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Functional Defects of RNA-Negative Temperature-Sensitive Mutants of Sindbis and Semliki Forest Viruses

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Defects in RNA and protein synthesis of seven Sindbis virus and seven Semliki Forest virus RNA-negative, temperature-sensitive mutants were studied after shift to the restrictive temperature (39°C) in the middle of the growth cycle. Only one of the mutants, Ts-6 of Sindbis virus, a representative of complementation group F, was clearly unable to continue RNA synthesis at 39°C, apparently due to temperature-sensitive polymerase. The defect was reversible and affected the synthesis of both 42S and 26S RNA equally, suggesting that the same polymerase component(s) is required for the synthesis of both RNA species. One of the three Sindbis virus mutants of complementation group A, Ts-4, and one RNA$^{\pm}$ mutant of Semliki Forest virus, ts-10, showed a polymerase defect even at the permissive temperature. Seven of the 14 RNA-negative mutants showed a preferential reduction in 26S RNA synthesis. The 26S RNA-defective mutants of Sindbis virus were from two different complementation groups, A and G, indicating that functions of two viral nonstructural proteins ("A" and "G") are required in the regulation of the synthesis of 26S RNA. Since the synthesis of 42S RNA continued, these functions of proteins A and G are not needed for the polymerization of RNA late in infection. The RNA-negative phenotype of 26S RNA-deficient mutants implies that proteins regulating the synthesis of this subgenomic RNA must have another function vital for RNA synthesis early in infection or in the assembly of functional polymerase. Several of the mutants having a specific defect in the synthesis of 26S RNA showed an accumulation of a large nonstructural precursor protein with a molecular weight of about 200,000. One even larger protein was demonstrated in both Semliki Forest virus- and Sindbis virus-infected cells which probably represents the entire nonstructural polyprotein.

The alphavirus 42S RNA genome (molecular weight, 4.3×10^6) is infectious and directs in the infected cells the synthesis of the proteins required for the replication of the viral RNAs. The earliest RNA product is the complementary strand of 42S RNA, which is used as the template for the synthesis of both new positive strands of 42S RNA and the subgenomic 26S RNA (molecular weight, 1.6×10^6), the messenger for the viral structural proteins (16, 27, 35).

As an intermediate in the synthesis of 42S RNA-positive strands, a replicative intermediate, RI_a, has been postulated which after exposure to RNase yields a double-stranded replicative form, RFI (molecular weight, about 8.8×10^6). The synthesis of 26S RNA takes place presumably through another replicative intermediate, RI_b, which after RNase treatment gives rise to RFII (molecular weight, 5.6×10^6) and RFIII (molecular weight, 3.2×10^6) (31, 32).

Several RNA-negative (RNA$^-$) mutants of Sindbis virus (1, 3, 4, 30) and Semliki Forest virus (SFV) (18, 19, 28) have been reported to show a specific defect in the synthesis of 26S RNA after shift to the restrictive temperature. We have studied one SFV mutant, ts-4, in more detail (15, 28, 29). When ts-4-infected cultures were shifted to the restrictive temperature (39°C), after initial incubation at the permissive temperature (28°C), the synthesis of 26S RNA was preferentially inhibited. At the same time the RI_b was converted to RI_a. Cultures shifted back to 28°C started the 26S RNA synthesis, even in the presence of cycloheximide, and part of RI_a was converted to RI_b (29). These results suggested that a virus-specific protein controls the conversion between RI_a and RI_b and thus the synthesis of 26S RNA (for review, see 16).

The overall RNA synthesis in ts-4-infected cells remained at the same level it had attained before the shift to 39°C, indicating that little if any active polymerase was made (assembled) at 39°C (15). At the same time a large nonstructural protein, about 200,000 daltons, accumu-

lated in the cultures shifted to 39°C. The interrelationship between these two defects could be explained by assuming that one (or more) of the nonstructural proteins in the 200,000-dalton precursor protein is the polymerase. In ts-4 it remained inactive due to a cleavage defect caused by a mutation in another nonstructural protein, the "interconversion protein" (30), a component of the same precursor.

It would be tempting to assume that if ts-4 is grown at 39°C from the beginning of infection, the same cleavage defect would give the mutant the RNA-negative phenotype.

Two mutants, Ts-21 and Ts-24, of Sindbis virus complementation group A have been reported to show a defect similar to that shown by our ts-4 (3, 30); after shift to the restrictive temperature 26S RNA synthesis declines, and a large nonstructural precursor protein accumulates. A similar finding has been reported also for another collection of Sindbis virus mutants (4). The ability of Ts-21 and Ts-24 to complement with other Sindbis virus RNA$^-$ mutants excludes the possibility that the cleavage defect of the nonstructural polyprotein is so absolute that it could explain the RNA-negative phenotype of these mutants.

The existence of four complementation groups among Sindbis virus RNA$^-$ mutants (6, 34) implies that four different functions, and probably four nonstructural proteins, are needed for RNA synthesis. If any of these functions is continuously impaired, RNA synthesis does not take place in the infected cell. This statement does not exclude that one or more of the proteins would have dual or even several functions.

In the present paper we have studied the RNA synthesis of 14 RNA-negative mutants of SFV and Sindbis virus, among them representatives of all four complementation groups of Sindbis virus. The mutant-infected cultures were first incubated at the permissive temperature to start the RNA synthesis and then shifted to the restrictive temperature to analyze temperature-sensitive defects in late RNA synthesis. The results show that dual functions for proteins regulating the synthesis of 26S RNA have to be predicted.

MATERIALS AND METHODS

Cells and viruses. Secondary cultures of specific-pathogen-free chicken embryo fibroblasts were used as 2-day-old monolayers on 50-mm petri dishes (Lux Scientific Co.). The origin and propagation of wild-type SFV and the mutants derived from it were as described before (19). The heat-resistant (HR) wild type of Sindbis virus and the mutants isolated from it (5) were kindly provided by Elmer Pfefferkorn. All Sindbis virus stocks used were secondary passages starting from one plaque each, formed under agar overlay, and propagated with low multiplicity of infection (1 PFU/100 cells) as described before for SFV (19). The abbreviation Ts is used for Sindbis virus mutants and ts is used for SFV mutants. The permissive and restrictive temperatures were 28 and 39°C, respectively.

