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Tryptic Peptide Analysis of Nonstructural and Structural Precursor Proteins from Semliki Forest Virus Mutant-Infected Cells

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Analysis of [^{35}S]methionine-labeled tryptic peptides of the large proteins induced by temperature-sensitive mutants of Semliki Forest virus was carried out. The 130,000-molecular-weight protein induced by ts-2 and ts-3 mutants contained the peptides of capsid protein and of both major envelope proteins E_1 and E_2. The ts-3-induced protein with molecular weight of 97,000 contained peptides of E_1 and E_2, whereas the 86,000 protein from the same cells contained peptides of the capsid and envelope protein E_2 but not those of E_1. Two proteins with molecular weights of 78,000 and 86,000 from ts-1-infected cells did not contain the peptides of the virion structural proteins. They are evidently expressions of the nonstructural part of the 42S RNA genome of Semliki Forest virus.

The 42S RNA genome of alphaviruses has an apparent molecular weight of about 4×10^6 (11, 20), which can code for about 400,000 daltons of proteins. The virion capsid protein (molecular weight, 30,000 to 33,000) (22, 26) and the envelope glycoproteins E_1 (molecular weight, 49,000), E_2 (molecular weight, 52,000), and E_3 (molecular weight, 10,000) have a total molecular weight of 145,000 with, and about 130,000 without, carbohydrates (5). They represent roughly one-third of the coding capacity of the 42S RNA.

In alphavirus-infected cells several nonvirion proteins have been detected using short amino acid pulses (2, 12), protease inhibitors (12, 14), amino acid analogues (12, 15), or temperature-sensitive mutants (9, 16, 17, 26, 27). Some of these proteins have been shown to be precursors for structural proteins by tryptic peptide mapping. The 130,000 protein from Sindbis (18) and Semliki Forest virus (SFV) (NVP 130) (9) temperature-sensitive mutant-infected cells has been shown to contain capsid protein and envelope proteins E_1 and E_2, whereas the B protein (molecular weight, 100,000) in Sindbis ts-2-infected cells contains amino acid sequences of E_1 and E_2 (17). The 62,000 protein (PE_2 or NVP 62), which can easily be detected even in the wild type-infected cells, has been shown to be the immediate precursor of E_2 (18, 23) and of E_3 (5). The order of the structural proteins in the precursors is not known.

As yet, no translational products of the nonstructural part of the 42S RNA genome have been identified, suggesting that this part of the 42S RNA is translated poorly, if at all, during the later phase of infection (25).

In the present study we have isolated the large proteins (molecular weight, 62,000 to 130,000) induced by three temperature-senstitive mutants of Semliki Forest virus. The tryptic peptide analysis of these proteins established the order of the main structural proteins in the polyprotein. It also revealed the presence of two nonstructural proteins which were induced by one of the mutants.

MATERIALS AND METHODS

Virus and cells. Semliki Forest virus, prototype strain (7), a cloned wild type of it, and three temperature-sensitive mutants, ts-1, ts-2 and ts-3, were used in this study. The isolation and properties of these mutants have been described previously (8, 9). BHK-21 cells and secondary, special pathogen-free chicken embryo fibroblasts were cultivated as before (8).

Virus purification. SFV prototype strain was grown in BHK-21 cells labeled with [^{35}S]methionine and purified as described previously (N. Glanville et al., submitted for publication).

Labeling of virus-specific proteins. Confluent monolayers of secondary chicken embryo fibroblasts, in 60-mm petri dishes, infected with the cloned wild type or the ts mutants, were incubated at the nonpermissive temperature (39 C) as described before (8). At 5 h postinfection 300 μCi of [^{35}S]methionine (210 to 510 Ci/mmol, Amersham) per plate was added for 60 min in methionine-free Eagle minimum essential medium. At 6 h postinfection the pulse medium was removed and the plates were washed with the chase medium containing a 10-fold concentration of unlabeled methionine and chased

in the same medium for a further 60 min. The whole cells were then taken into 1% sodium dodecyl sulfate at 65 C as described before (9). Actinomycin D (1 μg/ml) was present throughout the procedure.

Isolation of the proteins. Polyacrylamide gel electrophoresis was carried out using the discontinuous system described by Neville (13) with the following modification. The separating solution contained 18 g of acrylamide and 0.4 g of bisacrylamide per 100 ml of solution. Just prior to electrophoresis the samples containing 1% each of sodium dodecyl sulfate and 2-mercaptoethanol were boiled for 3 min, and a drop of glycerol was added to them. Electrophoresis was carried out on slab gels (3 by 85 by 150 mm) at 6 mA for 9 to 10 h.

Autoradiographs of wet slab gels were taken through a thin plastic film; some gels were dried on Whatman 3 MM paper before autoradiography. The protein bands, located using the autoradiographs, were cut from the gels. The slices were cut into pieces, and the proteins were eluted by shaking at 37 C in 0.1% sodium dodecyl sulfate overnight. Two successive elutions gave recoveries of 60 to 70%. After removal of the gel pieces by centrifugation the eluted proteins were precipitated overnight with 6 volumes of cold (−20 C) acetone using 1 to 3 mg of bovine serum albumin (Armour) as carrier protein. The precipitates were collected by centrifugation, washed with cold acetone, and dried in vacuo. Elution from the dried gels was in 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol at 100 C followed by two successive acetone precipitations after removal of the gel pieces.

Tryptic peptide analysis. The acetone-precipitated proteins were dissolved in methanol-formic acid, oxidized with performic acid, and treated with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington) in an enzyme-substrate ratio of 1 to 25 as described previously (24). The peptides were initially separated by high-voltage paper electrophoresis at pH 6.5. After autoradiography the bands to be compared were cut parallel to the origin line, sewn onto a new paper, and reelectrophoresed at pH 3.5, and again visualized by autoradiography as described previously (Glanville et al., submitted for publication). This enables direct comparison of several samples since they are run in the same paper at both pH 6.5 and pH 3.5.

RESULTS

Isolation of SFV ts mutant-induced proteins. Large proteins with molecular weights of 130,000 (NVP 130), 97,000 (NVP 97), 86,000 (NVP 86), 78,000 (NVP 78), and 62,000 (NVP 62) accumulate in cells infected with some of our temperature-sensitive mutants (9). Pulse-chase experiments suggested that the three largest proteins and NVP 62 are probably precursors of structural proteins. Since this method gives only suggestive evidence for their composition the large proteins were isolated for tryptic peptide analysis.

Infected chicken embryo fibroblasts maintained at the restrictive temperature (39 C) were pulsed with [^{35}S]methionine at 5 h postinfection for 1 h and then chased for an additional 1 h at the same temperature in the presence of excess unlabeled methionine. Thus only the kinetically more stable proteins were obtained. In attempting to separate these large proteins several polyacrylamide gel electrophoresis systems were tested, including the discontinuous system of Laemmli (10) with acrylamide concentrations of 7.5, 10, and 13% in the separating gels. The most satisfactory results were obtained using modifications of the discontinuous system described by Neville (13) (see above).

