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# Nonstructural Proteins of Semliki Forest Virus: Synthesis, Processing, and Stability in Infected Cells

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The synthesis of the nonstructural (ns) proteins of Semliki Forest virus was studied in vivo. The fourth ns protein, ns60, was identified and isolated. The order of translation ($NH_2$-ns70-ns86-ns60-ns72-COOH) was determined by using various labeling procedures after or in the presence of a hypertonic block of translation initiation. A sequential labeling procedure was devised to specifically label defined segments of the polyprotein. The specific labeling procedures allowed isolation of the four ns proteins in radiochemically pure form by gradient polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The four ns proteins were shown to have different primary structures by digestion with V8 protease of *Staphylococcus aureus*. The processing of the ns polyprotein and the stability of the mature ns proteins were studied by pulse-chase experiments. The cleavage of each of the proteins from the polyprotein took place within 2 to 3 min after the translation of the polypeptide chain. The N-terminal protein, ns70, appeared in its mature form later than ns86, which follows it in the polyprotein, suggesting that ns70 undergoes a post-translational modification. The migration of the C-terminal protein, ns72, immediately after a pulse was slightly faster than after a chase, suggesting that ns72 also undergoes a post-translational modification other than a cleavage. The half-life of ns72 was shorter than that of the other ns proteins.

The nonstructural (ns) proteins of alphaviruses are translated from the genomic 42S RNA (molecular weight, $4.3 \times 10^6$) as a 250,000- to 290,000-dalton polyprotein (8, 20, 22, 24) starting at a single initiation site (5, 8, 16) close to the 5′ end of the RNA molecule. The mature ns proteins cover about two-thirds of the genetic coding capacity of the 42S RNA. The viral structural proteins encoded by the remaining 3′ third of the genome are translated from a separate, subgenomic 26S mRNA (molecular weight, $1.6 \times 10^6$) as a polyprotein (molecular weight, 130,000) to yield capsid protein C and the three envelope glycoproteins, E3, E2, and E1, listed in the order of their translation. The envelope proteins E2 and E3 appear in the infected cells as a precursor form, p62 (molecular weight, 62,000), which is cleaved to the final envelope proteins at a late stage of virus maturation (for a review see Schlesinger and Kääriäinen [31]).

Various intermediate cleavage products of the ns polyprotein have been found in cells infected with RNA-negative temperature-sensitive mutants of both Sindbis virus and Semliki Forest virus (SFV) as well as in in vitro protein synthesizing systems. The precursor-product relationships among the ns polyprotein, its cleavage intermediates, and the final ns proteins have been demonstrated by peptide mapping (15, 24). The order of the ns proteins in the polyprotein has been determined based on (i) sequential translation of the ns proteins in vivo after release of a hypertonic block of initiation (2, 22), (ii) UV mapping of translation (10), and (iii) in vitro translation of 42S RNA (8).

The translation order proposed for SFV ns proteins is $NH_2$-ns70-ns86-ns72-ns60-COOH and for Sindbis virus proteins is $NH_2$-ns66-ns86-ns75-COOH. The numbers indicate the apparent weights of the proteins in kilodaltons. Based on the labeling kinetics after the release of a hypertonic block of initiation, a precursor molecule, ns155, has been localized at the N-terminus of the ns polyprotein of SFV followed by another precursor molecule, ns135 (22). Since ns155 contains tryptic peptides of ns70 and ns86 (15) and does not share peptides with ns135 (14), these two precursor molecules are likely to cover the entire polyprotein, and thus ns135 should represent the C-terminal half of the polyprotein and a precursor for ns72 and ns60.

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The fourth ns protein of SFV, ns60, has thus far been detected by indirect methods only, and an equivalent protein has not been reported for Sindbis virus. The problems in isolating ns60 are, first, that the ns proteins are synthesized in very low amounts and, second, that three other virus-specific proteins migrate at the same region (60 to 70K) in sodium dodecyl sulfate (SDS)-polyacrylamide gels. One of these proteins is the envelope protein precursor p62, which is a glycoprotein and migrates as a heterogenous rather wide band and disturbs the resolution of the ns proteins.

Here we describe specific labeling procedures that enable the isolation of each of the ns proteins of SFV in radiochemically pure form. Instead of wild-type SFV we used a temperature-sensitive mutant, ts-1, which at 39°C synthesizes the ns proteins in excess amounts compared to the wild-type virus (19). The envelope protein precursor, p62, is not cleaved in ts-1-infected cells and thus cannot be chased from the 60 to 70K region of the gels. To circumvent this, we inhibited the glycosylation of the envelope proteins with tunicamycin, thus changing the migration of p62. The specific labeling procedures allowed us to (i) identify ns60, (ii) determine the order of translation of the ns-proteins, (iii) isolate the ns proteins in radiochemically pure form, and (iv) study the processing and stability of the ns proteins. The fourth ns protein, ns60, is a unique entity as evidenced by limited proteolysis with *Staphylococcus aureus* V8 protease, and it is located in the polyprotein between the previously known proteins ns86 and ns72, the latter of which we showed to be the carboxy-terminal ns protein of SFV. Two of the ns proteins, ns70 and ns72, were suggested to undergo post-translational modification. The physical half-life of ns72 was shown to be shorter than that of the other ns proteins.

## MATERIALS AND METHODS

**Cells and virus.** Secondary, specified pathogen-free chicken embryo fibroblasts were used as 2-day-old monolayers on 50-mm petri dishes and were grown in medium 199 supplemented with 5% calf serum and 10% tryptose phosphate broth. The origin and propagation of the wild-type Semliki Forest virus, a prototype strain, and the mutant ts-1 have been described previously (18).

