Virology **275,** 244–248 (2000)
doi:10.1006/viro.2000.0427, available online at http://www.idealibrary.com on IDEAL®

RAPID COMMUNICATION

Early Alteration of Nucleocytoplasmic Traffic Induced by Some RNA Viruses

George A. Belov,*,† Alexandra G. Evstafieva,† Yuri P. Rubtsov,† Olga V. Mikitas,*
Andrey B. Vartapetian,† and Vadim I. Agol*,†,[1]

*M. P. Chumakov Institute of Poliomyelitis and Viral Encephalitides, Russian Academy of Medical Sciences, Moscow Region 142782; and †A. N. Belozersky Institute of Physical–Chemical Biology, Moscow State University, Moscow 119899, Russia

Received January 27, 2000; returned to author for revision March 14, 2000; accepted May 16, 2000

A HeLa cell line expressing the green fluorescent protein fused to the SV40 T-antigen nuclear localization signal (EGFP-NLS) was established. Fluorescence in these cells was confined to the nuclei. After poliovirus infection, cytoplasmic fluorescence in a proportion of cells could be detected by 1 h postinfection (p.i.) and in virtually all of the fluorescent cells by 2 h p.i. The relocation could be prevented by cycloheximide but not by inhibition of poliovirus replication by guanidine · HCl. Nuclear exit of a protein composed of three copies of GFP fused to the NLS also occurred upon poliovirus infection. A similar redistribution of EGFP-NLS took place upon infection with coxsakievirus B3 and, to a lesser extent, with vesicular stomatitis virus. The EGFP-NLS efflux was not due to the loss of NLS. Thus, some positive-strand and negative-strand RNA viruses trigger a rapid nonspecific relocation of nuclear proteins.

Reproduction of poliovirus takes place in the cytoplasm of the infected cell, with no essential requirement for nuclear functions. This notion is supported by such straightforward observations as the virus' ability to multiply in the nuclei-free cytoplasts (*1, 2*) or cytoplasmic extracts (*3*). On the other hand, several lines of evidence suggest that nuclei or nuclear components may be involved in virus multiplication. Thus, significantly lower amounts of infectious progeny were produced after infection of cytoplasts than after infection of the intact cells. Only the latter were able to support viral reproduction after infection with double-stranded poliovirus RNA (*4*). Moreover, several host proteins with predominantly nuclear localization were reported to specifically relocate into the cytoplasm upon poliovirus infection and to stimulate either translation (*5*) or replication (*6, 7*) of the viral genome. The goal of this study was to investigate nucleocytoplasmic traffic in poliovirus-infected cells by making use of enhanced green fluorescent protein (EGFP) fused to the SV40 T-antigen nuclear localization signal (NLS).

After discovering the rapid exit of this protein after poliovirus infection, we demonstrated that a similar phenomenon took place in cells infected with coxsakievirus B3 (CVB3) and vesicular stomatitis virus (VSV).

The EGFP-NLS-expressing plasmid (pEGFP-NLS) was constructed by in-frame ligation of the 74-bp-long *Hin*dIII–*Kpn*I fragment encoding the SV40 T-antigen NLS from pGAD424 (Clontech) into the multiple cloning site of pEGFP-C2 (Clontech) downstream of the EGFP sequence.

The transfection of HeLa-B (*8*) cells grown in DMEM supplemented with 10% bovine serum was carried out by the calcium–phosphate technique as described on the World Wide Web at http://www.sciencexchange.com/sxprotocols/molbiol/rapid.htm. The stable cell lines were established after 2 weeks of selection with 400 μg/ml of G-418 by pooling the resistant clones without additional subcloning.

Fluorescence in the overwhelming majority of positive cells was essentially confined to the nuclei (Fig. 1A), as could be expected from the presence of a nuclear localization signal in the EGFP-NLS. It may be noted that GFP lacking a NLS is known to exhibit no nuclear preference (*9*).

When HeLa/EGFP-NLS cells were infected with poliovirus (type 1, Mahoney strain; the input multiplicity of infection (m.o.i.) was from 10 to 500 PFU/cell), rapid redistribution of fluorescence could be observed. A significant number of cells exhibited detectable cytoplasmic fluorescence, along with nuclear fluorescence, as early as 1 h postinfection (p.i.) (Figs. 1B, 1C). Importantly, at the time of intense accumulation of fluorescence in the cytoplasm, no appreciable morphological alterations could be seen in the infected cells compared with uninfected cells, as evidenced by either phase-contrast microscopy of live cells or fluorescence microscopy of cells stained with permeable nuclear dye Hoechst-33342 (not shown).