For all experiments reported here, the cells were infected with 50 PFU/cell. The adsorption period was 1 h, after which the inoculum was removed and cells were washed once with 5 ml of Hanks balanced salt solution. The medium (5 ml/plate) was Eagle minimum essential medium supplemented with 0.2% bovine serum albumin and contained 1 μg of actinomycin D per ml, a generous gift from Merck, Sharp & Dohme. For the protein-labeling experiments, methionine was omitted from the medium. In the temperature shift experiments the medium was removed from the plates at the time of shift and replaced with new medium at the temperature to which the cells were shifted.

The RNA$^-$ phenotype of Sindbis virus mutants was verified by labeling with [^{3}H]uridine from 4 to 5 h during incubation at 39°C. No virus-specific RNA could be detected by sucrose gradient analysis.

Labeling and analysis of viral RNA. The RNA was labeled with [^{3}H]uridine ([5-^{3}H]uridine, 25 to 30 Ci/mmol; The Radiochemical Centre, Amersham, England), 10 to 20 μCi/plate, in 1 to 2 ml of fresh medium. After the labeling period the cells were drained and washed once with 5 ml of 0.01 M Tris-hydrochloride (pH 7.4)–0.15 M NaCl–0.001 M EDTA (TSE) and lysed in a 0.5-ml/plate portion of 2% sodium dodecyl sulfate in TSE, as described before (19). To measure the overall RNA synthesis, duplicate aliquots (20 μl) were precipitated with 5% trichloroacetic acid. The precipitates were collected on glass fiber filters (GF/C, Whatman), washed twice with 5% trichloroacetic acid, dried, immersed in toluene-based scintillation solution, and counted for radioactivity in a Wallac scintillation counter.

The RNAs were analyzed on 15 to 30% (wt/wt) sucrose gradients made in TSE containing 0.1% sodium dodecyl sulfate. The centrifugation was for 14 h at 24,000 rpm and 22°C in an SW27.1 rotor. Fractions (0.5 ml) were collected by pumping the sucrose out from the bottom via a capillary tube inserted through the gradient. The acid-insoluble radioactivity was measured as above.

Labeling and analysis of viral proteins. Cells infected at 28°C were shifted to 39°C at 6 h postinfection. At the time of shift or later, as indicated, the medium was removed and replaced with 335 mM NaCl in minimum essential medium without methionine. After 40 min of incubation at 39°C, the hypertonic medium was removed and the cells were labeled with 50 to 100 μCi of [^{35}S]methionine (665 to 1,350 Ci/mmol; The Radiochemical Centre) per plate in minimum essential medium without methionine containing 0.1 M sucrose for 30 min and chased either for 2 min ("pulse") or for 60 min ("chase") with minimum essential medium containing a 20-fold concentration of methionine. After this the medium was removed, and cells were washed once with 5 ml of 0.05 M Tris-hydrochloride (pH 7.4)–0.1 M NaCl. The cells were collected in 0.5 ml of 2% sodium dodecyl sulfate–2%

β-mercaptoethanol-9.5% glycerol-0.02% bromophenol blue per plate. The cell lysate was passed 10 times through a thin syringe needle to shear the DNA. The samples were boiled for 2 min immediately after collection and stored at −20°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 7.5% slab gels (21), using the discontinuous buffer system described by Neville (25) with pH 9.18 in the separation gel (10 cm long) and run with constant current, 15 mA/gel, for 6 h. The gels were stained according to Fairbanks et al. (9), and fluorographs were obtained as described by Bonner and Laskey (2). For the molecular weight determinations the continuous system of Weber and Osborn was used (36). The electrophoresis was in 3 or 5% gels (16.5 cm long) run with constant voltage, 60 V/gel. The following proteins were used as molecular weight markers: Myosin (a generous gift from Polly Etkind, Rockefeller University), β-galactosidase (Sigma), thyroglobulin (Sigma), phosphorylase A (Sigma). Dimethyl suberimidate and dimethyl adipimidate (Pierce) polymers of bovine serum albumin (Sigma), glutamate dehydrogenase (Miles Seravac), and ovalbumin (Sigma) were prepared (7), and the commercial molecular weight markers (442302R) of British Drug Houses Ltd. were also used. The marker proteins were localized by staining as above, and the labeled viral proteins were localized by autoradiography.

RESULTS

Analysis of viral RNA synthesis under temperature shift-up conditions. At 6 h postinfection at 28°C viral RNA synthesis is already going on in SFV-infected cells (15). The rate of RNA synthesis increases rapidly when the cultures are shifted to 39°C at 6 h postinfection (Fig. 1). There is a two- to fourfold increase in the rate of synthesis of both Sindbis virus and SFV RNA during the first hour after the shift. Thereafter the rate of synthesis levels off. (The significance of the different behavior of Ts-6 included in the figure will be discussed later.) The reason for the rapid initial increase is not known, but it may be due at least partly to an increased rate of polymerization.

The intracellular RNAs synthesized by Sindbis virus are shown in Fig. 2. The RNA profiles for SFV are essentially the same and therefore are not shown. Since the larger Sindbis virus-specific cytoplasmic RNA sediments together with SFV virion RNA at about 42S (Fig. 2B), both are designated 42S RNA. At 28°C both viruses make mainly 26S RNA, whereas at 39°C much more 42S RNA is synthesized than during the low-temperature incubation. Two hours after the shift-up the RNA pattern of 39°C incubation is already established. This temperature-dependent regulation of 42S/26S RNA ratios (19) has to be taken into account when mutants with deficient 26S RNA synthesis are looked for.

