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Resistance of Ribosomal Protein mRNA Translation to Protein Synthesis Shutoff Induced by Poliovirus

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Poliovirus infection induces an overall inhibition of host protein synthesis, although some mRNAs continue to be translated, suggesting different translation requirements for cellular mRNAs. It is known that ribosomal protein mRNAs are translationally regulated and that the phosphorylation of ribosomal protein S6 is involved in the regulation. Here, we report that the translation of ribosomal protein mRNAs resists poliovirus infection and correlates with an increase in $p70^{s6k}$ activity and phosphorylation of ribosomal protein S6.

Poliovirus infection results in a drastic shutoff of cellular protein synthesis, accompanied by a selective production of viral proteins (11, 54). This is achieved mostly at the level of translation by specific impairment of the cap-dependent initiation step (17, 28). In fact, the mRNA of poliovirus, and that of other picornaviruses, is uncapped and characterized by a long and structured 5′ untranslated region (5′UTR) where an internal ribosome entry site (IRES) can promote cap-independent translation initiation (19, 22, 43). One of the mechanisms responsible for the inhibition of cap-stimulated translation involves the modification and inactivation of the translation initiation factor eIF4F, due to the cleavage of the eIF4G subunit (11). On the other hand, the eIF4G cleavage products can facilitate the translation initiation of viral RNAs, mediated by an IRES, and of uncapped cellular RNAs (41). Although most of the host protein synthesis is inhibited in poliovirus-infected cells, the translation of some cellular mRNAs occurs. They include the heat shock protein (HSP) mRNAs and the immunoglobulin heavy-chain binding protein, c-myc, and eIF4G mRNAs, which use the mechanisms of internal initiation (25, 39, 50). The cellular modification induced by viral infection to cellular protein synthesis can help to identify the mechanisms that normally control mRNA translation.

We were interested in the regulatory mechanisms that control the translation of ribosomal protein (rp) mRNAs (rp-mRNAs) (1, 37). It is known that the translation of rp-mRNAs is regulated by elements contained in the 5′UTR of rp-mRNAs and, in particular, by a typical terminal oligopyrimidine segment (29, 34). Putative transacting factors can bind the 5′UTR of rp-mRNAs in mammalian and *Xenopus* cells (5, 26), where they were identified as the La protein and the cellular nucleic acid binding protein (44, 45). Furthermore, it was reported that in mitogen-stimulated cells, the efficiency of translation of mRNAs carrying a 5′-terminal pyrimidine tract is mediated by the activity of $p70^{S6k}$, the kinase responsible for the phosphorylation of r protein S6 (4, 23, 24, 55).

In this study, we have investigated the behavior of the class of rp-mRNAs under the translational conditions caused by poliovirus infection, in order to obtain information on the mechanisms that control their translation.

Translation of rp-mRNAs during poliovirus infection. We analyzed the mRNA distribution between polysomes and messenger ribonucleoprotein particles (mRNPs) in mock-infected and poliovirus-infected HEp-2 cells. Cells were infected with the poliovirus type 1 Mahoney strain and incubated for 4 h. Extracts corresponding to one plate of cell culture from mock-infected and poliovirus-infected cells were fractionated by sucrose gradient centrifugation, and the RNA was extracted from the fractions. Amounts corresponding to the same volumes of gradient fractions were analyzed by Northern blotting as previously described (32). A representative example of these experiments is given in Fig. 1, where the polysome-mRNP distribution of rp-mRNAs is compared to that of β-actin mRNA, a control mRNA subjected to shutoff. It should be noticed that in these experiments, only the distribution of the mRNAs along the gradients should be compared between mock-infected and infected cells and not the absolute amount of RNA. In mock-infected cells, about 70 to 80% of the mRNAs analyzed were loaded onto polysomes to be actively translated. In infected cells, β-actin mRNA was mostly displaced to mRNPs at the top of the gradient, as expected, while a large part of the L4, L32, and L11 rp-mRNAs was still associated with polysomes. However, these rp-mRNAs were associated with small polysomes, indicating that in infected cells, translation initiation might be less efficient than in uninfected cells. To obtain further information about the translational behavior of rp-mRNAs at different infection times, we analyzed the polysome-mRNP distribution of L4, L32, L11, and β-actin mRNAs at 90 min and 4 h after infection. Figure 2A shows graphically that, compared with mock-infected cells, the distributions of the rp-mRNAs and β-actin were soon quite different. At 90 min after infection, polysome-associated β-actin mRNA started decreasing and the dislocation of this mRNA to the top of the gradient reached 90% within 4 h. On the contrary, 90 min after infection, about 60% of the L4, L32, and L11 mRNAs was still associated with large polysomes and after 4 h they remained associated with polysomes which, however, were smaller. Since gradient analysis is intended to show the translational activity of the mRNAs and not to quantify their absolute amount, quantitative aliquots of each extract at different times of infection were taken before gradient loading for total RNA analysis by Northern blot hybridization to different