[1] To whom correspondence and reprint requests should be addressed. E-mail: viago@ipive.genebee.msu.su.

0042-6822/00 $35.00

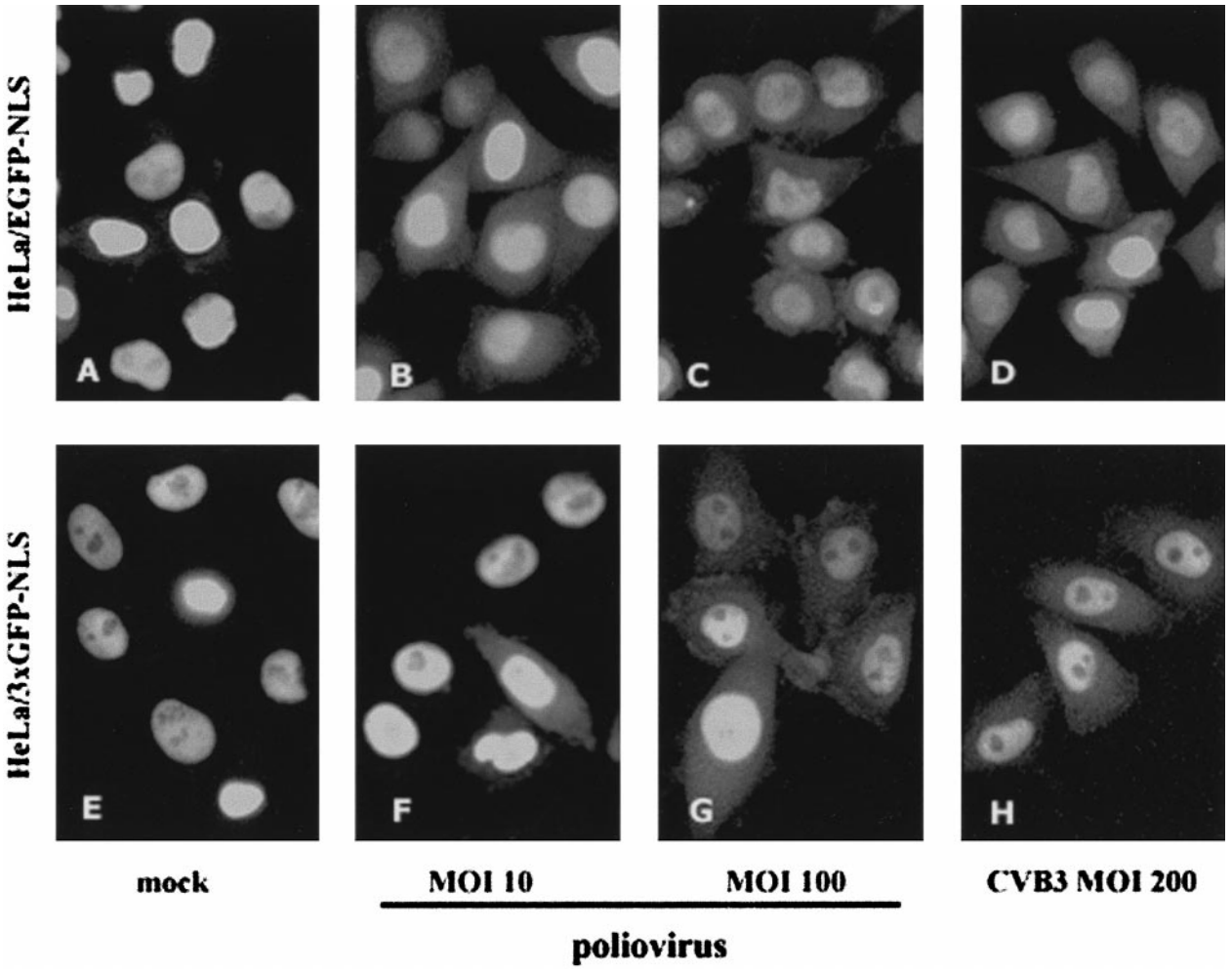

FIG. 1. Relocation of nuclear proteins triggered by enterovirus infection. HeLa/EGFP-NLS cells (A–D) and HeLa/3xGFP-NLS (E–H) cells were mock infected (A, E), infected with poliovirus at a m.o.i. of 10 PFU/cell (B, F) or 100 PFU/cell (C, G), or infected with CVB3 at a m.o.i. of 200 PFU/cell (D, H). Incubation was for 1 h p.i. at 37°C.

