JOURNAL OF VIROLOGY, May 1981, p. 728-736
0022-538X/81/050728-09$02.00/0

Vol. 38, No. 2

Shutoff of Neuroblastoma Cell Protein Synthesis by Semliki Forest Virus: Loss of Ability of Crude Initiation Factors to Recognize Early Semliki Forest Virus and Host mRNA's

HARRY VAN STEEG,* ADRI THOMAS, SJEF VERBEEK, MARCELLE KASPERAITIS, HARRY O. VOORMA, AND ROB BENNE

Department of Molecular Cell Biology, University of Utrecht, Utrecht, The Netherlands

Received 23 October 1980/Accepted 12 January 1981

A crude ribosomal wash containing the initiation factors of protein synthesis was isolated from mouse neuroblastoma cells 8 h after infection with Semliki Forest virus (SFV). The activity of this wash was compared with that of a wash from control cells in a cell-free protein-synthesizing "pH5" system, with early SFV mRNA (42S), late SFV mRNA (26S), encephalomyocarditis virus (EMC) mRNA, or neuroblastoma polyadenylated mRNA templates. A pronounced loss of activity (±80%) of the crude ribosomal wash from infected cells was observed with host mRNA (neuroblastoma polyadenylated mRNA) and early SFV mRNA, messengers which contain a cap structure at the 5′ terminus. However, these washes were only slightly less active in systems programmed with (noncapped) EMC mRNA and late SFV mRNA. Although late SFV mRNA (26S) is capped, the synthesis of late (= structural) proteins in infected lysates was insensitive to inhibition by cap analogs. Purified initiation factors eIF-4B (M_r, 80,000) and cap-binding protein (M_r, 24,000) from reticulocytes (but none of the others) were able to restore the activity of infected factors to about 90% of control levels in systems programmed with early SFV mRNA and host mRNA. These observations indicate that infection-exposed crude initiation factors have a decreased level of eIF-4B and cap-binding protein activity. However, after partial purification of these and other initiation factors from infected and control cells, we found no significant difference in activity when model assay systems were used. Furthermore, both eIF-4B and cap-binding protein from infected cells were able to restore the activity of these infection-exposed factors to the same level obtained when these factors isolated from control cells or reticulocytes were added. A possible mechanism for the shutoff of host cell protein synthesis is discussed.

The mechanism of host shutoff in virus-infected eucaryotic cells is still a matter of controversy (for a review see reference 3). However, some of the evidence accumulated in recent years points towards altered or inactivated initiation factors in infected cells (12, 13, 15, 22, 31). In extracts of reovirus-infected L-cells (26) and poliovirus-infected HeLa cells (31), capped mRNA's are translated at reduced efficiencies as compared with control extracts. The translation of capped mRNA's in poliovirus-infected HeLa cell extracts can be stimulated by the addition of eIF-4B from reticulocytes (22). Later, it was postulated that a contaminating protein with an M_r of 24,000 (restoring factor; cap-binding protein) was the active component in this eIF-4B preparation (31). However, Helentjaris et al. (12) showed that eIF-3, not eIF-4B, was inactivated, but these authors could not rule out the possibility that the 24,000-dalton protein was the inactivated component in their eIF-3 preparation. A later report by Trachsel et al. (31) indeed demonstrated that most of the cap-binding protein cosedimented with eIF-3 in low-salt sucrose gradients.

Semliki Forest virus (SFV) causes the shutoff of host protein synthesis in mammalian cells (11, 16). Both the 42S viral mRNA, which is utilized as an early mRNA for the synthesis of nonstructural proteins (14, 35), and the subgenomic 26S mRNA, which encodes for the structural proteins later in infection (14, 29), appear to be capped (21). Despite the fact that, late in infection, 50% of the mRNA population is still of cellular origin (32, 36), only virus-encoded proteins are synthesized in such cells. It was suggested (32, 36) that the host mRNA's are outcompeted by the late viral mRNA's because of the higher affinity of the viral mRNA's for the protein-synthesizing machinery. In such a

model, inactivation of one or more initiation factors would not be an absolute prerequisite for the shutoff of host cell protein synthesis.

This paper describes the effect of the infection of neuroblastoma cells by SFV on the activity of initiation factors of protein synthesis. We have isolated a crude initiation factor preparation from such cells, which were inactive in translation assays with early SFV mRNA and host mRNA but active with late SFV mRNA. Both purified initiation factors eIF-4B (virtually free of cap-binding protein) and cap-binding protein from reticulocytes were able to restore the activity of this inactivated initiation factor preparation. These findings imply that protein synthesis with capped mRNA's is inhibited in infected cells and that the dependence on the 5′ cap structure of the recognition of late viral mRNA's appears to be less stringent. Indeed, preliminary experiments indicate that the synthesis of structural proteins in infected lysates is relatively insensitive to cap analogs, in comparison with the synthesis of host proteins.

MATERIALS AND METHODS

Virus growth and cell culture. Subconfluent monolayer cultures of mouse neuroblastoma cells were grown in roller bottles (850 cm^2; Falcon Plastics, catalog no. 3027) in 80 ml of minimal essential medium supplemented with 10% fetal calf serum per bottle. For the preparation of initiation factors from infected cells, 50 PFU of wild-type SFV per cell, grown and isolated as described previously (33), were added. After 1 h, the inoculum (15 ml per bottle) was removed, and fresh medium was added (50 ml of minimal essential medium supplemented with 3% fetal calf serum). At 8 h postinfection, the cells were washed twice with 10 ml of ice-cold phosphate-buffered saline, harvested, and lysed by the method of Skup and Millward (25).