Extracts from cells infected with each of the mutants were electrophoresed on separate but similar slab gels. The proteins were localized by autoradiography (Fig. 1), the bands were cut, using the autoradiograph as a guide, and the proteins were isolated as described above. Aliquots of the isolated proteins were re-electrophoresed to check their purity (inset, Fig. 1).

Tryptic peptide analysis. The different [^{35}S]methionine-labeled proteins from both wild type and ts mutants were treated with trypsin, and the peptides were separated by high-voltage paper electrophoresis. For comparison tryptic peptides from the structural proteins were included. The first dimension was carried out at pH 6.5. The bands were located by autoradiography (Fig. 2 and 3), cut transversely, sewn to a new paper, and re-electrophoresed at pH 3.5 to resolve the peptides in each band (e.g., Fig. 4). Comigration of peptides in two dimensions was taken as the criterion for identity (e.g., Fig. 5 and 6). Under these conditions certain peptides derived from different proteins were not separated (e.g., Fig. 9, peptide a), showing that this criterion is not absolute. We therefore selected marker peptides for each of the structural proteins as indicators of the presence or absence of these proteins. The positions of these peptides in the first dimension are indicated in Fig. 2 and 3. One of the marker peptides for the capsid protein is easily identifiable already in the first dimension (band 1 in Fig. 2). The migration of peptide 1 and some other capsid peptides in the second dimension is shown in Fig. 5, 7, and 8 (peptides d and k).

The virion-derived E_1 and E_2 proteins were slightly cross-contaminated (Fig. 8, peptide g, and Fig. 9, peptide b). To enable us to select the marker peptides for these proteins a comparison was made with peptides derived from NVP 62 (the precursor of E_2) and from E_1 protein in those mutants in which NVP 62 is not cleaved (ts-1 and ts-2). Since these proteins were well separated in the gels (see Fig. 1), the contamination in the virion-derived proteins could be elim-

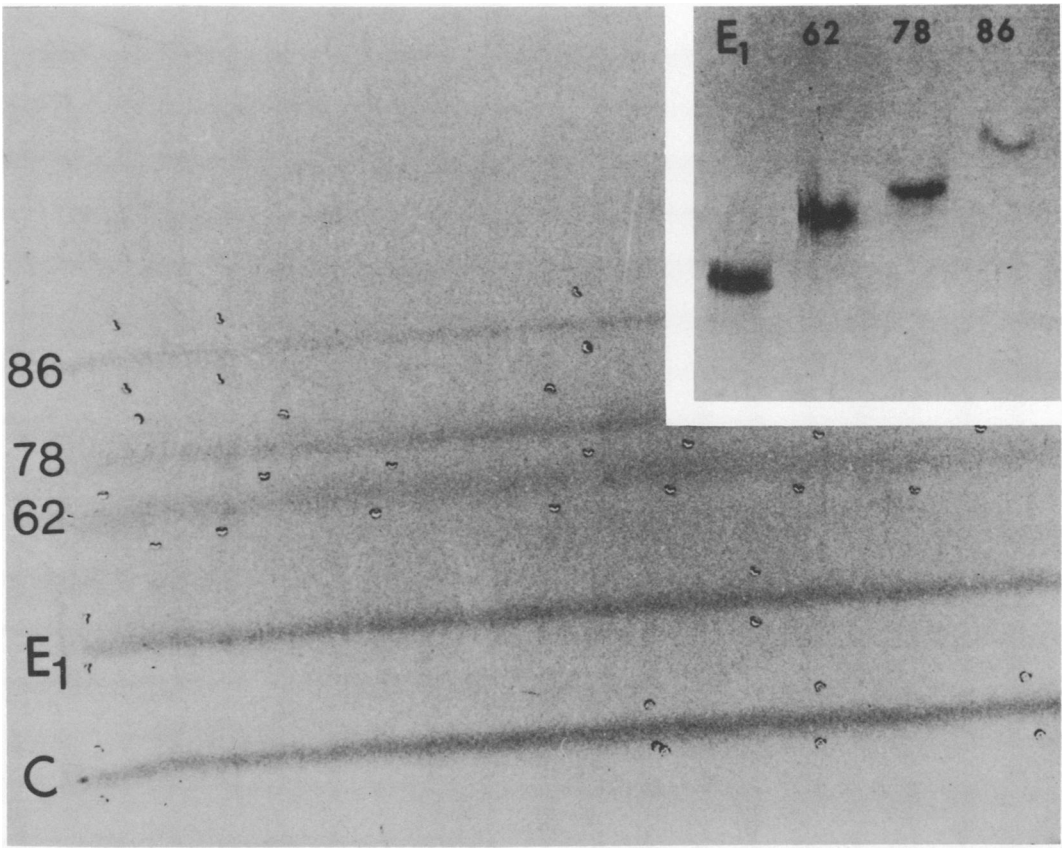

FIG. 1. *Discontinuous sodium dodecyl sulfate-polyacrylamide electrophoresis of cellular extract from ts-1 mutant-infected cells. Infected cells maintained at 39 C (restrictive temperature) were labeled for 60 min with [^{35}S]methionine 5 h postinfection followed by a 60-min chase at 39 C in the presence of excess of unlabeled methionine. The bands were located by autoradiography of the wet gel, cut according to the film (dots), and eluted. Aliquots of the proteins were re-electrophoresed under the same conditions (inset). Proteins: C (capsid, molecular weight, 33,000), E_1 (envelope E_1, molecular weight, 49,000), 62 (NVP 62, molecular weight, 62,000), 78 (molecular weight, 78,000), and 86 (molecular weight, 86,000).*

inated, and the source from which the peptides were derived was determined (e. g., Fig. 8, peptide g, which is E_1 specific). The second-dimension runs of some of the marker peptides are demonstrated in Fig. 4 and 8 for E_1 and Fig. 6 and 9 for E_2.

Structural constituents of the precursor proteins. The presence or absence of the selected marker peptides in the different proteins derived from cells infected with ts-1, ts-2, and ts-3 was recorded in altogether five first-dimension runs (pH 6.5), using different combinations of the proteins, followed by second-dimension runs (pH 3.5) of all bands. The results of the complete analysis are summarized in Table 1. It shows that NVP 130 from both ts-2 and ts-3 contains capsid, E_1, and E_2 protein marker peptides. The NVP 97 from ts-3-infected cells does not contain capsid peptides, but both the envelope proteins E_1 and E_2 are present (Fig. 3, Table 1). All the E_1 marker peptides were absent from the NVP 86 of ts-3. The presence of capsid and E_2 marker peptides in ts-3, NVP 130, and NVP 86 is also illustrated in Fig. 6, 7 and 10.

