JOURNAL OF VIROLOGY, May 1978, p. 510-521
0022-538X/78/0026-0510$02.00/0

Vol. 26, No. 2
Printed in U.S.A.

Control of Protein Synthesis in Extracts from Poliovirus-Infected Cells

I. mRNA Discrimination by Crude Initiation Factors

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Received for publication 28 December 1977

By using cell-free systems prepared from uninfected and poliovirus-infected cells, we have been able to demonstrate that crude preparations of initiation factors from infected cells do not stimulate the initiation of translation by polyribosomes containing endogenous host cell mRNA. When tested with polysomes containing endogenous viral mRNA, however, they were able to stimulate initiation of translation nearly as well as uninfected cell initiation factors. The uninfected cell initiation factor preparations were able to stimulate initiation of translation of both cell and viral mRNA. The results indicate an mRNA-specific activity present in crude initiation factor preparations from infected cells. Furthermore, the ability of eIF2 from infected cells to form a ternary complex with GTP and formyl [^{35}S]methionine-tRNA$_f^{met}$, an mRNA-independent step in initiation, was found not to be deficient. Implications of these data for proposed mechanisms of poliovirus-induced host cell shutoff are discussed.

The inhibition of host cell protein synthesis by picornaviruses has been recognized for some time (27), and yet the mediator and the mechanism of inhibition remain unclear. Steiner-Pryor and Cooper isolated poliovirus mutants that were temperature sensitive for the host cell protein synthesis shutoff function, all of which mapped in the coat protein region of the poliovirus genetic map (35). Subsequently, Wright and Cooper observed viral capsid proteins associated with infected cell ribosomes in vivo (39). From these observations they proposed the "equestron" model, whereby viral proteins somehow regulate translation in the infected cell by their ability to bind to the ribosome after translation. This model fails to account for the observed shutoff that occurs in the infected cell in the presence of guanidine, a compound that prevents detectable synthesis of viral proteins (20). In additon, defective picornaviruses containing deletions in the coat protein genes can effect normal inhibition of cellular protein synthesis (10).

Ehrenfeld and Hunt observed that a fraction from poliovirus-infected cells could inhibit protein synthesis by reticulocyte lysates and determined that the inhibitory activity resided in viral double-stranded (ds) RNA (12, 21). It was proposed that host cell shutoff might be caused by the production of dsRNA during infection; but later studies demonstrated that the inhibitory effects of dsRNA in HeLa cell extracts showed no specificity between viral and cellular protein synthesis, as would be expected for a mediator of host cell shutoff that permits virus-specific translation (7).

Analysis of the kinetics of the inhibition of host cell translation led Willems and Penman to suggest that host cell mRNA was specifically modified or degraded after infection (38). When later tested, however, Colby et al. could not find a decrease in the stability of cell mRNA from infected cells or any gross changes in size by polyacrylamide gel electrophoresis (PAGE) (9). Fernandez-Muñoz and Darnell (15) have also shown that the 5′ cap structure and the 3′ polyadenylic acid of cell mRNA remained essentially unchanged after infection, and no unusual base modifications were evident. Finally, Ehrenfeld and Lund (13) demonstrated that mRNA extracted from infected cells in which its translation was inhibited remained functional for translation in a cell-free wheat germ extract.

A finding by Nuss et al. (30) that hypertonic salt treatment of intact cells could block ribosome initiation on cell mRNA at concentrations not affecting viral mRNA translation prompted other workers to suggest this as a mechanism for host cell shutoff (6). Carrasco and Smith (6) and Egberts et al. (11) have documented that intracellular ion concentrations change in infected cells, but these changes appear to occur late in

infection, after inhibition has occurred, and the relevancy of this observation to host cell shutoff in vivo has not yet been convincingly demonstrated. A comparable phenomenon is the observation by Lawrence and Thach (25) that virion RNA is preferentially translated instead of cell mRNA when both are present at saturating levels in a cell-free translation system. This effect has been shown to be mediated by an mRNA-discriminating initiation factor, eIF4B (nomenclature according to that agreed upon at the International Symposium on Protein Synthesis, Bethesda, Md., 18–20 October 1976), and has led to the proposal by these workers that effective competition by viral mRNA for this initiation factor results in the observed synthesis of viral proteins and the exclusion of host cell mRNA translation. However, this model does not explain the occurrence of host cell shutoff in infected cells under conditions where no viral RNA is produced, i.e., ts mutants for RNA synthesis (35) and guanidine-treated infected cells (20).

To date, none of the proposals has convincingly accounted for all of the facts of picornavirus host cell shutoff and subsequent translation of viral proteins. Since the block in cell mRNA translation is recognized to be at the level of initiation of protein synthesis (26), we felt it was appropriate to examine the function of initiation factors in infected cells. Although two laboratories have reported previously that extracts prepared from uninfected and infected cells translate cell and viral mRNA equally well in vitro (1, 25), several considerations led us to reexamine the specificity of in vitro translation. First, non-preincubated extracts (S10s) prepared from infected cells synthesize only viral proteins (7) in response to endogenous mRNA, even though studies have now shown that endogenous cell mRNA functions perfectly well when removed from the infected cell and tested in cell-free systems (13, 23). Second, the systems reportedly showing nonselective translation of cell and viral mRNA's utilized purified RNAs as messengers. Proteins associated with messenger ribonucleoproteins, which might confer specificity to the translation of the two classes of mRNA, were removed by phenol extraction before testing. In addition, both studies used RNA extracted from virions as a source of viral mRNA; it is now known that viral mRNA associated with polysomes in the infected cell has a different 5′ terminus from that of virion RNA (15, 19, 28). Lastly, Kaufmann et al. (23) have demonstrated that the ribosomal salt wash prepared from cells early after infection, when inhibition of protein synthesis had occurred, but before significant viral translation, was unable to stimulate translation by polysomes carrying endogenous mRNA from uninfected cells. These latter results demonstrated that crude initiation factor preparations from infected cells no longer functioned in stimulating translation of endogenous cell mRNA, but their ability to stimulate translation of viral mRNA was not tested.

