JOURNAL OF VIROLOGY, Aug. 1975, p. 388-396

Vol. 16, No. 2
Printed in U.S.A.

# Proteins Synthesized by Semliki Forest Virus and Its 16 Temperature-Sensitive Mutants

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Received for publication 20 February 1975

The proteins synthesized in chicken embryo fibroblasts infected with wild-type Semliki Forest virus and 16 temperature-sensitive mutants derived from it were studied by polyacrylamide gel electrophoresis. In addition to the structural proteins, five nonvirion proteins (NVP) with molecular weights of 130,000, 97,000, 86,000, 78,000 and 62,000 were found in varying amounts in cells infected with the different RNA$^+$ mutants and also in the wild-type-infected cells. Pulse-chase experiments suggested that NVP 130, NVP 97, NVP 86, and NVP 62 are precursors presumably of the structural proteins. The amount of NVP 78 was not affected by the chase, and it may represent a translational product of the nonstructural part of the genome. The NVP 130 was shown to be a common precursor of the structural proteins by tryptic peptide mapping. Kinetic evidence from one of the mutants (ts-3) suggested that NVP 86 is one of the precursors of the capsid protein. A common feature of all the RNA$^+$ mutants was the inability to cleave the NVP 62 into $E_2$ and $E_3$, suggesting that that this cleavage is a crucial reaction in the virus maturation.

Semliki Forest virus (SFV), an alphavirus, is composed of a nucleocapsid surrounded by a membrane (14). The membrane consists of lipids and three different glycoproteins: $E_1$ (mol wt 49,000), $E_2$ (mol wt 52,000), and $E_3$ (mol wt 10,000) containing 7.5, 11.5, and 45.5% carbohydrate, respectively (10). The nucleocapsid contains one RNA molecule, 42*S* RNA (mol wt $4 \times 10^6$), and about 240 presumably identical capsid proteins (mol wt 32,000 to 34,000 [17]). The total molecular weight of the virion proteins is thus about 145,000 with, and 130,000 without, their carbohydrate components. In SFV-infected cells, the structural proteins predominate after long exposure to radioactive amino acids (8, 11, 21). Shorter pulses in both the presence and absence of amino acid analogues have revealed larger proteins considered to be precursors of the virion proteins (1, 17, 20). One of the larger proteins, NVP 62, has been shown to be the precursor of the envelope proteins $E_2$ and $E_3$ (10, 23).

The basic characteristics of 16 temperature-sensitive mutants of SFV isolated in our laboratory have recently been described (S. Keränen and L. Kääriäinen, Acta Pathol. Microbiol. Scand. Sect. B, in press). Seven of the mutants were unable to synthesize viral RNA at the restrictive temperature and have therefore been classified as RNA$^-$ mutants. As evidenced by temperature shift-up experiments, their defects are in the early phase of the virus growth cycle, presumably in the synthesis or function of the RNA polymerase(s). Seven of the mutants, synthesizing almost normal amounts of virus-specific RNA, were classified as RNA$^+$ mutants. Their temperature-sensitive defect is in the late phase of the growth cycle and thus probably in the structural proteins. Two mutants clearly made less virus-specific RNA and were designated as RNA$^\pm$ mutants.

In this paper we show that variable amounts of five nonvirion proteins with molecular weight between 60,000 and 130,000 accumulate at the restrictive temperature in the cells infected by the different RNA$^+$ mutants. Some of the proteins disappeared or decreased during the chase period with a concomitant increase of radioactivity in the structural proteins, suggesting that they are intermediates in the processing of virion proteins. The largest protein, NVP 130, contains peptides of capsid and envelope proteins showing that it is a common precursor of structural proteins.

## MATERIALS AND METHODS

**Virus and cell cultures.** The propagation of wild-type SFV and the temperature-sensitive mutants isolated from it has been described previously (Keränen and Kääriäinen, in press). Secondary special

pathogen-free chicken embryo fibroblasts were used, and the temperatures were 27 C (permissive) and 39 C (restrictive).