The characteristic capsid peptide (Fig. 2, band 1) was the only capsid peptide which was found neither in ts-3-induced NVP 130 nor in NVP 86 (Fig. 3, band 1). It was also absent from the ts-3 capsid protein isolated from the infected cells. Instead there was a slightly more slowly migrating peptide (Fig. 3, band 1^x) which was absent from the capsid preparations derived from SFV and from cells infected with wild-type virus and ts-1 and ts-2 mutants. Peptide 1 was present in the ts-1 and ts-2 capsid proteins and ts-2-induced NVP 130 (Table 1). The mobility difference between peptides 1 and

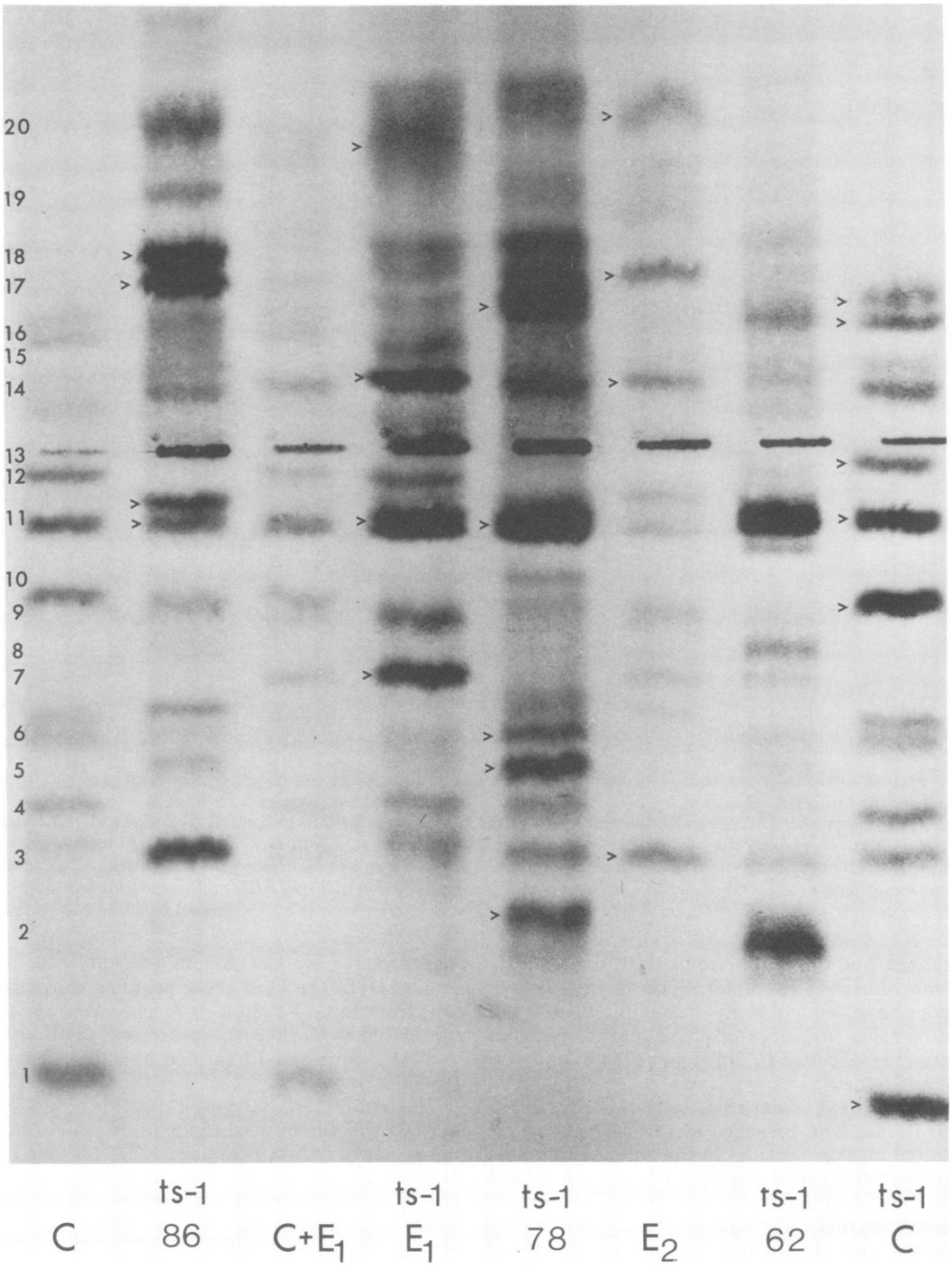

FIG. 2. *Autoradiograph of [^{35}S]methionine-labeled tryptic peptides derived from ts-1 mutant-induced proteins synthesized at 39 C. The peptides were separated in the first dimension at pH 6.5 by electrophoresis at 40 V/cm for 120 min. Peptides from the virion capsid (C) and envelope (E_1 and E_2) proteins were included for comparison. The bands analyzed further are numbered. The positions of the specific marker peptides used for identification are shown by arrow heads. Coding for the proteins is as in the legend to Fig. 1. Origin is at 13; cathode is at the bottom. Autoradiography was for 3 days.*

