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Specific Sindbis Virus-Coded Function for Minus-Strand RNA Synthesis

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The synthesis of minus-strand RNA was studied in cell cultures infected with the heat-resistant strain of Sindbis virus and with temperature-sensitive (*ts*) mutants belonging to complementation groups A, B, F, and G, all of which exhibited an RNA-negative (RNA^-) phenotype when infection was initiated and maintained at 39°C, the nonpermissive temperature. When infected cultures were shifted from 28°C (the permissive temperature) to 39°C at 3 h postinfection, the synthesis of viral minus-strand RNA ceased in cultures infected with *ts* mutants of complementation groups B and F, but continued in cultures infected with the parental virus and mutants of complementation groups A and G. In cultures infected with *ts*11 of complementation group B, the synthesis of viral minus-strand RNA ceased, whereas the synthesis of 42S and 26S plus-strand RNAs continued for at least 5 h after the shift to 39°C. However, when *ts*11-infected cultures were returned to 28°C 1 h after the shift to 39°C, the synthesis of viral minus-strand RNA resumed, and the rate of viral RNA synthesis increased. The recovery of minus-strand synthesis required translation of new proteins. We conclude that at least one viral function is required for alphavirus minus-strand synthesis that is not required for plus-strand synthesis. In cultures infected with *ts*6 of complementation group F, the syntheses of both viral plus-strand and minus-strand RNAs were drastically reduced after the shift to 39°C. Since *ts*6 failed to synthesize both plus-strand and minus-strand RNAs after the shift to 39°C, at least one common viral component appears to be required for the synthesis of both minus-strand and plus-strand RNAs.

As one of the initial steps in the infectious cycle of the alphaviruses Sindbis virus (SIN) and Semliki Forest virus, the parental 42S genome RNA is translated to yield a viral RNA-dependent RNA polymerase which utilizes the parental 42S plus-strand RNA as a template for the synthesis of 42S minus-strand RNA. The 42S minus-strand RNA in turn serves as a template for the synthesis of more 42S plus-strand RNA and of subgenomic 26S mRNA, which is translated into the viral structural proteins (15, 22, 29). During the first 3 h postinfection (p.i.), the rate of synthesis of plus-strand and minus-strand RNAs increases; however, at approximately 3 to 3.5 h p.i., the synthesis of minus strands ceases abruptly, whereas the synthesis of both 42S and 26S plus strands continues at a constant rate throughout the infectious cycle (3, 25). During the early period of infection, the synthesis of plus strands occurs in about a five-fold excess relative to minus-strand RNA synthesis (25).

The RNA-dependent RNA polymerase responsible for the synthesis of 26S and 42S plus-strand RNAs must be stable, since the addition of inhibitors of protein synthesis after 3 h postinfection does not significantly interfere with plus-strand RNA synthesis (13, 25, 26, 30). We have recently shown that the synthesis of minus-strand RNA in Semliki Forest virus-infected cells ceases within 15 to 20 min after the addition of inhibitors of protein synthesis (25). Thus, the polymerase responsible for minus-strand synthesis differs from the viral polymerase synthesizing plus-strand RNA by having a short half-life. We have suggested (25) that alphaviruses regulate the rate of transcription of viral RNA by regulating the number of minus-strand templates and that the synthesis of minus-strand RNA is regulated at the level of translation by a mechanism which utilizes one or more short-lived polymerase proteins. One possible mechanism could involve an unstable or rapidly turning-over polymerase protein that is required only for minus-strand synthesis. We report in this communication the identification of a temperature-

sensitive (*ts*) mutant of the heat-resistant strain of SIN (SIN HR) that selectively ceased minus-strand syntheis upon shifting to the nonpermissive temperature.

MATERIALS AND METHODS

Cell cultures. Secondary cultures of chicken embryo fibroblast (CEF) cells were prepared from primary cultures of 10-day-old embryos obtained from the eggs of leukosis-free flocks and were grown in plastic petri dishes in Dulbecco modified Eagle minimal essential medium (DMEM) supplemented with 5% fetal bovine serum and 5% tryptose phosphate broth. BHK-21 cells, a continuous cell line derived from baby hamster kidney cells, were grown in plastic petri dishes in DMEM supplemented with 5% fetal bovine serum.