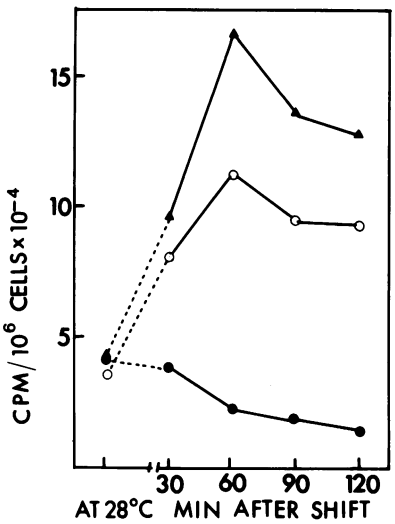

FIG. 1. *Rate of RNA synthesis after shift to 39°C. The cultures were infected at 28°C. At 6 h postinfection one set of plates was labeled at 28°C with 40 μCi of [³H]uridine per plate for 30 min. The rest of the plates were shifted to 39°C at 6 h postinfection. One set of plates was labeled as above at the moment of shift and the rest were labeled at 30-min intervals thereafter. The cells were harvested as indicated by lysing in 2% sodium dodecyl sulfate, and acid-insoluble radioactivity was measured. Symbols:* (○) *HR strain of Sindbis virus;* (●) *Ts-6 of Sindbis virus;* (▲) *wild-type SFV.*

In the following experiments the RNA and protein synthesis of all our RNA⁻ mutants of SFV (19) and representatives of each complementation group of RNA⁻ mutants of Sindbis virus (6, 34) have been studied after shift to the restrictive temperature, 39°C. Included are two RNA± mutants of SFV, ts-10 and ts-13, and one RNA⁺ mutant, ts-1, which is deficient in the synthesis of 26S RNA (19). The infections were started at 28°C (the permissive temperature for the mutants), and the cultures were shifted to 39°C 6 h postinfection. To avoid possible complications caused by the initial increase in the RNA synthesis rate upon temperature shift, the cultures were kept at 39°C for at least 1 h before labeling with [³H]uridine.

Sedimentation analysis similar to those shown in Fig. 2 was carried out for all Sindbis virus and SFV mutants pulsed with [³H]uridine between 7 and 8 h postinfection either at 28°C or after shift to 39°C. Figure 3A and B shows the RNA patterns of two SFV mutants with a defect in the synthesis of 26S RNA. One of these, ts-6, has a normal 42S/26S RNA ratio at 28°C, whereas ts-9 makes more 42S RNA even at the permissive temperature (Table 1). Figure 3C shows the RNAs from Sindbis Ts-6-infected cells. This mu-

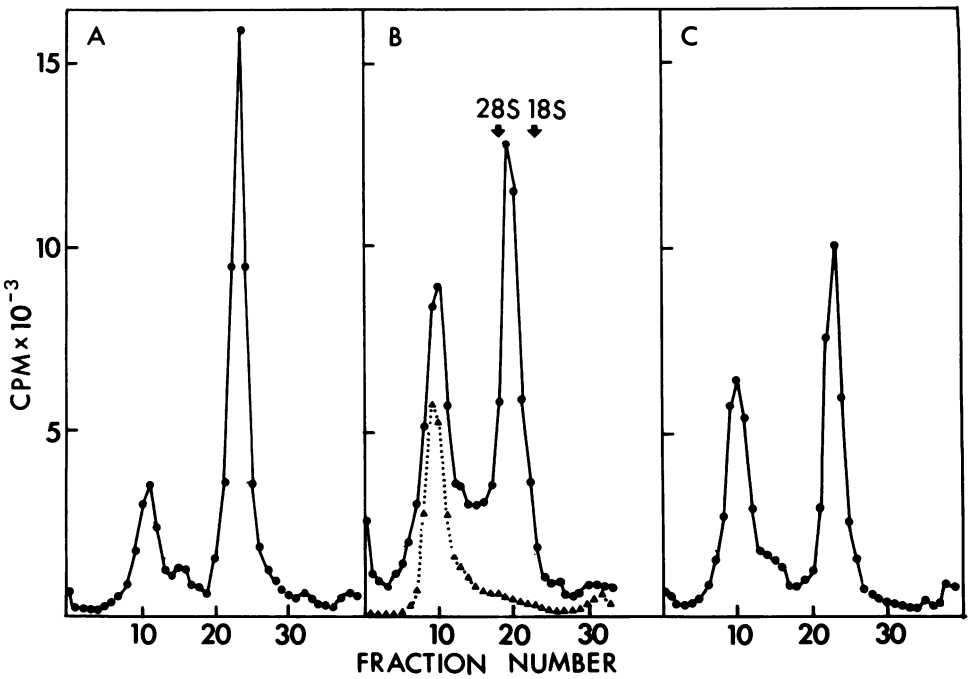

FIG. 2. *[^{3}H]uridine-labeled intracellular RNA profiles of the HR strain of Sindbis virus analyzed on 15 to 30% sucrose gradients. (A) Labeled at 28°C from 7 to 8 h postinfection. (B) Labeled at 39°C from 4 to 5 h postinfection. The triangles represent ^{32}P-labeled virion RNA of wild-type SFV present in the same gradient, and the arrows indicate the positions of the rRNA's monitored by UV absorbance at 254 nm. (C) Cells were infected at 28°C, shifted to 39°C at 6 h postinfection, and labeled at 39°C from 7 to 8 h postinfection. There is a total of 100,000 cpm of [^{3}H]uridine in each panel. Sedimentation is from right to left.*

tant is the only one which showed a clear reduction in the synthesis of both 42S and 26S RNAs after shift to 39°C (see also Table 2). Ts-15 of Sindbis virus shown in Fig. 3D is a representative of 26S RNA-deficient mutants. Cellular extract from the 28°C incubation shows an additional RNA peak sedimenting at about 36S, which could be a conformational variant of either 42S or 26S RNA (17, 33). Similar peaks were seen with some other mutants and occasionally with the HR strain, but only at 28°C.

Mutants with defects in RNA polymerase. Only one of the mutants studied, Ts-6 of Sindbis virus, showed a clear reduction in RNA synthesis after the shift to 39°C (Fig. 3, Table 2). Another Sindbis virus mutant, Ts-4, synthesized RNA poorly at both temperatures, indicating a temperature-independent defect in the function of the RNA polymerase. A puzzling finding was that Ts-4 showed a defect in the synthesis of 26S RNA at the permissive temperature. This defect did not become more pronounced upon incubation at 39°C, as was the case with other mutants showing a specific defect in 26S RNA synthesis (Fig. 4). SFV mutant ts-10, previously classified as RNA$^{\pm}$ (19), resembles Ts-4 in the sense that it also has a temperature-independent defect in RNA synthesis. It differs from Ts-4 since it has no specific deficiency in the synthesis of 26S RNA at either temperature (Table 1).