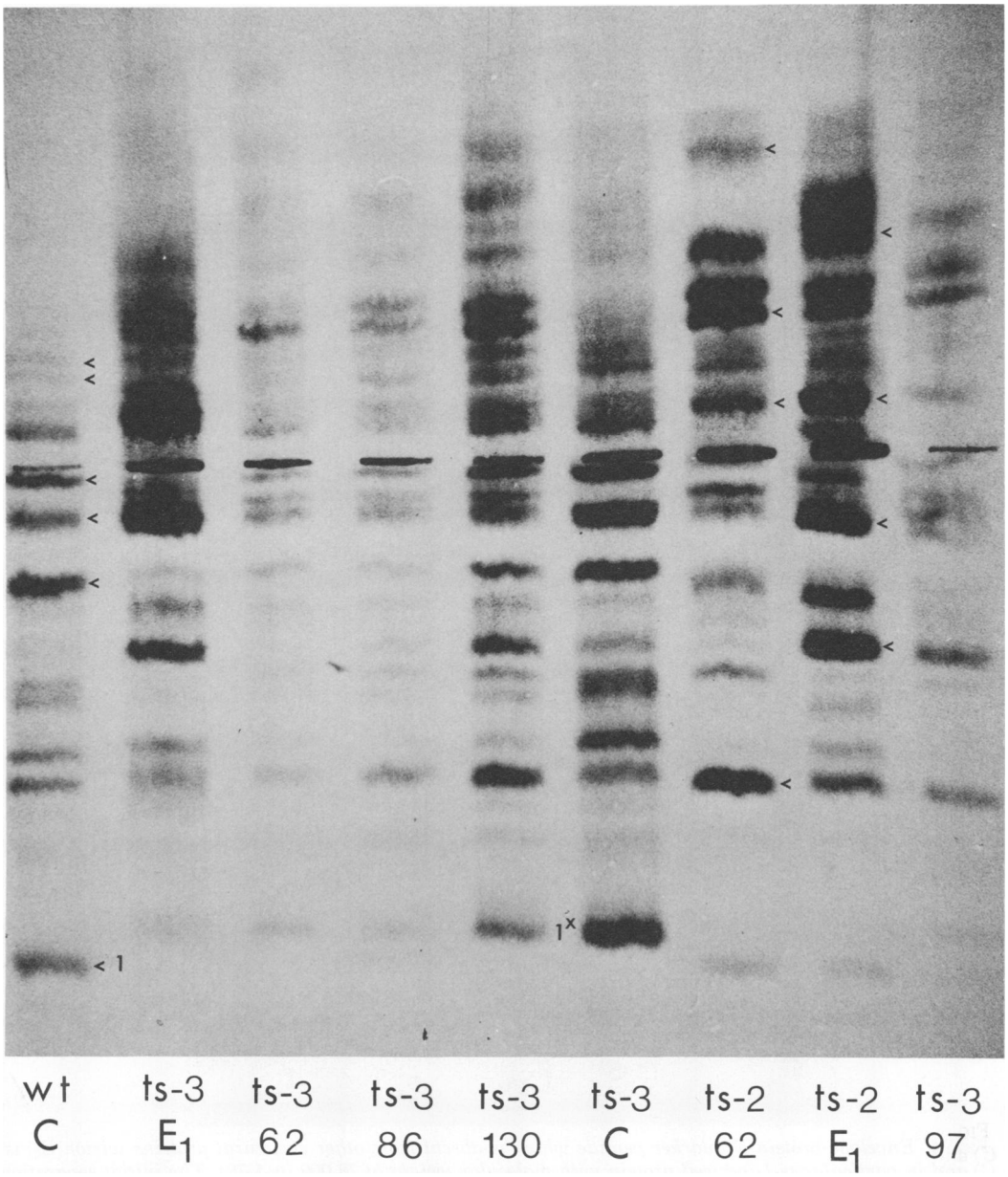

FIG. 3. *Autoradiograph of [^{35}S]methionine-labeled tryptic peptides derived from ts-3 mutant-induced proteins synthesized at 39 C. Electrophoresis was at pH 6.5 as in the legend to Fig. 2. The same marker peptides shown in Fig. 2 are indicated by arrow heads for the capsid from wild type-infected cells (wt C), ts-2 envelope (ts-2 E_1), and ts-2 NVP 62 (precursor of E_2) isolated from mutant-infected cells (ts-2 62). For bands 1 and 1^x, see text. The abbreviations used for the different proteins (ts-3 130, etc.) correspond to those used for Fig. 1 and 2. Cathode is at the bottom. Autoradiography was for 7 days.*

1^x (Fig. 3) was demonstrable only at pH 6.5. At pH 3.5 the peptides had similar mobilities.

Nonstructural proteins from ts-1-infected cells. Two proteins with molecular weights of 86,000 and 78,000 are the only proteins, apart from NVP 62, which are found after a chase in ts-1-infected cells maintained at the restrictive temperature (see also Fig. 1). These proteins are stable even if the cultures are shifted to the permissive temperature (9). The 86,000 protein clearly increases during the chase, suggesting that it is a cleavage product of a precursor,

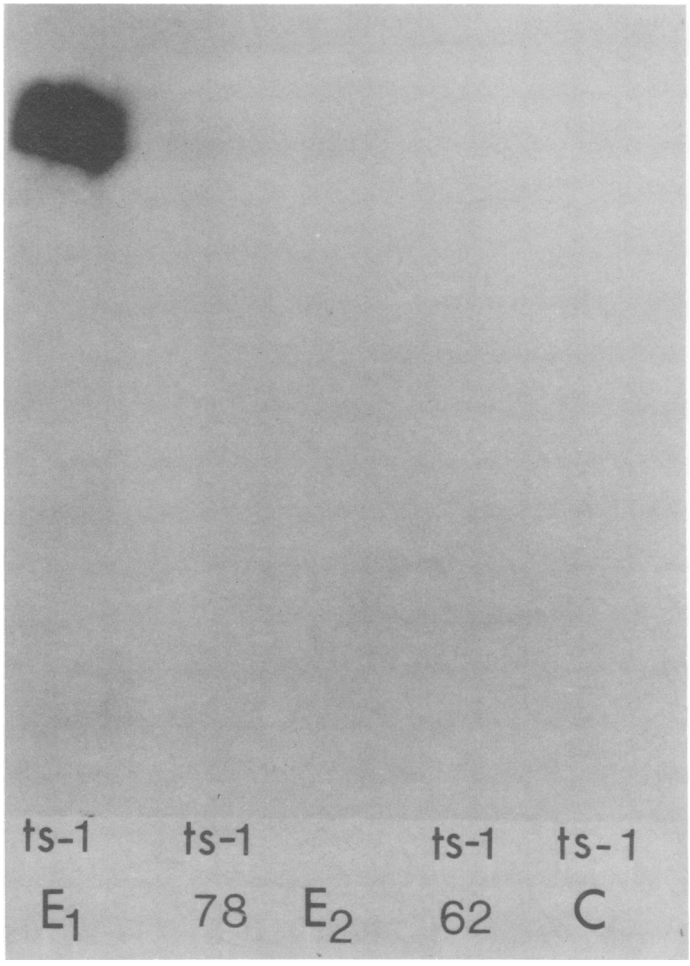

FIG. 4. *Envelope protein E_1 marker peptide which is absent from other structural proteins (virion E_2, ts-1 C) and in particular ts-1-induced protein with molecular weight of 78,000 (ts-1 78). The initial separation was at pH 6.5 for 120 min. Re-electrophoresis of band 7 (Fig. 2) was at pH 3.5 for 90 min. Origin is at the bottom; cathode is at the top. Autoradiography was for 14 days.*