The molecular mass of EGFP-NLS is 31.4 kDa, whereas the size limit for passive diffusion through nuclear pores is believed to be around 50 to 60 kDa. (*10*). To ascertain whether poliovirus infection promoted nuclear efflux of only protein species smaller than this limit, a plasmid (pEGFP-NLS-2xGFP$_F$) encoding two additional copies of a bright version of GFP (GFP$_F$) was engineered. To this end, the DNA fragment encoding GFP$_F$ was excised from pFRED 25 ΔNae gfp hyg (kindly provided by Dr. G. Pavlakis); two identical fragments were in-frame ligated and inserted into pGAD424 in-frame with the SV40 T-antigen NLS. The resultant NLS-2xGFP$_F$-encoding unit was then transferred into pEGFP-C2 to produce pEGFP-NLS-2xGFP$_F$. A stable HeLa-B line expressing this protein of ca. 90 kDa (HeLa/3GFP-NLS) was established. The nuclear fluorescence could only be detected in the positive cells (Fig. 1E). Again, poliovirus infection resulted in the relocation of a portion of the fluorescent material into the cytoplasm, the effect being more pronounced at a higher m.o.i. (compare Figs. 1F and 1G).

It has been reported recently that the nucleus-to-cytoplasm relocation of a portion of the La autoantigen in poliovirus-infected cells could be due to its limited proteolysis by the viral protease 3C, accompanied by the loss of a nuclear localization signal (*11*). Western blot analysis with rabbit polyclonal antibodies raised against EGFP failed to reveal any degradation of EGFP-NLS (Fig. 2, lane c) or 3xGFP-NLS (Fig. 2, lane f) isolated from poliovirus-infected cells compared to the mock-infected cells (Fig. 2, lanes d and e, respectively). These results suggested that the EGFP-NLS and 3xGFP-NLS relocations were not caused by the loss of their NLS and could hardly be due to passive diffusion of the proteins through intact nuclear pores.

Exit of EGFP-NLS and 3GFP-NLS into the cytoplasm was prevented when the infection with poliovirus was carried out in the presence of protein synthesis inhibitor cycloheximide (CHI; 100 μg/ml from the onset of infection; Figs. 3E, 3J). CHI at this concentration induces a strong apoptotic response in HeLa cells (*8*). Therefore, to prevent apoptosis, benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (zVAD.fmk), a cell-permeable broad-

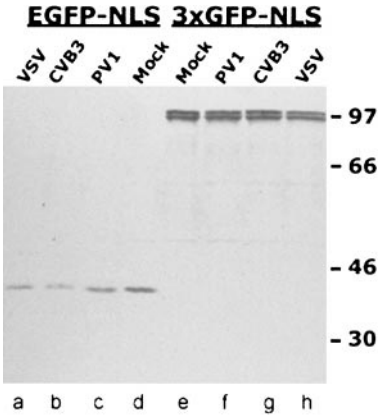

FIG. 2. Western blot analysis of extracts prepared from infected and mock-infected cells. HeLa/EGFP-NLS and HeLa/3xGFP-NLS cells were infected with poliovirus (PV1; m.o.i. 200 PFU/cell), CVB3 (m.o.i. 200 PFU/cell), or VSV (m.o.i. 60 PFU/cell) for 3 h. Cells were boiled in the SDS sample buffer (130 μl for 1 $\times$ 10^6 cells) for 5 min and 30-μl aliquots were subjected to electrophoresis through a 8% SDS–polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membrane and probed by rabbit polyclonal anti-GFP antibody, kindly donated by Dr. N. Kalinina. The blots were developed by an ECL chemiluminiscence system (Amersham, Pharmacia). The reason for the double band of 3xGFP-NLS is unknown but could be related to a conformational shift (cf., Ref. *12*).

range caspase inhibitor, was added to the samples simultaneously with CHI (*13*). zVAD.fmk itself exerted no effect on fluorescence relocation from the nuclei of infected cells (Figs. 3D, 3I), thus excluding the possibility of caspase involvement in the process. The exit was only slightly, if at all, retarded when the infection was carried out in the presence of guanidine · HCl (Figs. 3C, 3H), known to essentially prevent replication of the viral genome. It seemed likely therefore that the translation of parental RNA could generate sufficient product(s) to trigger the relocation. Thus, a low level of the poliovirus genome expression appeared to be sufficient to ensure exit of the proteins into the cytoplasm.