Protein synthesis in intact cells. Protein synthesis in SFV or mock-infected neuroblastoma cells grown on small tissue culture dishes (10 cm^2; Lux Plastics) was determined as described previously (33). At various time intervals, the cells were pulse-labeled for 1 h with 25 μCi of [^{35}S]methionine (1,000 Ci/mmol; New England Nuclear Corp.). The cells were lysed in 150 μl of sodium dodecyl sulfate sample buffer by the method of Laemmli (18), and methionine incorporation into protein was measured by counting 10-μl samples after hot trichloroacetic acid precipitation. Product analysis was performed on 12.5% polyacrylamide gels by the method of Laemmli (18). Fluorography was carried out as described by Bonner and Laskey (7).

Preparation of mRNA. Neuroblastoma polyadenylated [poly(A)] mRNA was prepared by standard procedures by oligodeoxythymidylic acid cellulose chromatography (17). SFV 42S mRNA was isolated as described earlier (33). Encephalomyocarditis virus (EMC) RNA was a kind gift from D. P. Leader.

For the preparation of late SFV mRNA (26S), neuroblastoma cells were infected with SFV and lysed after 8 h postinfection in a solution containing 20 mM Tris-hydrochloride (pH 7.4), 1 mM EDTA, 10 mM NaCl, 2 mM $Mg(OAc)_2$, 0.5% (vol/vol) Triton X-100, 0.5% (wt/vol) 1,5-naphthalene disulfonate, and 1 μg of cycloheximide per ml.

At 15 min before cell lysis, 100 μg of cycloheximide per ml was added to the medium. Cell debris and nuclei were removed by low-speed centrifugation (5 min, 1,000 × *g*), and the supernatant was brought to 100 mM KOAc and 4 mM $Mg(OAc)_2$ (final concentrations) and layered onto two sucrose cushions (1.0 and 1.5 M, respectively, in 20 mM Tris-hydrochloride [pH 7.4]–120 mM KOAc–4 mM $Mg(OAc)_2$–7 mM 2-mercaptoethanol–0.5% [wt/vol] 1,5-naphthalene disulfonate–1 μg of cycloheximide per ml). Centrifugation was at 18,000 rpm for 18 h at 4°C in a Beckman SW28 rotor.

The polysomes were suspended in buffer (20 mM Tris-hydrochloride [pH 7.4]–100 mM KOAc–4 mM $Mg(OAc)_2$–7 mM 2-mercaptoethanol) and immediately brought to 0.5% lithium dodecyl sulfate. RNA was deproteinized twice with a phenol-chloroform mixture (1:1). The aqueous phase, containing rRNA and late SFV mRNA, was applied onto a 15 to 30% (wt/vol) sucrose gradient as described by Pettersson et al. (21). Fractions (1 ml) containing 26S SFV RNA (contaminated with 28S rRNA) were collected and freed from rRNA on an oligodeoxythymidylic acid cellulose column (17). 26S mRNA was precipitated twice with ethanol and dissolved in water at 2 mg/ml.

Purification of initiation factors of protein synthesis. (i) Rabbit reticulocytes. Highly pure initiation factors from reticulocytes were obtained as described previously (5, 28, 30).

(ii) Mouse neuroblastoma. The neuroblastoma lysates of approximately 36 g of cells (80 ml) were centrifuged in a Beckman 60Ti rotor for 18 h at 30,000 rpm to collect the ribosomes. The supernatant (S_{100}) was used as a source of eIF-4A, since the factor occurs predominantly (90%) in this fraction in reticulocytes (30). The remaining factors (except eIF-1) were partially purified from the ribosomal pellet by standard procedures described extensively in the literature (5, 28, 30, 31). A summary of the procedures followed is presented below (see Fig. 4).

Protein synthesis in cell-free systems. (i) Neuroblastoma lysates. Neuroblastoma lysates were prepared as described previously for L-cell lysates (25). Incubation mixtures contained (per 25 μl) 6.25 μl of crude lysate, 0.05 U of creatine kinase, 100 mM KOAc, 1.5 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 100 μM spermine·$(HCl)_4$, 1 mM ATP$(Tris)_3$, 0.4 mM GTP$(Tris)_3$, 5 mM creatine phosphate·$(Tris)_2$, 19 unlabeled amino acids (50 μM each), and 0.2 μM [^{35}S]methionine (1,000 Ci/mmol), either in the absence or presence of 1 μM edeine (34). Incorporation of [^{35}S]methionine into protein was measured after 45 min at 37°C by hot trichloroacetic acid precipitation of 5-μl samples (1).

(ii) "pH5" systems. Protein synthesis was determined in a fractionated pH5 system as described previously (30, 33). A typical assay mixture of 25 μl contained 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.6), 1 mM ATP$(Tris)_3$, 0.4 mM GTP$(Tris)_3$, 1 mM dithiothreitol, 5 mM creatine phosphate$(Tris)_2$, 0.05 U of creatine

kinase, 2 mM $Mg(OAc)_2$, 120 mM KOAc, 100 μM spermine·$(HCl)_4$, 0.2 μM [^{35}S]methionine (1,000 Ci/mmol), 19 unlabeled amino acids (50 μM each), 2.5 μg of pH5 enzymes, 20 μg of crude initiation factor fraction A (0 to 40% ammonium sulfate fraction [23]), 30 μg of fraction BC (40 to 70% ammonium sulfate fraction [23]) from either uninfected or infected neuroblastoma cells, 0.08 unit of absorbance at 260 nm of 40S subunits (from rat liver) and 0.15 unit of absorbance at 260 nm of 60S subunits (from rat liver) plus either 2 μg of SFV 42S mRNA, 2 μg of SFV 26S mRNA, 1 μg of EMC mRNA, or 1 μg of neuroblastoma poly(A) mRNA. Incorporation of [^{35}S]methionine into protein was measured after 45 min at 37°C by hot trichloroacetic acid precipitation of 5-μl samples.

Other assay systems. To compare the activity of one specific initiation factor isolated from infected and uninfected cells, different assay systems were used.

(i) eIF-2. This factor was tested in the assay for ternary complex formation with Met-tRNA$_f$ and GTP as described by Thomas et al. (30). One unit of activity of eIF-2 was the amount of protein that bound 1 pmol of [^{3}H]Met-tRNA$_f$.