Virus. SIN HR and the RNA-negative (RNA^-) *ts* mutants used in this study were the generous gift of E. R. Pfefferkorn (Dartmouth Medical School, Hanover, N.H.) and have been characterized previously (5, 6, 22, 29). Growth and purification of each virus stock and determination of its infectivity by plaque assay at both the permissive (28°C) and the nonpermissive (39°C) temperatures were as previously described (16).

Infection and RNA labeling. In all of the experiments presented, with the exception of those in Fig. 1 and Table 1, we employed CEF monolayers in 50-mm petri dishes infected with either SIN HR or one of the mutants at a multiplicity of infection (MOI) of 50. After the monolayer was washed with Hanks balanced salt solution at 28°C, the virus was adsorbed to the CEF cells for 1 h at 28°C in an inoculum of 0.5 to 1.0 ml of complete DMEM (DMEM containing 0.2% bovine serum albumin, 22 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4], and 1 μg of actinomycin D per ml). At the end of the adsorption period, the virus-containing medium was removed, complete DMEM at 28°C was added, and the cultures were incubated at 28°C for the times indicated in the text. To accomplish the shift to 39°C, the monolayers were washed with Hanks balanced salt solution at 39°C before the addition of complete DMEM at 39°C. When cultures at 39°C were returned to 28°C, they were washed once with Hanks balanced salt solution at 28°C, followed by the addition of complete DMEM at 28°C. SIN RNA was labeled with [5,6-^{3}H]uridine at a final concentration of 250 μCi/ml per 50-mm petri dish, unless otherwise indicated in the text.

Since our studies involved the incubation of infected cells at two different temperatures, we determined the kinetics of SIN RNA synthesis at these two temperatures. At various times after infection, SIN HR-infected cells were labeled for 60 min with [^{3}H]uridine, and the cultures were solubilized with 2% sodium dodecyl sulfate in ET buffer (0.01 M EDTA, 0.01 M Tris-hydrochloride [pH 7.4]). DNA was sheared by passage several times through a 27-gauge needle. Samples were taken for protein determination by the method of Lowry et al. (17) and for total acid-insoluble radioactivity. We found, as have others (15, 16, 22), that the entire replication cycle takes approximately twice as long when incubation is at 28°C than when it is at 39°C. When the maximum number of transcription complexes had formed and RNA synthesis was at a constant rate (by 4 to 5 h at 39°C and by 8 to 9 h at 28°C), [^{3}H]uridine incorporation into RNA during a 1-h pulse-label in cultures maintained at 39°C was twice that obtained from cultures maintained at 28°C (data not shown). Therefore, to adjust for the effect of temperature on the observed rate of transcription, a pulse of 60 min was given to cultures to be labeled at 28°C, and a 30-min pulse was used for cultures labeled at 39°C.

Isolation of SIN RF RNA. At the times indicated, the infected cells were washed once with ice-cold phosphate-buffered saline, followed by the addition of 2 ml of lysis buffer (0.15 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4], 1.5 mM $MgCl_2$) containing 0.65% Triton X-100. The cells were scraped from the surface of the petri dish and transferred to 15-ml conical centrifuge tubes; the solution was vigorously blended in a Vortex mixer before being centrifuged at 250 × *g* for 10 min at 4°C to pellet the nuclei. The cytoplasmic supernatant was removed and adjusted to 1% sodium dodecyl sulfate. The solution was extracted twice with phenol, followed by two extractions with chloroform-isoamyl alcohol (96:4). RNA was precipitated from the aqueous phase by the addition of LiCl to 0.2 M and ethanol to 70%. After overnight storage at −20°C, RNA was collected by centrifugation in an SW41 rotor at 98,000 × *g* for 1 h at 0°C. The dried precipitate was dissolved in 0.2 ml of digestion buffer (0.15 M NaCl, 0.05 M Tris-hydrochloride [pH 6.8], 1 mM EDTA) and digested with 0.1 μg of pancreatic RNase per ml at room temperature for 15 min. A solution of 0.05 M Tris-hydrochloride (pH 6.8) and 1 mM EDTA was added to lower the NaCl concentration to 0.1 M, and ethanol was then added to a final concentration of 35% (vol/vol). The samples were immediately applied to columns of CF-11 cellulose by the procedure of Franklin (11) for isolation of replicative form (RF) RNA.

