JOURNAL OF VIROLOGY, Jan. 1976, p. 10-19

Vol. 17, No. 1
Printed in U.S.A.

Protein Synthesis in BHK-21 Cells Infected with Semliki Forest Virus

GERD WENGLER* AND GISELA WENGLER
Institut für Virologie, Justus Liebig-Universität, Giessen, Germany

Received for publication 16 July 1975

[^{3}H]leucine-labeled proteins synthesized in BHK-21 cells infected with Semliki Forest virus were fractionated by polyacrylamide gel electrophoresis (PAGE). Cellular and virus-specific proteins were identified by difference analysis of the PAGE profiles. The specific activity of intracellular [^{3}H]leucine was determined. Two alterations of protein synthesis, which develop with different time courses, were discerned. (i) In infected cultures an inhibition of overall protein synthesis to about 25% of the protein synthesis in mock-infected cultures develops between about 1 and 4 h postinfection (p.i.). (ii) The relative amount of virus-specific polypeptides versus cellular polypeptides increases after infection. About 80% of the proteins synthesized at 4 h p.i. are cellular proteins. Since significant amounts of nontranslocating ribosomes in polyribosomes were not detected up to 7 h p.i., the inhibition of protein synthesis is not caused by inactivation of about 75% of all polyribosomes but by a decreased protein synthetic activity of the majority of polyribosomes. Indirect evidence indicates that an inhibition of elongation and/or release of protein synthesis develops in infected cells, which is sufficient to account for the observed inhibition of protein synthesis. Inhibition of over-all protein synthesis developed when virus-specific RNA began to accumulate at the maximal rate. This relationship was observed during virus multiplication at 37, 30, and 25 C. A possible mechanism by which synthesis of virus-specific RNA in the cytoplasm could inhibit cellular protein synthesis is discussed. Indirect evidence and analysis of polyribosomal RNA show that the increased synthesis of virus-specific protein is brought about by a substitution of cellular by viral mRNA in the polyribosomes.

Infection of vertebrate cells in culture with the group A togaviruses Semliki Forest virus (SFV) or Sindbis virus drastically alters cellular protein synthesis. The amount of radioactivity incorporated from radioactive amino acids into protein during a series of labeling intervals decreases, and the ratio of virus-specific proteins to cellular proteins synthesized during these labeling intervals increases (2, 9, 24, 29). Both of these effects can be observed in BHK-21 cells infected with SFV. This system seems suitable for a more detailed study of these processes, since the structural proteins of SFV and the intracellular virus-specific proteins have been characterized (1, 10, 18, 27) and since the virus-specific mRNA molecules from which they are synthesized have been identified (4, 28, 31, 34). As a first step in a study of the alterations of protein synthesis induced in BHK-21 cells after infection with SFV, the results of an analysis of the synthesis of cellular and virus-specific proteins, of the polyribosomes, and of the accumulation of virus-specific RNA in infected cells are reported in this communication.

MATERIALS AND METHODS

Chemicals and isotopes. Sucrose (RNase free) was obtained from Schwarz/Mann. Sodium deoxycholate from Fluka (Switzerland) was purified from an ethanol solution by precipitation with hexane. Actinomycin D and Tween 80 were from Serva (Germany). Radiochemicals were all purchased from the Radiochemical Centre, Amersham (England). Phenol was p.a. grade from Merck (Germany) and was redistilled before use. DNase I was an RNase-free preparation from Worthington. Omnifluor was bought from New England Nuclear Chemicals GmbH and Triton X-100 was purchased from Röhn & Haas Deutschland GmbH (Frankfurt am Main, West Germany). Pactamycin was kindly provided by W. Kersten, Erlangen.

Solutions. The following solutions were used: lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM triethanolamine, pH 7.4); concentrated salt buffer

(500 mM KCl, 5 mM $MgCl_2$, 10 mM triethanolamine, pH 7.4); RNA buffer (50 mM NaCl, 10 mM Na_2-EDTA, pH 7.4); and citrate buffer (70 mM trisodium citrate, adjusted to pH 2.2 with HCl).

Preparation of confluent monolayers. The preparation of freshly confluent monolayers prelabeled with [^{14}C]uridine and their infection with SFV have been described (33). Cells were lysed, and the ribonucleoprotein particles were fractionated on sucrose density gradients as described earlier (31).

Labeling of protein with [^{3}H]leucine and analysis of protein by SDS-PAGE. Growth medium was replaced by labeling medium consisting of Eagle minimal essential medium, modified according to Dulbecco and Freeman (5), containing only 0.08 mM leucine with 10% fetal bovine serum and [^{3}H]leucine or [^{14}C]leucine, as indicated in the description of the experiments. After 30 min of incubation at 37 C, the cells were washed with cold Earle balanced salt solution and stored at −80 C. Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Lämmli (14). Cells were prepared for this analysis as follows. Cell pellets were thawed at 2 C, and 80 μl of the following buffer mixture was added: 100 μl of eightfold-concentrated spacer gel buffer of the electrophoretic system plus 100 μl of DNase I solution (1 mg/ml in water) plus 10 μl of RNase A solution (1 mg/ml in water) plus 400 μl of water plus 100 μl of glycerol. After 2 min, SDS was added to 0.2%, and the sample was incubated at 37 C for 1 min. After addition of 2 μl of 2-mercaptoethanol and 5 μl of a 20% SDS solution, the sample was heated to 100 C for 1 min and cooled to room temperature. Spacer and running gels contained 3 and 7.5% acrylamide, respectively. Electrophoresis was at room temperature at 4 mA/gel. Gels (diameter, 5 mm) were sliced into 1-mm slices and counted in a liquid scintillation counter.

Determination of the specific activity of the intracellular leucine pool. For each specific activity determination (6) monolayer cultures containing about 10^6 cells were taken 30 min after addition of [^{3}H]leucine to the growth medium and washed four times for 30 s with 50 ml of ice-cold Earle balanced salt solution at 4 C. Amino acids were extracted with 0.9 N perchloric acid for 2 min at 4 C. The total amount of radioactivity extracted was determined, the extract was neutralized with KOH, the precipitate of potassium perchlorate was removed by centrifugation, and the supernatant was lyophilized. The lyophilized material was taken up in 1.5 ml of citrate buffer (pH 2.2), an aliquot was used for determination of radioactivity, and 1 ml of the sample was used for fractionation and quantitation of the individual amino acids by chromatography in a Bio Cal amino acid analyzer.