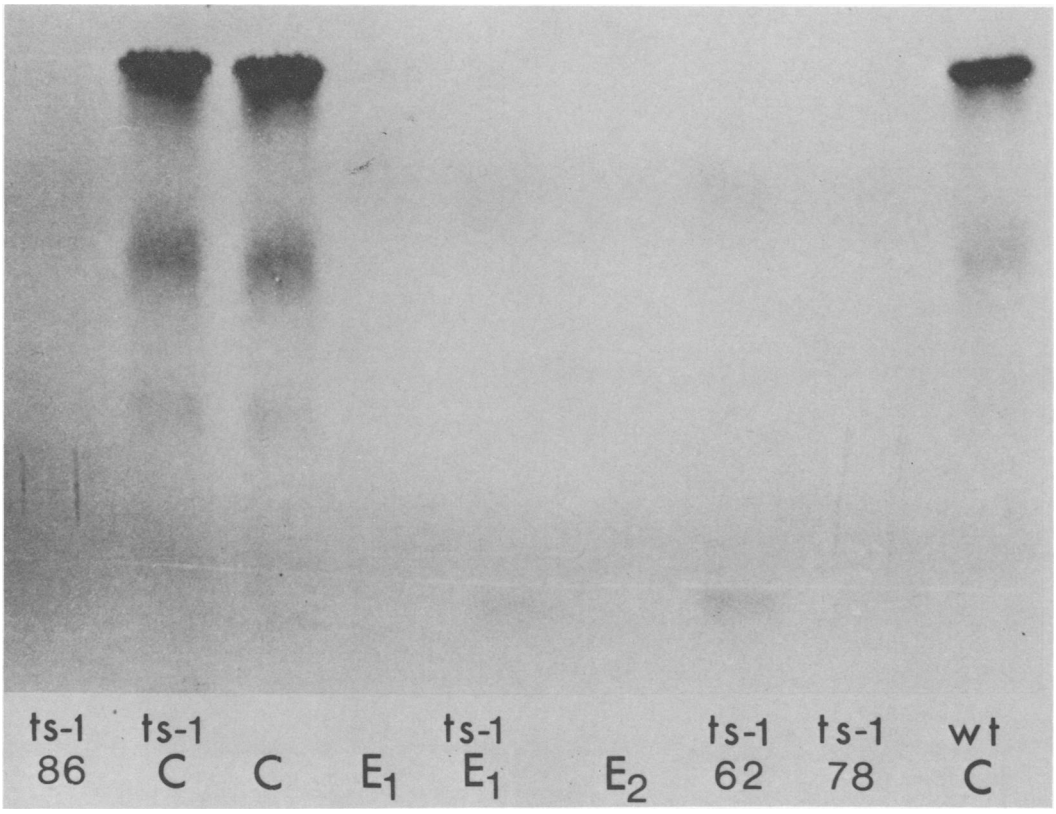

FIG. 5. *Comigration of a peptide from ts-1 capsid (ts-1 C) and wild-type capsid (wt C) from infected cells with a virion capsid (C) marker peptide. The peptide is clearly absent from the other proteins (note in particular ts-1-induced proteins with molecular weights of 86,000 [ts-1 86] and 78,000 [ts-1 78]). Initial separation was at pH 6.5 for 90 min. The band corresponding to band 1 in Fig. 2 was re-electrophoresed at pH 3.5 and 60 V/cm for 45 min. Origin is at the bottom; cathode is at the top. Autoradiography was for 14 days.*

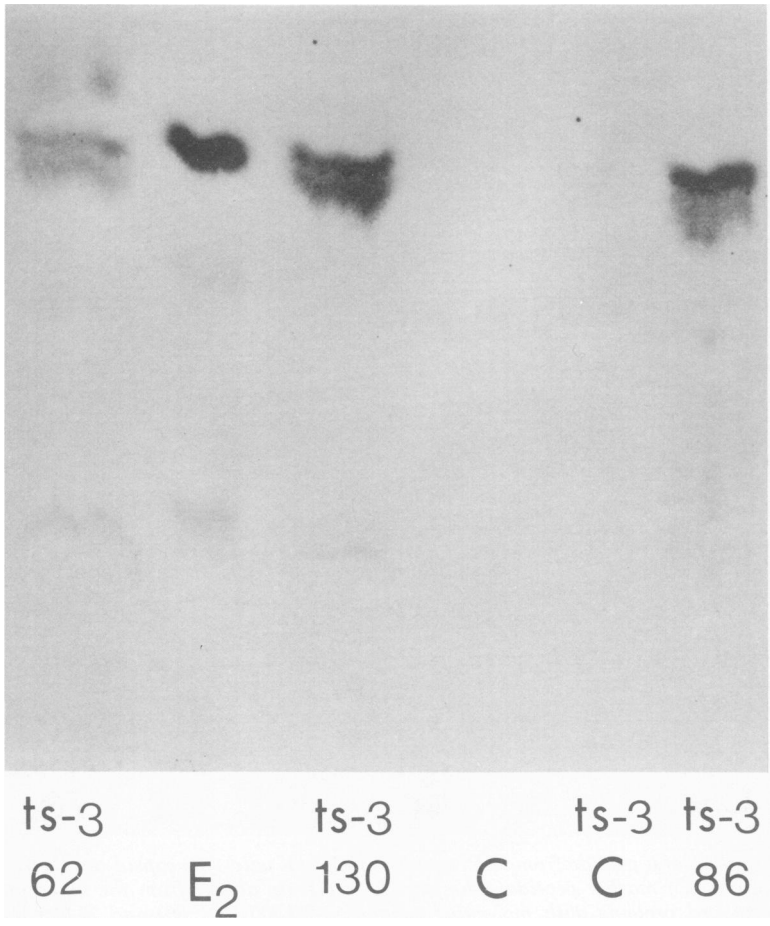

FIG. 6. *Comigration of peptides from ts-3 mutant-induced NVP 62, NVP 86, and NVP 130 from infected cells with a marker peptide for envelope protein E_2 (derived from the virion). Notice the absence of this peptide from the virion capsid protein (C). Initial separation was at pH 6.5 for 90 min, and re-electrophoresis of a band corresponding to band 17 in Fig. 2 at pH 3.5 was for 90 min. Origin is at the bottom; cathode is at the top. Autoradiography was for 14 days.*

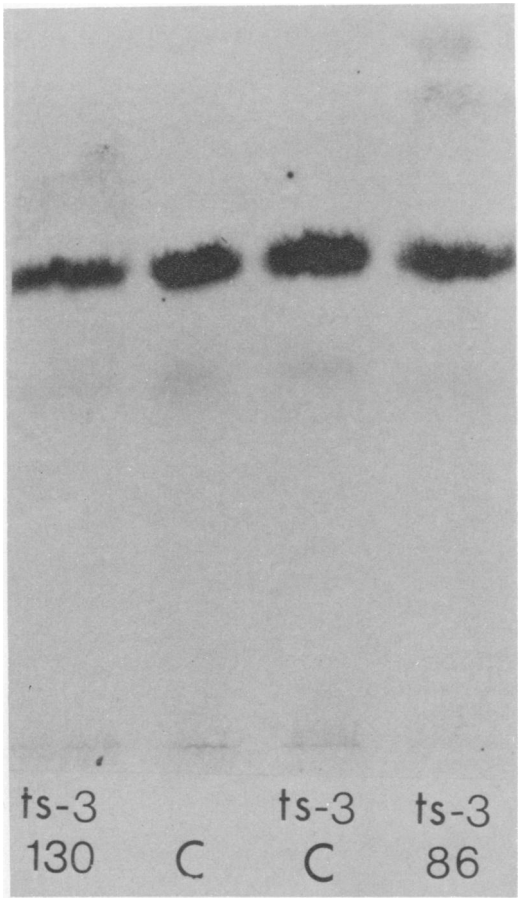

FIG. 7. *Comigration of peptides from ts-3 mutant-induced proteins capsid (ts-3 C), NVP 86 (ts-3 86), and NVP 130 (ts-3 130) isolated from infected cells with a marker peptide from the virion capsid (C). Initial separation was at pH 6.5 for 90 min. Re-electrophoresis of band corresponding to band 9 in Fig. 2 at pH 3.5 was for 90 min. Origin is at the bottom; cathode is at the top. Autoradiography was for 14 days.*

whereas the 78,000 protein remains almost constant. The possibility that these proteins are nonstructural could be tested by tryptic peptide analysis. As can be seen in Fig. 2 one of the characteristic capsid peptides (band 1) is missing from both of these proteins (see also Fig. 5), as are the other marker peptides for capsid protein (e.g., peptides d and k in Fig. 8). Nor were any of the marker peptides for E_1 or E_2 detected (see Fig. 4, 8, and 9): the lack of comigration between the structural marker peptides and peptides derived from the ts-1 mutant-induced 86,000 and 78,000 protein is shown in Table 1.

Some of the [^{35}S]methionine-labeled tryptic peptides from 86,000 and 78,000 proteins are shown in Fig. 8. Two of the peptides (f and i) from both proteins comigrate, whereas the others do not (peptides a, b, c, and e). Altogether 23 peptides derived from these proteins are listed in Table 2, which summarizes the results of the analysis. Only 4 peptides out of the 13 derived from each protein comigrate in two dimensions, suggesting that these two proteins are different entities.