In this report, we have isolated crude ribosomal salt wash preparations from cells synthesizing cellular (uninfected) or viral (late-infected) proteins and also from cells that had ceased synthesizing cellular proteins but which had not yet begun significant synthesis of viral proteins (early-infected). These crude initiation factor preparations were evaluated for their ability to stimulate translation of endogenous cell and viral mRNA in non-preincubated cell-free systems. The results demonstrate that the infected cell ribosomal salt wash possesses an mRNA-specific activity that blocks initiation of translation of cell mRNA but still stimulates initiation of viral mRNA.

MATERIALS AND METHODS

Cells and virus. The growth of HeLa S3 cells and the purification of the Mahoney strain of poliovirus type 1 have been described previously (18).

Measurement of rates of protein synthesis in infected cells. Cells (5×10^6/ml) in MEM (Eagle minimal essential medium) in Spinner culture were infected with virus at a multiplicity of infection of 200 PFU per cell. After 30 min, fetal calf serum was added to a concentration of 5%. Every 30 min from 0.5 to 5.0 h postinfection (p.i.), 0.5 ml of infected cells was removed and incubated with 0.25 μCi of [^{35}S]methionine ([^{35}S]met) (specific activity, 503 Ci/mmol, New England Nuclear Corp.) for 10 min at 37°C in Spinner culture. The incorporation was stopped by the addition of cold Earles salt solution, and the cells were harvested and washed by centrifugation. Cells were lysed in 1 ml of water, and protein was precipitated in 5% trichloroacetic acid onto Whatman GF/C filters. The incorporation of [^{35}S]met was determined by liquid scintillation spectroscopy.

Preparation of S10s. A total of 4 liters of cells at 5×10^5/ml was concentrated 10-fold and either mock-infected or infected at the indicated times. The cells were then washed in cold Earles solution, centrifuged at $700 \times g$ for 5 min, and suspended in two-cell pellet volumes of PSB (10 mM KCl–1.3 mM $Mg(OAc)_2$–0.5 mM dithiothreitol (DTT)–10 mM HEPES [*N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid], pH 7.4). Cells were disrupted in a Dounce homogenizer and centrifuged at $1,500 \times g$ for 3 min. The supernatant was collected, the nuclear pellet was washed in 2 ml of PSB and recentrifuged, and the two supernatants were combined. The combined supernatants were then centrifuged at $10,000 \times g$ for 15 min in a Sorvall SS34 rotor, and the supernatant was collected. For use as an unfractionated S10, the supernatant was adjusted to 15% glycerol and stored at −80°C.

For fractionated preparations, the system was pre-

pared in a manner similar to that of Kaufmann et al. (23) except that a fraction of pH 5 was substituted for an S200. The Sorvall supernatant from above was centrifuged in a Spinco 65 rotor at 48,000 rpm at 4°C for 60 min. The supernatant was removed, and 2 volumes of 10 mM β-mercaptoethanol was added to it. The pH was adjusted to 5.1 with 1 M acetic acid, and the resulting suspension was centrifuged at 1,500 × g for 10 min. The supernatant was discarded, and the pellet was suspended in 1/6 the original S10 volume of buffer containing 50 mM KCl-3 mM $Mg(OAc)_2$-30 mM Tris-hydrochloride (pH 7.4)-10 mM β-mercaptoethanol. The pH 5 fraction was stored at −80°C (14). The ribosome pellet from the 48K-rpm centrifugation was resuspended in 1.5 ml of PSB, the KCl concentration was adjusted to 0.5 M, and the sample was stirred at 4°C for 20 min. The ribosomes were then collected by centrifugation in a Spinco 65 rotor at 48,000 rpm at 4°C for 60 min suspended in 2 ml of 0.25 M sucrose-1 mM DTT-0.2 mM EDTA and stored in portions at −80°C. The salt wash was twice dialyzed against 2 liters of 5 mM Tris (pH 7.4)-100 mM KCl-0.05 mM EDTA-5 mM β-mercaptoethanol for 4 h and stored at −80°C.

Preparation of formyl [^{35}S]met-tRNA$_f^{met}$. Charged formyl [^{35}S]met-tRNA$_f^{met}$ was prepared as described previously (7). Specific activity of the final product was 3.14×10^5 cpm/μg of tRNA. Purity of the final product was analyzed by chromatography of the deacylated tRNA on Dowex 50 (5) and by electrophoresis at pH 3.5 of the RNase T1 digestion products (34). The preparation was shown to be greater than 98% radiochemically free of nonformylated [^{35}S]met-tRNA$_f^{met}$ and [^{35}S]met-tRNA$_m^{met}$.

In vitro protein synthesis assay. Incubation mixtures (100 μl contained either 40% unfractionated S10 or fractionated components as described, 1 mM ATP, 0.2 mM GTP, 25 mM creatine phosphate, 0.2 mg of creatine kinase per ml, 30 mM HEPES-KOH (pH 7.4), 3 mM $Mg(OAc)_2$, 80 mM KCl, 0.38 μM each of 19 unlabeled amino acids, 200 μM methionine, 1 mM DTT, 2.5 μCi of [^{3}H]leucine (40 to 60 Ci/mmol; New England Nuclear Corp.), and 6.6 μg of tRNA containing formyl [^{35}S]met-tRNA. The reaction mixtures were incubated at 32°C, and duplicate 40-μl samples were removed at various times, diluted with 400 μl of 0.1 M KOH, and incubated for 30 min at 32°C to deacylate the remaining charged tRNA. A 2-ml portion of 10% trichloroacetic acid was added to each sample, and the precipitates were cooled at 4°C before collection on Whatman GF/C glass fiber filters. Radioactivity was determined in a Beckman liquid scintillation counter.

SDS-PAGE. S10s were incubated in vitro, and newly translated proteins were labeled with either 3.0 μCi of formyl [^{35}S]met-tRNA$_f^{met}$ or 2.0 μCi of [^{35}S]methionine per 100-μl reaction for 30 min at 32°C. The samples were diluted with gel sample buffer and subjected to sodium dodecyl sulfate (SDS)-PAGE electrophoresis in a linear 7.5 to 30% polyacrylamide slab gel, according to the procedure of Laemmli (24). For examination of radioactively labeled proteins, the fluorography technique of Bonner and Laskey (3) was used. The gel was exposed to Kodak SB-4 X-ray film for various lengths of time before film development.