Other techniques. Phenol extraction of RNA at 60 C and measurement of total radioactivity of aqueous samples was done as described earlier (32, 33). Radioactivity incorporated into protein in cell cultures was determined as follows. Cells were washed with cold Earle balanced salt solution and suspended in 0.1 N NaOH. After 30 min of incubation at room temperature, the acid-precipitable material was precipitated by 10% trichloroacetic acid and filtered onto glass-fiber filters (Whatman GF/B). The dried filters were incubated with 2 ml of Soluence 350 solubilizer for 2 h at 60 C, 18 ml of toluene scintillation fluid was added, and the radioactivity was determined in a liquid scintillation counter. Autoradiography was performed as described by Perry (19).

RESULTS

Protein synthesis after infection. Infected and mock-infected cells were labeled for 30-min periods with [^{3}H]- or [^{14}C]leucine, respectively. The samples containing the [^{3}H]- and [^{14}C]leucine-labeled material synthesized at the same labeling interval were mixed, and the polypeptides were fractionated by SDS-PAGE. Results of such analyses are shown in Fig. 1. It can be seen that during the 8 h of the SFV growth cycle the amount of [^{3}H]leucine incorporated into protein in infected cells decreases and that the pattern of newly synthesized peptides is increasingly dominated by a few virus-specific proteins (2, 9, 18). Arrows C and E in Fig. 1 indicate the position of the viral core protein (1) and the two unresolved viral envelope glycoproteins E_1 and E_2 (1, 10), respectively. The NSP 68 protein is a precursor to the viral envelope glycoprotein E_2 (27). Proteins NSP 90 and NSP 103 have not been definitely characterized, but possibly both are also precursors for the synthesis of the viral envelope glycoprotein (18). The relative amounts of [^{3}H]leucine radioactivity incorporated into virus-specific and cellular polypeptides can be determined by difference analysis (35) from the analyses shown in Fig. 1. A region of normalization, which contains no significant amounts of virus-specific polypeptides, is chosen and consists of gel fractions containing the polypeptides migrating into the gel and migrating slower than the NSP 103 protein, as indicated by the bars in Fig. 1. The total ^{3}H radioactivity in this region is divided by the total ^{14}C radioactivity in the same region, and the ^{14}C radioactivity in each fraction of the gel is multiplied by this ratio. The resulting normalized ^{14}C profile, which is indicated in (A) and (C) of Fig. 1, is defined as host cell material, and the difference between ^{3}H radioactivity incorporated in infected cells and the normalized ^{14}C radioactivity is defined as virus-specific material (11).

Total [^{3}H]leucine radioactivity incorporated in infected cells into protein during different labeling intervals can be determined by trichloroacetic acid precipitation. Since the amount of radioactivity incorporated during 30-min label-