(ii) eIF-3, -4A, and -4B. These factors were assayed in a cell-free protein-synthesizing system as described previously (30). One unit of activity of these initiation factors was the amount of protein which stimulated the incorporation of 1 pmol of [^{35}S]methionine into proteins under the direction of neuroblastoma poly(A) mRNA.

(iii) Cap-binding protein. This factor was assayed in a pH5 system to which crude initiation factors (fractions A and BC) from infected cells were added (see above). One unit of activity was the amount of protein which stimulated the incorporation of 1 pmol of [^{35}S]methionine into proteins with neuroblastoma poly(A) mRNA as a messenger.

(iv) eIF-4C and -5. These factors were tested in an assay system for the formation of [^{3}H]methionyl puromycin as described by Thomas et al. (30). One unit of activity was the amount of protein which stimulated the formation of 1 pmol of [^{3}H]methionyl puromycin.

(v) Restoring assay. In this assay, factor preparations were tested for stimulatory activity in pH5 systems with infection-exposed washes and with neuroblastoma poly(A) mRNA or early SFV mRNA as a template.

Nomenclature. Initiation factors are named as agreed at the International Conference of Protein synthesis held in Bethesda, Md., in October 1976 (2). Cap-binding protein refers to the protein of M_r 24,000 (27).

RESULTS

Protein synthesis in neuroblastoma cells after infection with SFV. Infection with SFV results within 3 to 5 h in the shutoff of host protein synthesis in most cell lines studied (11, 16). The aim of this investigation was to determine whether such a shutoff is caused by an alteration or inactivation of one or more of the protein factors engaged in initiation of protein synthesis. For this purpose, the precise moment at which the shutoff occurs in the cell line studied (neuroblastoma cells from mice) had to be determined. Therefore, we observed cellular protein synthesis after infection with SFV and analyzed the proteins produced (Fig. 1). It is clear that at 3 h postinfection, total protein synthesis started to decline (Fig. 1A). At 4 h postinfection, the first viral structural proteins could be detected. Later in infection, a gradual suppression of host protein synthesis was observed; this suppression was virtually complete after 8 h, at which point the only proteins synthesized were of viral origin (p97, p62, E_1, and capsid protein, see Fig. 1B). An infection time of 8 h should therefore be sufficient for the detection of any virus-induced change in messenger specificity and activity of the initiation factors of protein synthesis.

Activity of crude ribosomal washes from infected cells. Crude ribosomal washes from control cells and from cells infected with SFV were prepared as described above. The washes were fractionated with ammonium sulfate in A and BC fractions as described previously (23). The dependence on these washes in a standard pH5 system for protein synthesis programmed with either neuroblastoma poly(A) mRNA, early SFV mRNA (42S), late SFV mRNA (26S), or EMC mRNA was determined (Table 1). It is clear that the transition from control to infection-exposed washes resulted in a severe inhibition of protein synthesis with host mRNA and early SFV mRNA [neuroblastoma poly(A)

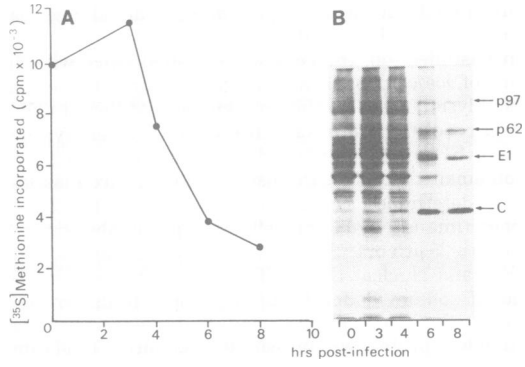


FIG. 1. *Protein synthesis in SFV-infected neuroblastoma cells. Dishes containing approximately 8 × 10^5 cells were infected with SFV (multiplicity of infection, 600 PFU per cell) and pulse-labeled with [^{35}S]methionine as described in the text. (A) Protein synthesis was monitored during infection by measuring [^{35}S]methionine incorporation into protein. (B) A 50-μl amount of cell lysate at each point of infection was analyzed on 12.5% sodium dodecyl sulfate gels. The figure is an 18-h autoradiogram of the dried gel. The arrows mark the position of the viral (precursor) proteins (10).*

TABLE 1. *Effect of washes from infected cells on protein synthesis in pH5 systems directed with different mRNA's*[a]

mRNA	[35S]methionine incorporation (cpm) in:		% Inhibition
	Washes from control cells	Washes from infected cells	
Early SFV (42S)	138,714	40,215	71
Late SFV (26S)	150,583	135,985	10
EMC	196,675	164,695	16
Host neuroblastoma poly(A)	84,855	9,429	89

[a] A 1-μg amount of neuroblastoma total poly(A) mRNA, 1 μg of EMC viral mRNA, 2 μg of late SFV 26S mRNA, or 2 μg of SFV 42S mRNA was translated in the pH5 system (25 μl) with 20 μg of fraction A and 30 μg of fraction BC from both infected and control cells (see text). Total protein synthesis was determined in 5-μl samples as described in the text.

mRNA and 42S mRNA], whereas systems programmed with EMC mRNA and late SFV mRNA (26S) showed little effect.

These findings also indicate that the alteration in the protein-synthesizing machinery which resulted in the shutoff of host and early viral mRNA translation occurred at the level of the initiation factors. Furthermore, these findings are in agreement with the generally accepted notion that 42S mRNA does not serve as a template for the synthesis of structural proteins (14), but is only translated at the early stages of infection, when no change in the specificity of initiation factors has yet occurred.

It is also noteworthy that the translation of late SFV mRNA, which is capped (21; data not shown), was equally unaffected by the transition from control to infection-exposed factors as was noncapped EMC RNA.

Figure 2 demonstrates that the inhibitory effect with early SFV mRNA and host mRNA is mainly localized in ammonium sulfate fraction A, since fraction A from infected cells is only 25% as active as its counterpart from noninfected cells (Fig. 2A). The loss of activity of fraction BC was much less severe and was apparent only when large amounts of this fraction were added to the assay system (Fig. 2B).