Ternary complex assay. The assay for ternary complex formation between eIF2, GTP, and [^{35}S]met-tRNA was performed according to the method of Safer et al. (32). Each microliter reaction contained 0.8 mM GTP, 40 mM Tris (pH 7.4), 40 mM KCl, 6.6 μg of tRNA containing formyl [^{35}S]met-tRNA, and varying amounts of crude ribosomal salt wash. Duplicate samples were incubated at 37°C for 10 min and then diluted with 1.5 ml of 20 mM Tris (pH 7.4) to 50 mM KCl-10 mM $MgCl_2$-1 mM methionine. The samples were filtered onto 0.45-μm nitrocellulose filters (Millipore Corp.), and the radioactivity was determined in a Beckman liquid scintillation counter.

RESULTS

Preparations of extracts. As previously described by this and other laboratories, the rate of protein synthesis in poliovirus-infected cells initially appears identical to that in uninfected cells until 1 h p.i., when a dramatic decrease in the rate of amino acid incorporation into trichloroacetic acid-precipitable material occurs. This phenomenon is depicted in Fig. 1 (●), which shows the results of a typical analysis of the rate of protein synthesis in vivo as a function of time after infection. At 2 h p.i., the rate of incorporation began to increase until it peaked at about 2.75 h, and then incorporation again fell off rapidly. SDS-PAGE analysis of the proteins synthesized subsequent to 2.0 h p.i. demonstrated that all were virus specific (data not shown).

To study protein synthesis in vitro by using cell extracts prepared during the various stages of virus-induced regulation of protein synthesis, three unfractionated S10s were prepared: an un-

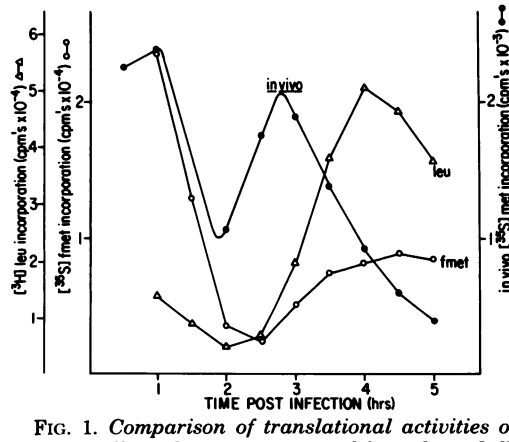

FIG. 1. *Comparison of translational activities of infected cells and extracts prepared from them. Cells were pulse-labeled with [^{35}S]methionine for 10 min at half-hour intervals p.i. and assayed for trichloroacetic acid-precipitable radioactivity* (●). *Unfractionated S10s were also prepared from infected cells at the same half-hour intervals and evaluated in vitro for incorporation of [^{3}H]leu* (△) *and formyl [^{35}S]met* (○) *into trichloroacetic acid-precipitable radioactivity.*

infected S10 (U-S10u); an early-infected S10 (E-S10u) prepared at 1.75 h p.i. (when cell mRNA translation was maximally diminished, but viral translation was not yet significant); and a late-infected S10 (L-S10u, prepared during the peak of viral translation, 2.75 h p.i., when only viral mRNA was being translated). Whereas the uninfected S10 was quite active in vitro and the early-infected S10 was inactive, as expected, the late-infected S10 was also found to be relatively inactive, despite the high rate of protein synthesis in the infected cells from which the extracts were prepared (data not shown). Attempts to manipulate the reaction conditions failed to increase the activity of the L-S10.

To examine this more closely, unfractionated S10s from infected cells were prepared every 30 min from 1.0 to 5.0 h p.i., and their activities in vitro were assayed by measuring incorporation of formyl [^{35}S]met-tRNA (to measure initiation of new proteins) and [^{3}H]leucine ([^{35}S]leu) (to measure elongation of both previously and newly initiated proteins). The data (Fig. 1) reveal a curious pattern. Although the activity of the extracts for incorporation of both formyl [^{35}S]met and leucine declined at 1.0 h p.i. as expected, the increase associated with viral translation was not seen until 3.0 h p.i. and did not reach a maximum until 4.0 to 4.5 h p.i., long after viral translation had diminished in infected cells. This disparity between the translational activities of infected cells and extracts prepared from those cells has been previously reported (11). The data in Fig. 1 also show that although the late-infected extracts possessed only about 30 to 40% of the initiation activity of uninfected extracts (formyl [^{35}S]met), they incorporated nearly four times as much [^{3}H]leu. Egberts et al. (11) gave a possible explanation for this phenomenon, demonstrating that leakage of ATP and critical ions occur late in infected cells, which might result in inefficient translation rates in vivo, which are corrected under the in vitro assay conditions. A discussion of their findings and its relationship to the data shown in Fig. 1 is presented below.

The products of the extracts described in Fig. 1 were analyzed by SDS-PAGE. Figure 2 shows that the overall pattern of host cell translation, shutoff, and later preferential viral translation seen in infected cells are reflected in extracts made from those cells, but the timing of these events is shifted, as suggested by the incorporation data. The use of both formyl [^{35}S]met-tRNA and [^{35}S]met shows that host cell proteins are made up to 1.5 h p.i.; at 2.0 and 2.5 h p.i. few proteins are synthesized, but most appear to be viral, and after 3.0 h p.i. only viral proteins are synthesized. The complexity of the pattern displayed by fmet-labeled proteins synthesized by late-infected extracts was somewhat surprising since poliovirus RNA is thought to have either one (36) or two (8) translational initiation sites. The reason for this complexity is not completely understood but may be due to incomplete cleavages or premature terminations. This problem is currently under study in this laboratory. As noted previously (30), some host proteins are more resistant to shutoff than others. In addition, at least one formyl [^{35}S]met-labeled viral protein (~115,000 daltons) appears to be preferentially synthesized at 3.0 to 3.5 h p.i., whereas most of the others are made at equal relative frequencies from 3.0 to 5.0 h p.i. This may represent some level of regulation not yet recognized in poliovirus replication.