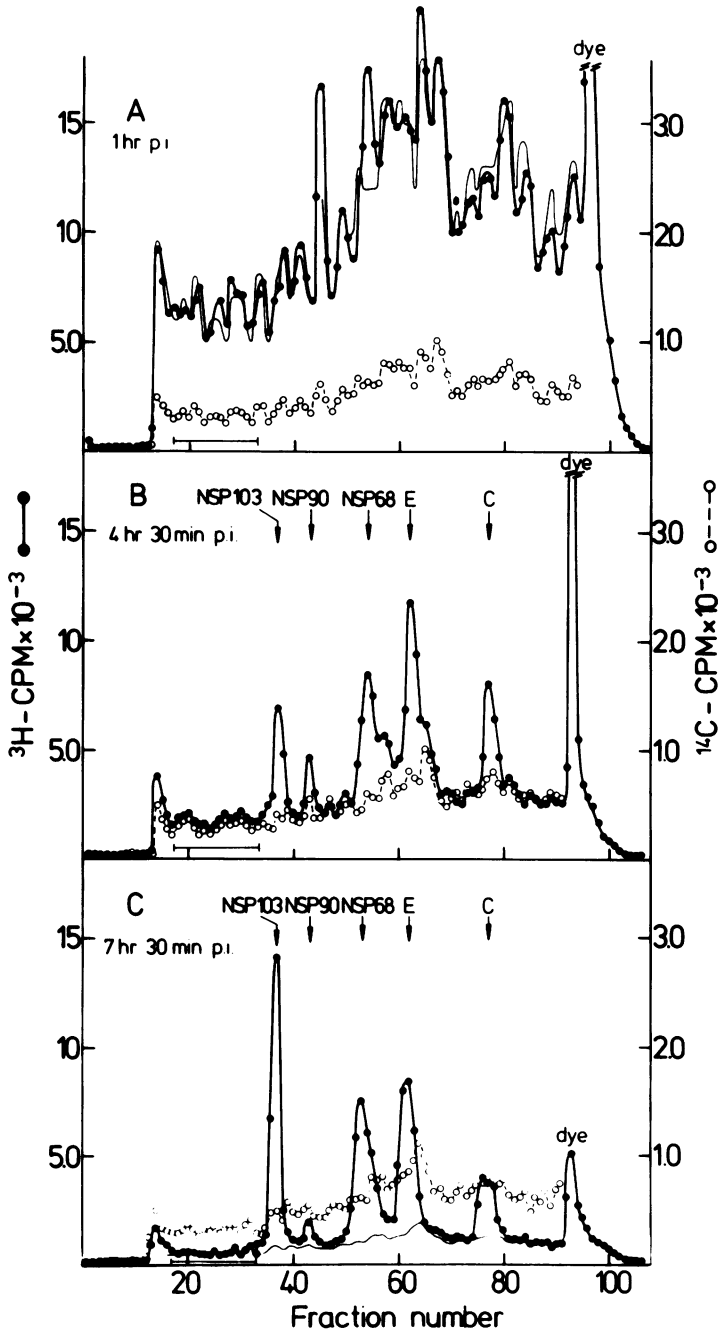

FIG. 1. *Polyacrylamide gel electrophoresis of proteins synthesized in BHK-21 cells after infection with SFV. Confluent monolayer cultures containing about 5 × 10^6 cells each were either infected with SFV or mock infected. After the 30-min period used for these treatments (time 0), all cultures were incubated with fresh growth medium at 37 C. Mock-infected cultures were labeled with [^{14}C]leucine (2 μCi/ml) and infected cultures were labeled with [^{3}H]leucine (25 μCi/ml) simultaneously for 30-min periods (see Materials and Methods) at various times after infection or mock infection, respectively. After the labeling period the cells were harvested, washed, and stored as pellets at −80 C. The cells were prepared for electrophoresis, aliquots of the samples containing [^{3}H]- and [^{14}C]leucine polypeptides synthesized at the same time were mixed, and the mixtures were analyzed on 7.5% polyacrylamide gels as described in Materials and Methods. Gels were sliced, and the radioactivity in the slices was measured. The direction of electrophoresis is from left to right. The gel fractions used for normalization for difference analysis are indicated by the bars. The normalized ^{14}C curves are shown only in (A) and (C), since the measured and normalized ^{14}C curves would be almost identical in the graphical representation in (B). Normalization is done as described in the text. Virus-specific proteins are indicated by arrows in (B) and (C). The analyses of the polypeptides synthesized between 1 and 1.5 h, 4.5 and 5 h, and between 7.5 and 8 h p.i. and post mock infection are presented in (A), (B), and (C), respectively. Symbols: (●) ^{3}H radioactivity (infected cells); (○) ^{14}C-radioactivity (mock-infected cells); (——) normalized ^{14}C radioactivity.*

ing intervals into acid-precipitable material in mock-infected cultures increases slightly with time (data not shown), the radioactivity incorporated in infected cells is expressed as percentage of radioactivity incorporated in mock-infected cells during a corresponding labeling interval (Fig. 2B). The results obtained by these analyses from a series of gels similar to those shown in Fig. 1 are presented in Fig. 2, together with the viral growth curve determined in the same experiment. It can be seen from the data presented in Fig. 2B that between 2 and 4.5 h postinfection (p.i.) the incorporation of [^{3}H]leucine into total protein rapidly declines to about 25% of the incorporation found in mock-infected cultures. At 4.5 p.i. about 80% of the [^{3}H]leucine is incorporated into cellular protein (Fig. 2A).

The data presented in Table 1 show that the specific activity of intracellular [^{3}H]leucine

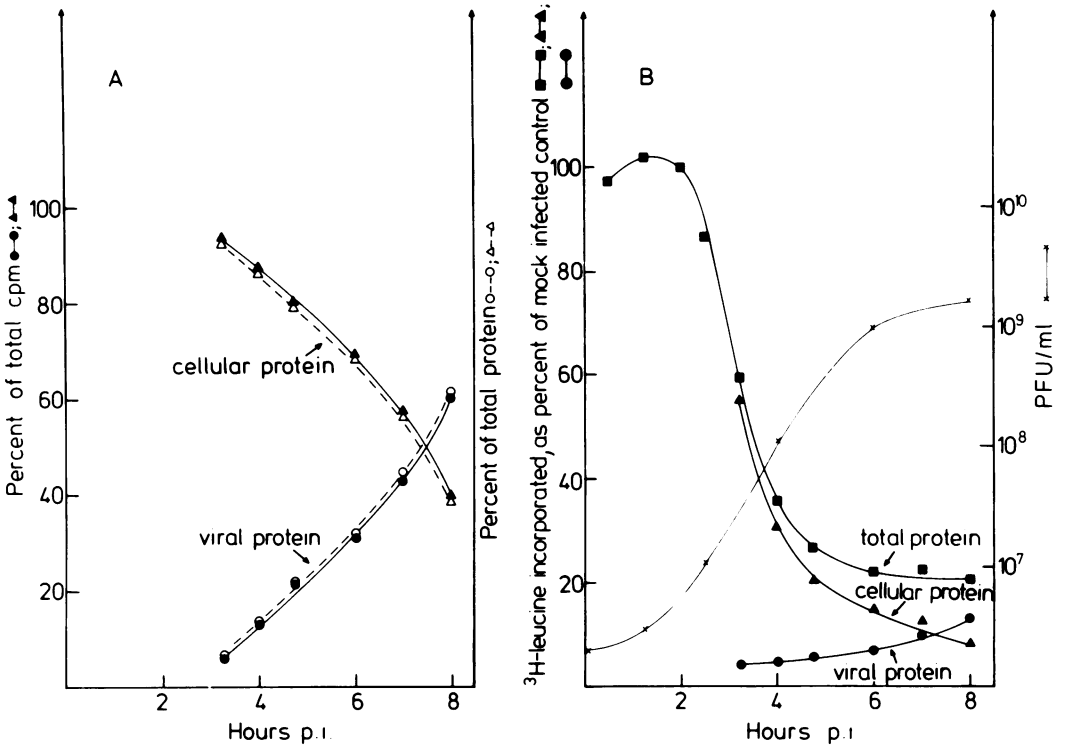

FIG. 2. *Incorporation of [^{3}H]leucine into protein and the synthesis of infectious virus particles at different times after infection. (A) Quantitative results of a series of gel electrophoretic analyses of pulse-labeled peptides similar to those shown in Fig. 1. Percentage of total [^{3}H]leucine radioactivity in viral (●) and cellular (▲) polypeptides has been determined by difference analysis as described in the text and in the legend to Fig. 1. The percentage of virus-specific protein synthesized (○) has been determined from the formula: percentage of viral protein/100% = counts per minute in viral proteins + 5% of counts per minute in viral proteins/counts per minute in total proteins + 5% of counts per minute in viral proteins. The percentage of cellular protein synthesized (△) is calculated from the formula: percentage of cellular proteins + percentage of viral protein = 100%. This calculation reflects the fact that about 5% less ^{3}H radioactivity is incorporated into viral protein than into the same amount of cellular protein because of the relative low leucine content of viral core protein (see text). (B) In the experiment presented in (A) the total amount of [^{3}H]leucine radioactivity incorporated during the various labeling intervals into protein in infected cultures has been determined by trichloroacetic acid precipitation on an aliquot of the labeled cultures (see Materials and Methods). A series of mock-infected cultures was labeled with [^{3}H]leucine under conditions identical to the infected cultures, and the amount of [^{3}H]leucine incorporated into protein was determined as indicated above. The amount of [^{3}H]leucine radioactivity incorporated into protein in infected cells during each labeling interval is then expressed as percentage of the [^{3}H]leucine radioactivity incorporated into protein in mock-infected cells during the corresponding labeling interval (■). The relative amounts of cellular (▲) and virus-specific (●) material snythesized at different times after infection are taken from the data given in (A) and are presented also as percentage of the total [^{3}H]leucine radioactivity incorporated in mock-infected cells. Virus multiplication was determined by plaque assay of the growth medium at various times after infection (×).*