A similar early nuclear efflux of both EGFP-NLS and 3xGFP-NLS was also observed after infection with another picornavirus, CBV3 (Fig. 1D, 1H). Moreover, relocation, though perhaps not so efficient, occurred in VSV-infected cells as well. In this case, cytoplasmic fluorescence in the EGFP-NLS-expressing cells was clearly evident by 2 h p.i. (Fig. 4C), when no significant alterations in nuclear morphology in the majority of cells could be revealed by using Hoechst 33342 (Fig.

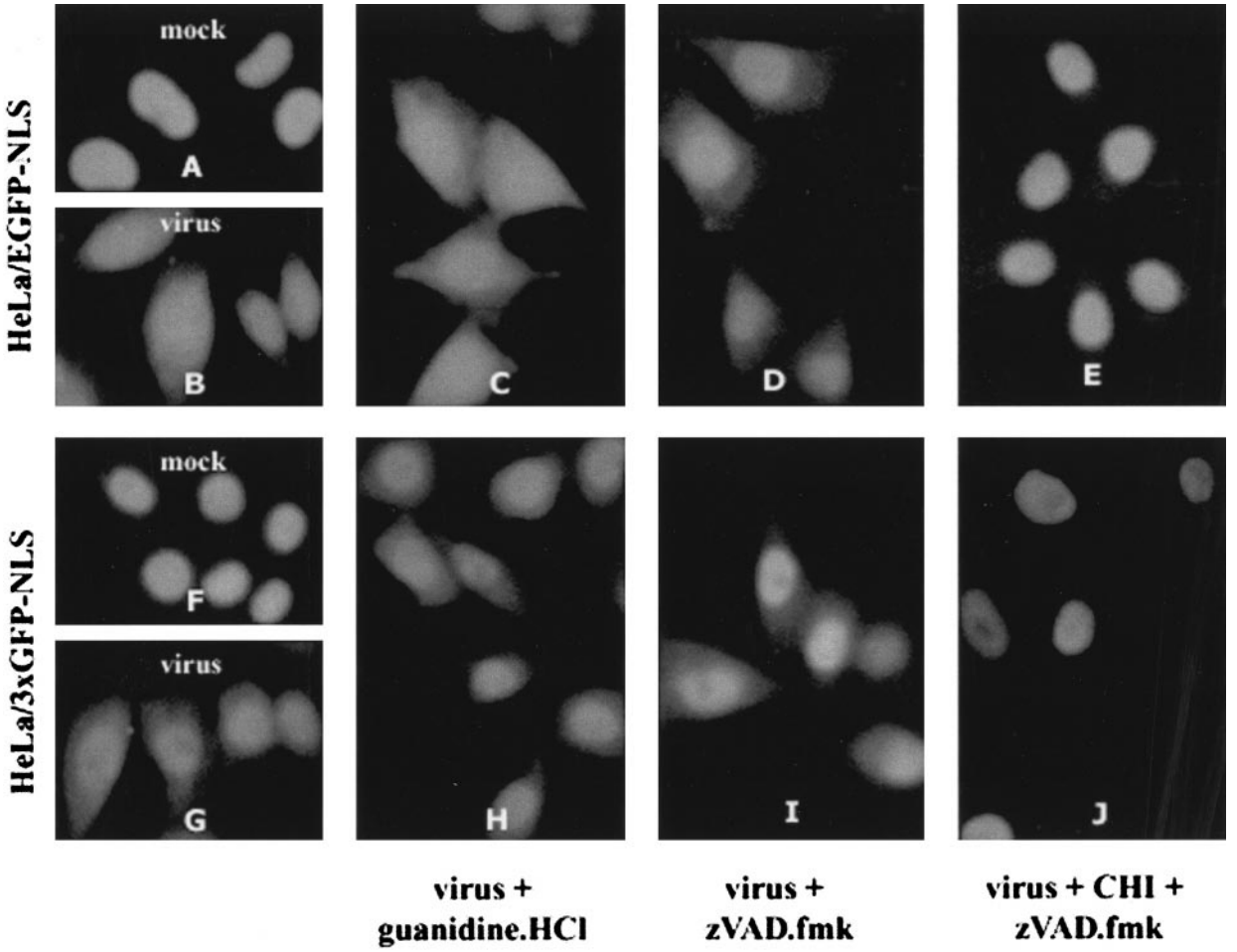

FIG. 3. Effect of inhibitors on nuclear efflux of NLS-containing proteins. HeLa/EGFP-NLS cells (A–E) and HeLa/3xGFP-NLS (F–J) cells were mock infected (A, F) and infected with poliovirus at a m.o.i. of 500 PFU/cell (B–E, G–J). Incubation was for 2 h without inhibitors (B, G), in the presence of 100 μg/ml of guanidine · HCl (C, H), 100 μM zVAD.fmk (D, I), or both 100 μg/ml of CHI and 100 μM zVAD.fmk (E, J).