**Labeling of the viral proteins.** For all experiments, the cells were infected with 100 PFU per cell at 39°C, the restrictive temperature for ts-1. After a 60-min adsorption period, the cells were washed once with Hanks balanced salt solution. The medium was Eagle minimum essential medium (MEM) without methionine supplemented with 0.2% bovine serum albumin, 10 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid)–NaOH, pH 7.0 to 7.2, and 1 μg of actinomycin D per ml, a generous gift from Merck Sharp & Dohme. To shut off the protein synthesis by hypertonic treatment, usually at 4.5 h postinfection, the medium was removed and replaced with medium containing 220 mM excess NaCl (final concentration, 335 mM). The details of the hypertonic treatment and the labeling with [$^{35}$S]methionine (Amersham International; 1,160 to 1,500 Ci/mmol) in 1 ml per plate for individual experiments are described below.

A 20-fold excess of the normal amount of methionine in MEM was added to the chase medium. For the labelings performed in the hypertonic medium, the chase medium was also hypertonic. When tunicamycin was used it was added (2 μg/ml) 2 h before labeling and was present in all media thereafter, until and including the pulse medium except for the 1-min initiation period for the "window labeling" experiments (for details, see below). Before harvesting, the medium was removed and cells were washed once with 0.05 M Tris-hydrochloride, pH 7.2–0.1 M NaCl, and the cells were lysed in 0.4 ml of 2% SDS. The cell lysate was passed 10 times through a 20-gauge needle to shear the DNA, boiled for 2 min, and stored at −70°C. The incorporation of [$^{35}$S]methionine was measured by precipitating portions with hot trichloroacetic acid (5 min, 100°C).

**SDS-polyacrylamide gel electrophoresis.** The proteins were separated on linear gradient polyacrylamide gels, using the discontinuous buffer system described by Laemmli (23). The analytical gels were fixed and stained by the modified procedure of Fairbanks et al. (9). For fluorography (1), the gels were equilibrated with dimethyl sulfoxide (2 × 30 min), impregnated with 20% (wt/wt) 2,5-diphenyloxazole for 2 h, and rinsed with running water for 1 h. The gels were dried and exposed to Kodak X-omat AR 5 film at −70°C.

**Isolation of the labeled proteins.** For localization of the proteins, the gels were exposed to Kodak X-omat AR 5 film after drying the gel on a dialysis membrane. Respective gel slices were cut out and the proteins were eluted in 0.1% formic acid for 16 to 18 h at room temperature. Bovine serum albumin (100 μg per sample) was used as carrier, and trichloroacetic acid was added to a final concentration of 10%. Protein precipitation was allowed to take place for 4 h at 0°C. The precipitates were pelleted by centrifugation for 30 min at 12,000 × *g* at 2°C, washed with −20°C acetone, and centrifuged as described above at −20°C. The precipitates were suspended in 100 μl of Clevelands digestion sample buffer for V8 protease digestion (see below).

**Peptide mapping by limited proteolysis.** The limited proteolysis in SDS was performed according to Cleveland et al. (7). The samples were boiled for 2 min and cooled. *S. aureus* V8 protease (Miles Laboratories) was added at an enzyme/substrate ratio of 1:5 or 1:2 and incubation at 37°C was for 15 or 30 min, respectively. After addition of 2-mercaptoethanol and SDS to final concentrations of 10 and 2%, respectively, the proteolysis was stopped by boiling the samples for 2 min. About 55 μl (12,000 to 16,000 cpm) of each sample was loaded into a sample well of a 10 to 20% linear polyacrylamide gel of the Laemmli system and electrophoresed at 6.5 V/cm for 16 h.

**Molecular weights.** Molecular weights were obtained from linear 7.5 to 15% gradient polyacrylamide gels (23). The standards were $^{14}$C-methylated proteins (Amersham International: myosin (200,000), phosphorylase b (92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and

lysozyme (14,300). The viral capsid protein and the envelope proteins and their apoproteins also served as marker proteins (11, 12).

**Quantitation of the proteins.** The proteins were quantitated by cutting the protein bands from the gel, eluting the radioactivity in NCS tissue solubilizer (Nuclear Chicago), and counting the radioactivity in toluene-based scintillation solution.

## RESULTS

The ns proteins of alphaviruses are first translated as a polyprotein and then cleaved to the mature ns proteins designated ns86, ns72, ns70, and ns60. So far, only indirect evidence has been obtained for ns60. It was first found by Lachmi and Kääriäinen (22) by labeling SFV-induced proteins in the presence of pactamycin, which specifically inhibits initiation of translation, and was localized to the carboxy terminus of the ns polyprotein. Additional indirect evidence for this protein has been obtained from peptide mapping analysis of the ns precursor proteins found in mutant-infected cells (14, 15, 17, 21, 24). However, ns60 has not been found under standard labeling conditions, probably because it is hidden by comigrating virus-specific proteins in SDS-polyacrylamide gels. Therefore, specific labeling procedures were designed (i) to identify the carboxy-terminal protein and (ii) to enhance labeling of different segments of the polyprotein to facilitate identification and isolation of the ns proteins.

**Identification of the carboxy-terminal ns protein of SFV by labeling with a 30-s pulse of [$^{35}$S]methionine.** Specific labeling of the C-terminal component of the ns polyprotein was attempted with a very short pulse of [$^{35}$S]methionine. Since the cleavage of an individual protein from the polyprotein is likely to occur a few minutes after its translation (8, 32), the first mature proteins appearing after the pulse should be those beyond which the translation is terminated, i.e., the carboxy-terminal proteins. Furthermore, we can expect to detect the carboxy-terminal ns protein before the mature form of the glycosylated envelope protein precursor, p62, which is assumed to migrate at the same position as ns60 (22).