TABLE 1. *Specific activity of intracellular [^{3}H]leucine in mock-infected and SFV-infected BHK-21 cells*[a]

Time (h) after mock infection or infection at which sp act was determined[b]	Sp act of intracellular [^{3}H]leucine (counts/min per nmol of leucine)[c]	
	Mock-infected cells	Infected cells
0.9	15,100	15,400
1.9	15,300	14,800
3	16,200	17,300
4	15,800	17,500
6	17,200	19,600
7.5	15,700	20,400

[a] Virus-induced inhibition of incorporation of [^{3}H]leucine radioactivity into protein has also been determined in this experiment. The time course of this inhibition was similar to the one presented in Fig. 2B and is therefore not shown.

[b] [^{3}H]leucine was added to the growth medium to 20 μCi/ml 30 min before determination of specific activity. At all times after infection and mock infection the [^{3}H]leucine pool reached its final specific activity within less than 8 min (data not shown).

[c] See Materials and Methods for a description of the experimental technique used. The specific activity of [^{3}H]leucine at various times in infected and mock-infected cells has been determined in two independent experiments with comparable results. The data obtained in one of these experiments are given in this table.

reached at various labeling intervals is rather constant at different times after infection and similar in infected and mock-infected cells. Therefore, the time course of the reduction of incorporation of [^{3}H]leucine into total protein presented in Fig. 2B is a quantitative measurement of the reduction of total protein synthesis occurring in infected cells.

To draw a conclusion concerning the relative amounts of cellular and viral proteins synthesized at different times after infection from the amount of [^{3}H]leucine incorporated into both types of proteins, their relative content of leucine has to be considered. The viral structural proteins E_1, E_2, and C have a relative content of 58, 60, and 38 mol of leucine, respectively, per 1,000 mol of amino acids (10, 13). The NSP 68 protein is a precursor to E_2 (27), and it seems plausible to assume a leucine content of 60 mol/per 1,000 for this protein, similar to the leucine content of E_2. A content of about 58 leucine molecules per 1,000 amino acids has been determined in hydrolysates of total protein of BHK-21 cells (data not shown). Therefore, only the leucine content of the viral core protein deviates significantly (by about 35%) from that of the average BHK-21 cell protein. Since about 15% of the [^{3}H]leucine radioactivity incorporated into virus-specific proteins is incorporated into viral core protein rather constantly at all times later than 3 h p.i. (data not shown; see Fig. 1B and C), the amount of [^{3}H]leucine incorporated into a certain amount of virus-specific protein is only about 5% (0.15 × 0.35 × 100%) lower than the [^{3}H]leucine radioactivity incorporated into the same amount of cellular protein. The relative amounts of viral and cellular proteins synthesized at different times after infection using this correction are presented in Fig. 2A. The relative amounts of [^{3}H]leucine radioactivity incorporated into virus-specific and cellular protein rather closely reflect the relative amounts of newly synthesized virus-specific and cellular proteins. From autoradiographic analyses of mock-infected and infected BHK-21 cells labeled with [^{3}H]uridine in the presence of actinomycin D (data not shown), it can be concluded that SFV multiplies in more than 95% of the cells of an infected monolayer culture.

Analyses of the polyribosomes of infected cells. Sedimentation patterns of polyribosomes isolated at various times after infection from untreated cells and from cells treated with pactamycin (30) or with low concentrations (2 μg/ml) of cycloheximide (8) have been determined. From the sedimentation profiles, the percentage of total ribosomal material found in polyribosomes can be calculated. Two precautions were taken to get reliable data. (i) The cells used for these experiments were prelabeled with [^{14}C]uridine in the rRNA and the radioactivity profiles were analyzed to avoid base line uncertainties of optical density measurements. (ii) The total cells, including the nuclei, were lysed, and the whole lysates were used for polyribosome analysis to avoid loss of ribosomal material in a nuclear fraction not analyzed for polyribosomes (33). The results obtained are presented in Fig. 3, together with the results of the analyses of the virus-induced alterations of cellular protein synthesis determined in the same experiment. The data show that the inhibition of cellular protein synthesis is not brought about by a reduction of the amount of ribosomes in polyribosomes. The polyribosomes of infected cultures were almost quantitatively disaggregated after treatment with pactamycin at all times up to 7 h p.i. (data not shown), a finding that excludes the existence of significant amounts of nontranslocating ribosomes in polyribosomes. Since increasing amounts of virus-specific polypeptides are synthesized in infected cells, these results would constitute indirect evidence for the substitution of cellular by viral