These results appear to be in line with the literature, since the three factors which have been implicated in host shutoff, eIF-3 (12), eIF-4B and cap-binding protein (31) all precipitated in fraction A.

Synthesis of viral structural proteins is cap analog insensitive. The results of the previous section are in agreement with reports from other groups on the shutoff of host protein

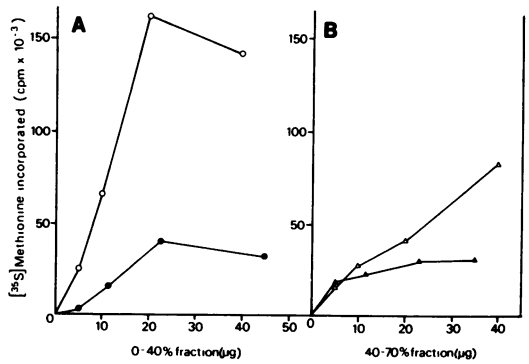


FIG. 2. *Stimulatory effect of crude ribosomal washes from infected and control cells on protein synthesis in pH5 systems. Increasing amounts of 0 to 40% fraction A (A) or 40 to 70% fraction BC (B) were added to the assay mixtures as indicated. [35S]methionine incorporation into protein in pH5 systems programmed with 2 μg of SFV 42S mRNA was determined as described in the text. (A) Addition of 30 μg of the 40 to 70% fraction from control cells. Symbols: ●, 0 to 40% fraction from infected cells; ○, 0 to 40% fraction from control cells. (B) Addition of 20 μg of the 0 to 40% fraction from control cells. Symbols ▲, 40 to 70% fraction from infected cells; △, 40 to 70% fraction from control cells.*

synthesis by infecting viruses (22, 25, 31; see above). In these cases, it was suggested that viral infection results in a shift to cap-independent protein synthesis. However, whereas the relevance of a redirection of the protein-synthesizing machinery towards noncapped messengers is obvious for the viruses studied, which are reproduced by using noncapped mRNA's, the situation with SFV appears to be more complex, since both the viral 42S mRNA and the subgenomic 26S mRNA, when isolated from infected cells, appear to be capped (21).

However, the possibility remains that the cap-structure is dispensable for efficient translation of the 26S mRNA. To further explore this hypothesis, we determined the effect of cap analogs, such as $m^7GpppGm$, on the synthesis of SFV capsid protein in a lysate prepared approximately 4 h after infection. At this stage, host protein synthesis was not shut off completely (Fig. 1), which enabled us to compare the effect of the cap analogs on the production of host and viral structural proteins (Fig. 3). Indeed, no inhibition of the cap analogs of the synthesis of capsid protein (as determined by immunoprecipitation) was observed. However, the translation of the host mRNA's was almost completely inhibited by the addition of cap analogs. Therefore, the cap structures on the mRNA's which serve as templates for SFV viral structural proteins do not appear to be essential for efficient

translation. Such a messenger would be relatively unaffected by a shift to cap-independent protein snthesis, which would result from a decrease in the activity of cap-specific initiation factors.

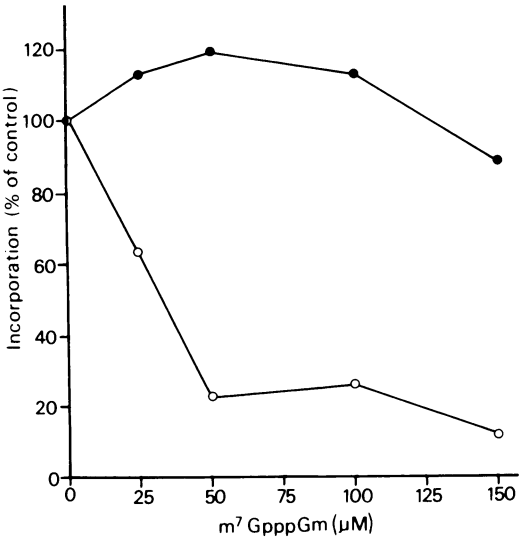


FIG. 3. *Effect of m^7GpppGm on protein synthesis in an infected lysate. Neuroblastoma lysate was prepared from infected cells 4 h postinfection. Protein synthesis was determined as described in the text. Total protein synthesis was measured in 5-μl samples. The amount of SFV capsid protein synthesized was determined by immunoprecipitation in 10-μl samples as described previously (33). Symbols: ●, capsid protein synthesis (percentage of incorporation relative to the value obtained in the absence of m^7GpppGm; 6,176 cpm = 100%); ○, total protein synthesis (percentage of incorporation relative to the value obtained in the absence of m^7GpppGm; 35,310 cpm = 100%). Values obtained in control experiments in the presence of edeine (an inhibitor of initiation of protein synthesis [34]) were subtracted (i.e., capsid protein synthesis, 6,096 cpm; total protein synthesis, 41,564 cpm).*

Activity of the infected ribosomal wash can be restored with purified eIF-4B and cap-binding protein from rabbit reticulocytes. The evidence presented so far indicates that, in infected cells, initiation of protein synthesis with host mRNA and early SFV mRNA is blocked. Table 1 and Fig. 2 suggest that this block is caused by the inactivation of one or more initiation factors. Therefore, we determined whether the addition of any of the purified factors from rabbit reticulocytes to the infected system (restoring assay) resulted in relief of inhibition (Table 2). Only the addition of eIF-4B and cap-binding protein resulted in significant stimulation of protein synthesis with washes from infected cells with both early SFV mRNA and poly(A) RNA from neuroblastoma cells (4.3- and 5.6-fold, respectively, provided that both factors were added). Stimulation by eIF-4B was more pronounced than that observed upon the addition of cap-binding protein. This most likely represented a true eIF-4B effect, since our eIF-4B preparations contained less than 1% cap-binding protein (see below). Stimulation by these factors in the control systems was rather small [1.1-fold for early SFV mRNA and 2.0-fold for neuroblastoma poly(A) RNA]. Additions of eIF-1, -2, -3, -4A, -4C, and -5 (both separate and combined) had no appreciable effect on the activity of the washes from infected cells.