For further examination three extracts were used: (i) a U-S10, which possesses and translates only cell mRNA; (ii) an E-S10, prepared at 2.0 h p.i., containing mostly cell mRNA and small amounts of viral mRNA, but which is intrinsically very inactive; and (iii) an L-S10 prepared at 4.0 h p.i., which possesses both types of mRNA but only translates viral mRNA. Unfractionated and fractionated S10s were prepared from uninfected and infected cells and evaluated in vitro for initiation and total protein synthesis. Activities of S10u's and reconstituted S10f's are shown in Fig. 3. The absolute levels of radioactivity incorporated by unfractionated and fractionated S10s were not comparable since the pool sizes of amino acids were probably quite different in the two preparations due to the removal of amino acids during the preparation of the pH 5 fraction for the S10f. Nevertheless, the fractionated S10s from uninfected and infected cells showed identical relative activities to those of the unfractionated S10s. fmet incorporation by both uninfected and late-infected preparations was relatively active, whereas the early-infected preparations were quite inactive. Comparing [^{3}H]leu incorporation, the uninfected S10s were more active than the early-infected S10s, and the late-infected S10s were three- to sixfold more active than either. Fractionated S10 extracts from uninfected and infected cells were dependent upon the addition of the ribosomal salt wash factors for initiation activity (see below).

Evaluation of crude initiation factors. By using the fractionated S10 systems from uninfected, early-, and late-infected cells, the ability of crude salt wash prepared from each extract to stimulate the initiation of translation of endogenous cell mRNA was evaluated. Fractionated S10s were reconstituted as described in Table 1. It is evident from the incorporation of fmet that both polysome preparations were very depend-

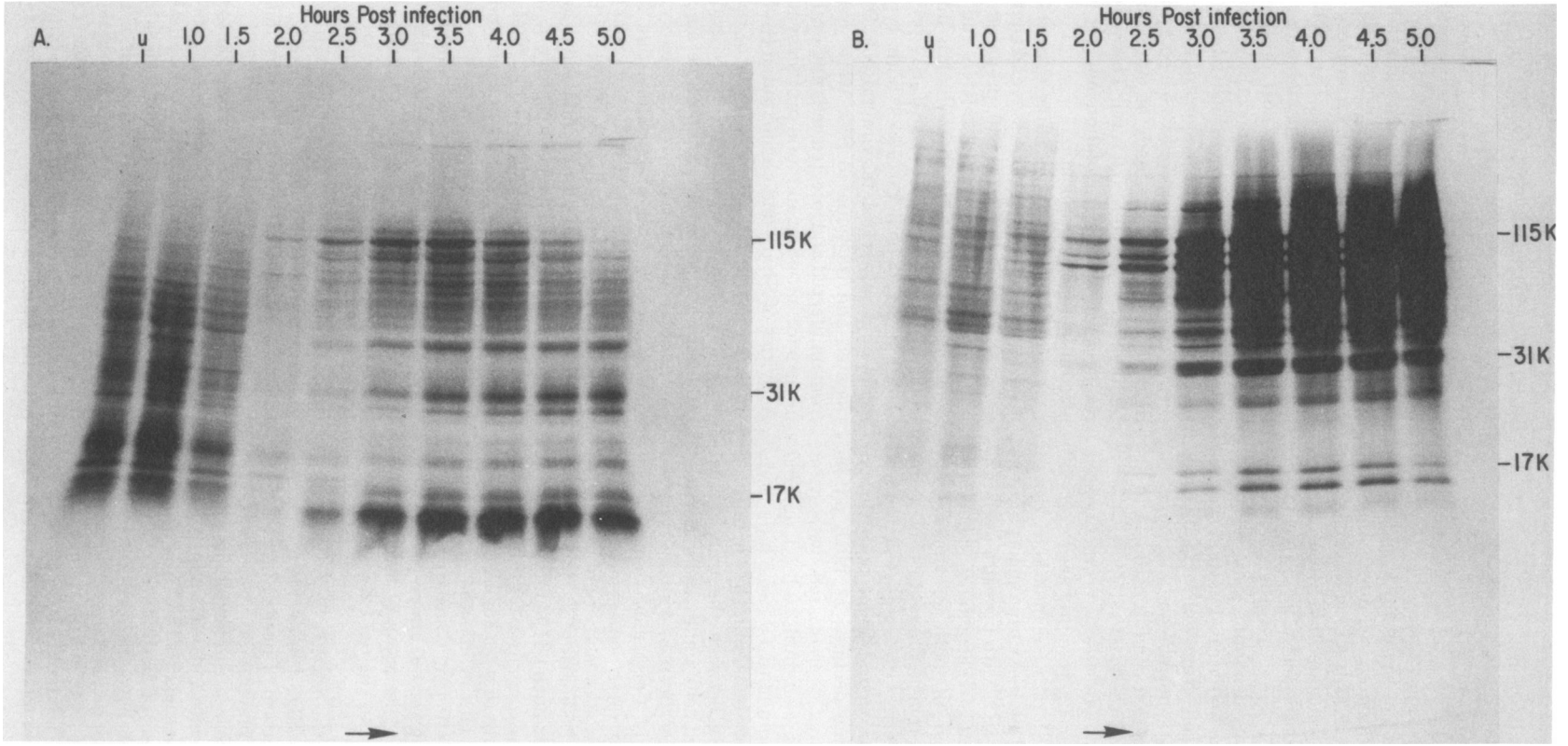

FIG. 2. *SDS-PAGE analysis of proteins synthesized by infected cell extracts. Unfractionated S10s were incubated in vitro with either formyl [^{35}S]met-tRNA (A) or [^{35}S]met (B), and the newly synthesized proteins were analyzed by SDS-PAGE and subsequent fluorography. The times at the top of the autoradiographs indicate the time p.i. at which the S10 was prepared; u represents an uninfected S10. The arrow at the bottom of each autoradiograph marks the bottom of the gel. Numbers to the right side represent molecular weights of poliovirus-infected cell proteins as determined in this laboratory.*