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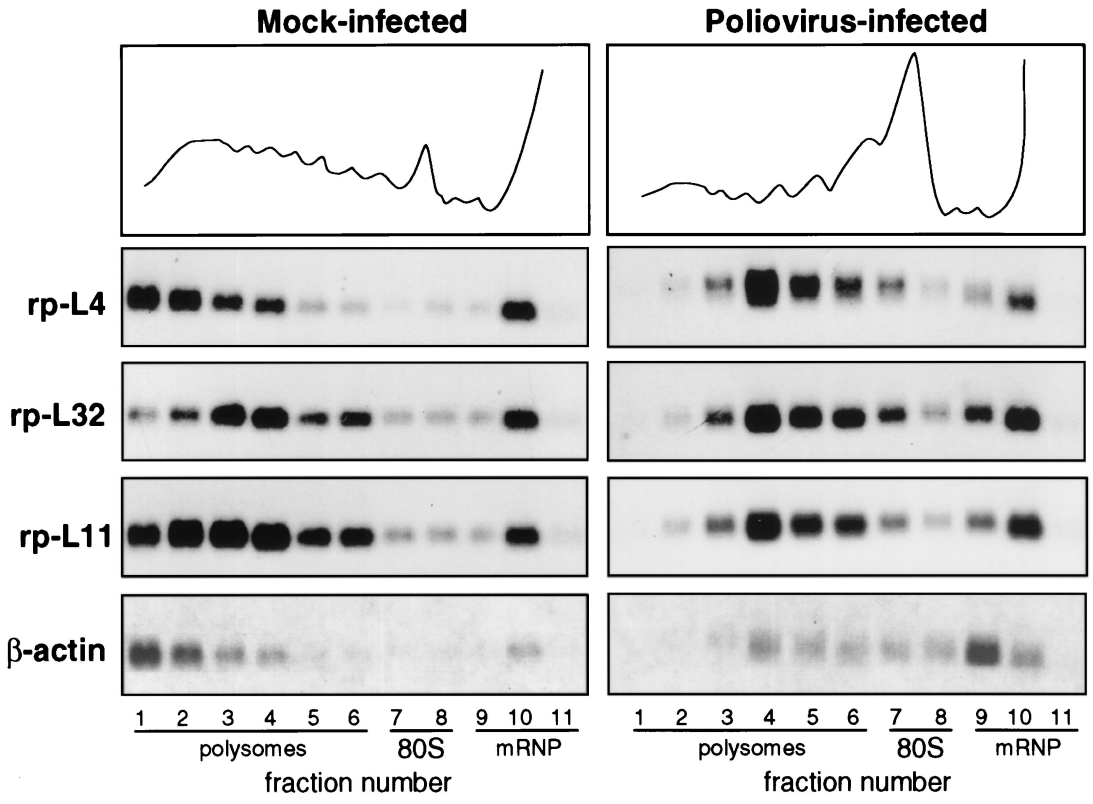