Partial purification and activity comparison of the initiation factors from infected and control cells. eIF-4B and cap-binding protein, which are both implicated in the recognition of capped mRNA's (20, 24, 31), stimulated the activity of the infected ribosomal wash (Table 2). The failure of these washes to support the translation of neuroblastoma poly(A)

TABLE 2. *Restoration of activity in washes from infected cells by purified initiation factors from rabbit reticulocytes*[a]

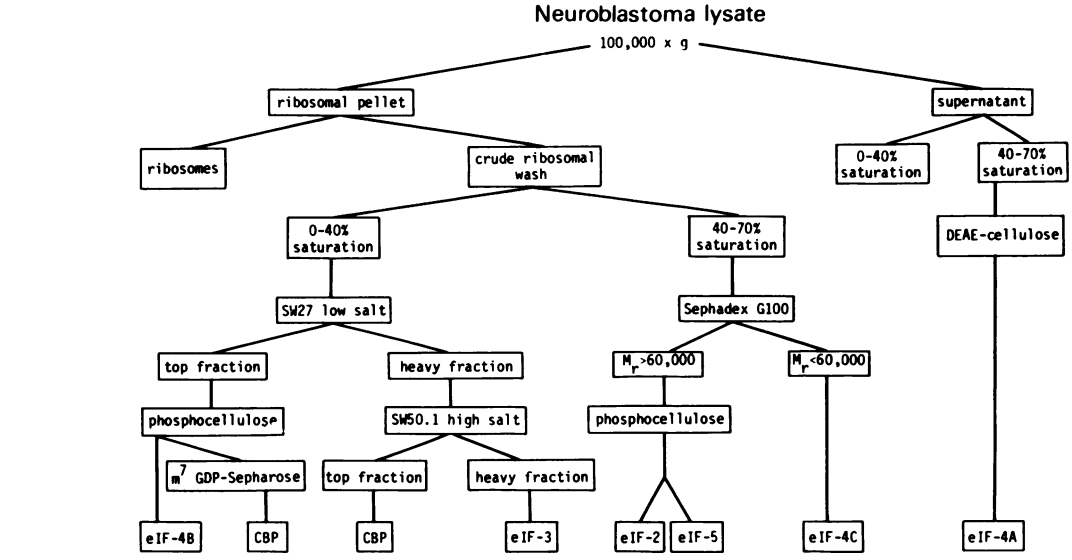
mRNA	Crude initiation factors from:	Radioactivity (cpm) with the following additions:				
		None	eIF-4B	CBP	eIF-4B + CBP	eIF-1, -2, -3, -4A, -4C, and -5
SFV	Uninfected cells	261,167	273,000	321,801	285,755	ND
	Infected cells	62,398	158,951	152,969	258,986	65,328
Neuroblastoma poly(A)	Uninfected cells	33,041	59,392	46,314	67,054	ND
	Infected cells	9,152	35,578	16,970	51,264	11,696

[a] Pure initiation factors of protein synthesis from rabbit reticulocytes were added as indicated to control and infected pH5 systems (25 μl) programmed with either 2 μg of SFV mRNA or 1 μg of neuroblastoma poly(A) mRNA (see Table 1, footnote *a*, and text). [^{35}S]methionine incorporation into protein was measured in counts per minute as described in the text. The following amounts (in micrograms) of factors were added: eIF-1, 0.6; eIF-2, 1.4; eIF-3, 3.5; eIF-4A, 2.0; eIF-4B, 2.0; cap-binding protein, 0.12; eIF-4C, 0.1; and eIF-5, 0.3. Blank incorporations obtained in the absence of exogeneous mRNA were subtracted. CBP, Cap-binding protein; ND, not determined.

mRNA and early SFV mRNA was most likely caused, therefore, by the inactivation of these factors in infected cells. On the other hand, it is possible that no direct inactivation occurred, but rather that an inhibitor(s) was formed which specifically blocked the action of eIF-4B and cap-binding protein. To discriminate between these two possibilities, we set out to purify these (and other) initiation factors from both infected and control cells. Standard procedures (5, 28, 30, 31) were used in abbreviated form to yield partially pure factor preparations with a low degree of cross-contamination.

A flow scheme of the procedure followed is shown in Fig. 4. Details are given above and in the legend to Fig. 4. The resulting preparations, with approximate purities between 1 and 10%, were tested in standardized assay systems (see above) derived from rabbit reticulocytes. Each factor from control cells was compared with its infected counterpart for both specific activity and total yield (Fig. 4). No significant difference between infected and control factors could be demonstrated, although the yields of eIF-2 and eIF-5 from infected cells appeared to be somewhat low. However, for reasons as yet obscure, about 60% of the total eIF-2 and eIF-5 activity precipitated in the 0 to 40% ammonium sulfate fraction from infected washes, a phenomenon not observed with control washes. The total yield, therefore, of eIF-2 plus eIF-5 from infected cells was approximately equal to that obtained from control cells.