Immediately after the 30-s pulse, most of the radioactivity was in nascent chains, but sharper bands were seen at the positions of the carboxy-terminal proteins E1 and ns135 as expected (Fig. 1). A faint band was seen also at the position of ns72, and this became more clear after a 0.5-min chase at which time no other ns protein was seen in its mature form. Protein ns86 became clearly visible after a 3-min chase, whereas ns70, preceding ns86 on the polyprotein, appeared only after 15 min. This suggests that ns70 is first present as a precursor form which is then modified to yield the mature ns70.

The capsid protein, known to be cleaved immediately after its synthesis (6, 13, 32), appeared after 0.5 min, and the envelope protein precursor, p62, appeared after a 2-min chase. p62 migrated in this gradient gel very close to ns72, whereas in our previous gels, 7.5% gel with the Neville buffers (25), it migrates below ns70 (20, 22, 24). Protein p62 is identified by pulse-chase labeling of wild-type SFV-infected cells; it is present after the pulse (Fig. 1b) and cleaved during the chase (Fig. 1c) to E2 and E3. E2 migrated just above E1 and E3 is not seen in Fig. 1. In ts-1-infected cells, p62 is not cleaved (19).

An additional protein migrating below ns70 was observed. When analyzed by 7.5% Neville gel (25), this band was not resolved (data not shown). We designated the new protein ns60. These results, however, do not support the location of ns60 to the C-terminus but rather suggest that ns72 is the carboxy terminal ns protein of SFV.

**Conditions to control the SFV-specific translation by hypertonic treatment.** By using the gradient gel, four virus-specific ns proteins could be resolved. However, the proteins migrating at the 60 to 70K region of the gel were not well

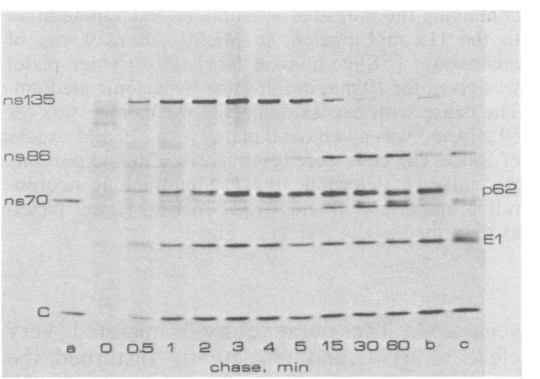


FIG. 1. Fluorograms of SFV ts-1-induced proteins labeled with [$^{35}$S]methionine for 30 s. The cells were infected with ts-1 at 39°C. At 4.5 h postinfection the cells were incubated in 335 mM NaCl for 30 min and then returned to isotonic medium. After 20 min of incubation, the cells were labeled with [$^{35}$S]methionine for 30 s and harvested in 2% SDS either immediately after the pulse or after the indicated chase periods and the proteins were analyzed by 7.5 to 15% linear polyacrylamide gradient gel electrophoresis in the presence of SDS, using the discontinuous buffer system described by Laemmli (23). The electrophoresis was with constant voltage (6.5 V/cm) for 20 h. The markers were ts-1-induced proteins labeled for 1 min and chased for 60 min (a) and wild-type SFV-induced proteins labeled for 15 min and chased for 2 min (b) or 60 min (c). All the markers were labeled in isotonic medium after a 30-min treatment with 335 mM NaCl.

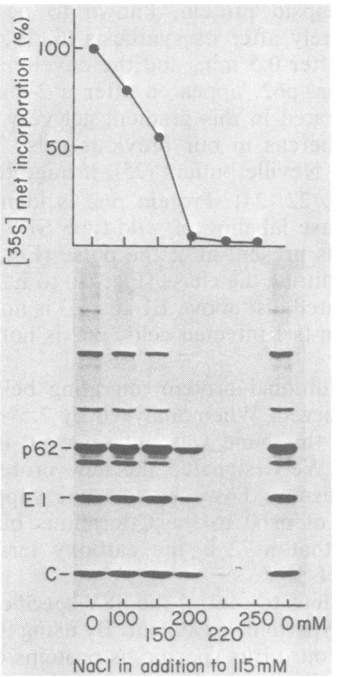


FIG. 2. Effect of different NaCl concentrations on the synthesis of SFV ts-1-induced proteins. Cells were infected with ts-1 at 39°C. At 4.5 h postinfection the medium was removed and replaced with medium containing the indicated amounts of NaCl in addition to the 115 mM present in MEM. After 30 min of incubation, [$^{35}$S]methionine label (20 μCi per plate) was given for 10 min in the same hypertonic medium. The chase with excess unlabeled methionine was for 60 min and was given to all plates with 220 mM excess of NaCl. The cells were lysed in 2% SDS and portions were taken to determine total [$^{35}$S]methionine incorporation (upper panel) and for gel analysis (lower panel) as described in the legend to Fig. 1.

separated. The putative ns60 migrated very close to ns70, and p62 greatly disturbed the resolution of ns72. Therefore, labeling conditions were sought to enhance labeling of the individual ns proteins. These procedures were based on the sequential mode of translation of the SFV proteins and took advantage of the hypertonic treatments by which the initiation of translation could be controlled.