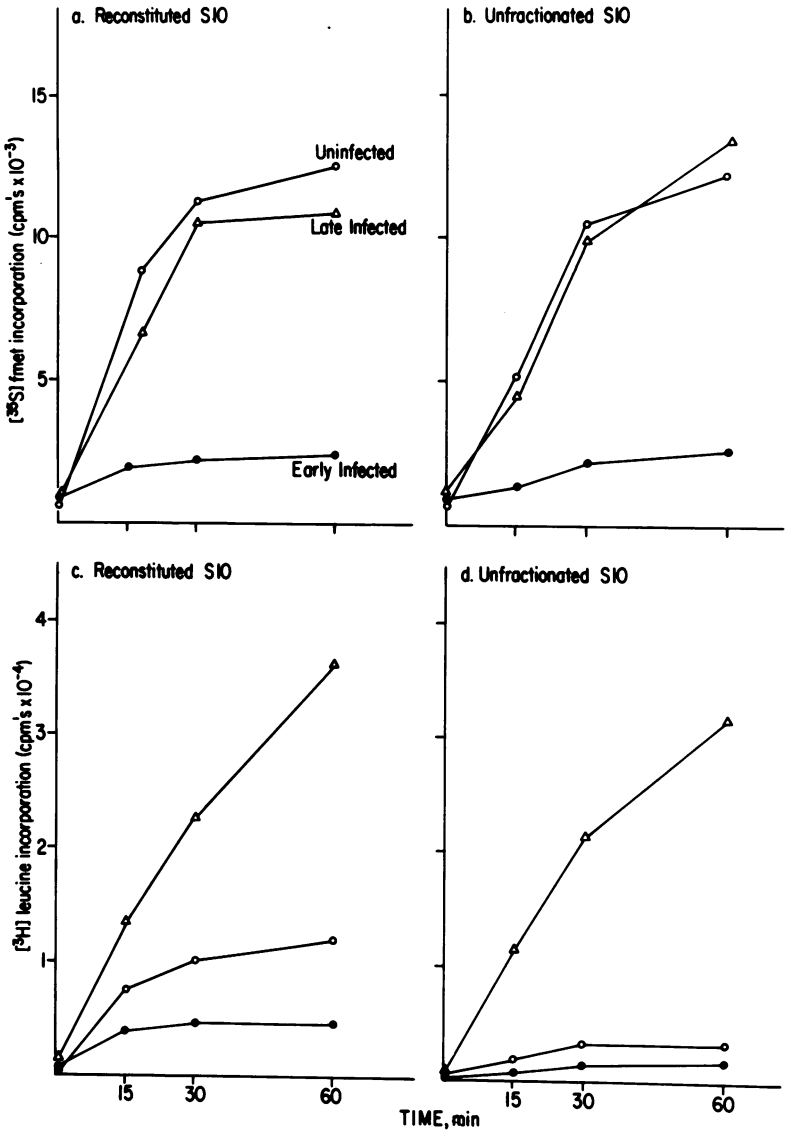

FIG. 3. *Translational activities of unfractionated S10s and reconstituted fractionated S10s. Unfractionated and fractionated S10s were prepared from uninfected cells* (○), *early-infected cells* (●), *and late-infected cells* (△) *and evaluated in vitro with either formyl [^{35}S]met-tRNA (a and b) or [^{3}H]leu (c and d). Reactions contained either 40% unfractionated extract or fractionated components (7.5-μl fraction [pH 5], 10 μl of polysomal fraction, 10 μl of salt wash). Samples of 40 μl were taken at 0, 15, 30, and 60 min and assayed for trichloroacetic acid-precipitable radioactivity.*

ent upon added salt wash for initiation activity (Table 1). For instance, incorporation by uninfected polysomes was stimulated approximately 25-fold by the addition of the homologous ribosomal salt wash. All of this increase due to addition of salt wash was completely sensitive to edeine, an inhibitor of initiation (data not shown). The activity of the uninfected ribosomal salt wash was independent of the source of polysomes; however, salt wash preparations from infected cells were completely inactive. In agreement with the data of Kaufmann et al. (23), the early-infected salt wash did not stimulate translation by either uninfected or early-infected polysomes (37 and 14%, respectively); but in addition, late-infected salt wash prepared from cells actively translating viral mRNA also did not function in stimulating the translation of cellular mRNA (10%). Although the early-infected salt wash was prepared from cells that

TABLE 1. *Effect of ribosomal salt wash from uninfected and infected cells on translation of cell mRNA*[a]

Source of salt wash	Formyl [^{35}S]met incorporation (cpm) by:		[^{3}H]leu incorporation (cpm) by:	
	Uninfected polysomes	Early-infected polysomes	Uninfected polysomes	Early-infected polysomes
None	363	278	14,800	3,659
Uninfected	8,801 (100)	8,407 (100)	24,256 (100)	11,175 (100)
Early-infected	3,466 (37)	1,386 (14)	19,468 (51)	5,043 (18)
Late-infected	1,175 (10)	992 (9)	12,848 (−21)	3,765 (1)

[a] Fractionated S10s were reconstituted with 7.5 μl of uninfected fraction (pH 5), 10 μl of polysomes, and 20 μl of crude salt wash. After 30 min of incubation, duplicate 40-μl samples were evaluated for trichloroacetic acid-precipitable radioactivity. The extracts were assayed for both leucine and formyl-methionine incorporation. Incorporation by polysomes with uninfected salt wash minus that obtained without salt wash was defined as 100% (in parentheses). Stimulation of incorporation by alternate salt washes with polysomes was then established as a percentage of that obtained for uninfected salt wash.

TABLE 2. *Effect of ribosomal salt wash from uninfected and infected cells on translation of viral mRNA*[a]

Source of salt wash	Formyl [^{35}S]-met incorporation (cpm) by polysomes from late-infected cells	[^{3}H]leu incorporation (cpm) by polysomes from late-infected cells
None	529	27,052
Uninfected	8,913 (100)	43,831 (100)
Early-infected	8,449 (95)	42,807 (94)
Late-infected	7,403 (82)	36,338 (55)

[a] Late-infected polysomes were evaluated for their response to different crude ribosomal salt washes. See footnote *a*, Table 1, for experimental details. Numbers in parentheses represent percentages.

were inactive in protein synthesis, and therefore might possibly be intrinsically inactive, the late-infected salt wash was prepared from cells that were actively translating; therefore, they should contain initiation factor activity. Thus, it appears unlikely that early-infected initiation factors are inactive simply because of the inactive state of the cells from which they were prepared, since they share this inability to stimulate initiation of cell mRNA with late-infected initiation factors prepared from cells in a highly active state of translation.

The ability of the crude initiation factor preparations to stimulate translation of viral RNA was then determined by similar assay of in vitro protein synthesis by polysomes from late-infected cells (Table 2). A comparison of the data from Tables 1 and 2 shows that although infected cell initiation factors were completely inactive with polysomes translating endogenous cell mRNA, they did stimulate translation by virus-specific polysomes. Uninfected cell salt wash was equally active with cell or viral mRNA. Early-infected initiation factors were 95% as efficient as uninfected cell initiation factors for stimulation of viral mRNA translation, and late-infected preparations showed 82% of the uninfected cell initiation factor activity.