An example of the specific activity determinations is presented in Fig. 5, in which the dependence of the assay systems on the most relevant factors is shown: eIF-4B (Fig. 5A) and cap-binding protein (Fig. 5B). It is clear that factors from control and infected cells triggered the same responses in the test systems in both cases. Obviously, the factors themselves were not inactivated in infected cells, but rather an inhibitory component specifically blocked their action (see below). Apparently this component



Source	C	I	C	I	C	I	C	I	C	I	C	I	C	I	C	I
Spec.Act. U/mg	35	37	0	0	5.6	6.2	157	187	350	350	6400	6200	1020	1420	19	20
Total Act. U	57	69	0	0	1.6	2.3	2100	1700	150	50*	2800	800*	2280	2180	160	130

FIG. 4. *Purification and activity comparison of initiation factors from control and infected neuroblastoma cells. The neuroblastoma lysates from infected and control cells (both 80 ml) were prepared as described in the text. The scheme shows the purification steps which were performed to acquire partially pure factors. All techniques have been extensively described in the literature (5, 28, 30, 31). Column sizes were adapted to the small amount of material used. The factors eIF-4B and eIF-2 plus eIF-5 were step-eluted from the phosphocellulose columns at 400 and 750 mM KCl, respectively, in standard phosphate buffer (5). Initiation factor eIF-4A was step-eluted from the DEAE-cellulose column at 400 mM KCl in standard Tris buffer (5). After sodium dodecyl sulfate gel electrophoresis and activity tests (see text), each factor was pooled and concentrated by ammonium sulfate precipitation. Protein concentrations were estimated by the methods of Layne (19) or Bradford (8). The specific activity of each factor was determined in model systems (see text) at suboptimal amounts of added factor. The asterisks indicate values where the losses of eIF-2 and eIF-5 were attributed to the fact that these factors partially precipitated only in fraction A of washes from infected cells (see text). C, Control cells; I, infected cells, CBP, cap-binding protein.*

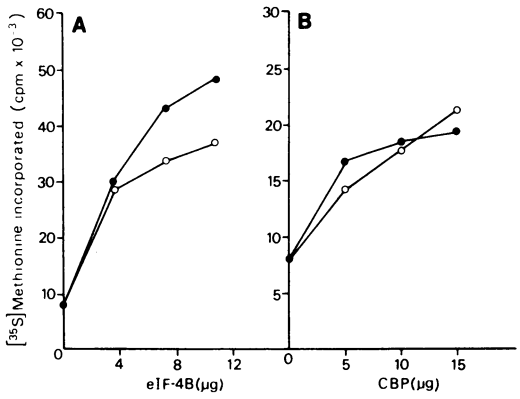


FIG. 5. *Comparison of the activities of eIF-4B and cap-binding protein (CBP) from infected and control neuroblastoma cells. (A) Increasing amounts of eIF-4B were added to the assay system for eIF-4B (described in the text). [^{35}S]methionine incorporation into protein was measured as described in the text. Symbols: ●, eIF-4B from infected cells; ○, eIF-4B from control cells. (B) Increasing amounts of cap-binding protein were added to the restoring assay system (described in the text). Symbols: ●, cap-binding protein from infected cells; ○, cap-binding protein from control cells. Both assay systems were programmed with 1 μg of neuroblastoma poly(A) mRNA; 5-μl samples from 25-μl reaction mixtures were counted.*

was lost upon purification.

Is cap-binding protein the active component in eIF-4B preparations? Trachsel et al. (31) have isolated from reticulocytes a factor which restored vesicular stomatitis virus mRNA-dependent protein synthesis in lysates from poliovirus-infected HeLa cells to control levels. This factor copurified with cap-binding protein. It has been suggested that cap-binding protein might be the active component in eIF-4B preparations (12, 31). The stimulatory effect of eIF-4B on protein synthesis with infected washes could, therefore, be due to contamination of this factor by cap-binding protein. Such a contamination has been demonstrated for other eIF-4B preparations (6, 27).

However, Table 3 and Fig. 6 show the results of an experiment from which a number of arguments can be derived against an exclusive role for cap-binding protein in the restoring of the infected washes. In this experiment, we performed a gel analysis of the eIF-4B and cap-binding protein preparations from reticulocytes and from infected and control neuroblastomas, and we determined their specific activities in both the eIF-4B and restoring assays.

Figure 6, lanes 1 through 3, shows a gel analysis of eIF-4B from reticulocytes and neuroblastomas. No protein of M_r 24,000 was discernible.

TABLE 3. *Specific activities of cap-binding protein and eIF-4B from reticulocytes and from control and infected neuroblastoma cells as determined by the eIF-4B assay and the restoring assay*[a]

Factor	Approx purity (%)	Sp act in eIF-4B assay	Sp act in restoring assay
eIF-4B (ret)	80	2,142	106
eIF-4B (NU)	2.0	1,500	135
eIF-4B (NI)	2.0	1,250	140
CBP (ret)	90	0	398
CBP (NU)	1.3	554	431
CBP (NI)	1.3	723	477

[a] The specific activities of eIF-4B and cap-binding protein (CBP) from different sources were measured in the model assay systems for eIF-4B and cap-binding protein (see text and legends to Fig. 4 and 5). To determine the purity of each factor, 12.5% sodium dodecyl sulfate gels (see Fig. 6) were scanned on a densitometer. The specific activities are expressed in units relative to the values obtained with 100% pure factor (see text). NU, Neuroblastoma uninfected; NI, neuroblastoma infected; ret, reticulocyte.

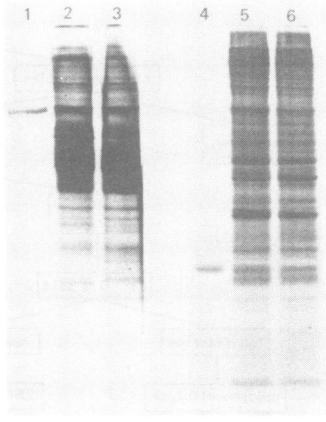


FIG. 6. *Gel analysis of cap-binding protein and eIF-4B from reticulocytes and from control and infected neuroblastoma cells. Lane 1, 2.5 μg of eIF-4B from reticulocytes; lane 2, 30 μg of eIF-4B from uninfected neuroblastoma cells; lane 3, 30 μg of eIF-4B from infected neuroblastoma cells; lane 4, 1.8 μg of cap-binding protein from reticulocytes; lane 5, 60 μg of cap-binding protein from uninfected neuroblastoma cells; lane 6, 60 μg of cap-binding protein from infected neuroblastoma cells.*

Prolonged exposure of autoradiograms of methyl-^{14}C-labeled eIF-4B (prepared as described previously [5]) revealed a 1% contamination by a protein of M_r 24,000. However, such a contamination cannot account for the stimulatory activity of eIF-4B in the restoring assay (Fig. 5B and Table 2).

eIF-4B from neuroblastomas did not contain cap-binding protein (Fig. 4); nevertheless, a pos-

itive effect in the restoring assays was observed upon the addition of the factor, which was comparable to the effect of purified eIF-4B from reticulocytes.