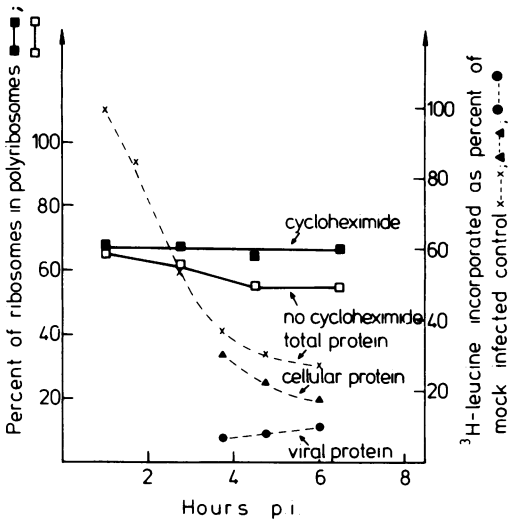

FIG. 3. *Polyribosomes and cellular and viral protein synthesis in infected cells. Freshly confluent BHK-21 monolayer cultures prelabeled with [^{14}C]uridine (0.1 μCi/ml) for 24 h were either mock infected or infected with SFV. At various times after infection, six infected cultures were taken, two of which were left untreated, whereas cycloheximide (2 μg/ml) or pactamycin (0.6 μg/ml) was added to two other cultures, respectively. After 20 min of incubation at 37 C, the cells were harvested and lysed as described in Materials and Methods, and polyribosomes and free ribosomes in the lysate were fractionated by 80-min centrifugation on linear 15 to 35% (wt/wt) sucrose density gradients in concentrated salt buffer at 40,000 rpm, 2 C, in the SW41 Spinco rotor. The gradients were fractionated, and the ^{14}C radioactivity of each fraction was determined. At the salt concentration obtained in the lysates and used during cell fractionation, the nonpolyribosome-associated ribosomes dissociate into subunits, whereas the mRNA-associated ribosomes are stable (36). From these polyribosome profiles the percentage of ribosomes in polyribosomes has been determined. The radioactivity found in the pellets of these gradients was always less than 15% of the total radioactivity sedimenting into the gradient and has not been included in this calculation. Infected or mock-infected cultures were also labeled for 30-min periods with [^{3}H]- or [^{14}C]leucine as described in the legend to Fig. 1. The polypeptides were fractionated by SDS-PAGE, and the inhibition of incorporation of [^{3}H]leucine into protein and the switchover to viral protein synthesis were analyzed as described in the legend to Fig. 2. The percentage of ribosomes in polyribosomes found in untreated cells and in cycloheximide-treated cells is indicated by (□) and (■), respectively. The [^{3}H]leucine radioactivity incorporated into total protein, cellular protein, or virus-specific protein during the various labeling intervals is indicated by the (×), (▲), and (●), respectively.*

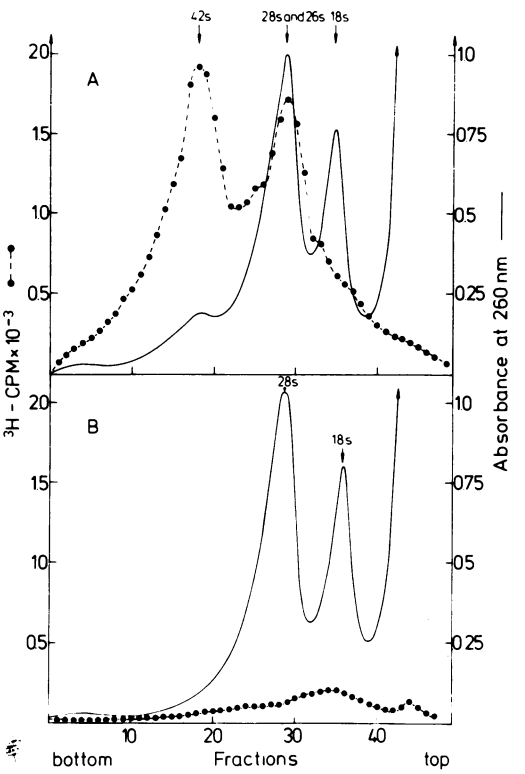

FIG. 4. *Analysis of polyribosomal RNA. Thirty confluent monolayer cultures were either mock infected or infected with SFV in the presence of actinomycin D (0.05 μg/ml). The cultures were then incubated at 37 C with fresh growth medium containing actinomycin D (0.05 μg/ml), [^{3}H]uridine (8 μCi/ml), and unlabeled uridine (20 μM). The cells were harvested at 6 h p.i. or post mock infection, respectively, and lysed as described in Materials and Methods. Each lysate was loaded onto two identical 15 to 35% (wt/wt) linear sucrose density gradients in concentrated salt buffer and fractionated by 80-min centrifugation in the SW41 Spinco rotor at 40,000 rpm, 2 C. The optical density distribution in the gradients was measured, the polyribosomes containing four or more ribosomes were pooled, and the RNA was immediately extracted from these polyribosomes with phenol at 60 C. The RNA was then fractionated by centrifugation on 10 to 30% (wt/wt) sucrose gradients in RNA buffer for 6 h at 40,000 rpm and 2 C in the SW41 Spinco rotor. The [^{3}H]uridine-labeled 26S virus-specific RNA and the unlabeled 28S rRNA are not separated from each other under these conditions of centrifugation. The optical density distribution in the gradients was measured, and total radioactivity was determined in an aliquot of each fraction. The results of the analysis of the RNA from polyribosomes of infected and uninfected cells are presented (A) and (B), respectively. Symbols: (——) absorbance at 260 nm; (●) [^{3}H]uridine radioactivity.*

mRNA in the majority of cellular polyribosomes if only stable proteins are synthesized in infected cells. If proteins translated from cellular mRNA would be short-lived, the SDS-PAGE patterns of proteins accumulated during 30-min labeling intervals could be dominated by more stable virus-specific proteins. The synthesis of short-lived polypeptides has been analyzed by measuring the time course of [^{3}H]leucine incorporation into protein in infected cells at various times up to 7 h p.i. [^{3}H]leucine was taken up into protein linearly with time during 15 min after addition to the growth medium, indicating that no significant amounts of short-lived polypeptides were synthesized in the infected cells (data not shown). Together with the data of Fig. 3 this constitutes indirect evidence for a gradual substitution of cellular by viral mRNA in the majority of cellular polyribosomes during virus multiplication.

Direct evidence for this mRNA substitution is obtained in the experiment presented in Fig. 4. Cells were either mock infected or infected with SFV and then labeled with [^{3}H]uridine in the presence of actinomycin D (0.05 μg/ml). This concentration of actinomycin D specifically inhibits the synthesis of rRNA (20). At 6 h post mock infection or p.i., respectively, the cells were harvested and lysed; the polyribosomes containing four or more ribosomes were isolated, and their RNA was extracted. The results of the gradient fractionation of these RNA preparations are presented in Fig. 4. Two species of [^{3}H]uridine-labeled, virus-specific RNA, which sediment at about 42*S* and 26*S*, respectively, cosediment with the polyribosomes of infected cells. It has been shown earlier (33) that the 26*S* RNA is released from the polyribosomes by EDTA and bands at the same density as the polyribosomes after fixation in cesium chloride density gradients, and therefore is a constituent of the polyribosomes. It can be determined from the optical density profile presented in Fig. 4A that about 6.4% of the

