical export pathways but overcoming the cellular control of RNA export (46). Adenoviruses (23) and herpesviruses (63) also express proteins that interact with the export machinery to help regulate the release of their RNA transcripts into the cytoplasm.

Viruses may also significantly modify the mechanisms of nucleocytoplasmic transport. For example the matrix protein of vesicular stomatitis virus was shown to inhibit active nuclear export and import by interaction with a nucleoporin (57; see reference 75 for review). SV40 changes the traffic capacity of nuclear pores in such a way that they become permeable for bigger cargoes (27, 28). The preintegration complex of human immunodeficiency virus type 1 not only exposes NLS (13, 36) but possibly also enters the nucleus through transient NPC-independent breakages in the nuclear envelope, induced by the viral Vpr protein (20). The unusual rearrangement of the nuclear envelope ("herniation") was previously described in reovirus-infected cells (39).

Picornaviruses, which accomplish all the steps of their multiplication in the cytoplasm, also exploit a variety of mechanisms to relocate viral and host proteins through the nuclear envelope. Some viral proteins, such as poliovirus 3CD (65) and cardiovirus 2A (5) and 3D (6), possess specific NLS targeting them to the nucleus. The cellular nuclear autoantigen, La, plays a role in poliovirus translation (17, 50, 69). This protein is known to relocate into the cytoplasm in poliovirus-infected cells (50) due to the loss of its NLS through a 3C^{pro}-mediated truncation (67). Similarly, the p65-RelA component of NF- κ B is cleaved in poliovirus-infected cells by 3C^{pro}, and this event perhaps plays a role in the capacity of the pathogen to overcome the innate cellular defensive NF- κ B system (54).

In addition to exploiting the canonical mechanisms of nucleocytoplasmic trafficking, some picornaviruses also trigger

pathological alterations in the machinery itself. For example, enteroviruses induce a bidirectional increase in permeability of the nuclear envelope accompanied by relocation of some nuclear proteins into the cytoplasm and cytoplasmic ones into the nucleus (8, 9). This effect appears to be due to opening of the nuclear pores, as revealed by electron microscopy (10). Some pathways of active protein import are additionally disturbed in poliovirus-infected and rhinovirus-infected cells (34, 35). Both of these phenomena, the enhanced permeability and inhibited active transport, are likely caused by the degradation of nucleoporins, in particular p62, Nup153, and Nup 98 (34, 35), through the action of the viral 2A^{pro} protease (10; K. Gustin et al., Abstr. Meeting of European Study Group on the Molecular Biology of Picornaviruses, 2005).

Cardioviruses have no homologue of the 2A^{pro} of enteroviruses and rhinoviruses. However, as shown here, these viruses can induce similar protein redistribution in the infected cells: nuclear proteins egress from and cytoplasmic ones regress into the nucleus. Our experiments with the Timer protein showed that "old" material that had been present in the nucleus prior to EMCV infection was transported to and accumulated in the cytoplasm afterwards. The nuclear envelope became leaky during infection, and lysates from infected cells or products of *in vitro* translation of viral RNA were able to increase the permeability of the nuclear envelopes from digitonin-treated, uninfected cells. These effects were dependent upon viral L protein, since the L-deficient viruses and replicons were severely impaired in their ability to affect protein redistribution. These data are consistent with those reported recently for Theiler's murine encephalomyelitis virus (19).

Our results show further that an intact zinc finger domain within L is a key component within the protein for its effect on permeability of the nuclear envelope and that phosphorylation of Thr47 also appears to be important. These effects were not due just to a decrease in the level of viral reproduction in HeLa