Furthermore, purified cap-binding protein from reticulocytes did not stimulate amino acid incorporation in an eIF-4B assay (data not shown).

Therefore, we conclude that both eIF-4B and cap-binding protein are capable of restoring the activity in ribosomal washes from infected cells, with eIF-4B being the more active of the two factors and their effects being additive (see Table 2). These observations suggest that the two factors are different entities. They are, however, similar in action at the molecular level and are both capable of promoting a cap-directed initiatory event.

DISCUSSION

This study demonstrates that infection of neuroblastoma cells by SFV results in a pronounced loss of activity in crude ribosomal washes when tested in a pH5 assay system for protein synthesis with host mRNA and early viral mRNA (Table 1 and Fig. 2). Most of the inhibition can be overcome by the addition of purified eIF-4B and cap-binding protein from reticulocytes (Table 2), factors which have been implicated in the recognition of the cap structure occurring at the 5′ terminus of messengers (20, 24, 31).

Apparently, the shift in initiation factor specificity which is thought to occur in HeLa cells upon infection with poliovirus (22) and in L-cells upon infection with reovirus (26) also takes place in neuroblastoma cells infected with SFV. This might be the general mechanism by which most RNA viruses shut off protein synthesis of their eucaryotic hosts, even if these viruses are reproduced with the aid of capped mRNA's, which is the case with SFV. Therefore, the relevance of a shift to cap-independent protein synthesis late in infection is not immediately obvious.

Nevertheless, the finding that the synthesis of structural proteins in infected lysates is insensitive to inhibition by cap analogs (Fig. 3) indicate that such a shift occurs in neuroblastoma cells infected with SFV. Apparently the cap structure is not essential for efficient translation of the 26S mRNA. This messenger should possess other features that promote its recognition by the protein-synthesizing machinery in infected cells. The inactivation or blocking of the cap recognition factors (eIF-4B and cap-binding protein) would, in this model, result in a preferential translation of the 26S mRNA and in a shutoff of host and early viral protein synthesis, since all mRNA's which depend more strictly on the cap structure for translation (e.g., host mRNA's and early SFV mRNA) would be translated at a low frequency. These results may be in line with the observations of Chroboczek et al. (9), who reported that the synthesis of brome mosaic virus structural proteins in wheat germ lysates is relatively insensitive to inhibition by cap analogs, although all known mRNA's of brome mosaic virus are capped.

Such a decrease in the dependence on the cap structure should result in a decreased requirement of 26S mRNA for eIF-4B and cap-binding protein. Indeed, preliminary experiments reveal a low need for eIF-4B and cap-binding protein of this mRNA, comparable to that displayed by EMC RNA and other noncapped mRNA's (4, 20, 22, 31).

It remains unclear by what mechanism the decrease of the level of eIF-4B and cap-binding protein activity is brought about. Obviously, eIF-4B and cap-binding protein isolated from infected cells have the same biological activity as the factors isolated from control cells, both in standard model systems and in the restoring assay (see Fig. 4, 5, and 6; Table 3). The ribosomal washes from infected neuroblastoma cells must therefore contain some virus-induced component(s) antagonistic to cap-binding protein and eIF-4B, which specifically incapacitates the protein-synthesizing machinery for proper recognition of host mRNA and early SFV mRNA. However, the precise identity and mode of action of such components remain obscure.

ACKNOWLEDGMENTS

We thank B. A. M. van der Zeyst for his advice on virus propagation and for a kind gift of rabbit anti-SFV serum.

This work was sponsored by the Netherlands Organization for the Advancement of Pure Research (ZWO), and financial aid was provided by the Netherlands Foundation for Chemical Research (SON).

LITERATURE CITED

1. **Amesz, H., H. Goumans, T. Haubrich-Morree, H. O. Voorma, and R. Benne.** 1979. Purification and characterization of a protein factor that reverses the inhibition of protein synthesis by HRI in rabbit reticulocyte lysates. Eur. J. Biochem. **98**:513–520.
2. **Anderson, W. F., L. Bosch, W. E. Cohn, H. Lodish, W. C. Merrick, H. Weissbach, H. G. Wittman, and I. G. Wool.** 1977. International symposium on protein synthesis. Summary of Fogarty center-NIH workshop held in Bethesda, Maryland, 18–20 October 1976. FEBS Lett. **76**:1–10.
3. **Bablanian, R.** 1975. Structural and functional alterations in cultured cells infected with cytocidal viruses. Prog. Med. Virol. **19**:40–83.
4. **Baglioni, C., M. Simili, and D. A. Shafritz.** 1978. Initiation activity of encephalomyocarditis virus RNA, binding to initiation factor eIF-4B and shut-off of host cell protein synthesis. Nature (London) **275**:240–243.
5. **Benne, R., M. L. Brown-Luedi, and J. W. B. Hershey.** 1979. Protein synthesis initiation factors from rabbit