The treatment of cells with hypertonic concentrations of salt blocks the initiation of translation but allows elongation and termination of previously initiated polypeptide chains (27). By allowing sufficient time for chain elongation before labeling the cells, the C-terminal component of a polyprotein can be labeled specifically. Restoration of isotonicity results in synchronous initiation of translation, and with short labeling periods, only the N-terminal part of the proteins are labeled. Both of these procedures were used to determine the translation order of SFV-specific ns proteins.

Since the resistance of mRNAs toward the hypertonic treatment varies (26), the conditions to control the initiation of translation in SFV-infected cells were determined first. A 335 mM concentration of NaCl which reduced total protein synthesis by 99% was chosen for further experiments to shut off the translation since the 5% synthesis occurring in the presence of 315 mM NaCl virtually consisted of viral structural proteins, which we especially wanted to get rid of (Fig. 2). Faithful initiation on both messengers was obtained after restoration of isotonicity as evidenced by the heaviest label in the N-terminal proteins: capsid protein and p62 on the structural messenger and ns70 and ns86 on the ns messenger (Fig. 3). No sign of other ns proteins was seen after 15 min of hypertonic treatment, suggesting that the ribosomes had already run off the messenger. The rather extensive labeling of the C-terminal structural protein, E1, until 25 min of treatment may indicate higher

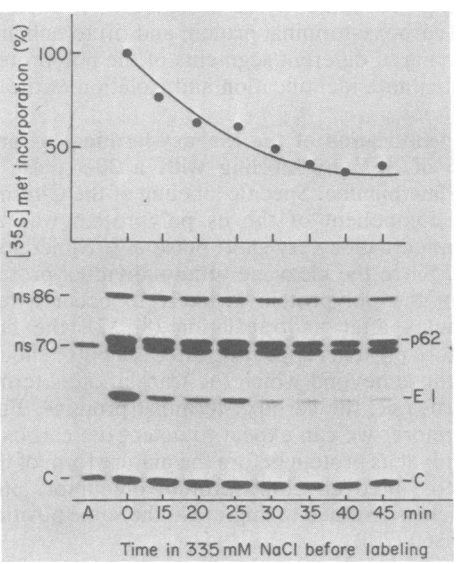


FIG. 3. Reinitiation of translation of SFV ts-1-induced proteins after release of the hypertonic block of initiation. Cells infected with ts-1 at 39°C were treated at 4 h postinfection with 335 mM NaCl as indicated and then labeled for 4 min with [$^{35}$S]methionine (40 μCi per plate) in isotonic medium and chased for 60 min. The cells were lysed in SDS and portions were taken to determine total [$^{35}$S]methionine incorporation (upper panel) and for gel analysis (lower panel) as described in the legend to Fig. 1. Lane A, ts-1-induced proteins labeled for 1 min in isotonic medium after a 30-min treatment with 335 mM NaCl and chased for 60 min.

resistance of the structural protein messenger to the hypertonic salt or that the termination of this messenger is retarded (27). To reduce this background, we used 30 to 40 min of hypertonic treatment in the following experiments.

**Identification of the carboxy-terminal ns protein by labeling under hypertonic salt conditions.** As another approach to identify the carboxy-terminal protein, the cells were labeled after addition of hypertonic medium. This procedure, analogous to the pactamycin treatment, should enhance the labeling of the carboxy-terminal part of the proteins. To improve the resolution at the 60 to 70K region of the gel, we included tunicamycin in the culture medium to prevent glycosylation of the envelope proteins. The unglycosylated form of p62 has a molecular weight of 54,000 (11), not in the range of the ns proteins.

The shutoff of initiation under these labeling conditions was demonstrated by the sequential disappearance of the labeled proteins (Fig. 4A). After 5 min of pretreatment with salt, ns70 was no longer detected, and the synthesis of the capsid protein also was reduced to the background level. The amount of p62 apoprotein was clearly diminished. After 10 min of treatment, only small amounts of E1 apoprotein and ns86 were detected. However, the labeling of ns60 was more extensive than that of ns72. This is contrary to the results obtained with the 30-s labeling experiment by which ns72 was suggested to be the carboxy-terminal component of the ns polyprotein (Fig. 1).

The rather weak labeling of ns72 in all samples suggested to us that ns72 may have a shorter half-life than the other ns proteins. Therefore, proteins in ts-1-infected cells were labeled as above after 10 min of treatment with 335 mM NaCl and chased for different time periods (Fig. 4B). After 0.5- and 10-min chases, the most heavily labeled proteins were ns72 and ns135. During longer chase periods, the amount of ns72 diminished, whereas the amount of ns60 remained more constant, which suggests that ns72 is degraded faster than ns60. This interpretation is supported by the results obtained on quantitation of the virus-specific proteins after pulse and chase (Table 2). We thus concluded that this labeling procedure also supports the idea that ns72 is the carboxy-terminal ns protein of SFV and ns60 precedes it. After the 0.5-min chase period, ns72 migrated slightly faster than after longer chases, suggesting post-translational modification other than a cleavage for ns72.