FIG. 1. Polysome-mRNP distribution of mRNA in mock-infected and poliovirus-infected cells. HEp-2 cells were grown at 37°C in Eagle's minimum essential medium supplemented with 10% fetal calf serum. When cells reached 80% confluence, the Mahoney strain of poliovirus type 1 was added at a multiplicity of infection of 50 PFU per cell. A 9-cm-diameter plate culture of mock-infected and infected cells was lysed at 4 h postinfection (10 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 1% Na-deoxycholate, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.4) to prepare cytoplasmic extracts (32). Cycloheximide, often used to prevent polysome runoff, was not added prior to extract preparation, as this drug sometime creates problems (38). Polysome protection can also be obtained by quickly preparing the extract and loading the sample on the gradient under strict temperature control. The extracts were separated on 15 to 50% sucrose gradients in gradient buffer (0.1 M NaCl, 10 mM $MgCl_2$, 30 mM Tris-HCl, pH 7). Gradient fractions were collected while the optical density profile at 260 nm was monitored (top), and the RNA was prepared by protease K-SDS-phenol extraction (32) of the fractions. The RNAs from equal volumes of mock-infected and infected gradient fractions were loaded onto two gels and analyzed by Northern blotting and autoradiography as previously described (32). Each filter was subsequently hybridized to probes for rp-L4 (2), rp-L32 (10), rp-L11 (12), and β-actin (6) mRNAs to obtain a reliable comparison of the distribution of the various RNAs along the same gradient. Probes were prepared by the random primer technique.

probes. The hybridization signals were quantified by comparison with 5S rRNA, which is structured in the ribosomes and therefore is expected to be fairly stable. Figure 2B shows that the level of all the mRNAs analyzed does not change appreciably up to 90 min and, with minor differences, decreases by about 30 to 35% at 4 h, indicating that rp-mRNAs and β-actin mRNA are subjected to similar rates of degradation.

To check the activity of polysome-associated mRNA after poliovirus infection, at 90 min and 3.5 h postinfection, [^{35}S]methionine-[^{35}S]cysteine was added for 30 min to HEp-2 cells to label synthesized r proteins. Thirty minutes of radioactive precursor administration was reasonable for detection of accumulated labeled proteins and to overcome the expected effect of r protein instability due to decreased rRNA synthesis, as will be discussed later. Considering the short labeling time and the fact that more extract could not be loaded onto the gel without pattern distortion, only faint spots can be expected on the two-dimensional (2D) gel, in accordance with data previously obtained with other systems (47). After incubation, protein extracts were prepared from mock-infected and infected cells and analyzed on a 2D gel optimized to resolve ribosomal proteins (58). Gels were Coomassie stained and fluorographed. Radioactive r proteins were identified on the 2D gel by their comigration with the purified human r proteins included in the sample (58). A polysome gradient experiment was performed with an aliquot of the extracts from mock-infected and infected labeled cells to check that the RNA distribution was as expected, namely, as in Fig. 1 (data not shown). At the same time, a protein labeling experiment was performed to check the pattern of total protein synthesis during infection. Figure 3A shows that at 2 h postinfection, the synthesis of r proteins is still efficient (arrowheads), in agreement with the rp-mRNA engagement with polysomes described above. However, at this time, the general inhibition of host cell protein synthesis is already detectable, as shown by the pattern of total protein synthesis at different times after infection (Fig. 3B). Note also the remarkable shutdown of two non-r proteins that can be seen in Fig. 3A (arrows). At 4 h postinfection, r protein labeling is no longer detectable (data not shown). This might be due to the severe inhibition of rRNA synthesis during poliovirus infection (7, 49) that becomes relevant with time, thus preventing accumulation of newly synthesized r proteins. Indeed, it has been observed in other systems that newly synthesized r proteins become unstable when rRNA needed for assembly is not available (8, 47, 58). It was recently reported that, following herpes simplex virus type 1 infection, r proteins continue to be synthesized during protein synthesis shutoff. However, in this system, where 60% rRNA synthesis persists in infected cells,