**Isotype labeling and extraction of the proteins.** Confluent monolayers in 50-mm petri dishes (Falcon Plastics) were infected at 50 PFU/cell in the presence of actinomycin D (2 μg/ml) as described (Keränen and Kääriäinen, in press). For [$^{35}$S]methionine labeling the medium was Eagle minimum essential medium (MEM) from which methionine was omitted. The label was given in 2 ml of fresh medium containing 15 to 20 μCi of [$^{35}$S]methionine per ml (120 to 240 Ci/mmol, Amersham) 5 h postinfection. For double labeling with [$^{3}$H]amino acids and [$^{35}$S]methionine the medium was MEM with 1/10 of the normal amino acid concentration. Fifteen minutes before the pulse the medium was removed and replaced with Hanks balanced salt solution containing 2 μg of actinomycin D per ml, and the pulse, given in 1 ml of the same medium at 5 h postinfection, contained 10 μCi of [$^{35}$S]methionine and 100 μCi of [$^{3}$H]amino acid mixture (Amersham or New England Nuclear) per ml. After the pulse the monolayers were washed twice with the chase medium, MEM, containing a 10-fold concentration of methionine for the [$^{35}$S]methionine label and MEM with a 10-fold concentration of all normal amino acids and of the nonessential amino acids of Eagle spinner medium for the double label.

Pulse sample plates were incubated for 2 min in chase medium immediately prior to harvesting. The chase sample plates, after a brief washing with the chase medium, were incubated in the same medium containing actinomycin D (2 μg/ml) for 50 min at 39 or 27 C or for 4 h at 27 C. At the end of the incubation the monolayers were rinsed once with phosphate-buffered saline and disrupted in hot (60 C) 1% sodium dodecyl sulfate (SDS) in water (Keränen and Kääriäinen, in press).

**Polyacrylamide gel electrophoresis.** Polyacrylamide gel electrophoresis (PAGE) was performed either using the continuous system described by Weber and Osborn (25) or the discontinuous system of Neville (19) as described previously (23). The samples for the run were treated with 1% SDS and 1% β-mercaptoethanol and heated for 3 min at 90 C. Continuous electrophoresis in a 5% slab gel was used for the molecular weight determinations. The gel slab was stained with Coomassie brilliant blue and destained (7) for localization of the marker proteins included in each slot. After this the gel slab was dried and autoradiographed. The stained marker proteins used were β-galactosidase (Sigma; mol wt 130,000) (4), phosphorylase A (Sigma; mol wt 92,500) (4), human serum albumin (Kabi; mol wt 68,000) (25), glutamate dehydrogenase (Miles Seravac; mol wt 53,000) (25), ovalbumin (Sigma; mol wt 25,700) (25), and bovine pancreatic ribonuclease (Sigma; mol wt 13,700) (25). In addition to these dimethylsuberimidate polymers (9) of bovine serum albumin (BSA; Armour; mol wt 68,000) (25), ovalbumin, and glutamate dehydrogenase were used.

Continuous cylindrical gels were cut into 2-mm slices, treated with NCS, and counted in toluene-based scintillation fluid as described before (10). Discontinuous electrophoresis was done in cylindrical gels. After the run the gels were sliced longitudinally. The center slice (1 mm thick) was dried, autoradiographed, and then scanned with a Joyce Loebl chromoscan apparatus. For quantitation of the discontinuous gels the center slice or a longitudinally halved gel on a filter paper was cut in 1-mm slices, treated with NCS, and counted as above.

**Tryptic peptide mapping.** Cells were infected at 39 C as above and exposed to [$^{35}$S]methionine, 300 μCi/plate, and the monolayeres were disrupted in hot SDS as above. The samples were electrophoresed in the discontinuous PAGE system. The protein bands were localized by autoradiography of the wet gels wrapped in a thin plastic film. The bands were cut, and the proteins were eluted in SDS, precipitated with 5% trichloroacetic acid after addition of BSA as a carrier, washed with acetone, oxidized with performic acid, and treated with trypsin. The tryptic peptides were separated by high-voltage paper electrophoresis first at pH 6.5 and then at pH 3.5 as previously described (24).

## RESULTS

**Proteins induced by SFV ts mutants.** Protein synthesis in the mutant-infected cells was first studied at 39 C by giving a 30-min pulse of [$^{35}$S]methionine at 5 h postinfection and disrupting the cells in hot SDS. The proteins were analyzed on a discontinuous SDS-PAGE.

The protein synthesis pattern of most of the RNA$^-$ mutants was indistinguishable from that of the uninfected cells as shown for ts-4 in Fig. 1, indicating that viral proteins are not synthesized to a significant degree and that host protein synthesis is not inhibited. Two RNA$^-$ mutants (ts-8 and ts-14) showed a faint band at the position of the capsid protein. The two RNA$^\pm$ mutants, ts-10 and ts-13, which were able to make a somewhat intermediate amount of RNA, showed a peak corresponding to the capsid protein (Fig. 1, ts-13). Apparently some viral proteins are synthesized along with the host proteins.