**Order of translation of the ns proteins of SFV determined by cumulative N-terminal labeling.** Cells infected with ts-1 were treated with 335 mM NaCl for 30 min and then labeled in the isotonic medium for increasing time periods and chased for 1 h to allow maturation of the proteins to their final size (Fig. 5). After 1 min of labeling, only the N-terminal proteins, capsid protein C and ns70, were seen. The second protein on the structural protein messenger, p62, was seen above ns70 after a 2-min labeling period and the third, E1, after a 4-min labeling period. The second ns protein, ns86, became clearly visible after 4 min of labeling. After 6 min of labeling, a protein band appeared below ns70 but was not well resolved from it. This should represent ns60. At 8 min, ns72 also became visible but remained weak due to a long chase period. No further proteins were seen even after 15 min of labeling (data not shown). To enhance the resolution at the 60 to 70K region badly disturbed by the presence of p62, we repeated the experiment in the presence of tunicamycin. During the first 2-min labeling period, there was

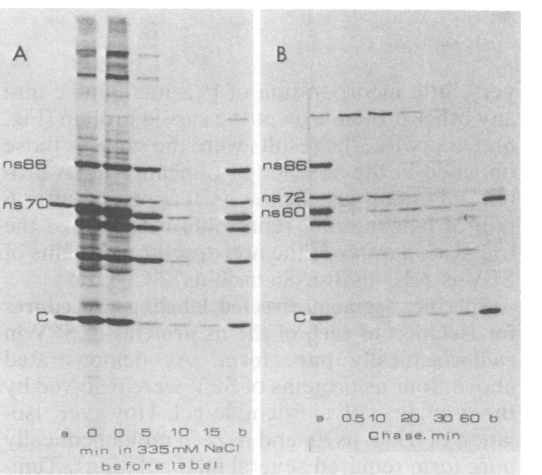


FIG. 4. Fluorograms of SFV ts-1-induced proteins labeled in hypertonic medium. Cells were infected with ts-1 at 39°C and tunicamycin (2 μg/ml) was included in the medium at 3 h postinfection. At 4.5 h postinfection, the medium was removed and new medium was added containing 335 mM NaCl, and after the indicated incubation periods (A) or after 10 min (B) the cells were labeled with [$^{35}$S]methionine (20 μCi per plate) for 4 min and chased for 60 min (A) or as indicated (B) in the presence of 335 mM NaCl. The cells were then lysed in SDS and the proteins were analyzed by a 7.5 to 15% polyacrylamide gel as described in the legend to Fig. 1. (A) The second lane from the left contains lysate from cells labeled in isotonic medium without any treatment with NaCl and chased for 60 min. The markers for (A): (a) ts-1-induced proteins labeled for 1 min in isotonic medium after 30 min treatment with 335 mM NaCl and chased for 60 min; (b) ts-1-induced proteins labeled with the window labeling procedure from 6 to 12 min in the presence of tunicamycin as described in the legend to Fig. 7 and chased for 60 min. The markers for (B): (a) like (b) in (A) and (b) like (a) in (A).

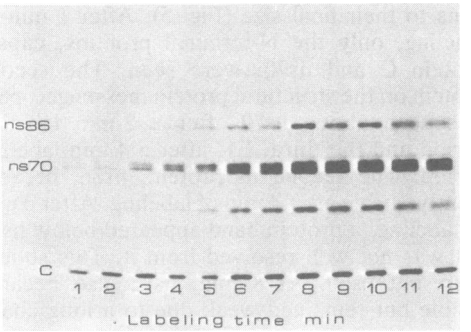


FIG. 5. Fluorograms of SFV ts-1-induced proteins appearing during cumulative N-terminal labeling after release of the hypertonic block of initiation. Cells infected with ts-1 were treated at 4.5 h postinfection with 335 mM NaCl for 30 min and then labeled with [$^{35}$S]methionine as indicated in isotonic medium and chased for 60 min. The cells were lysed in SDS and the proteins were analyzed on 7.5 to 15% polyacrylamide gel as described in the legend to Fig. 1.

very little incorporation of [$^{35}$S]methionine into any other protein except the capsid protein (Fig. 6). Otherwise, the results were the same as those obtained in the absence of tunicamycin. A protein migrating just below ns70 appeared after 6 min of labeling and represents ns60. Thus, the translation order of the nonstructural proteins of SFV is $NH_2$-ns70-ns86-ns60-ns72-COOH.

**Specific, segment-directed labeling procedures for isolation of each of the ns proteins of SFV in radiochemically pure form.** As demonstrated above, four ns proteins of SFV were resolved by the gradient polyacrylamide gel. However, isolation of ns60, ns70, and ns72 in radiochemically pure form required several manipulations. Tunicamycin was used to chase out p62. After release of the hypertonic block of initiation, ns70 was specifically labeled and should have been entirely translated in 4 min. (Fig. 5). At this time, the second protein, ns86, was already made but it was well resolved from ns70. The isolation of ns60, however, was not possible after cumulative labeling since it migrated very close to ns70. Therefore, a specific labeling procedure was developed for the isolation of ns60. The effects of hypertonic/isotonic treatments on the initiation of translation seem to be immediate (27) and should allow labeling of the giant ns polyprotein of SFV at defined segments as follows. Protein synthesis is first shut off by hypertonic treatment. When all the ribosomes have run off the messenger, translation is allowed to initiate for a very short period of time in isotonic medium, after which the cells are returned to the hypertonic medium. Elongation of the chains initiated during the brief isotonic treatment is allowed but new initiation should be blocked. Thus a "translation window" would travel along the messengers. Addition of the label at different times after the initiation should result in labeling of different segments of the polyprotein.