The kinetics of Sindbis Ts-6 RNA synthesis after the shift to 39°C was studied in more detail. The rate of RNA synthesis of Ts-6 starts to decline right after shift-up, with no initial increase, suggesting that the function of the polymerase is inactivated rather efficiently (Fig. 1). To see whether the defect of Ts-6 is reversible, infected cultures were shifted to 39°C at 6 h postinfection as usual, but to half of them cycloheximide(100 μg/ml) was added at the moment of shift (Fig. 5). After 2 h of incubation at the restrictive temperature, part of the cultures was shifted back to 28°C. As a control, HR infected cultures were treated similarly. In all cases [^{3}H]uridine incorporation was measured using 60-min labeling periods. The addition of cycloheximide had no effect on the overall RNA synthesis of the HR, infected cells. At 39°C there was a reduction in the rate of RNA synthesis due to the cytopathic effect of the virus. In Ts-6-infected cultures 2 h of incubation at the permissive temperature restored more than half of the RNA-synthesizing activity, in both the pres-

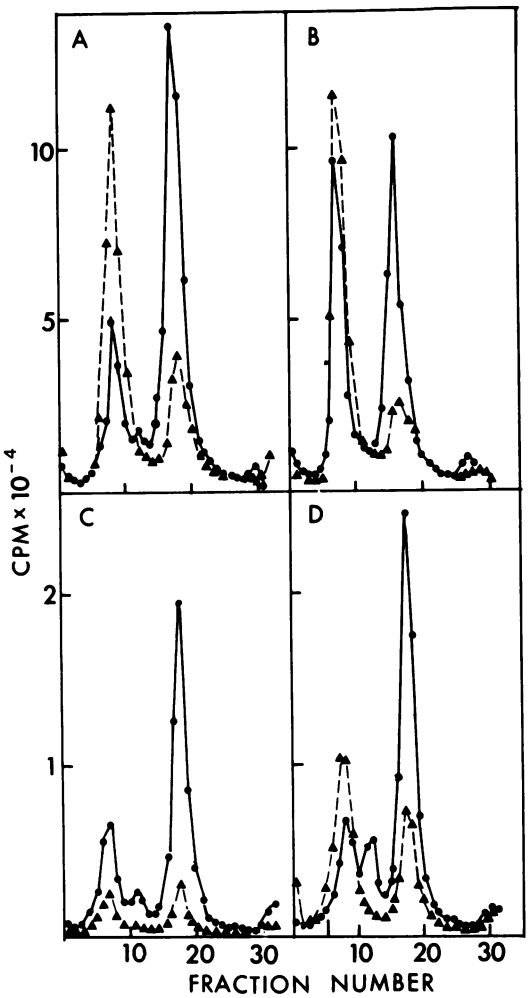

FIG. 3. *[³H]uridine-labeled intracellular RNA profiles of temperature-sensitive mutants of SFV and Sindbis virus. The cells were infected at 28°C and labeled from 7 to 8 h postinfection at either 28 or 39°C after shift-up at 6 h postinfection. The cells were lysed in 2% sodium dodecyl sulfate and analyzed on 15 to 30% sucrose gradients. (A) ts-6 of SFV; (B) ts-9 of SFV; (C) Ts-6 of Sindbis virus; (D) Ts-15 of Sindbis virus. Symbols: (●) labeled 28°C; (▲) labeled 39°C. Sedimentation is from right to left.*

ence and absence of cycloheximide, indicating that the defect was reversible. Analysis of the RNAs from the restored cultures of Ts-6 showed that both 42S and 26S RNA were again synthesized in their normal ratio. Thus, Ts-6 did not show any signs of a specific defect in 26S RNA synthesis at 28°C, after shift to 39°C or after shift back to 28°C (see also Table 2). We would therefore regard Ts-6 as having a real temperature-sensitive defect in RNA polymerase.

Mutants with defects in 26S RNA synthesis. SFV mutants with specific defects in the synthesis of 26S RNA are presented in Table 1. The data are based on analysis of extracts from infected cells on sucrose gradients as illustrated in Fig. 2 and 3. Three mutants of SFV, one RNA^+ (ts-1), one $RNA^\pm$ (ts-13), and one RNA^- (ts-9), have defects in the 26S RNA synthesis manifesting even at the permissive temperature. Three mutants, ts-4, ts-6, and ts-11, show this defect only after shift to the restrictive temperature. One of these, ts-4, has been studied more extensively before (15, 28, 29).

Among the Sindbis virus mutants (Table 2), three (Ts-15, Ts-18, and Ts-24) have a temperature-sensitive defect in 26S RNA synthesis and one mutant (Ts-4) shows the defect possibly only at the permissive temperature. Because the Sindbis virus mutants continued to synthesize proportionally more of 26S RNA than the SFV mutants after shift-up, their RNA synthesis was studied upon longer incubation at the restrictive temperature (Fig. 4). The 26S RNA synthesis continued to decrease during the prolonged incubation with all these mutants, except Ts-4,

TABLE 1. *Properties of SFV mutants*

Mutant	Phenotype	RNA synthesis[a]			
		At 28°C		After shift to 39°C	
		% of control[b]	Molar ratio 42S/26S RNA[c]	% of control	Molar ratio 42S/26S RNA
ts-1	RNA^+	100	0.60	91	1.1
ts-4	RNA^-	88	0.14	67	0.98
ts-6	RNA^-	140	0.12	104	0.91
ts-8	RNA^-	131	0.12	116	0.42
ts-9	RNA^-	66	0.33	49	1.17
ts-10	$RNA^\pm$	25	0.16	35	0.43
ts-11	RNA^-	95	0.13	82	0.82
ts-12	RNA^-	87	0.11	108	0.48
ts-13	$RNA^\pm$	89	0.30	91	1.16
ts-14	RNA^-	112	0.11	125	0.53
Wild-type SFV		81	0.13	101	0.50

[a] Cells were infected at 28°C, and part of the cultures were shifted to 39°C at 6 h postinfection. All cultures were labeled with [³H]uridine from 7 to 8 h postinfection. The RNAs from cell lysates were analyzed on sucrose gradients as described in the text, and sedimenting radioactivity was used as the basis for the calculations.