DISCUSSION

The accumulation of large nonvirion proteins in cells infected with temperature-sensitive mutants of Semliki Forest virus allowed analysis of their tryptic peptides and comparison with those derived from the structural proteins of the wild-type SFV. The results summarized in Table 1 help to establish the order in which the structural proteins are synthesized. The absence of E_1 from the ts-3 NVP 86 shows that it must be located at one end of the polyprotein. The absence of C protein from NVP 97 establishes that it is at the other end. The recent results from translation of SFV 26S RNA in vitro strongly suggest that the C protein is N-terminal (Glanville et al., submitted for publication). Similar results have been obtained in vivo using pactamycin to inhibit initiation (18) and by synchronizing the initiation by high salt treatment of the infected cells (3).

The order of the structural proteins in the polyprotein and the different cleavage products shown in this study are presented schematically in Fig. 11. The cleavage resulting in capsid protein and NVP 97 (the B protein) has been shown to take place in Sindbis virus-infected BHK cells (26) and also in vitro (21). The resulting B protein, which is not glycosylated in the cells (19), is presumably degraded (4). Our NVP 86 from ts-3-infected cells yields capsid protein but not NVP 62 during chase at restrictive temperature (9), suggesting that the latter is degraded. In normal infection both cleavages of capsid and envelope E_1 take place rapidly leaving NVP 62, which is then cleaved more slowly, probably during the budding process (6), to yield envelope proteins E_2 and E_3. The order of these proteins in NVP 62 still remains open.

The absence of the fastest migrating capsid protein marker peptide from the two ts-3-induced, capsid protein-containing precursors and capsid protein was a constant finding. Since there was another peptide which migrated slightly more slowly at pH 6.5 and was absent from the other capsid proteins or capsid protein precursors it is possible that peptide 1 (Fig. 3) in ts-3 is actually mutated, resulting in peptide 1^x. This mutation may be responsible for the observed defects of the ts-3 mutant, i.e., the accu-

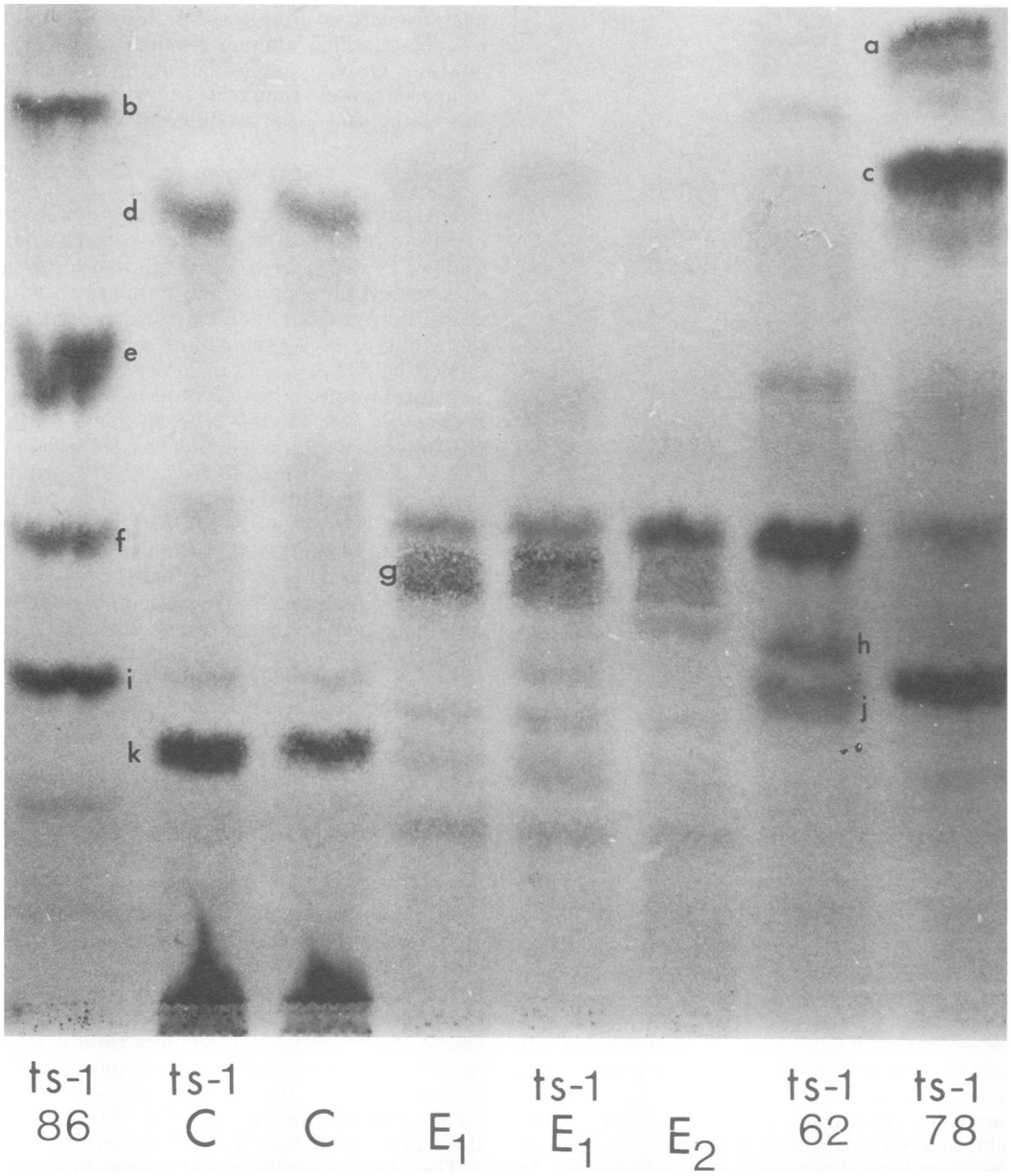

FIG. 8. *Analysis in second dimension of a band corresponding to band 11 (Fig. 2). Initial separation was at pH 6.5 for 90 min, and re-electrophoresis was at pH 3.5 for 90 min. Peptide k is one of the capsid protein marker peptides. The smeary peptide hardly moved from the origin is a typical capsid protein peptide (see also Fig. 10). For the nonstructural peptides a, b, c, and e see text and Table 2. Peptide g is an E_1 envelope protein marker peptide. Origin is at the bottom; cathode is at the top. Autoradiography was for 14 days.*