When such an experiment was performed with ts-1-infected cells, very clear sequential labeling of the ns proteins was obtained (Fig. 7). In samples labeled from 2 to 4 min postinitiation, ns70 and ns86 were the dominant ns proteins. When labeled from 4 to 6 min, the radioactivity in ns70 clearly diminished and that in ns86 increased. At this time ns60 also became clearly visible and was more extensively labeled during the labeling periods from 6 to 8 min and from 8 to 10 min. In the sample labeled from 8 to 10 min, ns72 became clearly labeled and at the same time ns86 was already fading out. The most extensive labeling of ns72 was obtained when labeled from 10 to 12 min and from 12 to 14 min, at which time the labeling of ns60 was already getting weaker. Thus, very clearly, the ns proteins of SFV were labeled in the expected sequential order of their translation. The sequential appearance and disappearance of the ns proteins shows that translation initiation was rapidly inhibited by the reexposure of the cells to the hypertonic medium after the 1-min initiation period, and thus a translation window travelled along the messenger. Similar sequential translation of the structural proteins was seen but was not equally evident. Rather high back-

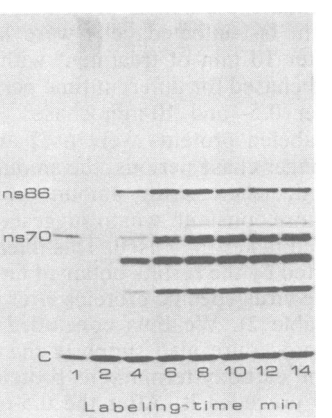


FIG. 6. Fluorograms of SFV ts-1-induced proteins labeled in the presence of tunicamycin after release of the hypertonic block of initiation. The labeling conditions were as described in the legend to Fig. 5 except that tunicamycin (2 μg/ml) was included in the medium at 3 h postinfection. The 1-min labeling was in the absence of tunicamycin. The proteins were analyzed on 7.5 to 15% polyacrylamide gel as described in the legend to Fig. 1.

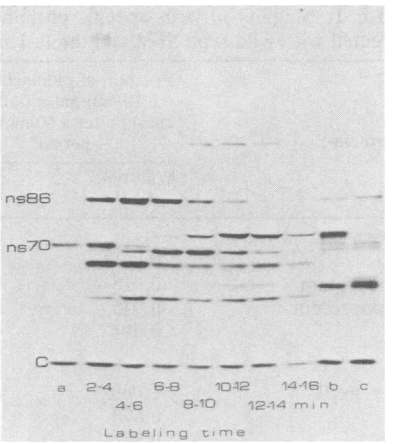


FIG. 7. Fluorograms of SFV ts-1-induced proteins labeled by the window labeling procedure. The cells were infected with ts-1 at 39°C, and tunicamycin (2 μg/ml) was included in the medium at 3 h postinfection. At 4.5 h postinfection the medium was removed and replaced with medium containing 335 mM NaCl. After 40 min, the hypertonic medium was removed and isotonic medium was given to the cells for 1 min beginning at time point 0 min. At 1 min, the isotonic medium was removed and replaced again with medium containing 335 mM NaCl. At this time the cell monolayer was first washed once with 4 ml of and then incubated in another 4 ml of the hypertonic medium. At indicated time points, the cells were labeled with [$^{35}$S]methionine for 2 min and chased with excess unlabeled methionine for 60 min in the presence of 335 mM NaCl. The cells were then lysed in 2% SDS, and the proteins were analyzed on 7.5 to 15% polyacrylamide gel as described in the legend to Fig. 1. The markers were ts-1-induced proteins labeled for 1 min and chased for 60 min (a) and wild-type SFV-induced proteins labeled for 15 min and chased for 2 min (b), or for 60 min (c). All the markers were labeled in isotonic medium after a 30-min treatment with 335 mM NaCl.

ground translation of the structural proteins was seen all the time. It was important to give the chase in the hypertonic medium, otherwise labeling of ns70 was obtained any time, indicating that the effect of the tonicity on the initiation of translation takes place more rapidly than the methionine in the chase medium is available for charging the tRNA.

As evidenced in Fig. 5, 6, and 7, it should be possible to isolate the ns proteins of SFV in radiochemically pure form by using the segment-directed labeling procedures: ns70 after a short (4 min) N-terminal labeling (Fig. 5); ns60 labeling with the window labeling procedure (Fig. 7) or in the hypertonic medium after about 5 min of pretreatment with the salt (Fig. 4A); and ns72 with several labeling possibilities, provided that the chase period is not too long. For isolation of these three proteins, tunicamycin must be used to prevent the glycosylation of p62. The easiest to isolate should be ns86 since it is well separated from the other proteins.

**Characterization of the specifically labeled ns proteins of SFV.** The molecular weights of the specifically labeled ns proteins were determined on the gradient polyacrylamide gel. The apparent molecular weight values obtained (ns86, 86,000; ns72, 68,000; ns70, 64,000; ns60, 61,000) for ns70 and ns72 were lower than those previously reported. The gels with the discontinuous buffer systems may not be the most reliable means for molecular weight determinations, but this was the only way we could resolve these proteins. With the continuous buffer system of Weber and Osborn (34), the only ns protein resolved from the others was ns86, and the other three proteins migrated as one peak or were masked by p62 as was the case also with the 11% Neville gel (19).

The sequential labeling kinetics of the ns proteins of SFV suggested that the four proteins are different entities. This was further verified by labeling the proteins specifically by using the above described procedures and isolating them and subjecting them to limited proteolysis with V8 protease of *S. aureus*. The ns proteins obtained by using the specific labeling procedures were reasonably pure (Fig. 8). The V8 protease patterns supported the conclusion that the four ns proteins of SFV have different primary structures.

**Stability of the virus-specific proteins in the infected cells.** The rather weak labeling of ns72 in many of the above experiments, especially after longer chase periods, suggested that ns72 may have a shorter half-life than the other proteins. Therefore, the stability of the virus-specific proteins was studied in more detail by a pulse-chase experiment performed in isotonic medium. (Table 1).