This observation that the infected cell ribosomal salt wash functions actively to stimulate initiation of translation of viral mRNA, but not cell mRNA, defines an mRNA-specific activity in the preparation from infected cells. These results are also consistent with those obtained by examining the incorporation of [^{3}H]leu (Tables 1 and 2). Although the effects of exogenous salt washes were not as prominent because of large amounts of previously initiated protein synthesis, especially by late-infected polysomes, whose runoff was not affected by added salt wash, infected cell initiation factors still appear to stimulate initiation only with viral mRNA.

Similar effects were observed by testing the ability of exogenous ribosomal salt washes to stimulate unfractionated S10s. Although unfractionated extracts possess endogenous initiation factor activity, exogenous salt washes are capable of stimulating initiation above the level supported by endogenous factors. Unfractionated S10s were tested with and without the addition of salt wash from uninfected and infected cells (Table 3). Salt wash was added to levels that provided maximal stimulation. The early- and late-infected cell salt washes stimulated an uninfected unfractionated S10 only slightly (3 and 3%, respectively), but these same preparations were quite active with late-infected unfractionated S10 extracts (59 and 125%, respectively). Both results were similar to those found in the fractionated systems.

Although the uninfected extracts contain only cell mRNA, the infected S10s contain a mixture of both cell and viral RNA. It has been demonstrated previously (7) and in Fig. 2 that infected cell extracts, using endogenous initiation factors, synthesize only viral proteins. To test whether the addition of excess uninfected cell salt wash could relieve the block in initiation of cell proteins by the infected S10, fmet- and met-labeled

reaction products synthesized with and without added salt wash were analyzed by SDS-PAGE. In no case did the addition of uninfected cell salt wash to an infected cell translation system stimulate the initiation of host cell proteins (data not shown).

These reconstitution experiments show that although uninfected salt wash at the level used would stimulate translation of either cell or viral mRNA, infected cell salt washes have been altered so much that, although they function well with viral mRNA, they no longer stimulate initiation directed by cell mRNA. However, since mRNA-specific initiation factor requirements have been previously reported for α and β globin mRNA's (29) and for encephalomyocarditis virus RNA (37), but were subsequently shown to represent only different rate associations and hence quantitative requirements of mRNA's for initiation factors (2, 22), a saturation experiment was performed to determine whether the addition of excess infected cell salt wash would eventually stimulate the uninfected S10f extract. Figure 4 shows the ability of increasing concentrations of uninfected and late-infected salt wash to stimulate initiation of cell and viral mRNA. In this experiment, increasing amounts of uninfected and late-infected salt wash were added to either 10 μl of uninfected (Fig. 4a) or 10 μl of infected (Fig. 4b) cell polysomes, and the incorporation of formyl [^{35}S]met was determined. Figure 4a shows that uninfected polysomes respond almost linearly to increasing amounts of uninfected salt wash from 0 to 40 μl, the highest concentration allowable in this system for mechanical reasons. On the other hand, formyl [^{35}S]met incorporation increased negligibly with increasing amounts of infected salt wash. When examined with infected cell polysomes (Fig. 4b), the uninfected salt wash stimulated initiation as well as with uninfected polysomes; however, as seen previously (Table 2) the infected salt wash also stimulated these polysomes. The activity of the infected cell salt wash was equal to that of the uninfected cell salt wash up to about 20 μl of salt wash per 100 μl of reaction, although at higher concentrations it appeared to be less active. This difference in activity between the two salt wash preparations at high concentrations on infected polysomes was slightly variable in different experiments. A concentration of 10 μl of salt wash per 10 μl of ribosomes represents the

TABLE 3. *Stimulation of unfractionated S10s by exogenous ribosomal salt wash*[a]

Source of salt wash	Formyl [^{35}S]met incorporation (cpm) by:		
	Uninfected S10	Early-infected S10	Late-infected S10
None	7,736	1,997	16,397
Uninfected	38,219 (100)	21,480 (100)	31,705 (100)
Early infected	8,546 (3)	4,706 (14)	25,437 (59)
Late infected	8,518 (3)	10,110 (42)	35,592 (125)

[a] Unfractionated S10s were tested as described in the text with and without additional salt wash (40 μl per 100 μl of reaction). After 30 min of incubation, duplicate 40-μl samples were evaluated for trichloroacetic acid-precipitable radioactivity. Incorporation by S10s with uninfected salt wash minus that obtained without salt wash was defined as 100% (in parentheses). Stimulation of incorporation in S10s by alternate salt washes was then established as a percentage of that obtained for uninfected salt wash stimulation.

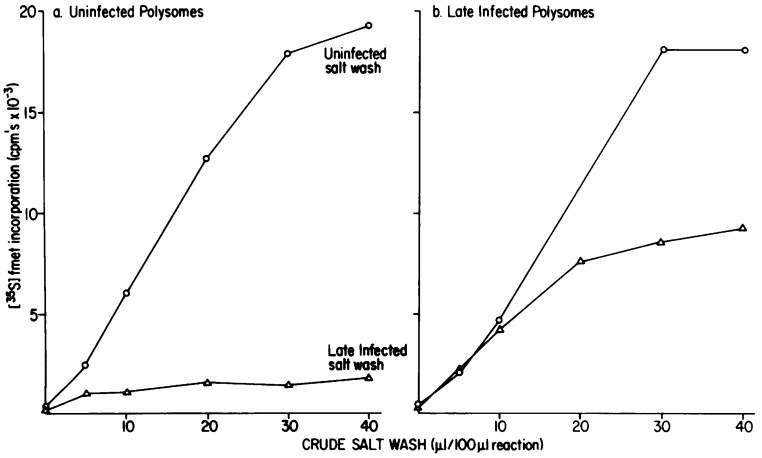

FIG. 4. *Response of uninfected and late-infected cell polysomes to variable amounts of salt wash. Uninfected (a) and late-infected (b) polysomes were tested in vitro for their response to different amounts of crude ribosomal salt washes. To 10 μl of polysomes and 7.5 μl of uninfected fractions (pH 5) were added different volumes of either uninfected* (○) *or late-infected crude ribosomal* (△) *salt washes. These reactions were incubated in vitro and assayed for their incorporation of formyl [^{35}S]met into trichloroacetic acid-precipitable material.*

approximate reconstitution of an unfractionated S10; these larger amounts might reflect unnatural ratios of initiation factors and mRNA not likely to be found in vivo.