[b] The mean incorporation values of wild-type SFV and those mutants showing no defect in RNA synthesis served as the control. ts-1 is from a separate experiment; shift-up is compared with ts-1 at 28°C.

[c] The molecular weights used in the calculation were 4.3×10^6 for 42S RNA and 1.6×10^6 for 26S RNA.

TABLE 2. *Properties of Sindbis virus mutants*

Mutant	Complementation group	RNA synthesis[a]			
		At 28°C		After shift to 39°C	
		% of control[b]	Molar ratio 42S/26S RNA[c]	% of control	Molar ratio 42S/26S RNA
Ts-4	A	14	0.24	18	0.38
Ts-6	F	71	0.15	11	0.32
Ts-7	G	103	0.09	81	0.27
Ts-11	B	76	0.16	78	0.38
Ts-15	A	85	0.11	71	0.58
Ts-18	G	98	0.06	116	0.46
Ts-24	A	47	0.14	157	0.77
HR		84	0.10	180	0.28

[a] See footnote *a*, Table 1.

[b] The mean incorporation values from different experiments by HR and those mutants showing no defect in RNA synthesis served as the control.

[c] See footnote *c*, Table 1.

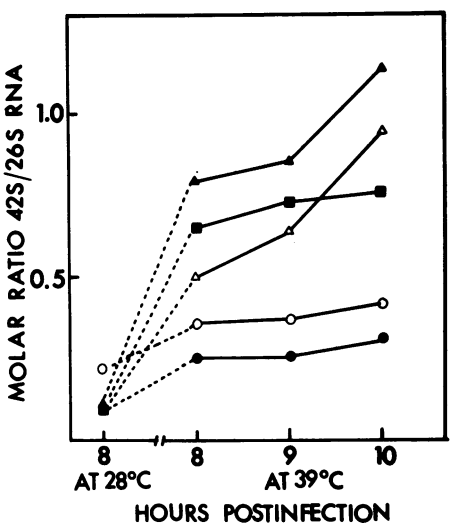

FIG. 4. *42S/26S RNA ratios of the HR strain and temperature-sensitive mutants of Sindbis virus. Cells were infected at 28°C and labeled with [^{3}H]uridine for 60-min periods at either 28 or 39°C after shift-up at 6 h postinfection. At the indicated times cells were lysed in 2% sodium dodecyl sulfate and analyzed on 15 to 30% sucrose gradients. Symbols:* (●) *HR;* (○) *Ts-4;* (▲) *Ts-15;* (△) *Ts-18;* (■) *Ts-24.*

approaching the values obtained with the 26S RNA-deficient SFV mutants (Table 1).

The reversibility of the defect in 26S RNA synthesis was studied by incubating the infected cultures first for 2 h at 39°C after the shift-up. Cycloheximide (100 μg/ml) was added to the cultures at the moment of shift. After 2 h of incubation at 39°C, half of the cultures were shifted back to 28°C and incubated for 1 h before labeling with [^{3}H]uridine between 10 and 11 h postinfection at 28°C (Table 3). The defects of Sindbis virus mutants were readily reversible (see also Table 2), whereas the normal 42S/26S RNA ratios at 28°C incubation (Table 1) were not obtained with the SFV mutants. However, all mutants started to synthesize more 26S RNA after the shift back to 28°C, and therefore their defects are considered to be reversible.

Large nonstructural proteins in cells infected with 26S RNA-defective mutants. It has been shown previously that a large nonstructural protein with a molecular weight of about 200,000 accumulates at 39°C in cells infected with SFV ts-4 (15) and with Ts-24 and Ts-21 of Sindbis virus (3). To see whether similar large proteins accumulate in cells infected with other Sindbis virus and SFV mutants, we screened them under the following experimental conditions. The mutants were grown for 6 h at 28°C as before and then shifted to 39°C. Immediately after the shift, hypertonic medium was added for 40 min followed by a 30-min pulse of [^{35}S]-methionine in 0.1 M sucrose. Part of the cultures were chased for 60 min before harvest. Under these conditions the host protein synthesis is efficiently shut off (15).

Figure 6 shows a pulse-chase experiment with SFV wild type-, ts-1-, ts-6-, and mock-infected cultures. No radioactivity could be detected from the mock-infected cells when the maximum amount of cell lysate was applied to the gel. After the pulse, the previously reported nonstructural proteins ns155 and ns135 can be seen also in the wild-type-infected cells. The largest known protein in ts-4-infected cells after the chase, ns220 (15), is also seen. This protein is found in ts-1- and wild-type-infected cells only after the pulse, but remains there in ts-6-infected cells even after the 60-min chase period. In ts-6-infected cells an even larger protein, with a molecular weight of at least 250,000 (ns250), is clearly seen after the pulse. After similar pulse-labeling, this protein is present in trace amounts with all our RNA$^-$ mutants of SFV.