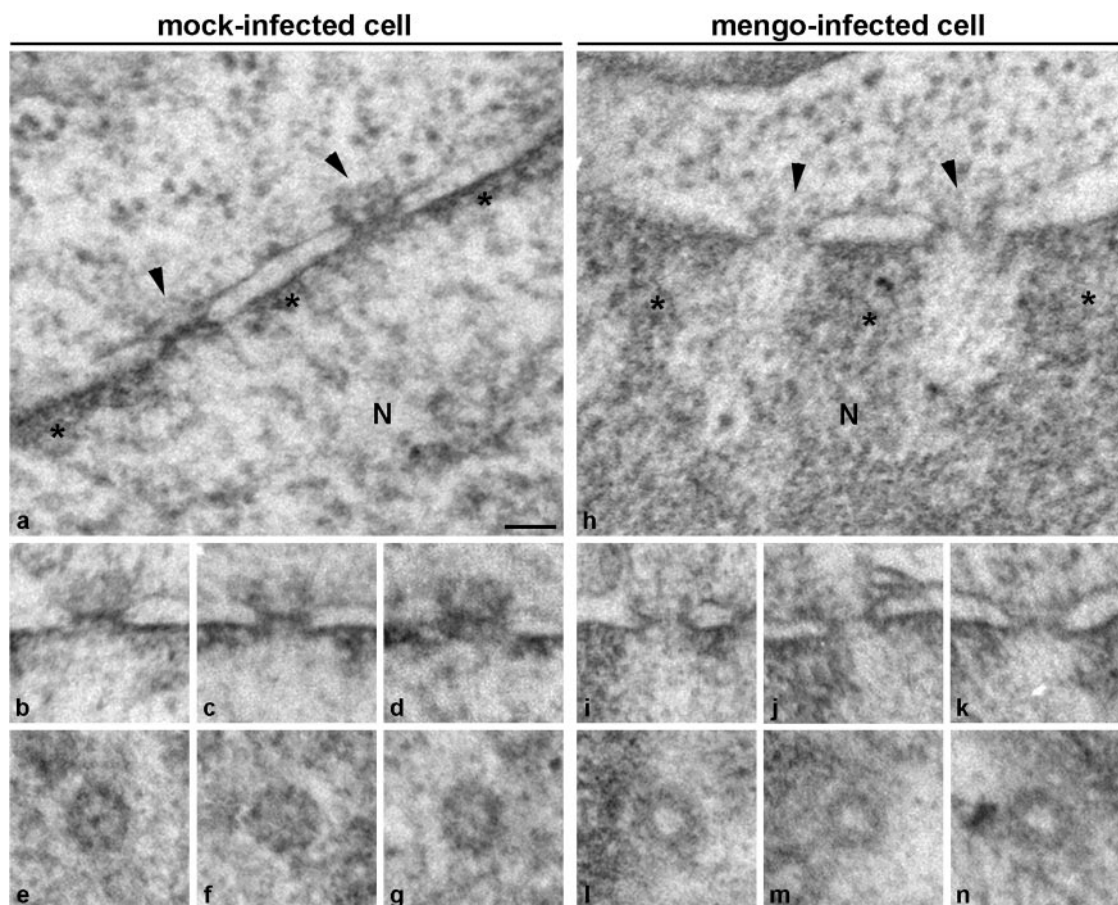


FIG. 11. Electron microscopy of nuclear pores (arrowheads) in uninfected (a to g) and infected (h to n) HeLa cells. Pictures were taken at 5 h p.i. Shown are cross-sections (a to d and h to k) and tangential sections (e to g and l to n) of the nuclear envelope. N, nucleus; asterisk, condensed chromatin. Bar, 50 nm.

cells caused by the various mutations, since similar phenotypes were observed in BHK-21 cells, where the L mutations barely affected the growth potential of the virus. The dispensability of viral replication for the nuclear trafficking disorders follows also from our experiments in which a replicon with a defective polymerase ($\Delta 3D$) retained its effect on the nuclear envelope. All these lines of evidence demonstrate that the mutations in L must have altered the effect of EMCV on the nucleocytoplasmic traffic through a pathway not directly related to the efficiency of viral reproduction.

Since the leader protein has no known enzymatic activity, it seems that its effect on the NPC must be accomplished through interaction with some NPC components directly or through an NPC-impairing pathway. One candidate pathway would be a perturbation in the caspase-dependent apoptotic program that is well known to disturb nucleocytoplasmic trafficking (14, 26, 30). But the failure of a broad-range caspase inhibitor (zVAD.fmk) to prevent cardiovirus-induced nuclear envelope permeabilization suggests that this particular pathway may not play a critical role in the phenomenon observed.

Both the Zn finger domain and the highly acidic domain (the protein pI is 3.8) of the cardiovirus L surely confer multiple options for specific binding opportunities with other viral or cellular proteins, but the only partner identified to date is

EMCV protein 2A (pI 10.3) (A. C. Palmenberg, unpublished data). However, a replicon lacking 2A efficiently induced the efflux of nuclear protein, showing that 2A is dispensable for disruption of nucleocytoplasmic traffic.

The inhibitory effect of staurosporine on the nuclear envelope-damaging activity suggests the involvement of a cellular protein kinase(s). The nature of this enzyme is yet to be established, but it seems appropriate to note that phosphorylation is a key regulatory reaction in mitotic disassembly of NPC (47).

Remarkably, unlike the situation in cells infected with poliovirus and rhinovirus, where permeabilization of the nuclear envelope is accompanied by degradation of certain nucleoporins (34, 35), the same nucleoporins appeared to be intact in cardiovirus-infected cells. On the other hand, the nuclear pores appear to be significantly altered in both poliovirus- and cardiovirus-infected cells, suggesting that in these two types of infection, the pathways triggered by different proteins and accomplished through different mechanisms may converge and hit the same target.