The above data demonstrate that infected cell salt wash is inactive in the initiation of translation directed by uninfected cell mRNA, but is active in stimulating the translation of viral mRNA. It does not distinguish between a defective initiation factor activity or the presence of an inhibitor of this activity. To examine this latter possibility, a small amount (10 μl) of uninfected cell salt wash, which would allow a low level of initiation, was added to both uninfected and infected cell polysomes. In addition, increasing amounts of either uninfected or infected salt wash were added to observe their effect on the initiation activity of the first 10 μl of uninfected salt wash (Fig. 5). The addition of increasing amounts of uninfected salt wash to the 10 μl of starter uninfected salt wash and uninfected polysomes (Fig. 5a) again demonstrated the linear response of uninfected polysomes to increasing amounts of uninfected salt wash. However, when increasing amounts of infected salt wash were added to this system, there was no increase, but an actual decline in stimulation of initiation. If the infected salt wash were merely defective in some activity, which could have been compensated for by the starter uninfected initiation factors, an increase in incorporation might have been expected. Alternatively, a defective and rate-limiting activity might have produced no effect on fmet incorporation. The finding that the rate of incorporation actually declined with increasing amounts of infected salt wash suggests, but does not prove, the presence of an inhibitor in the infected salt wash. As expected, the addition of infected salt wash to infected polysomes and starter uninfected salt wash (Fig. 5b) did not cause a decline but rather an increase in the incorporation of fmet, reaffirming the mRNA specificity of the infected salt wash.

Ternary complex formation. The evidence presented so far indicates that crude initiation factor preparations from infected cells possess a specificity of function that depends on the translation of mRNA. An early step in the initiation process is ternary complex formation among eIF2, GTP, and [^{35}S]met-tRNA$_f^{met}$ (32), and this step is thought to be independent of mRNA. If the inhibition of host cell protein synthesis expressed in infected cells is specific for classes of mRNA, ternary complex formation should proceed normally. We have therefore tested the eIF2 activity in our crude ribosomal salt wash preparations from uninfected and infected cells. GTP and formyl [^{35}S]met-tRNA$_f^{met}$ were incubated at 37°C with either uninfected or infected salt wash preparations, and the reactions were then assayed for ternary complex formation by the nitrocellulose filter binding assay. Table 4 shows that late-infected salt wash possessed nearly twice the eIF2 activity of the uninfected salt wash. Increasing concentrations of salt wash above those shown gave no further increase in ternary complex formation, and the relative eIF2 activities of the uninfected and infected cell preparations were similar at all concentrations tested. These experiments were performed with the same formyl [^{35}S]met-tRNA preparations as those used in the translation assays reported above. Although the effect of methionine formylation on eIF2-dependent ternary complex

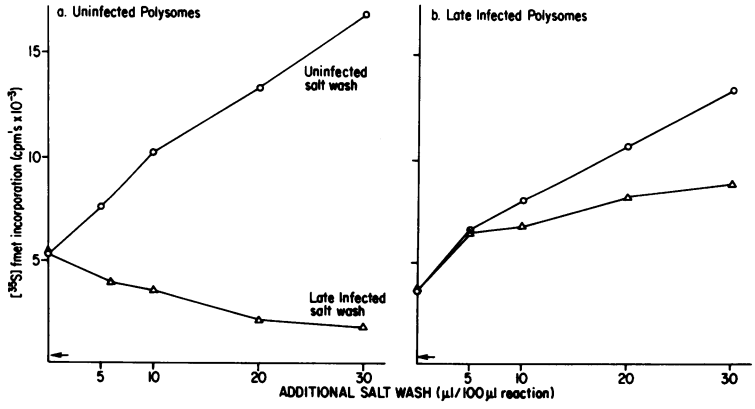

FIG. 5. *Effect of mixing ribosomal salt washes from uninfected and late-infected cells. To 7.5 μl of uninfected fraction (pH 5), 10 μl of uninfected cell salt wash, and either 10 μl of uninfected polysomes (a) or 10 μl of late-infected polysomes (b) were added different amounts of either uninfected* (○) *or late-infected* (△) *crude ribosomal salt washes. The reactions were incubated in vitro for 30 min when duplicate 40-μl samples were assayed for trichloroacetic acid-precipitable radioactivity.*

TABLE 4. *Ternary complex formation*[a]

Source of salt wash (μl/50-μl reaction)	Formyl [^{35}S]met-tRNA bound to nitrocellulose filters (cpm)
None	2,100
Uninfected	
1	4,300
10	10,200
Late-infected	
1	6,000
10	17,500

[a] eIF2 activity was measured in crude ribosomal salt washes from uninfected and late-infected cells by determining their ability to form a ternary complex with GTP and formyl [^{35}S]met-tRNA as described in the text. Ternary complex formation was dependent upon GTP in the reaction.

formation is not known, we conclude that the inactivity demonstrated by infected cell protein synthesis must occur at a later step in translation than ternary complex formation.

DISCUSSION

Use of infected cell extracts. The preparation of cell-free extracts from poliovirus-infected cells for use in in vitro translation studies posed several puzzling problems. First, infected cell extracts, prepared when virus-specific protein synthesis in vivo was maximal (2.75 h p.i.), were inactive (Fig. 1). Analysis of extracts prepared at half-hour intervals throughout the viral replication cycle revealed that the activity of extracts, prepared soon after infection, declined, as did the cells' rates of protein synthesis, but the subsequent peak of viral protein synthesis seen in infected cells was not reflected in the activity of the extracts until after a delay of about 90 min. At the times after infection when extracts were maximally active in vitro, the rates of protein synthesis in the cells from which the extracts were made were already declining due to completion of the replication cycle. Thus, two apparent inconsistencies occurred in the timing of extract preparation: (i) cells harvested at the peak of viral protein synthesis yielded extracts that were inactive, and (ii) cells harvested late in infection when viral protein synthesis has been completed yielded extracts that were highly active for in vitro translation of viral proteins.