Similar pulse-chase analysis was carried out with Sindbis Ts-15 and the HR strain, comparing the protein patterns with those of ts-1 and ts-6 of SFV. The largest protein, ns250, is seen as a faint band in Sindbis Ts-15-infected cells analyzed after the pulse (Fig. 7). ns220 is a dominating nonstructural protein in Ts-15-infected cells and can still be seen after the 60-min chase period. It is present also in the HR-infected cells but only after the pulse. The two largest proteins (ns220 and ns250) in SFV- and Sindbis virus-infected cells migrate with the

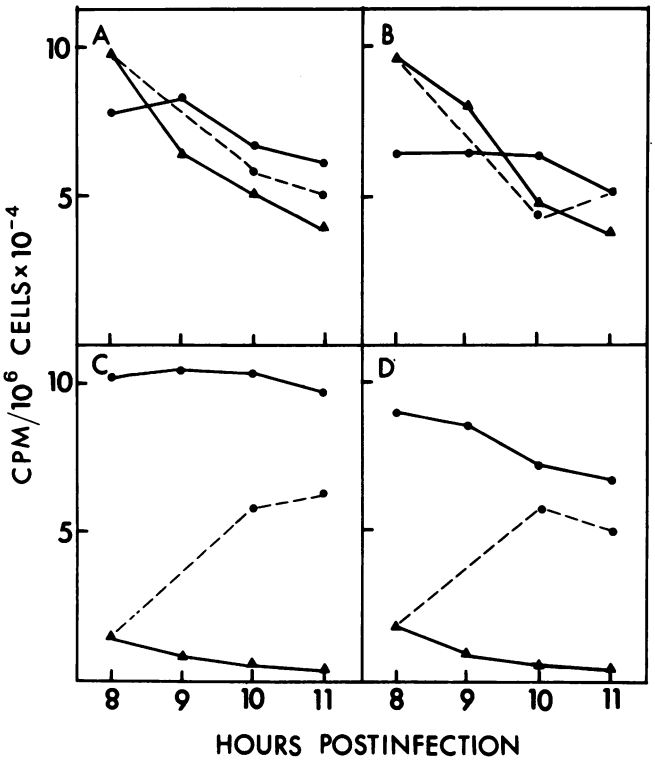

FIG. 5. *Reversibility of the defect of Sindbis virus Ts-6. Cells were infected at 28°C. At 6 h postinfection one-half of the plates received medium with 100 μg of cycloheximide per ml, and part of the cultures were shifted to 39°C. Cells were labeled with 20 μCi of [^{3}H]uridine per plate for 60-min periods and collected at the indicated times by lysing in 2% sodium dodecyl sulfate. One set of plates was transferred back to 28°C at 8 h postinfection, represented by the dashed lines. (A) HR; (B) HR with cycloheximide; (C) Ts-6; (D) Ts-6 with cycloheximide. Symbols: (●) labeled at 28°C; (▲) labeled at 39°C.*

TABLE 3. *Reversibility of the defect in 26S RNA synthesis*[a]

Virus	Molar ratio, 42S/26S RNA	
	Labeled at 39°C	Labeled at 28°C
SFV		
Wild type	0.48	0.13
ts-1	1.17	0.47
ts-4	1.68	0.47
ts-6	1.24	0.21
ts-9	2.29	1.21
ts-11	1.09	0.30
Sindbis		
Ts-15	0.53	0.08
Ts-18	0.44	0.05
Ts-24	0.47	0.09

[a] Cells were infected at 28°C and shifted to 39°C at 6 h postinfection. Cycloheximide, 100 μg/ml, was added at the moment of shift. Half of the cultures were labeled with [^{3}H]uridine from 8 to 9 h postinfection at 39°C. The others were transferred back to 28°C at 8 h postinfection and labeled from 10 to 11 h postinfection.

same mobility in our standard Neville gels. In HR-infected cells there are two smaller proteins which migrated slightly differently from ns155 and ns135 of SFV (22) and which disappeared during the chase period.

To be able to judge which proteins really accumulate in the infected cells, we analyzed the mutants after a 60-min chase period. SFV mutants ts-4 and ts-6 accumulate ns220 in large amounts (Fig. 8), and only two Sindbis virus mutants, Ts-15 and Ts-18 (Fig. 9), showed clear accumulation of ns220. Ts-24, which previously has been reported to accumulate protein of about this size (3), showed only a small amount of ns220, which could be due to differences in experimental conditions. The series of proteins slightly smaller than ns220 may be partial degradation products since they were not detected regularly.

The presence of ns86 in cells infected with mutants showing the accumulation of ns220 implied that the cleavage defect of the nonstructural polyprotein is not absolute (Fig. 8 and 9).

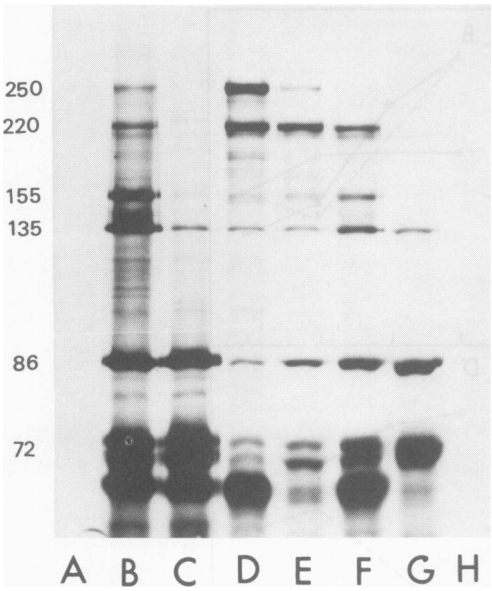

FIG. 6. *Fluorograms of nonstructural proteins of SFV. Cells were infected at 28°C and shifted to 39°C at 6 h postinfection. At the moment of shift 335 mM NaCl was added for 40 min, after which the cells were labeled with [^{35}S]methionine in 0.1 M sucrose for 30 min, chased with excess unlabeled methionine, and harvested as described in the text. The proteins were analyzed by discontinuous, 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis run with 15 mA/gel for 6 h. By this time the structural proteins of the virus had come out of the gel. Equal amounts of radioactivity were added to each slot except to the mock-infected samples, which received the maximum amount of cell lysate, as did the wild-type chase. (A) Mock pulse; (B) ts-1 pulse; (C) ts-1 chase; (D) ts-6 pulse; (E) ts-6 chase; (F) wild-type pulse; (G) wild-type chase; (H) mock pulse. The numbers on the left indicate various nonstructural proteins expressed by their apparent molecular weights × 10^3.*