Most of the RNA$^+$ mutants gave protein patterns similar to that of the wild-type-infected cells (Fig. 1 and 2). In all cases, C and $E_1$ proteins were present together with NVP 62, the precursor of $E_2$ and $E_3$ (13, 18, 21). $E_2$, which is clearly seen in the wild-type-infected cells (data not shown), was absent from most of the RNA$^+$ mutant-infected cells (Fig. 1), and when it was present (ts-2 and ts-5) only trace amounts were seen. In the wild-type-infected cells four minor proteins migrating more slowly than NVP 62 were regularly detected. They are shown in Fig. 2 after a 10-min pulse of [$^{35}$S]methionine. Many of these proteins were

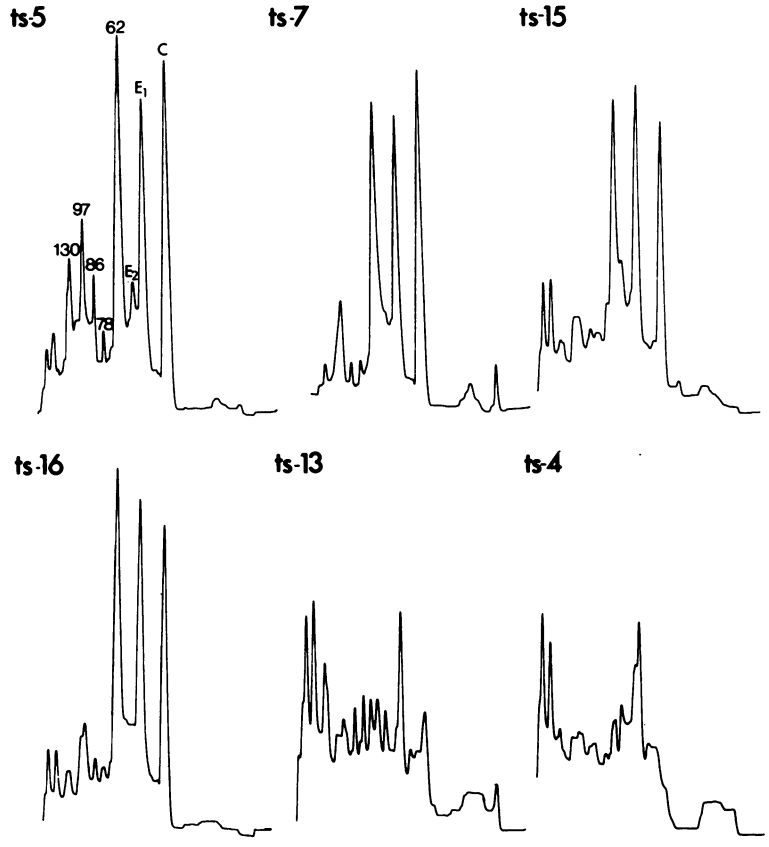


FIG. 1. *Absorbance tracings of autoradiograms of proteins from infected chicken embryo cells labeled with [$^{35}$S]methionine from 5.5 to 6 h postinfection and separated on 11% discontinuous SDS-PAGE. Mutant ts-4 is a RNA$^-$ mutant, ts-13 is a RNA$^\pm$ mutant, and the others are RNA$^+$ mutants. Capsid protein (C), envelope protein $E_1$, and the nonvirion proteins (NVP 62, NVP 78, NVP 86, NVP 97, and NVP 130) are indicated for ts-5 only. An equal amount of radioactivity was applied onto the gels and the exposure time was the same for all of them.*

accentuated in cells infected with the different RNA$^+$ mutants (Fig. 1 and 2). The molecular weights of these proteins were determined on slab gels by continuous SDS-PAGE. Several different molecular weight markers were used and visualized by staining (see above). Among these were polymers of BSA, ovalbumin, and glutamate dehydrogenase. The apparent molecular weights of the SFV-induced nonvirion proteins were: 130,000, 97,000, 86,000, 78,000, and 62,000. They are hereafter referred to as NVP followed by the molecular weight in thousands.

The two most slowly migrating proteins seen in the uninfected and in the RNA$^-$ mutant-infected cells (Fig. 1, ts-4) are reduced in SFV-infected cells (Fig. 2), indicating that host cell protein synthesis is efficiently inhibited. This inhibition was pronounced also with mutants ts-1, ts-2, ts-3 (Fig. 2), and ts-7 (Fig. 1).