The first inconsistency was the most surprising. A possible explanation is based on the previously reported observation that the majority of viral protein synthesis at midcycle occurs on polysomes that are bound to the endoplasmic reticulum (31). If these polysomes are released from membranes at later times, then the procedure to prepare S10 extracts would have resulted in our discarding the majority of active polysomes from extracts at the peak of viral protein synthesis, but not from those prepared at later times. In preliminary studies not reported here, we tested the activities of crude cytoplasmic extracts with and without a 10,000 × *g* centrifugation step from infected cells for their translational capacity in vitro. The results were consistent with the presence of a significantly larger fraction of activity in the discarded S10 pellet at the time of in vivo maximal viral protein synthesis when compared with S10 pellets from preparations at later times in infection. This possibility was not pursued further.

The second inconsistency deals with the increased activity of late-infected extracts, prepared from cells that were no longer active in viral protein synthesis. Egberts et al. (11) demonstrated that ATP and other factors critical for the maintenance of translation in the cell begin to leak from infected cells near the time of peak viral protein synthesis; they postulated that the resulting altered intracellular environment may be the cause of the decline in virus-directed protein synthesis late in infection. When extracts are prepared from such cells, relatively active preparations, which could be the result of an artificial restoration of ATP and other ionic conditions to optimal levels, are obtained. The results of these investigators suggest that as the rate of viral protein synthesis in late-infected cells declines, elongation is affected more drastically than initiation. This results in an accumulation of polysomes whose runoff is slow under in vivo conditions. The results reported here support this interpretation since the ratio of incorporated [^{3}H]leu to incorporated formyl [^{35}S]met by late-infected cell extracts is much higher than that by uninfected extracts ([^{3}H]-leu/formyl [^{35}S]met = 6.0 [infected] and 0.7 [uninfected] [Tables 1 and 2]). In addition, the majority of [^{3}H]leu incorporation in fractionated infected cell extracts is not dependent upon the addition of ribosomal salt wash (Table 2).

In light of previous reports from other laboratories (1, 25) that extracts from infected and uninfected cells show no specificity of translation for cellular and viral mRNAs, we have attempted to use translation systems that retained, as much as possible, the characteristics of the infected cell. At this time, we have no data regarding the failure of other reported extracts prepared from infected cells, which contain initiation factors comparable to our late-infected ribosomal salt wash, to restrict the

translation of host cell mRNA. However, we have tested our uninfected and infected initiation factor preparations in another translation system using rat liver ribosomal subunits and a pH 5 fraction and rabbit reticulocyte globin mRNA and have obtained results identical to those reported here (John Hershey and Tim Helentjaris, unpublished data).

mRNA specificity of infected cell extracts. The report by Kaufmann et al. (23) that ribosomal salt washes prepared from early-infected cells were inactive in stimulating translation of cell mRNA suggested the attractive hypothesis that poliovirus could inhibit host cell translation while permitting viral protein synthesis by a mechanism involving alteration of initiation factor(s) so that they remained functional only with viral mRNA. Whether the viral mRNA can utilize the "altered" factor(s) or whether it merely bypasses the requirement for an "inactivated" factor(s) was not distinguished. At least two alternative hypotheses, however, were also consistent with their data. First, the early-infected ribosomal washes were prepared from cells that were translationally inactive and which contained very few polysomes; thus, it was possible that such cells would yield inactive initiation factors regardless of the mechanism for inhibition of protein synthesis. By this reasoning, an inactive initiation factor preparation would be the result, rather than the cause, of translation inhibition. Second, it has been suggested that early inhibition of host cell protein synthesis is a nonspecific effect of viral infection, and that, subsequent to the synthesis of sufficient quantities of viral mRNA, viral protein synthesis can occur to the exclusion of host cell protein synthesis due to effective viral mRNA competition for a limiting factor (17, 25).

These possibilities could be tested by evaluating both the function of initiation factors, prepared from cells actively translating viral mRNA later in infection, to stimulate the initiation of cell mRNA translation, and also the ability of early- and late-infected initiation factors to function with viral mRNA. The results in both cases demonstrate that the ribosomal salt wash preparation from infected cells is inactive for initiation of cellular translation but retains activity for virus-directed protein synthesis. These data are entirely consistent with our first hypothesis.

The data reported here, taken together with other observations that UV irradiation of poliovirus prevents the inhibition of host cell protein synthesis (18) whereas guanidine restriction of all detectable viral RNA and protein synthesis does not prevent host cell shutoff at high multiplicities of infection (20), leads us to conclude that some viral product, which is responsible for the expression of host cell shutoff phenotype, is produced early in infection, possibly in quite small amounts. This product may itself act catalytically or may induce the host cell to produce an inhibitory activity found in the ribosomal salt wash from infected cells. Although the experimental design of the work presented here limits examination of the polysome fraction for the presence of cell mRNA-specific inhibitory activity, both we and Kaufmann et al. (23) have been unable to detect its presence in the post-ribosomal supernatant from early-infected cells. We do, however, detect a similar inhibitory activity in the postribosomal supernatant from late-infected cells (data not shown), but have not studied it further.

The presence in infected cells of a ribosomal salt wash factor, which restricts initiation of translation of cellular mRNA but not viral RNA, could readily account for both host cell shutoff and subsequent preferential translation of viral mRNA. An apparent mechanism for this regulation might be the exploitation of the fact that picornavirus mRNA alone among known animal cell mRNA's possesses no "cap" structure at its 5′ end (15, 19, 28). Recent reports of a tentative identification of "cap"-binding proteins (16), the inhibition of eIF4B binding to mRNA by free cap structures (33), and the requirement for the cap structure on other mRNA's for optimal translation (4) would all support such an hypothesis. Alternatively, the site of action could be eIF4A, which previously has been demonstrated to control rates of initiation of different mRNA's (2, 22). Studies are in progress to purify the discriminating factor(s) from infected cell ribosomal salt washes and to identify it as either one of the known eukaryotic initiation factors or possibly another cellular factor.

ACKNOWLEDGMENTS

This work was supported by grants PCM76-22065 from the National Science Foundation and by Public Health Service grant AI 12387 from the National Institute of Allergy and Infectious Diseases T.H. is supported by Public Health Service Genetics Training Grant 5T32 GM0746401 from the National Institute of General Medical Sciences. E.E. is the recipient of a Teacher-Scholar Award from the Dreyfus Foundation.

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