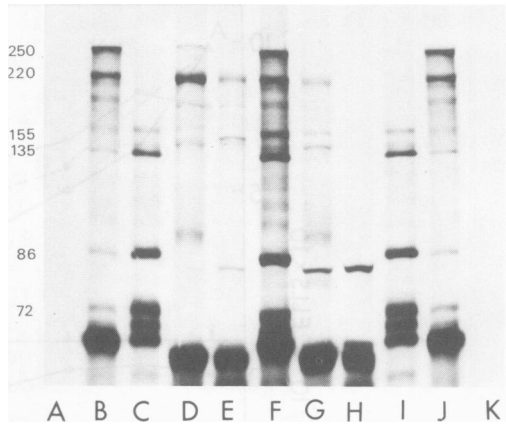

FIG. 7. *Fluorograms of nonstructural proteins of Sindbis virus. Experimental conditions as in the legend to Fig. 6. SFV ts-1 and ts-6 proteins, same as in Fig. 6, were used as the markers. Equal amounts of radioactivity were added to each Sindbis virus slot. A maximum amount of cell lysate was added to the mock samples and to the HR pulse. (A) Mock pulse; (B and J) SFV ts-6 pulse; (C and I) SFV ts-1 chase; (D) Sindbis Ts-15 pulse; (E) Sindbis Ts-15 chase; (F) SFV ts-6 pulse and ts-1 chase; (G) HR pulse; (H) HR chase; (K) mock chase. The numbers on the left indicate various nonstructural proteins of SFV expressed by their apparent molecular weights × 10^3.*

The least amount of ns86 was found in cells infected with Sindbis Ts-18.

In conclusion, we can say that accumulation of ns220 was seen only with mutants having a specific defect in 26S RNA synthesis. These two defects were not always connected, so that not all 26S RNA-deficient mutants showed accumulation of ns220.

DISCUSSION

Since the RNA-negative mutants are unable to initiate their replication cycle at the restrictive temperature, or at least the RNA replication stops very early, their defects can be studied most conveniently by starting the infection at the permissive temperature. We selected 6 h for the time of shift-up. This means that essentially

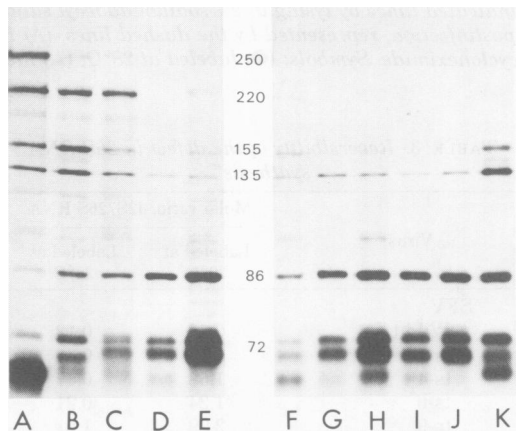

FIG. 8. *Fluorograms of nonstructural proteins of RNA-negative mutants of SFV. Experimental conditions were as in the legend to Fig. 6, except that only the chased samples are shown. The markers on slots A and K are pulse samples. Equal amounts of radioactivity were added to each slot, except the markers. (A) ts-4 (pulse); (B) ts-4; (C) ts-6; (D) ts-8; (E) ts-9; (F) ts-11; (G) ts-12; (H) ts-13; (I) ts-14; (J) wild type; (K) ts-1 (pulse). The numbers in the middle indicate various nonstructural proteins expressed by their apparent molecular weights × 10^3.*

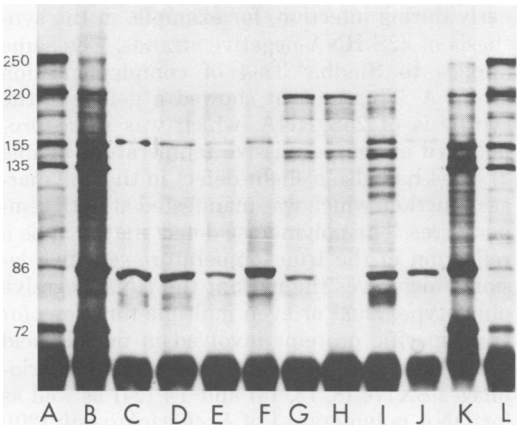

FIG. 9. *Fluorograms of nonstructural proteins of RNA-negative mutants of Sindbis virus. Experimental conditions were as in the legend to Fig. 6, except that the Sindbis virus-infected cells were incubated at 39°C for 60 min before the hypertonic treatment and labeling. Only the chased samples are shown. The SFV ts-1 and ts-4 markers on the left and right are pulse samples. Equal amounts of radioactivity were added to each Sindbis virus slot. (A and L) SFV ts-4 (pulse); (B and K) SFV ts-1 (pulse); (C to I) Sindbis virus mutants. (C) Ts-4; (D) Ts-6; (E) Ts-7; (F) Ts-11; (G) Ts-15; (H) Ts-18; (I) Ts-24; (J) HR. The numbers on the left indicate various nonstructural proteins of SFV expressed by their apparent molecular weights × 10^3.*

all the components of the RNA-synthesizing machinery have already been synthesized and assembled (also see Fig. 5). Thus, our study concentrates on putative temperature-sensitive events in the synthesis of 42S RNA-positive strands and of 26S RNA. The results are summarized in Table 4. Out of the 17 mutants studied only Ts-6 of Sindbis virus, the only member of complementation group F (34), had a temperature-sensitive component in the polymerase. The defect was reversible, allowing the RNA synthesis to start when the mutant-infected cultures were shifted back to the permissive temperature. Under all experimental conditions both 42S and 26S RNA were synthesized in their normal ratio, suggesting that the same polymerase component is required for the synthesis of both RNA species. This is supported also by the fact that no mutants have been found with a specific defect in 42S RNA synthesis. The temperature sensitivity of Ts-6 polymerase explains the low yields of virus after the shift-up, as suggested by Pfefferkorn and Burge (26). Another Sindbis mutant, Ts-4 from complementation group A, and one SFV mutant, ts-10, seemed to be somewhat defective in their RNA synthesis, but the defect was manifested even at the permissive temperature.