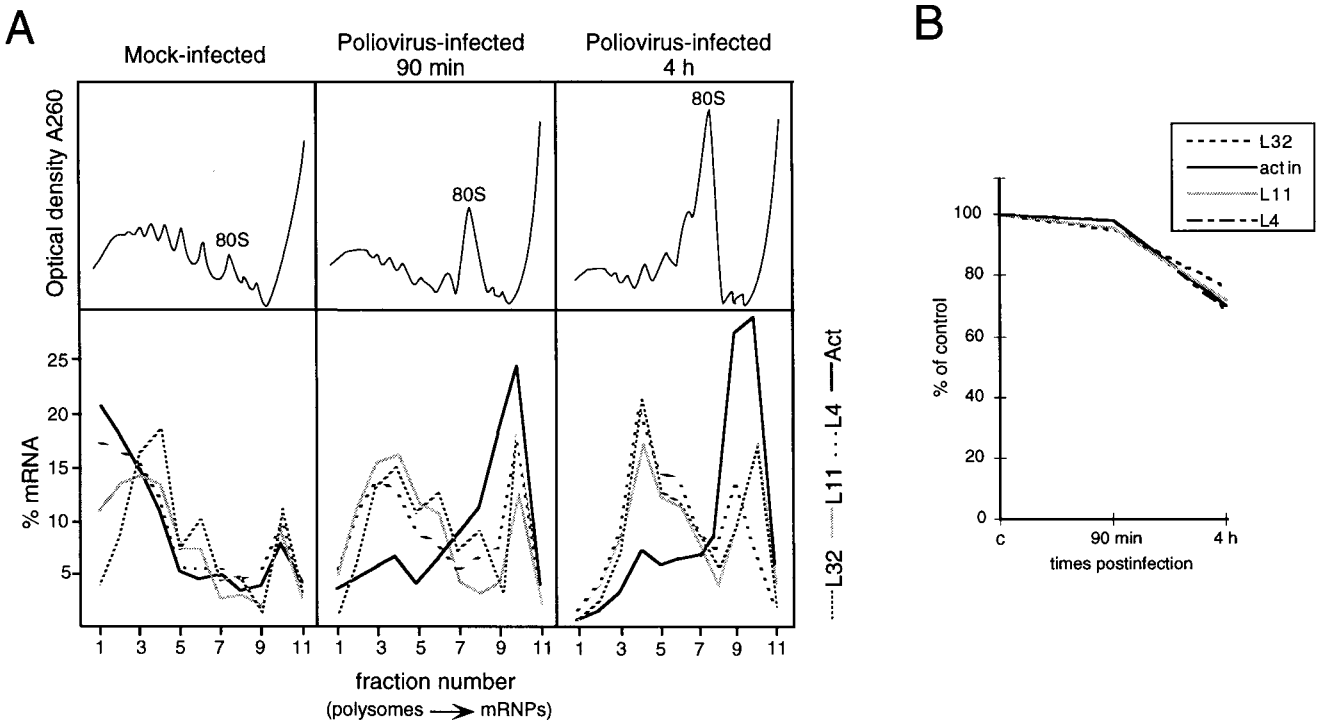

FIG. 2. Time course of polysome-mRNP distribution and accumulation of mRNAs during poliovirus infection. (A) Mock-infected and poliovirus-infected cells were processed at the times indicated as described in the legend to Fig. 1. Northern blots were subsequently hybridized with rp-L4, rp-L11, rp-L32, and β-actin probes. Measurement of radioactivity, reported as the percentage of mRNA in each fraction, was done by PhosphorImager (Molecular Dynamics) analysis. The optical density profiles of the sucrose gradients were monitored at 260 nm (top), and the positions of the 80S monomers are indicated. These experiments were performed at least three times, and the results were consistently similar. (B) One tenth of each extract, prepared at the indicated time postinfection, was taken before gradient loading. The RNA was extracted, and equivalent amounts were analyzed by Northern blotting as described in the legend to Fig. 1. The filters were subsequently hybridized to rp-L4, rp-L32, rp-L11, β-actin, and 5S RNA probes (46). Measurement of radioactivity was done by PhosphorImager (Molecular Dynamics) analysis, and the values obtained were normalized to the signal of the 5S rRNA probe. The level of each mRNA is expressed as a percentage of the amount measured in mock-infected cells (c).

r proteins are stable, as they can assemble with rRNA into ribosomes (52). To ascertain rp-mRNA association with polysomes later in infection as well, some control experiments were carried out. Cytoplasmic extracts were prepared 4 h postinfection and treated with EDTA to dissociate polysomes. Compared to untreated infected cells, a typical rp-mRNA such as L4 was shifted by EDTA treatment to the top of the gradient, implying association with polysomes (Fig. 3C, top and middle). Similar results were obtained for the other rp-mRNAs (data not shown). Moreover, to show that polysome association was due to active translation, cells were treated with pactamycin for 30 min at 3.5 h postinfection. This drug, a translation inhibitor at the initiation step, caused a decrease in polysome size which resulted in the accumulation of L4 mRNA in the dimer and 80S fractions (Fig. 3C, bottom). The same occurred to other rp-mRNAs (data not shown). Rehybridization of the filters with the viral probe showed a signal of the expected size peaking on fraction 3. This might represent the viral particles, which migrate close to, but never coincide with, the rp-mRNAs (data not shown). These results support the hypothesis that rp-mRNAs associate with polysomes, a view further strengthened by the fact that at 2 h postinfection, a similar RNA distribution along the gradient corresponds to protein synthesis activity.