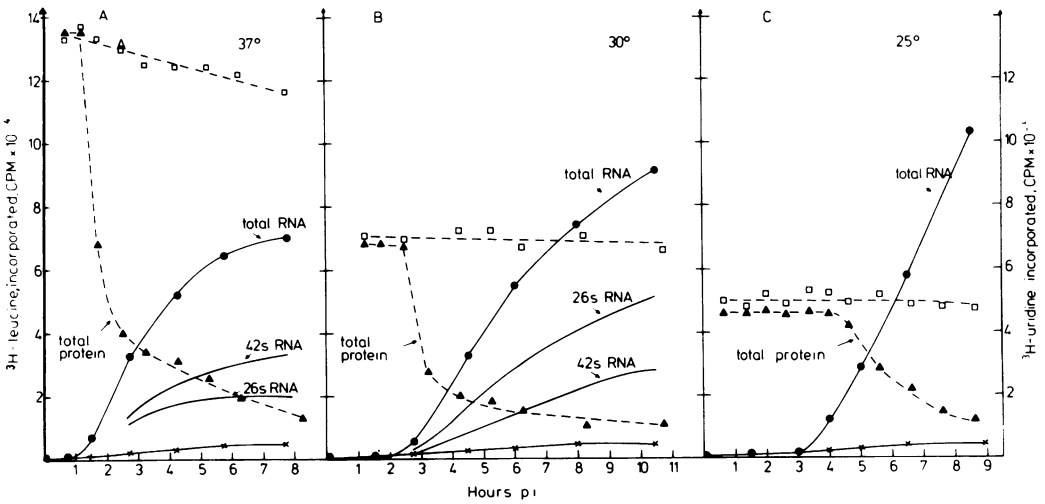

FIG. 5. *Virus-induced inhibition of protein synthesis and synthesis of virus-specific RNA at different temperatures. Confluent monolayer cultures, each containing about 15 × 10^5 cells, were either mock infected or infected with SFV in the presence of actinomycin D (1 μg/ml). Infection was done for 30 min at 37 C in all experiments described in this figure. The cultures were then (time 0) covered with growth medium containing actinomycin D (1 μg/ml) and unlabeled uridine (0.03 mM) and incubated at the temperature of the particular experiment. The time course of RNA synthesis in the cultures was determined as follows. [^{3}H]uridine was added to some of the cultures at zero time, and cells were harvested at various times for the determination of total acid-precipitable radioactivity and extraction of RNA. The amount of [^{3}H]uridine radioactivity incorporated into 42S and 26S RNA, respectively, was determined by sucrose density gradient analysis. The time course of protein synthetic activity was determined by measuring the incorporation of [^{3}H]leucine into acid-precipitable material during 30-min labeling intervals. In all experiments [^{3}H]uridine was added to 10 μCi/ml, and the amount of [^{3}H]uridine and [^{3}H]leucine radioactivity incorporated in one monolayer culture is indicated in the figure. (A) 37 C; [^{3}H]leucine labeling: 10 μCi/ml, 30 min. (B) 30 C; all other conditions as in (A). (C) 25 C; [^{3}H]leucine labeling: 10 μCi/ml, 40 min. The total [^{3}H]leucine radioactivity incorporated in mock-infected or infected cultures is indicated by (□) and (▲), respectively. The [^{3}H]uridine incorporated into RNA in infected cells is indicated by (●) and (——) as indicated in the figure. [^{3}H]uridine incorporated into RNA in mock-infected cells is indicated by (×).*

combined mass of 28S, 26S, and 18S RNA in this gradient is in 42S RNA. It is reasonable to assume that the virus-specific 42S and 26S RNA species have similar specific radioactivities, since both are made at the same time (Fig. 5) and have a similar content of uridine (12). Since the ratio of radioactivity in 26S RNA to radioactivity in 42S RNA in the gradient shown in Fig. 4A is 0.68, it can be calculated that 4.38% of the combined mass of 28S, 26S, and 18S RNA in the gradient is in 26S RNA. From these results and an estimation of the size of the polyribosomes from which the RNA was isolated, it can be calculated that about 50% of the polyribosomes, the RNA of which has been analyzed in the experiment shown in Fig. 4A, contained virus-specific 26S RNA as follows. It has been estimated from optical density analyses of the gradients used for isolation of polyribosomes that the majority of these polyribosomes contained between 4 and 10 ribosomes. An average number of 7 ribosomes per polyribosome has therefore been used for the following calculation. A polyribosome containing 7 ribosomes and one molecule of 26S RNA as mRNA contains about $7 \times 2.4 \times 10^6$ daltons of rRNA (17, 21) plus 1.6×10^6 daltons of 26S RNA (16). Of the total amount of 18.4×10^6 daltons of polyribosomal RNA, therefore, 1.6×10^6 daltons, corresponding to 8.6%, are in 26S RNA. Since 4.38% of the total mass of 28S, 26S, and 18S RNA consisted of 26S RNA, about $4.38 \times 100/8.6 = 51\%$ of the polyribosomes contained 26S RNA. Since a significant percentage of the 42S RNA molecules sedimenting with polyribosomes at 6 h p.i. is not bound to the polyribosomes (33), the amount of polyribosomes containing 42S RNA cannot be calculated from the data presented in Fig. 4.

Time course of protein synthesis and synthesis of virus-specific RNA. SFV multiplies in actinomycin-treated BHK-21 cells with a similar time course and to a similar titer of infectious virus particles as in cells not treated with the drug. The accumulation of virus-specific RNA can therefore be followed by labeling infected cells with [^{3}H]uridine in the presence of actinomycin D. In the experiment presented in Fig. 5 the time course of the virus-induced inhibition of protein synthesis and the accumulation of virus-specific RNA have been determined at three different temperatures. In two of the experiments RNA has also been extracted from the infected cells, and the amount of radioactivity in 42S and 26S virus-specific RNA has been measured. It can be seen that a drastic inhibition of overall protein synthesis develops in the infected cells at all three temperatures, that the time at which this inhibition starts is temperature dependent, that the linear phase of accumulation of virus-specific RNA and the protein synthesis inhibition both start at about the same time, and that both processes follow different kinetics, the inhibition of protein synthesis occurring more rapidly than the accumulation of virus-specific RNA. In contrast to the situation at 37 C, at 30 C more radioactivity is incorporated into 26S RNA than into 42S RNA at all times after infection.