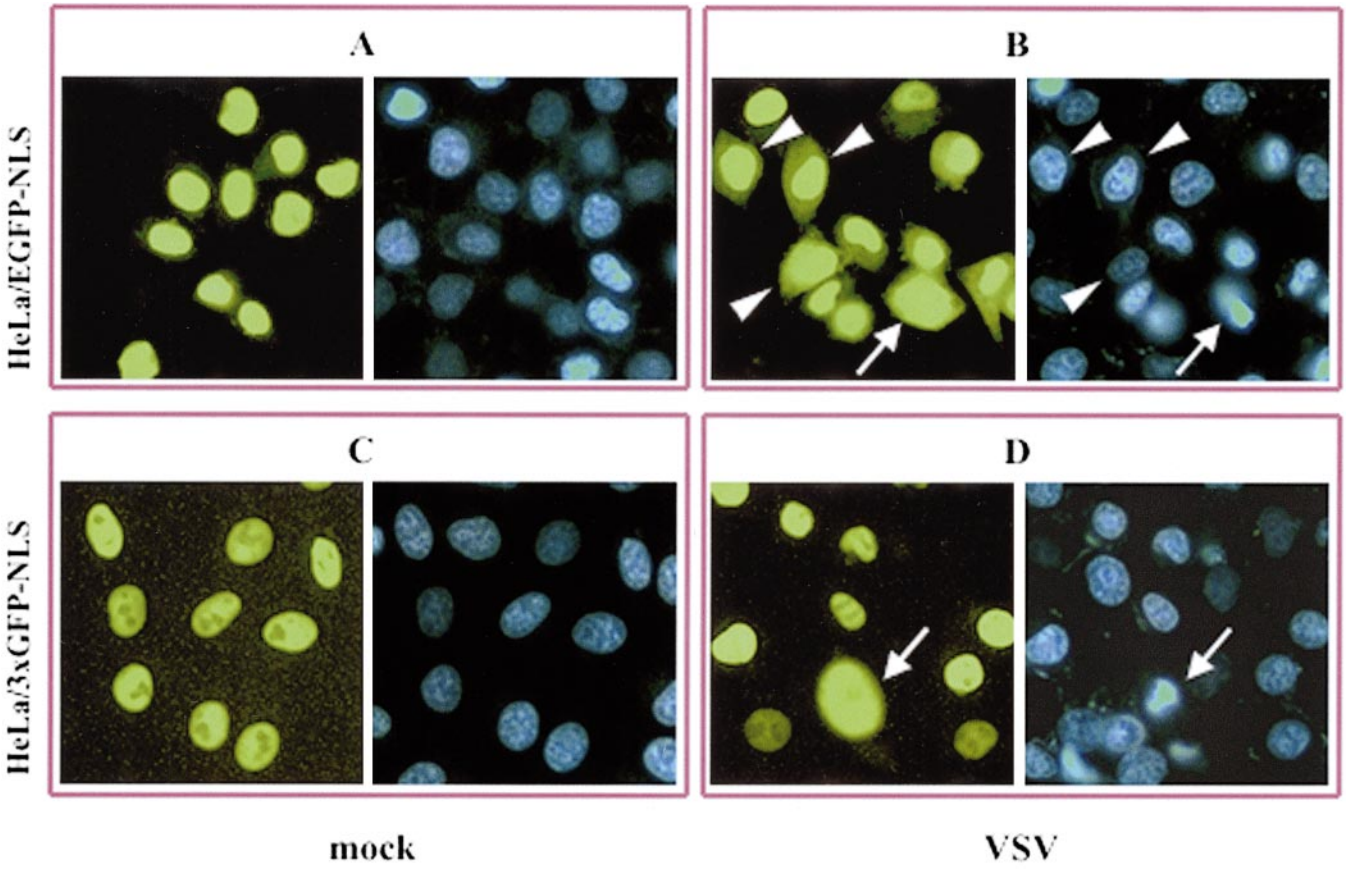

FIG. 4. Relocation of nuclear proteins triggered by VSV. HeLa/EGFP-NLS (A, B) and HeLa/3xGFP-NLS (C, D) cells were mock infected (A, C) or infected with VSV at a m.o.i. of 60 PFU/cell (B, D) and incubated for 2 h. Hoechst 33342 (a final concentration of 5 μg/ml) was added 30 min prior to the end of incubation. Each field was observed with an epifluorescent microscope Leica DMLS equipped with filter cubes I3 (for GFP green fluorescence, left) and A (for Hoechst blue fluorescence, right). Cytoplasmic fluorescences in some cells with intact-looking and damaged nuclei are marked with arrowheads and arrows, respectively. Note that not all of the G-418-resistant cells are expressing functional EGFP derivatives.

4, arrowheads). The efflux of 3xGFP-NLS at this time could be observed only in cells with markedly damaged nuclei (Figs. 4G, 4H, arrows). No degradation of the fluorescent proteins (and consequently no loss of their NLS) after either CBV3 or VSV infection was revealed by Western blotting. (Fig. 2).

The nature of the viral function involved in the alteration of nucleocytoplasmic trafficking is yet to be determined. Our preliminary attempts to identify the relevant poliovirus function by using plasmids expressing, under a cytomegalovirus promoter, different viral nonstructural proteins have thus far provided no conclusive results. The nature of the alterations in the structure and/or function of nuclear membranes also remains unknown. It has been reported previously that VSV M protein inhibited the Ran-dependent transport of RNA and proteins between the nucleus and cytoplasm of *Xenopus laevis* oocytes (*14*). The relation, if any, between this fact and the early efflux of nuclear protein in VSV-infected cells described here is unknown. Similar changes in protein trafficking triggered by such unrelated viruses as positive-RNA-strand picornaviruses and negative-RNA-strand VSV may suggest that the observed phenomenon represents a component of an unknown general cellular response to infection.