CF-11 cellulose columns were poured in 3-ml plastic syringes (2.5 ml of packed CF-11 cellulose) and were washed first with modifed STE buffer (0.1 M NaCl, 0.05 M Tris-hydrochloride [pH 6.8], 10 mM EDTA) containing 1% beta-mercaptoethanol, followed by extensive washing with STE buffer (0.1 M NaCl, 0.05 M Tris-hydrochloride [pH 6.8], 1 mM EDTA) containing 35% ethanol. The samples were applied to the columns and washed with 10 to 15 ml of STE buffer containing 35% ethanol, followed by an equal volume of STE buffer containing 15% ethanol; RF RNA was eluted in STE buffer. We routinely recovered 2 to 5% of the applied radioactivity as RF RNA. RF RNA was collected by ethanol precipitation in the presence of 0.2 M LiCl and 25 μg of carrier rat liver RNA.

This procedure quantitatively recovered RF RNA from cells infected with SIN HR or *ts* mutants of SIN HR. The same amount of RF RNA was obtained by either the sucrose gradient method used previously (24, 25) or the CF-11 cellulose method of Franklin (11). Furthermore, both of these preparations of RF RNA contained the same proportion of labeled minus-strand RNA and sedimented at 15S to 20S in sucrose gradients. RF RNA isolated by two cycles of sucrose gradient centrifugation (24, 25) bound to CF-11 cellulose, and 100% of applied RF RNA was recovered in

the STE buffer fraction. Less than 0.1% of labeled single-stranded viral RNA was found to elute in the STE buffer fraction after treatment with this concentration of pancreatic RNase. Cellular DNA eluted in STE buffer containing 15% ethanol and thus could be efficiently removed from the RF RNA fraction.

Quantitation of minus-strand RNA synthesis. Minus-strand RNA was measured by determining the amount of [^{3}H]uridine incorporated into RF RNA that hybridized to unlabeled 42S plus-strand RNA. The conditions of hybridization were identical to those reported by us previously (24, 25). Labeled RNA that was protected from pancreatic RNase digestion was taken to be labeled minus-strand RNA. It should be noted that this minus-strand RNA represented that which remained after treatment of the replicative intermediates with low levels of pancreatic RNase to obtain RF RNA. Such RNase digestion would remove any single-stranded, nascent minus-strand or plus-strand RNA in the replicative intermediates.

Materials. [5,6-^{3}H]uridine (53 Ci/mmol) was purchased from Amersham Radiochemicals, London, England. Cycloheximide was obtained from Calbiochem, La Jolla, Calif., and CF-11 cellulose was obtained from Whatman, Inc., Clifton, N.J. All other materials were from previously described sources (23–25).