DISCUSSION

Two virus-induced alterations of cellular protein synthesis can be detected in SFV-infected BHK-21 cells: (i) a rapid inhibition of overall protein synthetic activity to about 25% of the activity found in mock-infected control cultures, which developed between 2 and 4.5 h p.i. in the experiment presented in Fig. 2, and (ii) a switchover from the synthesis of cellular proteins to the synthesis of virus-specific proteins, which proceeds more slowly than the protein synthesis inhibition described above and does not lead to a significant recovery of cellular protein synthetic capacity. That SFV infection leads to a decrease of the incorporation of radioactivity from labeled amino acids into protein and to increasing incorporation of radioactivity into virus-specific, as compared to cellular, proteins has already been described (2, 9, 18). Similar effects have also been observed in Sindbis virus-infected cells (23, 24, 29), albeit in Sindbis virus-infected chicken embryo fibroblasts maximal inhibition of overall protein synthesis and maximal synthesis of virus-specific proteins were observed at about the same time (29).

The results concerning the structure of polyribosomes at different times after infection give some indication of which step of protein synthesis is inhibited. No significant disaggregation of polyribosomes occurred up to 7 h p.i., and treatment of the infected cells with pactamycin resulted in polyribosome disaggregation.

Therefore, the virus-induced inhibition of cellular protein synthesis is not brought about by a disaggregation or inactivation of cellular polyribosomes but by a decrease of their protein synthetic activity. This decrease can result from a slow down of initiation or elongation or release of proteins from polyribosomes, or by a combination of these effects. If initiation of translation would be selectively inhibited, the amount of ribosomes in polyribosomes would decrease,

the residual polyribosomes would contain fewer ribosomes per polyribosome, and treatment of the cells with low concentrations of cycloheximide should significantly reverse both changes by specifically slowing down the movement of ribosomes along the mRNA (8). The size of polyribosomes did not decrease in infected cells (data not shown), and the data of Fig. 3 show that both other phenomena are observed only to a very limited extent after infection. Therefore, the 70% inhibition of total protein synthesis observed in the experiment presented in Fig. 3 at 4 h p.i. cannot result from a virus-induced specific inhibition of initiation of protein synthesis. Double-stranded RNA inhibits initiation of protein synthesis in vitro (7, 22), and it has been suggested that the inhibition of cellular protein synthesis observed in poliovirus-infected cells is the consequence of virus-specific, double-stranded RNA-induced inhibition of initiation of protein synthesis (7). The inhibition of protein synthesis observed in SFV-infected BHK-21 cells cannot be explained by such a mechanism. A similar conclusion has been reached for Sindbis virus-infected chicken embryo fibroblasts by a different approach by Shenk and Stollar (25). Therefore either the rate of elongation and/or of release of the finished peptide chains from polyribosomes must be inhibited at 4 h p.i. At present we have no experimental evidence to distinguish between these possibilities.

In the experiments presented above, inhibition of protein synthesis and linear accumulation of virus-specific RNA started at about the same time during virus growth at 37, 30, and 25 C. These findings might be coincidental, but they could indicate that the initial phase of synthesis of virus-specific RNA at its maximal rate is involved in the virus-induced inhibition of cellular protein synthesis. A possible mechanism, currently analyzed in our laboratory, by which synthesis of virus-specific RNA in the cytoplasm could inhibit cellular protein synthesis is that consumption of cytoplasmic ribonucleoside triphosphates might lead to a reduction of the amount of these triphosphates available for protein synthesis.

Indirect evidence and the analysis of the RNA extracted from polyribosomes show that the shift from the synthesis of cellular to that of viral proteins is the result of a substitution of cellular by viral mRNA in the cellular polyribosomes. The amount of virus-specific proteins synthesized in infected cells increases during the 3- to 8-h p.i. time period (Fig. 2). The data presented in Fig. 5A show that the 26*S* RNA, which presumably is the mRNA for all viral structural proteins (3, 4, 31), accumulates in the infected cells during a similar time interval. From the sucrose density gradient fractionation of the RNA extracted in the experiment presented in Fig. 5A at 7 h and 45 min p.i. from infected cells, it has been calculated that 8.5% of the mass of rRNA plus 26*S* RNA consisted of virus-specific 42*S* and 26*S* RNA (data not shown). Since the ratio of radioactivity in 42*S* RNA to that in 26*S* RNA is 1.57 in this sample (Fig. 5A), it can be concluded that 3.3 and 5.2% of the total mass of rRNA plus 26*S* RNA is presented as 26*S* and 42*S* RNA, respectively. If it is assumed that about 50% of cellular ribosomes are found in polyribosomes (Fig. 3), it can be estimated that about 6.6% of the mass of polyribosomal rRNA plus 26*S* RNA is found as 26*S* RNA in these cells. It has been calculated that 26*S* RNA constitutes 8.6% of the mass of rRNA plus 26*S* RNA of a polyribosome containing seven ribosomes and one molecule of 26*S* RNA (see Results). This calculation indicates that sufficient 26*S* RNA accumulates in infected cells to substitute the cellular mRNA in the majority of cellular polyribosomes. Analyses of cellular ribonucleoprotein particles at various times after infection have shown that the great majority of the 26*S* RNA accumulating in infected cells is, in fact, bound to polyribosomes (33; unpublished data). To us the most straightforward explanation of these findings seems to be the assumption that the 26*S* RNA outcompetes the cellular mRNA for a component necessary for initiation of protein synthesis on all cellular mRNA molecules. Such a process would lead to a gradual substitution of cellular by viral mRNA, which would parallel the accumulation of 26*S* RNA in the infected cells and the final extent of which would depend on the amount of 26*S* RNA made in the infected culture. Since the virus-specific mRNA molecules of SFV-infected cells recently have been isolated and characterized (4, 28, 31, 34), it might be possible to clarify the involvement of such a mechanism in the mRNA substitution observed in vivo by experiments studying the in vitro translation of viral and cellular mRNA, similar to the experiments reported recently for the translation of encephalomyocarditis virus mRNA (15).

ACKNOWLEDGMENTS

We thank R. Rott for his interest and support during this work, L. Rohrschneider for reading of the manuscript, and C. Ebert (University of Marburg) for the determination of the concentrations of amino acids in the amino acid analyzer.

This study was supported by the Sonderforschungsbereich 47 (Virologie).