In both wild-type- and ts-1-infected cells, a reduction of about 10 to 15% in the radioactivity of most of the proteins was seen after a 1-h chase period. An exception was a slight increase in the amount of ns60/ns70, quantitated together because their separation is not good enough to allow individual analysis. The increase in ns60/ns70 could be explained by cleavage from precursors not quantitated in this study. The gel patterns (not shown) indicated that the intensity of ns70 especially increased during the chase period, which is in accordance with the finding that ns70 did not appear in its mature form immediately after its cleavage from the polyprotein (Fig. 1). The greatest reduction was observed in the amount of ns72, and its degradation was even more evident in ts-1-infected cells than in wild-type-infected cells.

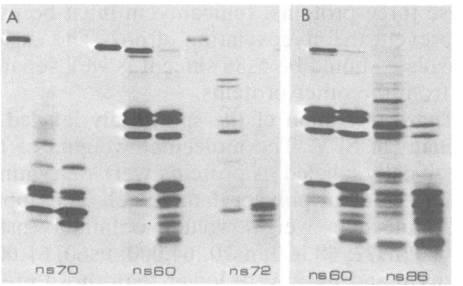


FIG. 8. Fluorograms of V8 protease peptides of SFV ns proteins. [$^{35}$s]methionine-labeled proteins were separated by 7.5 to 15% gel electrophoresis as described in the legend to Fig. 1. The gels were dried on dialysis membrane and the protein bands were localized by autoradiography on X-ray film, cut out, and eluted in 0.1% formic acid and precipitated with 10% TCA as described in the text. The proteins were incubated at 37°C at an enzyme/substrate ratio of 1:5 for 15 min or 1:2 for 30 min. (A) The left-hand lane in each case contains undigested protein, the middle lane contains less extensively digested protein, and the right-hand lane contains more extensively digested sample. (B) For ns86, only the digested samples are shown compared with those of ns60. The left lane contains less extensively digested sample and the right lane contains more extensively digested sample. The separation was on a linear, 10 to 20% polyacrylamide gel with the Laemmli (23) buffers. Electrophoresis was at 6.5 V/cm for 16 h. All labelings were done in the presence of tunicamycin and were preceded by 30 min of treatment with 335 mM NaCl and followed by a 60 min chase. Labeling conditions: in isotonic medium for 4 min (ns70); in isotonic medium for 30 min (ns86); window labeling from 6 to 14 (ns60 and ns72).

The window labeling procedure yielded an additional protein (molecular weight, 51,000) migrating just above the glycosylated form of E1 (Fig. 7). We do not know whether this protein is a specifically activated and labeled host protein, an abnormally glycosylated form of E1 due to the presence of tunicamycin, or a cleavage product of one of the ns proteins. The fact that this protein is most abundant in samples specifically labeled for ns72 may indicate that the 51,000-dalton protein is a cleavage product of ns72.

The amounts of the virus-specific proteins induced by ts-1 in comparison with those induced by wild-type SFV are given in Table 2. This quantitation confirms a previous observation (19) that, in ts-1-infected cells, the ns proteins are made in increased amounts, excluding ns72. The lower amount of ns72 may reflect its faster degradation in ts-1-infected cells. In addition, the synthesis of the structural proteins by ts-1 is clearly less than that by the wild-type virus, which may result from the reduced synthesis of structural protein messenger by ts-1 (18). The ratio of ns to structural proteins in ts-1-infected cells is clearly higher than in wild-type-infected cells, which makes ts-1 a good tool to study the synthesis of the ns proteins.

TABLE 1. Stability of virus-specific proteins in cells infected with wild type SFV and the ts-1 mutant

| Protein | Fraction of radioactivity of a 10-min pulse (+2 min chase) after a 60-min chase period[a] | |
|---|---|---|
| | Wild-type SFV | ts-1 |
| Structural | | |
| C | 0.895 | 0.868 |
| E1 apoprotein | 0.918 | 0.886 |
| p62 apoprotein | 0.935 | 0.748 |
| Total | 0.914 | 0.832 |
| ns | | |
| ns60 + ns70 | 1.069 | 1.133 |
| ns72 | 0.678 | 0.569 |
| ns86 | 0.871 | 0.978 |
| Total | 0.908 | 0.964 |
| Total virus-specific proteins | 0.913 | 0.868 |

[a] Cells infected with ts-1 or wild-type SFV at 39°C were labeled in the presence of tunicamycin with [$^{35}$S]methionine at 4 h postinfection and chased with excess unlabeled methionine. The radioactivity in protein bands separated on 7.5 to 15% gradient polyacrylamide gel was quantitated as described in the text.

## DISCUSSION

The existence of four complementation groups among the Sindbis virus temperature-sensitive

TABLE 2. Synthesis of virus-specific proteins in cells infected with the ts-1 mutant of SFV

| Protein | % of wild-type SFV protein synthesis[a] after 10-min pulse a chase of (min): | |
|---|---|---|
| | 2 | 60 |
| Structural | | |
| C | 61 | 59 |
| E1 apoprotein | 78 | 75 |
| p62 apoprotein | 74 | 59 |
| Total | 70 | 63 |
| ns | | |
| ns60 + ns70 | 126 | 134 |
| ns72 | 95 | 80 |
| ns86 | 105 | 118 |
| Total | 112 | 119 |
| Total virus-specific proteins | 77 | 74 |

[a] Protein labeling and quantitation are described in Table 1, footnote *a*.

mutants with defects in the RNA synthesis (3, 4, 33) suggests that four virus-specific proteins may be needed for alphavirus RNA replication. Since the viral structural proteins are not required for RNA synthesis, these four complementation groups must represent ns proteins. Three ns proteins have been previously detected in both Sindbis virus- (2, 8, 10) and SFV-infected (22) cells, and indirect evidence has been obtained for an additional protein, ns60, for SFV. The translation order previously proposed for the SFV specific proteins was $NH_2$-ns70-ns86-ns72-ns60-COOH.