From the results described above, it appears that rp-mRNAs are fairly resistant to protein synthesis shutoff compared to β-actin mRNA. Therefore, in spite of the complete cleavage of eIF4G which already occurs 1 h after infection (Western blot in Fig. 4), rp-mRNAs can still initiate protein synthesis. Similarly, HSP mRNAs are resistant to poliovirus-induced shutoff and it has been proposed that they may utilize cap-independent initiation (48). The limited secondary structure of the HSP 5′UTR, as well as the short and unstructured 5′UTR of rp-mRNAs, may determine a lower dependence on initiation factors compared to more-structured mRNAs (9, 20, 53). In line with this, it has been reported that the efficiency of translation of rp-mRNAs is regulated independently of the level or activity of eIF4E (51), whereas the selective translational repression of mRNAs bearing extensive secondary structure in the 5′UTR is relieved by the overexpression of this factor (27). It was recently reported that eIF4GII, a functional homolog of eIF4G (hence called eIF4GI), can persist longer in poliovirus-infected cells, as shown by the fact that about 30% of the entire form still persists at up to 2 h after infection (14, 15). It can be argued that the rp-mRNA association with polysomes and the r protein synthesis described here could be sustained by eIF4GII in infected cells. However, later in infection, when eIF4GII is completely cleaved, rp-mRNAs are still associated with polysomes, suggesting that other elements are also involved.

Analysis of $p70^{S6k}$ activity and S6 phosphorylation during poliovirus infection. As mentioned above, a relationship among translation of rp-mRNAs, activity of $p70^{S6k}$, and phosphorylation of r-protein S6 has been reported (23, 24, 55). To investigate the state of $p70^{S6k}$ activity after poliovirus infection, we performed in vitro kinase assays by using $p70^{S6k}$ immunoprecipitated from equal amounts of mock-infected and infected-cell extracts and 40S ribosomal subunits as a substrate (16). As