LITERATURE CITED

1. Acheson, N. H., and I. Tamm. 1970. Structural proteins of Semliki Forest virus and its nucleocapsid. Virology **41**:321-329.
2. Burrell, C. I., E. M. Martin, and P. D. Cooper. Posttranslational cleavage of virus polypeptides in arbovirus-infected cells. J. Gen. Virol. **6**:319-323.
3. Cancedda, R., R. Swanson, and M. J. Schlesinger. 1974. Effects of different RNAs and components of the cell-free system on in vitro synthesis of Sindbis viral proteins. J. Virol. **14**:652-663.
4. Clegg, C., and J. Kennedy. 1975. Translation of Semliki Forest virus intracellular 26 S RNA. Characterization of the products synthesized *in vitro*. Eur. J. Biochem. **53**:175-183.
5. Dulbecco, R., and G. Freeman. 1959. Plaque production by the polyoma virus. Virology **8**:396-397.
6. Eagle, H., K. A. Piez, and M. Levy. 1961. The intracellular amino acid concentration required for protein synthesis in cultured human cells. J. Biol. Chem. **236**:2039-2042.
7. Ehrenfeld, E., and T. Hunt. 1971. Double-stranded polio virus RNA inhibits initiation of protein synthesis by reticulocyte lysates. Proc. Natl. Acad. Sci. U.S.A. **68**:1075-1078.
8. Fan, H., and S. Penman. 1970. Regulation of protein synthesis in mammalian cells. II. Inhibition of protein synthesis at the level of initiation during mitosis. J. Mol. Biol. **50**:655-670.
9. Friedman, R. M. 1968. Protein synthesis directed by an arbovirus. J. Virol. **2**:26-32.
10. Garoff, H., K. Simons, and O. Renkonen. 1974. Isolation and characterization of the membrane proteins of Semliki Forest virus. Virology **61**:493-504.
11. Hightower, L. E., and M. A. Bratt. 1974. Protein synthesis in Newcastle disease virus-infected chicken embryo cells. J. Virol. **13**:788-800.
12. Kääriäinen, L., and P. J. Gomatos. 1969. A kinetic analysis of the synthesis in BHK 21 cells of RNA's specific for Semliki Forest virus. J. Gen. Virol. **5**:251-265.
13. Kennedy, S. I. T., and D. C. Burke. 1972. Studies on the structural proteins of Semliki Forest virus. J. Gen. Virol. **14**:87-98.
14. Lämmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) **227**:680-685.
15. Lawrence, C., and R. E. Thach. 1974. Encephalomyocarditis virus infection of mouse plasmacytoma cells. I. Inhibition of cellular protein synthesis. J. Virol. **14**:598-610.
16. Levin, J. G., and R. M. Friedman. 1971. Analysis of arbovirus ribonucleic acid forms by polyacrylamide gel electrophoresis. J. Virol. **7**:504-514.
17. McConkey, E. H., and J. W. Hopkins. 1969. Molecular weights of some HeLa ribosomal RNA's. J. Mol. Biol. **39**:545-550.
18. Morser, M. J., and D. C. Burke. 1974. Cleavage of virus specific polypeptides in cells infected with Semliki Forest virus. J. Gen. Virol. **22**:395-409.
19. Perry, R. P. 1969. Autoradiography of cultured cells, p. 242-249. *In* Karl Habel and Norman P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
20. Perry, R. P., and D. Kelley, 1968. Persistent synthesis of 5 S RNA when production of 28 S and 18 S RNA is inhibited by low doses of actinomycin D. J. Cell Physiol. **72**:235-244.
21. Peterman, M. L., and A. Pavlovec. 1966. The subunits and structural ribonucleic acids of Jensen Sarcoma ribosomes. Biochim. Biophys. Acta **114**:264-276.
22. Robertson, H. D., and M. B. Mathews. 1973. Double stranded RNA as an inhibitor of protein synthesis and as a substrate for a nuclease in extracts of Krebs II ascites cells. Proc. Natl. Acad. Sci. U.S.A. **70**:225-229.
23. Schlesinger, M. J., and S. Schlesinger. 1973. Large-molecular-weight precursors of Sindbis virus proteins. J. Virol. **11**:1013-1016.
24. Schlesinger, S., and M. J. Schlesinger. 1972. Formation of Sindbis virus proteins: identification of a precursor for one of the envelope proteins. J. Virol. **10**:925-932.
25. Shenk, T. E., and V. Stollar. 1972. Effect of double-stranded RNA's from Sindbis virus infected cells on rabbit reticulocyte and chick embryo cell-free protein synthesis. Biochim. Biophys. Acta **287**:501-513.
26. Simmons, D. T., and J. H. Strauss. 1972. Replication of Sindbis virus. I. Relative size and genetic content of 26 S and 49 S RNA. J. Mol. Biol. **71**:599-613.
27. Simons, K., S. Keranen, and L. Kääriäinen. 1973. Identification of a precursor for one of the Semliki Forest virus membrane proteins. FEBS Lett. **29**:87-91.
28. Smith, A. E., T. Wheeler, N. Glanville, and L. Kääriäinen. 1974. Translation of Semliki Forest virus 42 S RNA in a mouse cell-free system to give virus-coat proteins. Eur. J. Biochem. **49**:101-110.
29. Strauss, J., H. Jun, B. W. Burge, and J. E. Darnell. 1969. Sindbis virus infection of chick and hamster cells. Synthesis of virus specific proteins. Virology **37**:367-376.
30. Tabert, R., D. Rèkosh, and D. Baltimore. 1971. Effect of pactamycin on synthesis of polio virus proteins: a method for genetic mapping. J. Virol. **8**:395-401.
31. Wengler, G., M. Beato, and B. A. Hackemack. 1974. Translation of 26S virus specific RNA from Semliki Forest virus infected cells *in vitro*. Virology **61**:120-128.
32. Wengler, G., and G. Wengler. 1972. Medium hypertonicity and polyribosome structure in HeLa cells Eur. J. Biochem. **27**:162-173.
33. Wengler, G., and G. Wengler. 1974. Studies on the polyribosome-associated RNA in BHK 21 cells infected with Semliki Forest virus. Virology **59**:21-35.
34. Wengler, G., and G. Wengler. 1975. Comparative studies on polyribosomal, non-polyribosome-associated and viral 42 S RNA from BHK 21 cells infected with Semliki Forest virus. Virology **65**:601-605.
35. Zweerink, H. J., and W. K. Joklik. 1970. Studies on the intracellular synthesis of reovirus. Virology **41**:501-518.
36. Zylber, E. A., and S. Penman. 1970. The effect of high ionic strength on monomers, polyribosomes and puromycin treated polyribosomes. Biochim. Biophys. Acta **204**:221-229.