RESULTS

Blocking of minus-strand RNA synthesis in SIN HR-infected cells by inhibition of protein synthesis. Figure 1 shows the effect of blocking translation on the synthesis of viral RNA (Fig. 1A) and minus-strand RNA (Fig. 1B) in cells infected with SIN HR. In untreated cells, the overall rate of viral RNA synthesis increased exponentially beginning at 1 to 1.5 h p.i. and continued until 3.5 to 4 h p.i., after which time it became constant or slowly decreased (Fig. 1A). The rate of synthesis of minus-strand RNA increased at the same time that the overall rate of viral RNA synthesis increased and ceased at the time that the overall rate of viral RNA synthesis became constant (Fig. 1B). When cycloheximide was added during the period in which the rates of synthesis of viral plus-strand and minus-strand RNAs were increasing, the overall rate of synthesis of viral plus-strand RNA stopped increasing and became constant or slowly declined (Fig. 1A), and the synthesis of minus-strand RNA ceased within 30 min after the addition of cycloheximide (Fig. 1B). Cycloheximide had no effect on the overall rate of viral RNA synthesis when it was added after the normal cessation of minus-strand synthesis had occurred and after the overall rate of viral RNA synthesis had become constant (data not shown). These results, which were the same regardless of whether CEF or BHK-21 cells were used for the infection, demonstrated that minus-strand transcription in SIN-infected cells was shut off selectively by cycloheximide in a manner identical to that observed in BHK-21, HeLa, or Vero cells infected with Semliki Forest virus (25). The synthesis of alphavirus minus-strand RNA, therefore, appears to require the continued synthesis of pro-

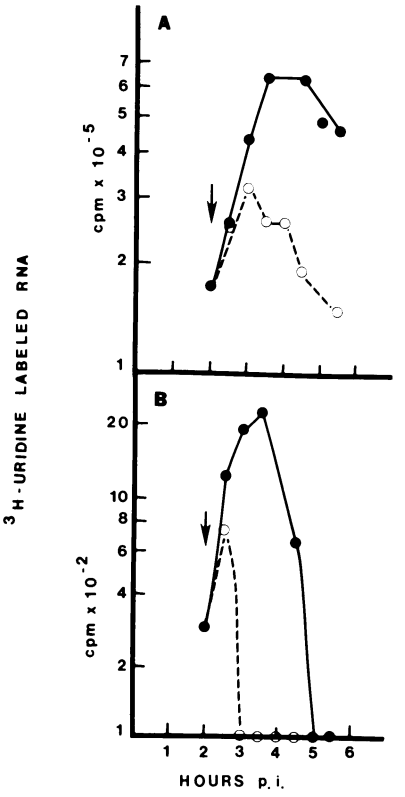

FIG. 1. *Rate of SIN HR RNA synthesis in the presence and absence of cycloheximide. BHK-21 monolayers in 50-mm petri dishes were infected with SIN HR (MOI, 50) and were maintained at 37°C in the presence of actinomycin D. At 2 h p.i., medium containing cycloheximide (100 μg/ml) was added to one set of cultures (arrow). Both sets of cultures were pulsed with [^{3}H]uridine (250 μCi/ml) at 37°C for 30-min periods between 2 and 5.5 h p.i. and were harvested immediately after the pulse. (A) Total [^{3}H]-uridine incorporation into viral RNA. Acid-insoluble radioactivity was determined by counting a sample after trichloroacetic acid precipitation. The values shown represent the total [^{3}H]uridine in each sample; duplicate experiments gave similar results. (B) Total [^{3}H]uridine incorporation into minus-strand RNA. RF RNA in each sample was isolated by CF-11 cellulose chromatography as described in the text, denatured by heating at 100°C, and allowed to reanneal in the presence of an excess of unlabeled SIN HR 42S virion RNA. RNase-resistant, radiolabeled RNA formed during the hybridization reaction was taken as labeled minus-strand RNA. Symbols: ●, untreated cultures; ○, cultures treated with cycloheximide.*

teins. To determine whether a viral protein is responsible for minus-strand transcription, we screened several *ts* mutants of SIN HR to identify mutants possessing a *ts* defect that manifests itself in the failure to synthesize minus-strand RNA, but not plus-strand RNA, at the nonpermissive temperature.