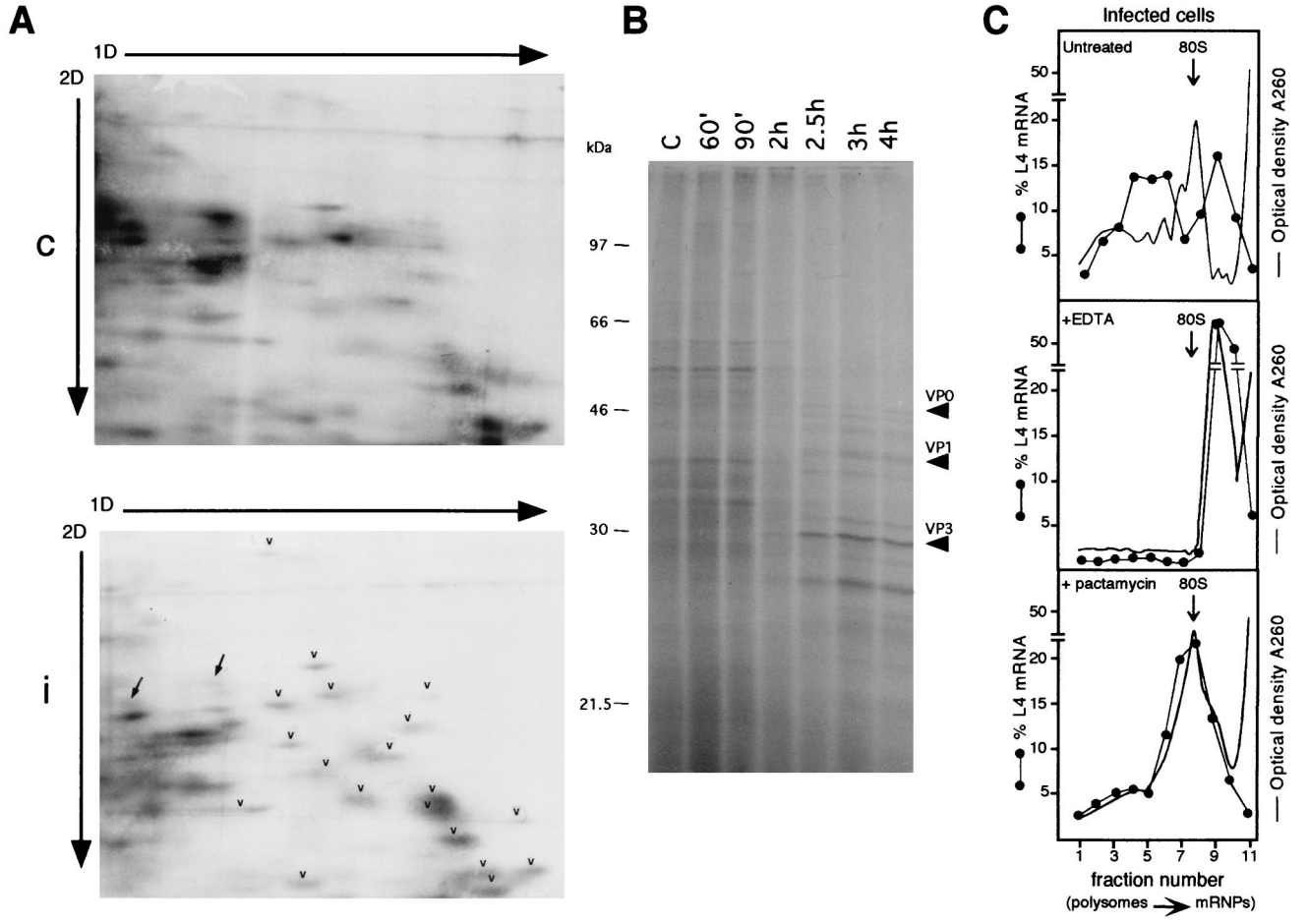

FIG. 3. Analysis of the activity of polysome-associated RNA in infected cells by metabolic labeling of proteins and by EDTA and pactamycin treatment. (A) HEp-2 cells were grown and infected as described in the legend to Fig. 1. At 90 min postinfection, mock-infected and infected cells were incubated for 30 min with [^{35}S]methionine-[^{35}S]cysteine (Pro-mix; Amersham; >1,000 Ci/mmol) at a concentration of 0.1 mCi/ml. Cells were harvested in phosphate-buffered saline and homogenized in 0.5 ml of ice-cold rp buffer (0.1 M NaCl, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5), acid extracted (58), and processed by the 2D gel electrophoresis method optimized to resolve r proteins, with the exception that the second-dimension (2D) gel was 13% polyacrylamide (58). A 200-μg sample of r proteins purified from HEp-2 ribosomes (58) was added as markers to an equal amount of control (c) or infected-cell (i) protein extract (200 μg). Gels were fluorographed and exposed to X-ray film for the same time. Arrowheads point to some r proteins, and arrows point to non-r proteins. 1D, first dimension. (B) HEp-2 cells, grown and infected as indicated above, were labeled with Pro-mix (40 μCi/ml) for 10 min at the indicated times after infection. Cells were lysed as described above, and 5 μg of each extract was loaded onto an SDS–12.5% PAGE gel and autoradiographed. Arrows point to viral proteins. Lane C, control. (C) Untreated infected cells (top) were lysed at 4 h postinfection and analyzed on a sucrose gradient as described in the legend to Fig. 1. For the EDTA treatment (middle), cytoplasmic extracts, brought to a concentration of 50 mM EDTA, pH 7.4, were incubated in ice for 5 min, loaded onto sucrose gradients containing 10 mM EDTA instead of magnesium, and analyzed as described in the legend to Fig. 1. To test the effect of pactamycin (bottom), cells were incubated at 3.5 h after infection with 30 ng of pactamycin per ml for 30 min, thus reaching the 4-h infection time of untreated cells, and then processed as described in the legend to Fig. 1. When the gradient fractions were collected, the optical density at 260 nm was monitored. The profiles are shown as a continuous line, and the 80S monomers are indicated by the arrows. Northern blots were hybridized with an rp-L4 probe. Measurement of radioactivity, reported as a percentage of the mRNA in each fraction, was done by PhosphorImager (Molecular Dynamics) analysis.