reticulocytes: purification, characterization and radiochemical labeling. Methods Enzymol. **60**:15-35.
6. **Bergmann, J. E., H. Trachsel, N. Sonenberg, A. J. Shatkin, and H. F. Lodish.** 1979. Characterization of rabbit reticulocyte factor(s) that stimulates the translation of mRNAs lacking 5'-terminal 7-methylguanosine. J. Biol. Chem. **254**:1440-1443.
7. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83-88.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.
9. **Chroboczek, J., L. Puchkova, and W. Zagorski.** 1980. Regulation of brome mosaic virus gene expression by restriction of initiation of protein synthesis. J. Virol. **34**: 330-335.
10. **Clegg, J. C. S., and S. I. T. Kennedy.** 1975. Initiation of synthesis of the structural proteins of Semliki Forest virus. J. Mol. Biol. **97**:401-411.
11. **Hay, A. J., J. J. Skehel, and D. C. Burke.** 1968. Proteins synthesized in chick cells following infection with Semliki Forest virus. J. Gen. Virol. **3**:175-184.
12. **Helentjaris, T., E. Ehrenfeld, M. L. Brown-Luedi, and J. W. B. Hershey.** 1979. Alterations in initiation factor activity from poliovirus-infected HeLa cells. J. Biol. Chem. **254**:10973-10978.
13. **Helentjaris, T., and E. Ehrenfeld.** 1978. Control of protein synthesis in extracts from poliovirus-infected cells. I. mRNA discrimination by crude initiation factors. J. Virol. **26**:510-521.
14. **Kääriäinen, L., and H. Söderlund.** 1978. Structure and replication of α-viruses. Curr. Top. Microbiol. Immunol. **82**:15-69.
15. **Kaufmann, Y., E. Goldstein, and S. Penman.** 1976. Poliovirus-induced inhibition of polypeptide initiation in vitro on native polyribosomes. Proc. Natl. Acad. Sci. U.S.A. **73**:1834-1838.
16. **Keränen, S., and L. Kääriäinen.** 1975. Proteins synthesized by Semliki Forest virus and its 16 temperature-sensitive mutants. J. Virol. **16**:388-396.
17. **Krystosek, A., M. L. Cawthon, and D. Kabat.** 1975. Improved methods for purification and assay of eukaryotic mRNAs and ribosomes. J. Biol. Chem. **250**:6077-6084.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
19. **Layne, E.** 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Methods Enzymol. **3**: 451-454.
20. **Padilla, M., D. Canaani, Y. Groner, J. A. Weinstein, M. Bar-Joseph, W. C. Merrick, and D. A. Shafritz.** 1978. Initiation factor eIF-4B (IF-M3) dependent recognition and translation of capped versus uncapped eukaryotic mRNAs. J. Biol. Chem. **253**:5939-5945.
21. **Pettersson, R. F., H. Söderlund, and L. Kääriäinen.** 1980. The nucleotide sequences of the 5'-terminal T1 oligonucleotides of Semliki Forest virus 42S and 26S RNAs are different. Eur. J. Biochem. **105**:435-443.
22. **Rose, J. K., H. Trachsel, K. Leong, and D. Baltimore.** 1978. Inhibition of translation by poliovirus: inactivation of a specific initiation factor. Proc. Natl. Acad. Sci. U.S.A. **75**:2732-2736.
23. **Schreier, M. H., and T. Staehelin.** 1973. Initiation of mammalian protein synthesis: the importance of ribosome and initiation factor quality for the efficiency of in vitro systems. J. Mol. Biol. **73**:329-349.
24. **Shafritz, D. A., J. A. Weinstein, B. Safer, W. C. Merrick, L. A. Weber, E. D. Hickey, and C. Baglioni.** 1976. Evidence for role of m^7G^5-phosphate group in recognition of eukaryotic mRNA by initiation factor IF-M3. Nature (London) **261**:291-294.
25. **Skup, D., and S. Millward.** 1977. Highly efficient translation of messenger RNA in cell-free extracts prepared from L cells. Nucleic Acids Res. **4**:3581-3587.
26. **Skup, D., and S. Millward.** 1980. Reovirus-induced modification of cap-dependent translation in infected L cells. Proc. Natl. Acad. Sci. U.S.A. **77**:152-156.
27. **Sonenberg, N., M. A. Morgan, W. C. Merrick, and A. J. Shatkin.** 1978. A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 5'-terminal cap in mRNA. Proc. Natl. Acad. Sci. U.S.A. **75**:4843-4847.
28. **Sonenberg, N., K. M. Rupprecht, S. M. Hecht, and A. J. Shatkin.** 1979. Eukaryotic mRNA cap binding protein: purification by affinity chromatography on Sepharose coupled m^7GDP. Proc. Natl. Acad. Sci. U.S.A. **76**: 4345-4349.
29. **Strauss, J. H., and E. G. Strauss.** 1977. Togaviruses, p. 111-116. *In* D. P. Nayak (ed.), The molecular biology of animal viruses. Marcel Dekker, New York.
30. **Thomas, A., H. Goumans, H. Amesz, R. Benne, and H. O. Voorma.** 1979. A comparison of the initiation factors of eukaryotic protein synthesis from ribosomes and from the post-ribosomal supernatant. Eur. J. Biochem. **98**:329-337.
31. **Trachsel, H., N. Sonenberg, A. J. Shatkin, J. K. Rose, K. Leong, J. E. Bergmann, J. Gordon, and D. Baltimore.** 1980. Purification of a factor that restores translation of vesicular stomatitis virus mRNA in extracts from poliovirus-infected HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **77**:770-774.
32. **Tuomi, K., L. Kääriäinen, and H. Söderlund.** 1975. Quantitation of Semliki Forest virus RNAs in infected cells using ^{32}P equilibrium labeling. Nucleic Acids Res. **2**:555-565.
33. **van Steeg, H., M. H. Pranger, B. A. M. van der Zeyst, R. Benne, and H. O. Voorma.** 1979. In vitro translation of Semliki Forest virus 42S RNA. Initiation at two different sites. FEBS Lett. **108**:292-298.
34. **Vazquez, D.** 1979. Inhibitors of protein synthesis. Mol. Biol. Biochem. Biophys. **30**:31-32.
35. **Wengler, G., and G. Wengler.** 1975. Comparative studies on polyribosomal, nonpolyribosomal-associated and viral 42S RNA from BHK 21 cells infected with Semliki Forest virus. Virology **65**:601-605.
36. **Wengler, G., and G. Wengler.** 1976. Protein synthesis in BHK-21 cells infected with Semliki Forest virus. J. Virol. **17**:10-19.