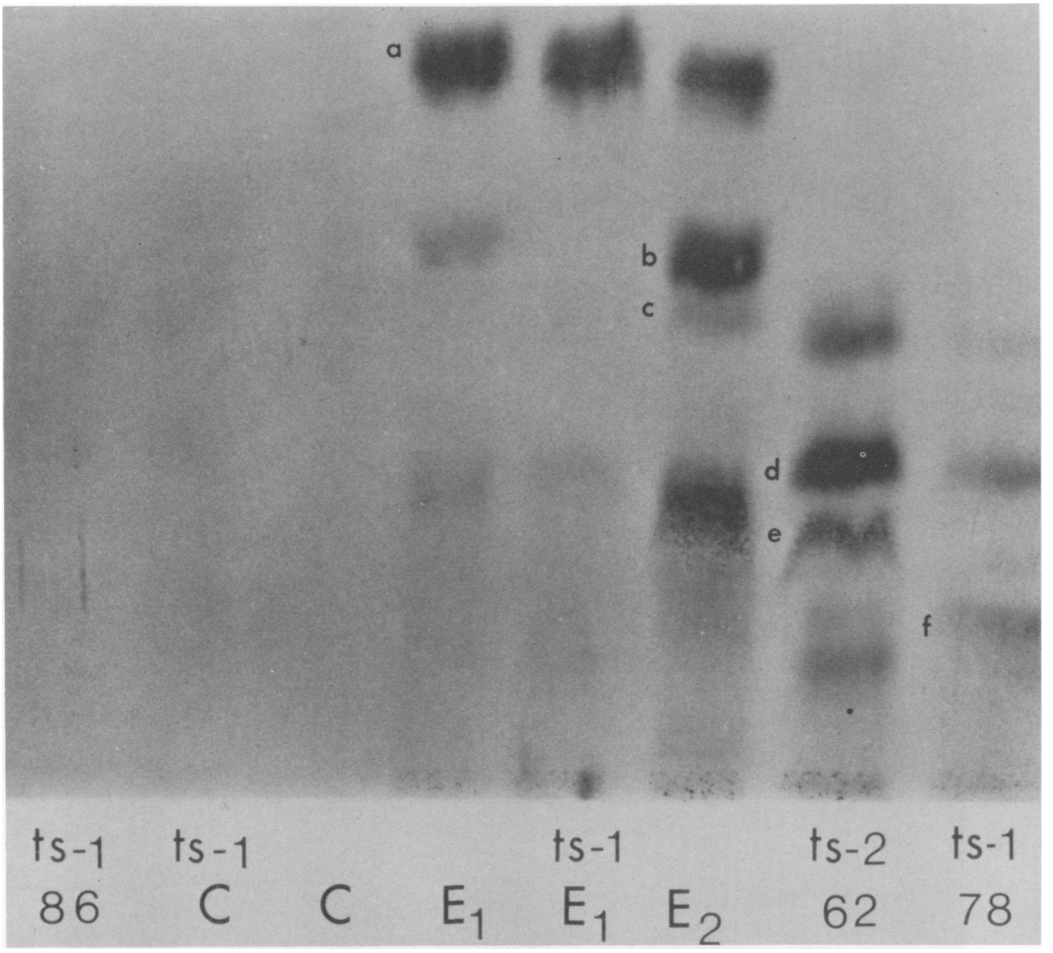

FIG. 9. *Absence of peptides specific for envelope protein E_2 (b, c, and e) from ts-1-induced proteins with molecular weights of 86,000 (ts-1 86) and 78,000 (ts-1 78). Peptide b is one of the E_2 marker peptides. Initial separation was at pH 6.5 for 90 min. Re-electrophoresis of band corresponding to band 20 in Fig. 2 was at pH 3.5 for 90 min. Origin is at the bottom; cathode is at the top. Autoradiography was for 14 days.*

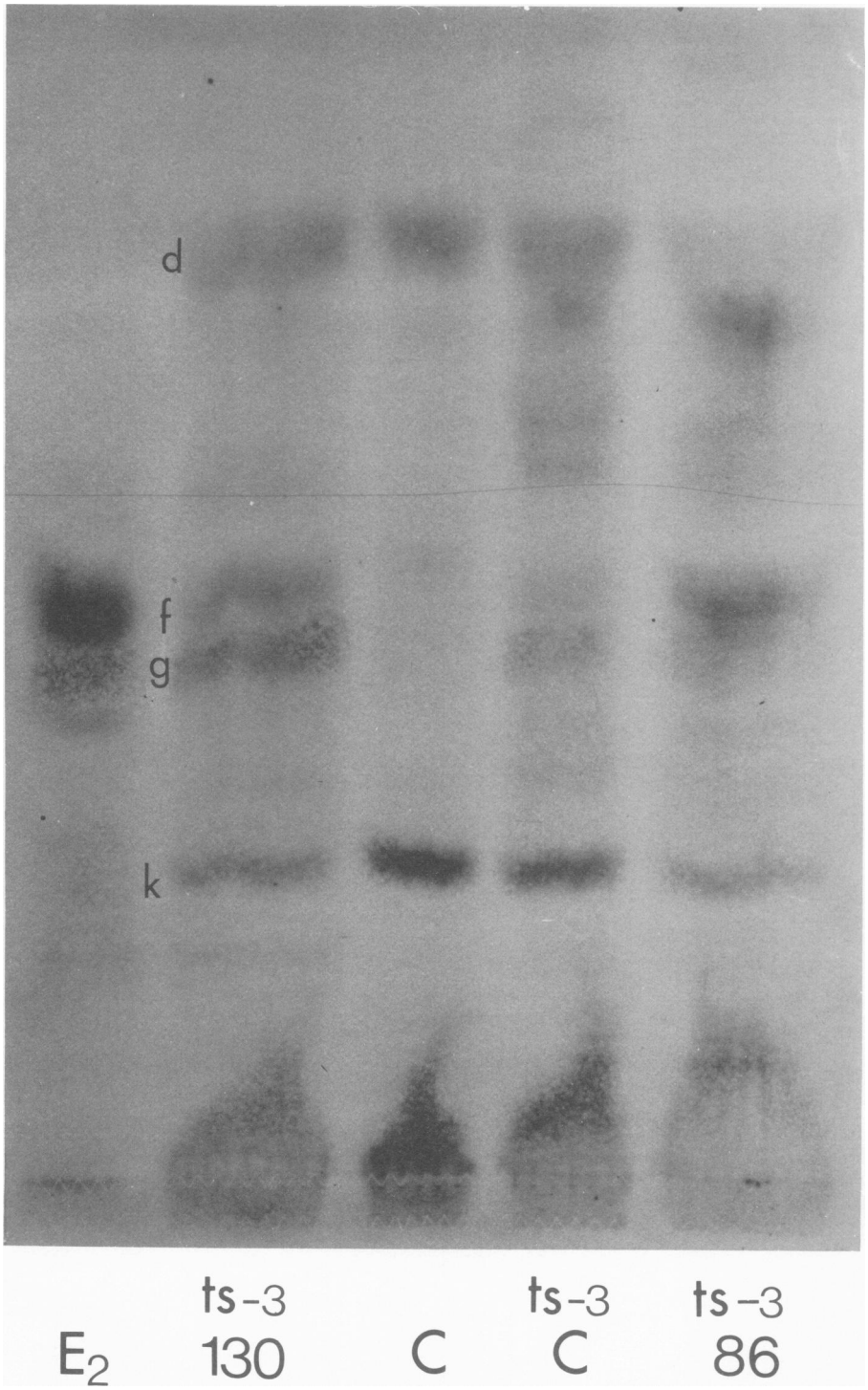

FIG. 10. *Presence of capsid and E_2 peptides in NVP 130 and NVP 86 from ts-3-infected cells. Initial separation was at pH 6.5 for 90 min, and re-electrophoresis of band corresponding to band 11 in Fig. 1 was for 90 min at pH 3.5. Origin is at the bottom; cathode is at the top. Autoradiography was for 14 days. The capsid peptide pattern is very similar to that shown in Fig. 8. The same lettering of peptides is used in both figures despite the fact that the nonstructural peptides a, b, c, e, and i are not present either in ts-3 NVP 86 or in ts-3 NVP 130, showing that these proteins do not contain nonstructural peptides.*