Screening for SIN HR *ts* mutants that are defective in minus-strand RNA synthesis. We screened seven representatives of SIN HR *ts* mutants that have been assigned to four complementation groups that give an RNA$^-$ phenotype. These RNA$^-$ mutants fail to synthesize detectable amounts of viral RNA when the infection is initiated and maintained at 39°C, the nonpermissive temperature. To identify *ts* mutants of SIN HR that are selectively unable to synthesize minus-strand RNA at the nonpermissive temperature, the cells must be infected at the permissive temperature. The infected cells are then shifted to the nonpermissive temperature at a time when minus-strand RNA transcription is detectable. Thus, the RNA$^-$ mutants can be characterized by their ability or inability to continue synthesizing plus-strand or minus-strand RNA. Nascent viral RNA and all minus-strand RNAs are found as part of the replicative intermediates. During a pulse-label with [^{3}H]-uridine, the proportion of radiolabel incoporated into minus-strand RNA relative to that in plus-strand RNA in the replicative intermediates indicates the relative level of synthesis of minus-strand RNA. During the period in which minus strands are being accumulated, 40 to 50% of the [^{3}H]uridine incorporated into RF RNA derived from replicative intermediates is in minus strands, and this proportion is constant for pulse periods of 15 min to 4 h (24, 25).

Monolayers of CEF cells were infected with either SIN HR or RNA$^-$ *ts* mutants and were maintained at 28°C for 3 h before being shifted to 39°C. After 30 min at 39°C, [^{3}H]uridine-containing medium was added to the cultures which were maintained at 39°C for an additional 2.5 h before harvest. Viral double-stranded RF RNA was isolated, RNA was denatured by heating at 100°C, and the proportion of labeled minus-strand RNA in RF RNA was determined by hybridization in the presence of an excess of unlabeled SIN HR virion RNA. Table 1 shows three *ts* mutants that appeared to be unable to continue synthesis of minus-strand RNA after the shift to 39°C: *ts*4, *ts*6, and *ts*11. RF RNA isolated from *ts*11-infected cells contained the least proportion of labeled minus-strand RNA (about 6%); RF RNA isolated from *ts*4- and *ts*6-infected cells contained only about 10% labeled minus-strand RNA. These results were in contrast with those obtained with RF RNA isolated from the remaining mutants (20 to 36% labeled minus strands) and SIN HR (40% labeled minus strands).

TABLE 1. *Analysis of SIN RNA$^-$ mutants for defects in minus-strand synthesis*[a]

Virus	Complementation group	% Labeled RF RNA in minus-strand RNA
SIN HR		40.4
*ts*4	A	9.8
*ts*6	F	8.8
*ts*7	G	20.3
*ts*11	B	6.2
*ts*15	A	24.6
*ts*18	G	31.6
*ts*24	A	36.2

[a] Monolayers of CEF cells in 50-mm petri dishes were infected with either SIN HR or the *ts* mutants as described in the text. An MOI of 50 was used for the B and G group mutants, and an MOI of 100 was used for the A and F group mutants. SIN HR was tested at both MOIs and gave equivalent results for minus-strand synthesis. The different MOI was used to maximize incorporation into *ts*4 and *ts*6 RNAs because of the reported effect of the shift to 39°C on RNA synthesis (16). At 3 h p.i., the monolayers were shifted to 39°C and were further incubated at 39°C in fresh medium. Beginning 30 min after the shift to 39°C, [^{3}H]uridine-containing medium was added (500 μCi/2 ml per petri dish), and the incubation continued for 2.5 h. The infected cell extracts were prepared, and RNase-resistant RF RNA was isolated and hybridized to an excess of unlabeled 42S virion RNA. The hybridization values shown represent the average of from two to five determinations. A control preparation of RF RNA from SIN HR-infected cells labeled with [^{3}H]uridine from 1 to 5 h p.i. at 37°C contained 49% of the labeled RNA in the RFs in minus-strand RNA.