**Stability of nonvirion proteins.** The stability of the mutant-induced proteins at the restrictive temperature was analyzed by pulse-chase experiments. The infected cells were pulsed for 10 min with [$^{35}$S]methionine, and part of the cultures was removed immediately whereas the other part was incubated for a further 50 min in the presence of excess unlabeled methionine.

In no case was there an increase in hot acid-insoluble radioactivity after the chase, which indicates that [$^{35}$S]methionine incorporation after the pulse was minimal. With the wild-type virus and mutants ts-1, ts-2, and ts-3 the experiment was repeated, including a 50-min chase also at the permissive temperature (27 C). The results are shown in Fig. 2 as densitometric tracings of autoradiograms, which show the intensities of different proteins semiquantitatively.

In the wild-type-infected cells, NVP 130 and

NVP 97 were clearly accentuated after the pulse (Fig. 2A to C) and disappeared almost completely during the chase period. At the same time NVP 62 diminished and $E_2$ appeared.

In the ts-1-infected cells, both NVP 130 and NVP 97 (seen only as a shoulder in Fig. 2D) disappeared almost completely during the chase at 39 C (Fig. 2E). There was an increase

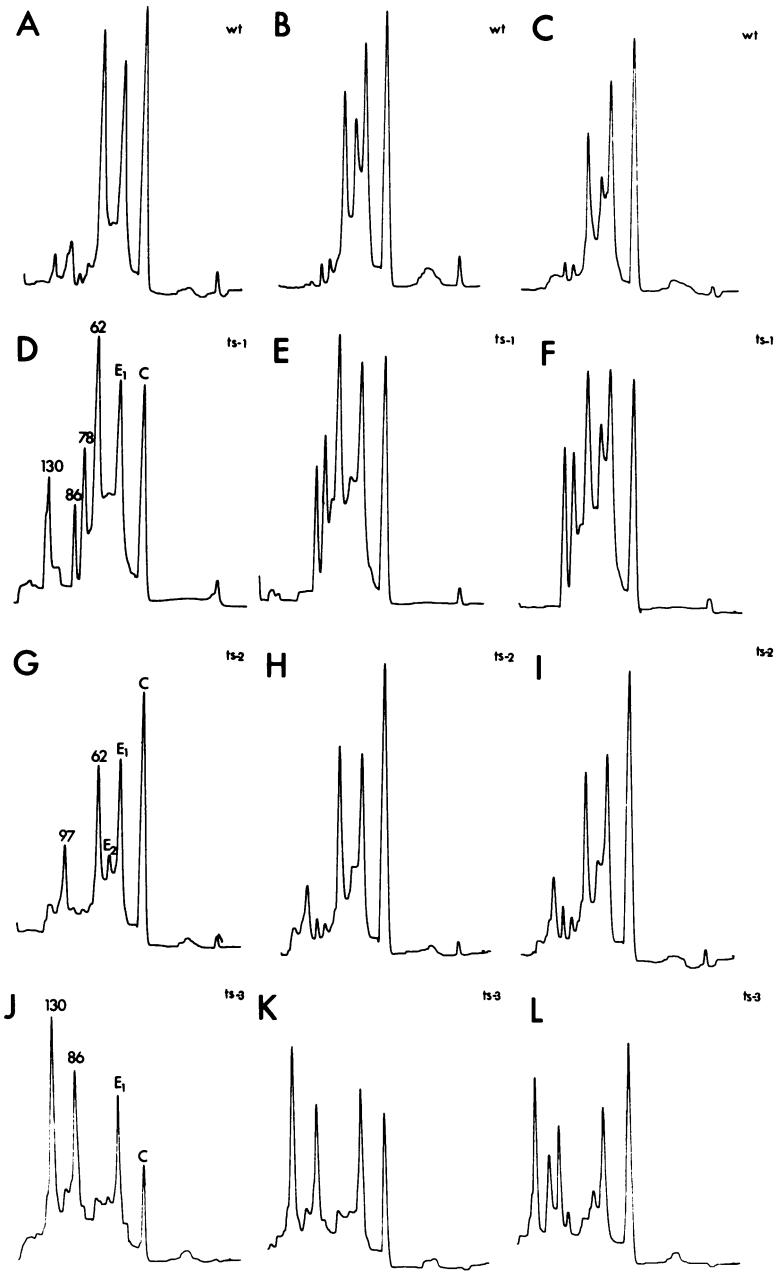


FIG. 2. *Absorbance tracings of autoradiograms of [$^{35}$S]methionine-labeled proteins from infected chicken embryo cells separated on 11% discontinuous SDS-PAGE. Wild type (A, B, C), ts-1 (D, E, F), ts-2 (G, H, I) and ts-3 (J, K, L). A 10-min pulse was given at 39 C 5 h postinfection (A, D, G, J) followed by a 50-min chase at 39 C (B, E, H, K) and a 50-min chase at 27 C (C, F, I, L). In all figures the prominent peak at the right is the capsid protein (C) followed by $E_1$, $E_2$, NVP 62, NVP 78, NVP 86, NVP 97, and NVP 130 (if present) as indicated in (D), (G), and (J). Exposure for each gel was 5 × 10$^6$ (= hours × counts/min).*