Regardless of the mechanism, the fact of a significant change in the nucleocytoplasmic trafficking in virus-infected cells should have important implications for the reproduction of the virus, as well as for the fate of the infected cell. This phenomenon may be related to the above-mentioned observations of relocation of certain nuclear proteins to the cytoplasm during poliovirus infection. Although the infection-induced protein redistribution is, as shown here, nonspecific, it is not general. Certain nuclear proteins [e.g., TATA-binding protein (*6*), splicing factor SC-35 (*5*), or mutated Sam68 (*15*)] did not change their locations. It seems likely that the fate of a protein depends on its ability to bind to other cytoplasmic or nuclear macromolecules. If the nuclear location of a protein depends solely on the presence of a NLS, this protein is expected to be allowed to exit the nucleus because of the alterations in the nucleocytoplasmic trafficking caused by infec-

tion. However, existence of other strong enough anchors, such as DNA in the case of DNA-binding proteins, may prevent relocation of the relevant protein.

Although not studied here directly, the observed deterioration of the nucleocytoplasmic relationships may facilitate not only the nuclear exit but also the entry of certain cytoplasmic proteins into the nucleus. Nuclear accumulation of virus-specific proteins in poliovirus-infected cells has indeed been documented (*16, 17*). It is also known that poliovirus infection is accompanied by the shutoff of host transcription due to the degradation of certain transcription factors caused by viral proteinases 3C (*18–21*) and 2A (*22*). Early (by 1 h p.i.) nuclear accumulation of VSG G protein in the infected cells has also been reported (*23*). Whether viral infections may promote nuclear uptake of cytoplasmic proteins by a mechanism similar to that responsible for enhanced nuclear release has yet to be determined.

This study adds another dimension to the known pattern of profound deterioration of the whole intracellular infrastructure that occurs upon viral infection. Other facets of this deterioration involve, in the case of poliovirus, suppression of the trafficking of intracytoplasmic membranes and inhibition of the secretory pathway (*24, 25*) as well as accumulation of membranous vesicular structures (*26*).

ACKNOWLEDGMENTS

We thank an anonymous reviewer for valuable suggestions. This study was supported by grants from INTAS and the Russian Foundation for Basic Research. V.I.A. is a Soros Professor.

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