exemplified by the experiment of Fig. 5A, the ability of $p70^{S6k}$ to phosphorylate S6 was maintained and even increased 1 h after infection, reaching a level of two- to three-fold at 5 h. Similar results were consistently obtained by either immunoprecipitation or direct incubation with 40S ribosomes of equal amounts of control and infected-cell extracts. Although we cannot determine whether this was due to higher $p70^{S6k}$ activity or to an increased amount of it, the experiments reproducibly showed an increase in the capacity of $p70^{S6k}$ to phosphorylate S6 in infected cells. Then, to analyze S6 phosphorylation in vivo, cells were labeled with [^{32}P]orthophosphate for 90 min at 2.5 h postinfection, thus reaching a total infection time of 4 h, when the in vitro phosphorylating activity was still increasing (Fig. 5A). Proteins were analyzed by 2D gel electrophoresis as previously described (33), with the exception that in the second dimension, a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis (PAGE) gel was used. The 2D gel electrophoresis conditions were set up to map S6 phosphorylated forms, as indicated in the legend to Fig. 5B. In this system, the hyperphosphorylated forms migrate slower in the first dimension. Figure 5B shows two identical gels loaded with extracts from [^{32}P]orthophosphate-labeled control and infected cells. Compared to control cells, infected cells show a slight shift of the S6 spot toward the anode, as measured by the relative positions of the radioactive S6 and stained, purified r proteins included in each sample. A small decrease in hypophosphorylated forms and a small increase in hyperphosphorylated forms can be seen. This finding, observed in repeated experiments, suggests that in infected cells, the level of S6 phosphorylation is maintained and indeed slightly increased, compared to that in control cells, in line with the result of the $p70^{S6k}$ kinase assay. Similarly, the activity of the

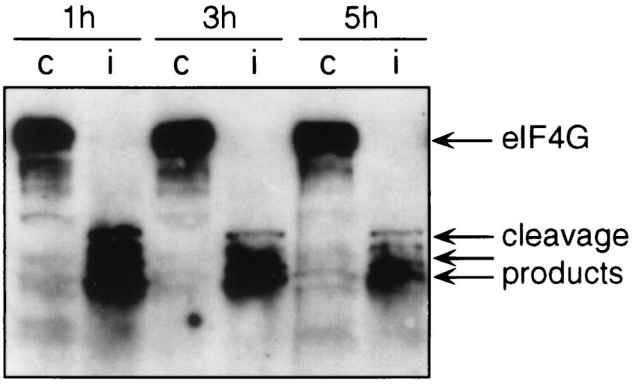

FIG. 4. eIF4G cleavage in poliovirus-infected cells. Cytoplasmic extracts from mock-infected (lanes c) and poliovirus-infected (lanes i) cells were prepared at the times indicated as described in the legend to Fig. 1. Aliquots of the cytoplasmic extracts were precipitated with acetone for protein analysis by SDS-PAGE and Western blotting using the anti-eIF4G antibody as previously described (18).

S6 kinase and S6 phosphorylation are stimulated following herpes simplex virus type 1 infection (21, 35).

It is known that both $p70^{S6k}$ activation and the phosphorylation of the initiation factor 4E binding protein (4E-BP1) are mediated by the mTOR-FRAP signalling pathway (3, 31, 57). The different phosphorylation state of 4E-BP1 affects eIF4E activity, since the dephosphorylated form can sequester eIF4E, thus blocking cap-dependent initiation (30, 42). It has been recently proposed that the $p70^{S6k}$–4E-BP1 phosphorylation pathway bifurcates immediately upstream from $p70^{S6k}$ (56). Interestingly, it has been found that 4E-BP1 becomes dephosphorylated after poliovirus infection (13) while our data indicate that $p70^{S6k}$ activity and S6 phosphorylation do not decrease but are maintained and somewhat stimulated in infected cells. If this is the case, these observations might suggest that poliovirus infection either influences the $p70^{S6k}$–4E-BP1 kinase pathway downstream from the bifurcation or differentially regulates specific phosphatases.