The fact that most of the RNA^- mutants were able to continue RNA synthesis after shift-up is

TABLE 4. *Summary of the properties of the mutants of Sindbis virus and SFV*

Virus	Complementation group/ RNA phenotype	Observed defect in late RNA synthesis	Temperature sensitivity of the defect	Reversibility of the defect	Accumulation of ns220 after shift to 39°C[a]
Sindbis					
Ts-4	A	(26S RNA synthesis; polymerase?)	–	ND[b]	–
Ts-6	F	Polymerase	+	+	–
Ts-7	G	None			–
Ts-11	B	None			–
Ts-15	A	26S RNA synthesis	+	+	+
Ts-18	G	26S RNA synthesis	+	+	+
Ts-24	A	26S RNA synthesis	+	+	+?[c]
SFV					
ts-1	RNA^+	26S RNA synthesis	–	ND	–
ts-4	RNA^-	26S RNA synthesis	+	+	+
ts-6	RNA^-	26S RNA synthesis	+	+	+
ts-8	RNA^-	None			–
ts-9	RNA^-	26S RNA synthesis	+[d]	+	–
ts-10	$RNA^\pm$	Polymerase?	–	ND	–
ts-11	RNA^-	26S RNA synthesis	+	+	–
ts-12	RNA^-	None			–
ts-13	$RNA^\pm$	26S RNA synthesis	+[d]	ND	–
ts-14	RNA^-	None			–

[a] Since in both SFV- and Sindbis virus-infected cells the proteins comigrate in sodium dodecyl sulfate-polyacrylamide gels, both are designated ns220.
[b] ND, Not determined.
[c] Reported by Bracha et al. (3).
[d] The defect is manifested also at the permissive temperature.

in accordance with the previous studies on virus yields (26) and in vitro RNA polymerase activity measurements (23). These results all indicate that with the majority of the mutants the RNA polymerase is functional at the restrictive temperature once it has been synthesized and assembled at the permissive temperature.

Three of seven Sindbis virus and four of seven SFV RNA-negative mutants showed a specific defect in the synthesis of 26S RNA. The high proportion of these mutants could be understood if we assume that more than one of the viral proteins are responsible for the regulation of the 26S RNA synthesis. This assumption is supported by the finding of 26S RNA-deficient mutants of Sindbis virus from two different complementation groups: Ts-18 from group G and Ts-15 and Ts-24 from group A.

With most mutants the decrease in the synthesis of 26S RNA after shift-up did not affect the overall RNA synthesis (Tables 1 and 2), indicating that the functions of the 26S RNA-regulating proteins are not needed in the synthesis of 42S RNA-positive strands. The existence of four complementation groups among RNA-negative mutants of Sindbis virus (34) suggests, however, that four (nonstructural) proteins may be needed for the replication of alphavirus RNAs. This in turn means that the proteins regulating 26S RNA synthesis must have some other function as well.

We have isolated an RNA-positive mutant, ts-1, of SFV which is deficient in 26S RNA synthesis. The 26S RNA defect is manifested also at the permissive temperature, similarly to ts-9 of SFV (Table 1) and Ts-4 of Sindbis virus (Table 2), making it improbable that a defect in a structural protein of ts-1 could cause the deficiency in 26S RNA synthesis. If we therefore assume that the mutational lesion of ts-1 affecting 26S RNA synthesis is in one of the nonstructural proteins normally involved in the regulation of 26S RNA synthesis, the above-postulated other function of this protein must be intact, allowing RNA synthesis at the restrictive temperature.

The idea of a dual function of a given polypeptide would explain the somewhat unexpected results obtained with the Sindbis virus mutants of complementation groups A and G. Ts-7 of group G had no defect in the 26S RNA synthesis, and we can assume that the temperature-sensitive defect of "polypeptide G" in Ts-7 is confined to another functional center of the polypeptide distinct from that involved in 26S RNA synthesis. This function could be required either in the assembly of the polymerase (if this polypeptide is a component of the polymerase complex) or early during infection, for example, in the synthesis of 42S RNA-negative strands. The same applies to Sindbis Ts-4 of complementation group A. This mutant showed a defect in the synthesis of 26S RNA, which was more pronounced at the permissive temperature (Table 2). Ts-4 had also a slight defect in the polymerase function, which was manifested at both temperatures. The polymerase defect may well be a reflection of the true temperature-sensitive lesion which gives the mutant the RNA-negative phenotype. Dual or even multiple functions for virus-specific proteins involved in nucleic acid synthesis have been described for bacteriophages ϕX174 (8, 13, 14) and T4 (24) as well as for DNA polymerase I of *Escherichia coli* (20). In the above cases mutants have been isolated which have a temperature-sensitive lesion in one of the functions while the other function(s) is intact.

A large nonstructural protein with a molecular weight of about 200,000 (ns220) was synthesized at 39°C in cells infected with many of the mutants having a defect in the synthesis of 26S RNA (Tables 1 and 2). A protein similar in size has previously been reported in cells infected with Sindbis Ts-21 and Ts-24 of complementation group A (3). The ns220 in SFV ts-4-infected cells was labeled with short pulses after synchronous initiation of protein synthesis (15), indicating that this protein represents the amino-terminal part of the nonstructural polyprotein. ns220 should therefore have the amino acid sequences of ns70, ns86, and ns72 starting from the amino terminus (22).

The existence of an even larger nonstructural protein has so far been based on indirect evidence (10–12). In pulse-labeled cells infected with SFV mutants ts-4 and ts-6, a prominent large protein with a molecular weight of at least 250,000 was seen, which may well turn out to be the primary translational product of the nonstructural proteins. A protein of the same size was seen transiently in cells infected with all SFV mutants and the wild type. It was also seen less clearly in Sindbis Ts-15-infected cells after the pulse (Fig. 7).

We assume that the accumulation of ns220 in cells infected with the mutants defective in 26S RNA synthesis reflects the importance of certain amino acid sequences for the proper cleavage of the polyprotein. These sequences are probably also necessary for the proper functioning of regulation of 26S RNA synthesis. Another possibility would be that proteins involved in the regulation of 26S RNA synthesis have autoproteolytic activity, responsible for the cleavage of the nonstructural polyprotein. A mutational lesion

would inactivate the proteolytic activity at the restrictive temperature and lead to the accumulation of ns220.

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