TABLE 1. *Number of marker peptides of capsid and envelope E_1 and E_2 proteins present in mutant-induced proteins isolated from infected cells*

Specificity and no. of marker peptides[a]	No. of marker peptides														
	ts-1					ts-2				ts-3					
	C	E_1	NVP 62	NSP 78	NSP 86	C	E_1	NVP 62	NVP 130	C	E_1	NVP 62	NVP 86	NVP 97	NVP 130
Capsid (5) envelope	5	0	0	0	0	5	0	0	5	4[b]	0	0	4	0	4
E_1 (4)	0	4	0	0	0	0	4	0	4	0	4	0	0	4	4
E_2 (4)	0	0	4	0	0	0	0	4	4	0	0	4	4	4	4

[a] Marker peptides were selected as described in the text.
[b] One of the five marker peptides of capsid protein (C) is absent, presumably due to a mutation.

TABLE 2. *Comparison of [^{35}S]methionine-labeled tryptic peptides derived from two ts-1-induced nonstructural proteins synthesized at restrictive temperature*

Peptide code	Presence of tryptic peptide[a]	
	78,000[b]	86,000
2 a[c]	+	+
5 a	+	−
5 c	+	−
5 f	+	−
5 g	+	−
6 h	−	+
9 a	+	−
9 b	−	+
11 a[d]	+	−
11 b	−	+
11 c	+	−
11 e	−	+
11 f	+	+
11 i	+	+
14 ?	+	+
16 a	+	−
16 c	+	−
16 e	−	+
17 a	−	+
17 c	+	−
18 e	−	+
19 b	−	+
20 d	−	+

[a] +, Presence of the given peptide; −, absence of the given peptide.
[b] Molecular weight of the protein.
[c] Peptides were initially separated at pH 6.5, and the numbers refer to bands corresponding to those indicated in Fig. 2. The letters refer to migration at the second dimension (pH 3.5), the farthest migrating peptide being "a."
[d] Peptides 11 a through 11 i are shown in Fig. 8.

mulation of NVP 30 and NVP 86 and the inability to form nucleocapsid (8).

In ts-1-infected cells two stable proteins with molecular weights of 86,000 and 78,000 accumulate at the restrictive temperature. Together they account for 15 to 20% of the protein-associated radioactivity (9). The stability of the 78,000 protein in pulse-chase experiments suggested that it may not be the precursor of the structural proteins. The tryptic peptide analysis reported in this study showed that both 86,000 and 78,000 proteins from ts-1-infected cells are actually nonstructural proteins. We therefore suggest that they should be designated NSP 86 and NSP 78 to distinquish them from, for example, NVP 86 derived from ts-3-infected cells which contains structural proteins.

Since most of the [^{35}S]methionine-labeled tryptic peptides from the NSP 86 and NSP 78 did not comigrate in two-dimensional electrophoresis, it seems that they are indeed different proteins with little if any overlapping amino acid sequences. Their molecular weights then add up to about 165,000, and, with the structural proteins (total molecular weight of about 130,000), would account for approximately 3/4 of the coding capacity of the 42S RNA genome. The possibility that NSP 86 and NSP 78 are specifically induced host proteins cannot at present be excluded. We regard this as improbable since the host protein synthesis in ts-1-infected cells is inhibited by about 90% (unpublished data). Also the synthesis of NSP 86 and NSP 78 is temperature dependent (9) (L. Kääriäinen, B. Lachmi, and N. Glanville, submitted for publication). However, only in vitro translation of the proteins under the direction of SFV RNAs will definitely prove their viral origin.

Traces of NSP 78 are found regularly in cells infected with wild-type SFV and mutants other than ts-1 (9), suggesting that small amounts of nonstructural sequences are translated at the exponential phase of the growth cycle. Whether NSP 86 is also translated in the wild type-infected cells cannot be determined because of the difficulty in separating it from NVP 86. We think that the accumulation of both NSP 86 and

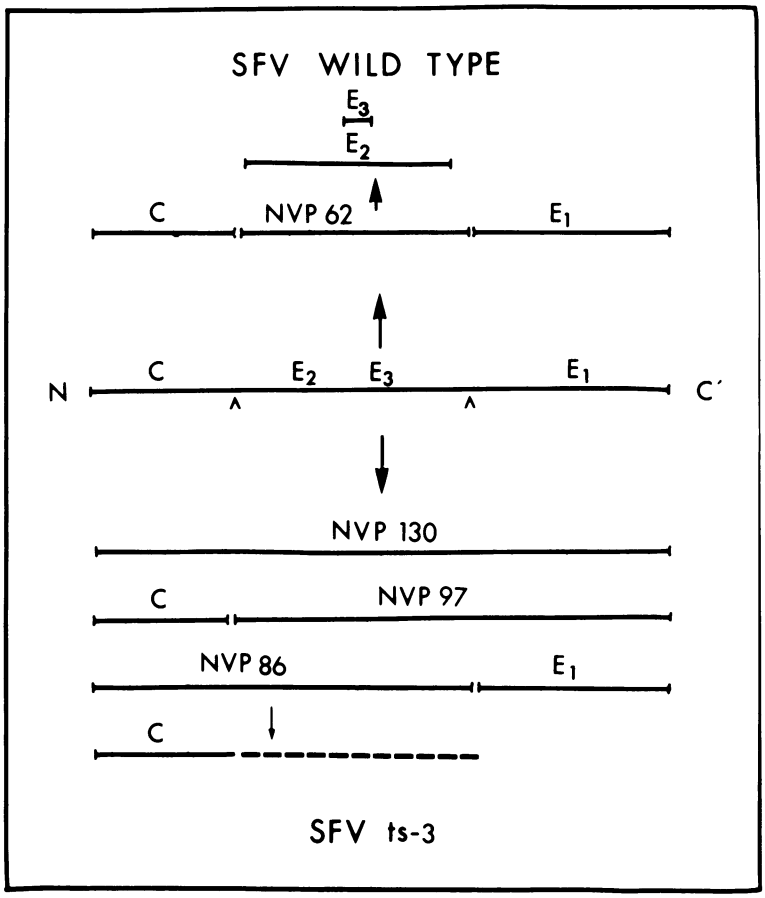

FIG. 11. *Schematic drawing of cleavage products of the hypothetical polyprotein (in the center) isolated from wild-type SFV and ts-3 mutant-infected cells. The cleavage points are indicated by arrow heads. N' and C' refer to N- and C-terminus of the polyprotein. The broken line indicates degradation. The order of E_2 and E_3 is not known.*

NSP 78 in ts-1-infected cells represents a disturbance in the regulation of protein synthesis. This may be purely at the level of transcription since in ts-1-infected cells the 42S-26S RNA molar ratio is about five compared to the wild type and other RNA^+ mutants for which it is about one (8).

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