in NVP 86 but little, if any, change in NVP 78. No change in the amount of NVP 62 was noticed at 39 C, whereas at 27 C this protein seemed to decrease slightly with a concomitant increase in $E_2$ (Fig. 2F). The small-molecular-weight material migrating faster than the C protein was absent from ts-1-infected cells. This material is seen regularly in cells infected with wild-type virus and other $RNA^+$ mutants.

In ts-2-infected cells (Fig. 2G to I) the only change during the chase at both temperatures was a slight increase in NVP 86. In ts-3-infected cells there was an increase in C protein even at 39 C. At the same time NVP 86 seemed to decrease (Fig. 2J and K). After the chase at 27 C NVP 130 had decreased together with NVP 86. Simultaneously NVP 97 and C protein increased (Fig. 2L).

The wild-type virus and mutants ts-1, ts-2, and ts-3 were also grown at the permissive temperature (27 C), and a 10-min pulse was given at 10 h postinfection. Part of the cultures was chased for 2 h at the same temperature. The protein pattern of the wild-type virus was almost identical to that at 39 C both after the pulse and the chase, and the mutants did not differ from it, as revealed by densitometric tracings.

The protein pattern of ts-5-infected cells both after the pulse and chase was very similar to that of ts-2. The changes in ts-7-infected cells were comparable to those seen with ts-1. Both the large proteins NVP 130 and NVP 97 disappeared. In the ts-15- and ts-16-infected cells the inhibition of the host protein synthesis was incomplete. However, in both cases NVP 130 seemed to disappear.

To summarize, NVP 130 disappeared during the chase in most cases. All mutants except ts-3 showed an increase in NVP 86, whereas NVP 78 remained unchanged. At the restrictive temperature the decrease in NVP 62 was not found with any of the mutants, and $E_2$ was detectable, if at all, only in trace quantities.

**Quantitation of the proteins.** Because the densitometric tracings do not allow exact quantitation of the proteins, the dried longitudinal gel slices were cut into 1-mm pieces, treated with NCS, and counted in liquid scintillation counter (Fig. 3). This technique does not resolve the individual proteins as sharply as the tracing because the bands are often slightly curved. In some experiments tritiated amino acids were used together with [$^{35}$S]methionine and analyzed also on continuous SDS-PAGE. After a 10-min pulse the cultures were chased both at 39 and 27 C as before. To ensure that all the proteins undergoing modification had reached their final stage, the chase at 27 C was extended to 4 h in certain experiments.

The distribution of radioactivity in [$^3$H]amino acid-[$^{35}$S]methionine-labeled proteins of the wild-type virus is presented in Table 1. The chase at 27 C is omitted since the result