Although the exact role and mechanism(s) of the leader protein action in infected cells remain unknown, the protein has been implicated in several important aspects of the virus-cell interaction, such as control of viral (24) and host (81) translation as well as suppression of interferon production (19,

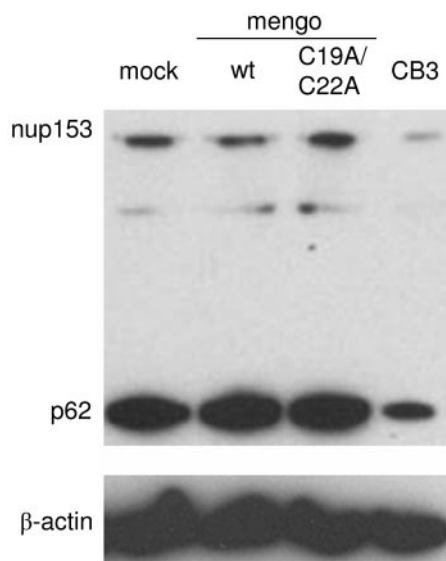


FIG. 12. The nucleoporins degrading upon enterovirus infection remain intact in mengovirus-infected cells. Cells infected with wild-type and zinc finger domain-mutated mengoviruses were analyzed by Western blotting for nucleoporin integrity. Coxsackievirus B3 infection was used as a positive control.

82; S. Hato et al., submitted). One may speculate that at least some of these events are somehow coupled to the nuclear envelope leakiness, e.g., through alterations in the transcription regulation which is frequently associated with the nucleocytoplasmic relocation of transcription factors.

Enhanced nucleocytoplasmic traffic would appear to be an advantage to the virus by (i) facilitating access to the nuclear factors that might stimulate cytoplasmic viral reproduction or (ii) helping to counteract an up-regulation of cellular defensive measures. Nevertheless, permeabilization of the nuclear envelope is not an essential requirement for viral reproduction, as evidenced by the (nearly) normal growth of mengovirus mutants lacking functional L (and hence unable to modulate nucleocytoplasmic traffic) in BHK-21 cells (Fig. 10). Nevertheless, reproduction of the mutants unable to modify the nucleocytoplasmic traffic is not as efficient in many situations as that of their wild-type counterparts.

It is worth emphasizing that different picornaviruses induce very similar alterations in the nucleocytoplasmic traffic through the use of different mechanisms involving unrelated viral proteins, such as poliovirus 2A^{Pro} and cardiovirus L. No doubt, the capacity to enhance the permeability of the nuclear envelope was independently acquired by cardioviruses, on the one hand, and enteroviruses and rhinoviruses, on the other. The very fact of the apparent multiple independent acquisitions of a similar function may be taken as an additional argument for its adaptive character. Nevertheless, it does not seem likely that the permeabilization of the nuclear envelope will eventually prove to be the most important function of these proteins. This activity is just another manifestation of the multifunctional, versatile character of proteins of viruses with relatively small genomes, such as, for example, picornaviruses (2).

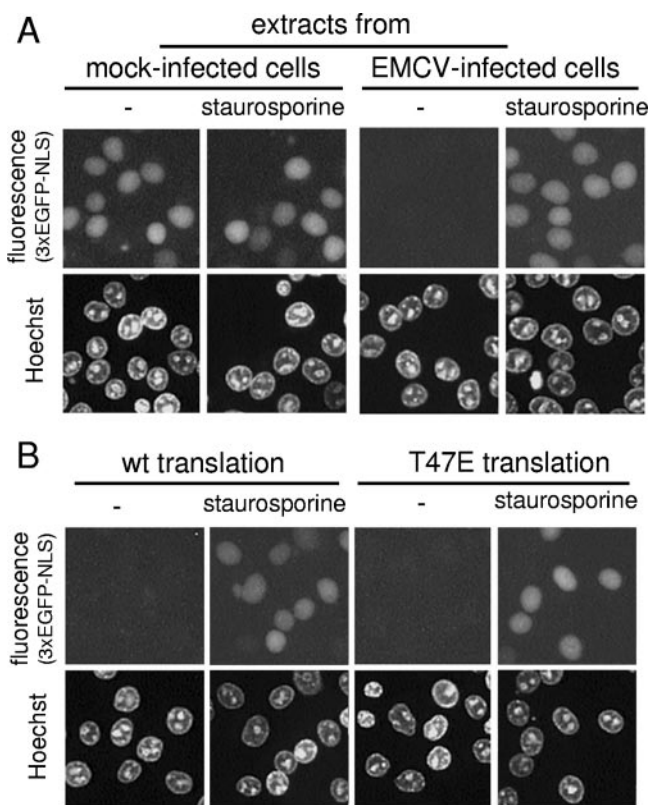


FIG. 13. Inhibitory effect of staurosporine on the nuclear efflux-triggering effect of extracts from EMCV-infected cells. (A) S-100 lysates from either mock-infected or EMCV-infected cells, diluted 1:10, were added to digitonin-permeabilized HeLa-3E cells and incubated for 1 h in the presence or absence of 1 μ M staurosporine. (B) The products of in vitro translation of viral wild-type (wt) and T47E mutant RNAs were assayed in the presence and absence of staurosporine (1 μ M) essentially as described in legend of Fig. 9.

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