Now we have isolated the four ns proteins of SFV in radiochemically pure form and have shown that they have different primary structures. The revised translation order ($NH_2$-ns70-ns86-ns60-ns72-COOH) was obtained by several segment-directed labeling procedures. The translation order reported for the closely related Sindbis virus is the same except that a protein analogous to either ns60 or ns72 has not been detected. It may well be that the fourth protein of Sindbis virus is similarly covered by other virus-specific proteins in the gels. We were not able to separate the ns protein of SFV with either 10, 7.5, or 5% gels with the Laemmli system, only the gradient gel was able to resolve them. The erroneous location of ns60 previously to the carboxy-terminus of the polyprotein (22) may be explained partly by a lack of proper separation of the proteins and partly by specific degradation of ns72 during longer chase periods.

The specific labeling procedures and the improved gel analysis allowing unequivocal identification of the proteins enabled us to study in more detail some aspects of the translation and processing of the ns proteins.

The translation rate after release of the hypertonic block of initiation was estimated based on the apparent molecular weights and the sequential appearance of the ns proteins during cumulative N-terminal labeling (Fig. 5). The last time point when a particular protein was not detected was taken as the time the ribosomes needed to reach this protein, i.e., to translate the preceding protein(s). In the original fluorograms, each protein was seen 1 min earlier than in the photoreproduction, and based on those time points translation rates of 290, 340, and 320 amino acids per min were obtained for the translations of ns70, ns70 plus ns86, and ns70 plus ns86 plus ns60, respectively. Taking 320 amino acids per min as an average translation rate, the time required to read the four ns proteins would be about 8 min, which is considerably shorter than the time estimated for translation of the polio virus messenger of about the same size (27). Our higher translation rate, also in comparison with that previously estimated for the structural proteins of SFV (6), may be partly explained by the higher temperature, 39°C instead of the normal 37°C, but it would also be affected by errors in the apparent molecular weight values of the proteins.

The coding capacity theoretically available for the translation of the ns proteins of SFV in one reading frame is close to 300,000 daltons of protein. The combined molecular weight of the four ns proteins would thus leave space for a polypeptide of 20,000 daltons maximum. The translation rate estimate for ns70 was lower than that for the other proteins, and thus additional sequences could be translated before or after ns70, but no evidence was found for such a protein by the sequential labeling experiments. It may also be that the initiation of translation after the release of the hypertonic block is slightly retarded and that this is reflected in the lower translation rate obtained for ns70.

The processing of the ns polyprotein was revealed by the 30-s pulse labeling experiment (Fig. 1). Whereas in other experiments described the label was directed to defined segments of the polyprotein and usually the proteins were allowed to mature before analysis, in this experiment the label was incorporated randomly, and the proteins were analyzed at different stages of maturation. The appearance of the mature proteins tells how fast they are cleaved from the precursors after their translation. The first protein appearing was ns72, which was localized to the carboxy-terminus of the polyprotein by several other labeling procedures. After a 2- to 3-min chase, ns60 and ns86 were clearly visible, indicating that by this time all the cleavages between the ns proteins had taken place, although part of ns135 remained uncleaved for prolonged periods. Surprisingly, ns70 was detected in its mature form only after a 15-min chase. In a more detailed study with 1-min N-terminal label, ns70 appeared after a 5- to 6-min chase period (data not shown). Since ns70 is N-terminal and ns86 follows it in the polyprotein, this means that during a few minutes after the cleavage ns70 is present as a precursor form which is then modified to yield the mature form of the protein. The nature of the modification remains open. As discussed above, the coding capacity and the translation rate estimates would allow additional protein sequences on either side of ns70. However, if ns70 has an N-terminal extension, this could be at most 50 to 60 amino acids long since the mature form of ns70 becomes labeled with a 10-s N-terminal pulse of [$^{35}$S]methionine (data not shown).

During longer chase periods, ns72 was shown to be degraded, whereas the other ns proteins were stable. The negative-strand RNA synthesis of SFV requires continuous protein synthesis,

whereas the positive-strand synthesis does not (28). Exploring the defects of the RNA-negative temperature-sensitive mutants of Sindbis virus (30), we found one mutant (ts-11) which was specifically defective in the synthesis of the minus-strand RNA. Thus, a virus-specific protein is required for the minus-strand synthesis in addition to the components needed for the plus-strand RNA synthesis. This protein was shown to have a short functional half-life. Therefore, ns72 would be a likely candidate for this negative-strand-specific viral protein. Although it has been shown that the inactivation of the minus-strand-specific protein is reversible for ts-24 of Sindbis virus (29) and therefore is not likely to be a cleavage, it is still possible that a cleavage occurs with the wild-type virus.

The segment-directed labeling procedures described in this study allow more detailed analysis of the synthesis and functions of the ns proteins of alphaviruses and should be applicable to other similar translation systems. They also facilitate isolation of these closely migrating proteins in radiochemically pure form and thus enable the determination of the $NH_2$-terminal amino acid sequences of these proteins, which is a prerequisite for the nucleotide sequence analysis of this part of the SFV genome which is currently being carried out in our laboratory.

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