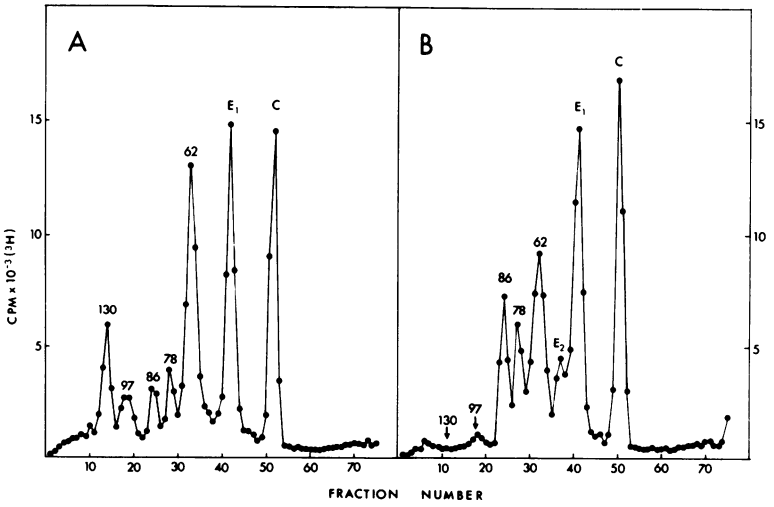


FIG. 3. *Quantitation of [$^3$H]amino acid-labeled proteins from ts-1-infected CE cells separated on 11% discontinuous SDS-PAGE. The longitudinal half of the gel was dried on Whatman 3MM paper cut in 1-mm slices, and counted in NCS toluene. The cells were labeled at 39 C for 10 min (A) 5 h postinfection with [$^3$H]amino acids and [$^{35}$S]methionine (not shown in figure). A batch of the cells was chased for 4 h (B) in the presence of unlabeled amino acids at 27 C (see also Table 2). The viral structural proteins and the nonvirion protein are indicated.*

was essentially the same as at 39 C. Most of the radioactivity (70%) immediately after the pulse is found in the proteins NVP 62, $E_1$, and C. During the chase some increase in $E_1$ and C takes place. The increase in $E_2$ corresponds well to the decrease in NVP 62. NVP 97 showed a clear decrease during the chase. There was also a decrease in NVP 130 as well as in the larger material. This may indicate that some bigger virus-specific proteins are synthesized. The $^{35}S$-$^{3}H$ ratio is the same in most of the proteins both after the pulse and chase, indicating homogeneity. The exceptions were the $E_2$ and C proteins. In the latter case the relative proportion of methionine increased slightly during the chase. This is consistent with our earlier report in which the relative increase of methionine-labeled C protein during the chase was greater than that measured by [$^{14}$C]amino acids (21).

Quantitative analysis of the ts-1 pulse-chase experiment (Fig. 3 and Table 2) confirms the finding from the densitograms, namely the decrease in both NVP 130 and NVP 97 and increase in NVP 86 and also NVP 78 during the chase even at the restrictive temperature. Again there is a decrease in the amount of material larger than NVP 130 and an increase in both $E_1$ and C proteins. During the chase at the permissive temperature $E_2$ increases, but there is no respective decrease in its precursor, NVP 62, indicating that cleavage of the larger precursors is partly compensating for the loss.

The quantitation of the dried discontinuous gels of ts-3-induced proteins shown in Fig. 2J to L is in good agreement with the densitograms, showing an increase in C protein even during the chase at 39 C and a concomitant decrease in NVP 86. After the chase at 27 C there is a decrease in NVP 130 and NVP 86 with a concomitant rise in NVP 97 and C protein. The same result was obtained with continuous SDS-PAGE analysis. In another experiment the total amount of NVP 130 was higher as measured with both [$^{35}$S]methionine and [$^{3}$H]amino acid labels (Table 3), and there was a smaller increase in C protein during the chase period at both temperatures. NVP 97 seemed to increase, but the resolution between NVP 86 and NVP 97 was not sufficient for us to conclude that NVP 86 had decreased. Since both the above described experiments were done with the same stock virus preparation, the reason for the different results remains open.

**Tryptic peptide analysis of NVP 130.** [$^{35}$S]methionine-labeled NVP 130 from ts-3-infected cells was eluted from a slab gel. The protein was digested with trypsin, and the peptides were separated by two-dimensional paper electrophoresis first at pH 6.5 and thereafter at pH 3.5. Tryptic digests of total envelope protein fraction and of capsid protein were analyzed in parallel. The result is shown in Fig. 4. Both envelope and capsid protein peptides seemed to be present in NVP 130, indicating

TABLE 1. *Distribution of [$^{3}$H]amino acid-[$^{35}$S]methionine-labeled proteins from SFV wild-type-infected cells after pulse and chase analysis on discontinuous SDS-PAGE*

| Exp conditions | Radioactivity (%) in: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | > NVP 130 | NVP 130 | NVP 97 | NVP 86 | NVP 78 | NVP 62 | $E_2$ | $E_1$ | C |
| [$^{3}$H]amino acid labeling | | | | | | | | | |
| 10-min pulse at 39 C | 2.3 | 3.0 | 8.9 | 2.9 | 2.3 | 22.8 | 4.1 | 23.1 | 20.1 |
| 50-min chase at 39 C | 1.1 | 1.6 | 4.8 | 2.0 | 2.7 | 17.8 | 9.6 | 26.4 | 22.2 |
| [$^{35}$S]methionine labeling | | | | | | | | | |
| 10-min pulse at 39 C | 1.6 | 2.4 | 7.6 | 2.7 | 2.5 | 22.0 | 4.8 | 19.6 | 26.6 |
| 50-min chase at 39 C | 0.9 | 1.3 | 4.1 | 1.7 | 2.7 | 16.9 | 9.3 | 21.3 | 30.0 |