In conclusion, we have identified a new class of cellular mRNAs that, besides HSP (39), and some IRES-containing cellular mRNAs recently analyzed with an approach similar to the one used in this study (25), can be translated in poliovirus-infected cells. Moreover, we have shown that the selective translation resistance of rp-mRNAs correlates with the maintenance of $p70^{S6k}$ activity and with a small, but consistent, phosphorylation increase in r protein S6. It is hard to believe that r protein synthesis resistance is an advantage for the virus, as rRNA synthesis is inhibited and ribosome assembly is not possible. It is more likely that r protein mRNA translation can continue because the conditions required are still sufficient, in spite of the drastic damage to cellular translation initiation generated by the infection. This translation survival, that so far can be identified as lower dependence on initiation factors, may reveal a feature of the normal mechanism governing rp-mRNA translation in the cell. It is possible to speculate that this mechanism can be due to the short and typical rp-mRNA 5′UTR; to the utilization of specific ribosomes containing hyperphosphorylated S6, a feature that appears to persist under infection conditions; and to auxiliary factors that specifically bind the 5′UTR of rp-mRNAs (44, 45). Interestingly, one of these binding factors, La protein, is known to have a positive role in poliovirus RNA translation in vitro (36). Study of the mechanisms that govern cellular mRNA resistance to poliovirus-induced shutoff of protein synthesis adds to our knowledge of the cellular response to viral infection and should provide important clues to understand the translational regulation of mRNAs.

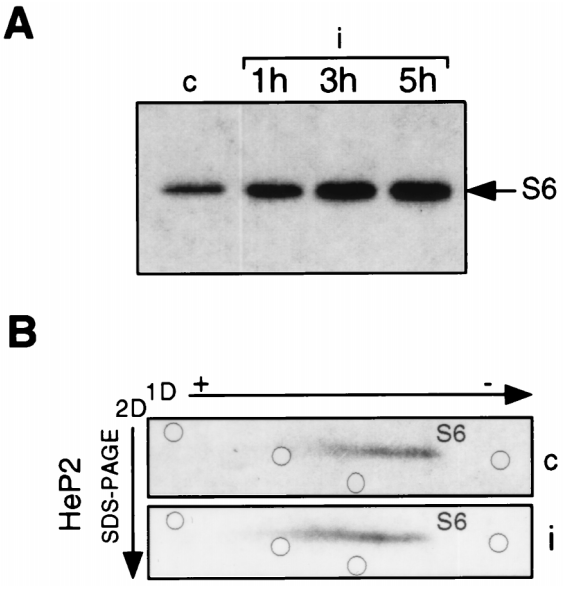

FIG. 5. $p70^{S6k}$ activity and S6 phosphorylation during poliovirus infection. (A) S100 cytoplasmic extracts from mock-infected (lane c) and poliovirus-infected (i) cells were prepared at the times indicated in the presence of a phosphatase inhibitor as previously described (40). Protein concentration was determined by the Bio-Rad Protein Assay kit. Eight-microgram extract samples were used for immunoprecipitation of $p70^{S6k}$ with M6 antibody, and the immunocomplex was assayed for in vitro kinase activity in the presence of 40S ribosomes at an A_{260} of 0.45 U as previously described (16). Proteins were separated by SDS–PAGE and autoradiographed. Phosphorylated S6 protein is indicated by the arrow. (B) Mock-infected and poliovirus-infected cells (c and i, respectively) were labeled at 2.5 h after infection in a phosphate-free medium with 20 μCi of [^{32}P]orthophosphate per ml for 90 min. Ribosomes from labeled cells were purified, and ribosomal proteins were extracted in the presence of 40 mM β-glycerophosphate (58). Proteins were analyzed by 2D electrophoresis as previously described (33), with the exception that the second dimension (2D) was an SDS–15% PAGE gel. A 200-μg sample of unlabeled purified HEp-2 ribosomal proteins was added to each sample before gel loading for Coomassie staining and used as position markers in the gel. The positions of stained ribosomal proteins in the S6 area are indicated by open circles. To standardize 2D gel electrophoresis conditions and to map the positions of S6 phosphorylated forms, preliminary experiments were carried out by loading gels with purified 40S subunits phosphorylated in in vitro kinase assays by extracts from quiescent and serum-stimulated Swiss 3T3 cells (16). The level of S6 phosphorylation by quiescent cell extracts was very low and increased 20-fold after serum stimulation. Accordingly, an evident shift in migration of S6 in the 2D gel was observed (data not shown). 1D, first dimension.

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