TABLE 2. *Distribution of [$^{3}$H]amino acid-labeled proteins from SFV ts-1 mutant-infected cells after pulse and chase analysis on discontinuous SDS-PAGE*

| Exp conditions | Radioactivity (%) in: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | >NVP 130 | NVP 130 | NVP 97 | NVP 86 | NVP 78 | NVP 62 | $E_2$ | $E_1$ | C |
| 10-min pulse at 39 C | 3.9 | 9.3 | 6.5 | 4.2 | 6.1 | 20.4 | 4.4 | 20.0 | 16.7 |
| 50-min chase at 39 C | 1.9 | 1.7 | 2.5 | 7.6 | 7.0 | 21.9 | 4.9 | 23.9 | 20.7 |
| 50-min chase at 27 C | 2.9 | 1.7 | 3.6 | 8.9 | 8.0 | 21.0 | 6.1 | 22.0 | 18.0 |
| 4-h chase at 27 C | 2.1 | 1.2 | 2.4 | 9.8 | 7.5 | 18.6 | 7.2 | 22.5 | 19.1 |

TABLE 3. *Distribution of [$^3H$]amino acid-[$^{35}S$]methionine-labeled proteins from SFV ts-3 mutant-infected cells after pulse and chase analysis on continuous SDS-PAGE*

| Exp conditions | Radioactivity (%) in: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | >NVP 130 | NVP 130 | NVP 97 | NVP 86 | NVP 78 | NVP 62 | $E_2 + E_1$ | C |
| [$^3$H]amino acid labeling | | | | | | | | |
| 10-min pulse at 39 C | 9.7 | 43.6 | 7.0 | 11.1 | 3.8 | 4.1 | 10.8 | 3.6 |
| 50-min chase at 39 C | 8.3 | 40.3 | 6.8 | 12.8 | 5.4 | 3.9 | 11.1 | 5.3 |
| 50-min chase at 27 C | 7.0 | 40.0 | 8.9 | 12.5 | 3.9 | 3.9 | 11.1 | 6.5 |
| 4-h chase at 27 C | 6.4 | 36.7 | 9.4 | 12.9 | 4.7 | 4.5 | 11.6 | 7.4 |
| [$^{35}$S]methionine labeling | | | | | | | | |
| 10-min pulse at 39 C | 12.7 | 43.0 | 6.5 | 10.8 | 3.5 | 3.8 | 9.1 | 4.4 |
| 50-min chase at 39 C | 11.5 | 38.9 | 6.4 | 12.5 | 5.0 | 3.6 | 9.3 | 6.6 |
| 50-min chase at 27 C | 11.5 | 38.7 | 7.9 | 11.6 | 3.4 | 3.5 | 9.0 | 8.2 |
| 4-h chase at 27 C | 9.2 | 35.2 | 8.6 | 12.2 | 4.4 | 4.1 | 9.7 | 9.7 |

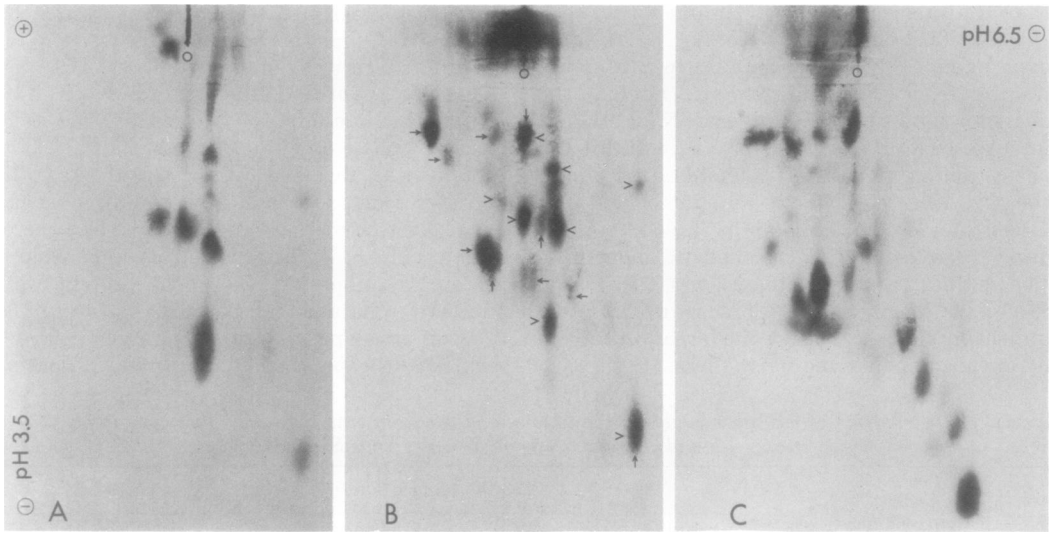


FIG. 4. *Tryptic peptides of SFV proteins separated in two dimensions (first pH 6.5, 40 V/cm for 90 min and second pH 3.5, 60 V/cm for 90 min) by high-voltage paper electrophoresis. Wild-type virion capsid protein (A) and envelope protein (C) peptides are indicated by (>) and (→), respectively, in the NVP 130 (B) isolated from ts-3-infected CE cells. O, Origin.*

that it is a common precursor to viral structural proteins.

## DISCUSSION

Proteins induced by the RNA$^-$ mutants in the infected cells were both qualitatively and quantitatively indistinguishable from those of the uninfected cells, except for the two mutants (ts-8 and ts-14) which showed a band migrating at the position of the capsid protein. This may be due to a slight leakiness in RNA synthesis. Since the host cell protein synthesis was not affected by the RNA$^-$ mutants, it seems clear that the establishment of inhibition needs synthesis of viral RNA. Double-stranded RNA has been shown to be a potent inhibitor of protein synthesis both in vivo and in vitro (3, 5, 6). The double-stranded RNA formed during virus replication has been proposed as a possible mechanism for the inhibition of host protein synthesis (3). Recent studies with poliovirus (2) do not support this idea, however. In SFV-infected CE cells the RNA synthesis itself is not enough to inhibit the cellular protein synthesis, as shown by our two RNA$^\pm$ mutants (ts-10 and ts-13), which are able to make 10 to 20% of the amount of the wild-type RNA without grossly affecting the host protein synthesis. Apparently larger

amounts of RNA are required to effect inhibition as is the case with most of the RNA+ mutants (ts-1, ts-2, ts-3, ts-5, and ts-7) which make normal amounts of RNA (Keränen and Kääriäinen, in press).

The larger nonvirion proteins (NVP 130, NVP 97, NVP 86, NVP 78, and NVP 62) detected in ts mutant-infected cells are most probably virus specific. Proteins migrating similarly were also found in wild-type-infected cells, although in smaller quantities.

It thus seems that these proteins are not qualitative artifacts due to mutations but rather represent increased amounts of the larger viral products of normal infection. All these proteins could be accumulations of otherwise short-lived precursors of the structural proteins. Due to mutation the cleavage is retarded to a greater or lesser extent, giving rise to different intermediates according to the mutant.

The precursor nature of NVP 130 and NVP 97 was suggested by pulse-chase experiments with several of the mutants as well as with the wild-type virus. Comparison of the tryptic peptides from NVP 130 with those of the structural proteins strongly suggests that this protein is a common precursor for capsid and envelope proteins as is the case for a similar-sized protein in Sindbis virus-infected cells (22). NVP 86 was the only protein in ts-3-infected cells in which the radioactivity decreased during the chase at the restrictive temperature while the capsid protein increased, suggesting that it is one of the precursors of C protein. In cells infected with other mutants this protein increased during the chase, indicating that it, in turn, is formed from a precursor. Comparison of NVP 86 from ts-1 and ts-3 in the same slab gel has shown a slight migration difference (unpublished data), leaving some doubts as to the identity of these proteins. The precursor nature of NVP 78 remains unestablished since it did not decrease during the chase either at restrictive or at permissive temperature with the wild-type or any of the mutants. The possibility that some of the proteins like NVP 78 and perhaps NVP 86 from ts-1-infected cells are proteins which do not contain amino acid sequences of structural proteins cannot be excluded at present.

The interesting finding that $E_2$ was virtually absent from cells infected with all the RNA+ mutants after a 30-min labeling period would indicate that NVP 62 is not cleaved at the restrictive temperature. This finding is supported by the results of pulse-chase experiments carried out at the same temperature. The final maturation of alphaviruses may thus be linked to the cleavage of NVP 62 as proposed also for Sindbis virus (12).

## ACKNOWLEDGMENTS

We thank Mirja Langer and Ritva Saarlemo for excellent technical assistance.

This work was supported by grants from Sigrid Juselius Foundation, Finnish Academy, and Emil Aaltonen Foundation. Actinomycin D was a gift from Merck Sharp & Dohme.

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