A closer examination of these mutants was undertaken. Pulses of [^{3}H]uridine were given for 30 min at the time of the shift to 39°C and at 30-min intervals thereafter to cultures infected with SIN HR mutants *ts*4, *ts*6, *ts*11, and *ts*15 and with the parental SIN HR. The rate of RNA synthesis in SIN HR-infected cells increased approximately 20-fold over the 3-h period after the shift to 39°C (Fig. 2). However, in all of the mutant-infected cultures, raising the temperature to 39°C at 3 h p.i. resulted in a failure to increase substantially the rate of RNA synthesis. The rate of RNA synthesis in *ts*11-infected cultures increased only two- to threefold over this period, remained essentially constant in *ts*4- and *ts*15-infected cultures, and slowly decreased in *ts*6-infected cultures. We examined the other RNA$^-$ mutants shown in Table 1, and all of them failed to increase significantly the rate of RNA synthesis after the shift to 39°C at 3 h p.i.; their rate of synthesis either was constant or slightly decreased over the 3-h period after the shift to

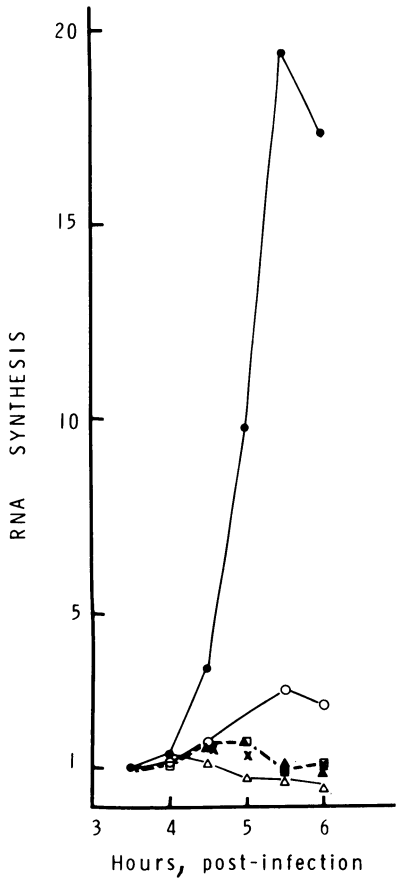

FIG. 2. *Relative RNA synthesis in SIN HR- and mutant virus-infected cultures at 39°C. CEF cells in 50-mm petri dishes were infected with SIN HR or one of the ts mutants at an MOI of 50 and maintained at 28°C until 3 h p.i., at which time they were shifted to 39°C. [^{3}H]uridine incorporation into RNA at 39°C was determined with pulses of [^{3}H]uridine (250 μCi/ml) for 30 min between 3 and 6 h p.i. The cells were harvested as described in the text, and samples were taken for acid-insoluble radioactivity. The amount of incorporation observed in cultures pulsed between 3 and 3.5 h p.i. (shown below in parentheses) was taken as 1.0, and the amount of incorporation observed in the subsequent pulse periods for each mutant and the parental virus is expressed relative to this amount. During the infectious cycle, [^{3}H]uridine incorporation into SIN HR RNA during a 30-min pulse-label increased by an average of 22-fold (five experiments ranging from 9- to 33-fold). Cultures were infected with: ▲, ts4 (133, 181 cpm); △, ts6 (183, 848 cpm); ○, ts11 (273, 600 cpm); ×, ts15 (230, 854 cpm); ●, SIN HR (241, 270 cpm); and □, SIN HR incubated in the presence of cycloheximide from the time of the shift to 39°C (241, 029 cpm).*

39°C at 3 h p.i. (data not shown). The inability of the RNA$^-$ mutants to increase significantly the level of viral RNA synthesis after the shift to 39°C at 3 h p.i. was not unexpected. The failure to increase the rate of viral RNA synthesis could have resulted from a failure to synthesize additional minus-strand templates, a failure to accumulate functional polymerase components, and the inactivation of previously formed polymerase components that are temperature sensitive in these mutants. Therefore, the relative level of minus-strand RNA synthesis was determined from RF RNA isolated from each of the datum points shown in Fig. 2 (Fig. 3). Between 40 and 50% of RF RNA synthesized in SIN HR-infected cells between 1 and 2 h after the shift to 39°C hybridized to unlabeled virion RNA and decreased to less than 1% by 3 h after the shift. Therefore, these results again showed that the increasing rate of SIN HR plus-strand RNA transcription correlated with the synthesis of minus-strand RNA. Cells infected with SIN *ts*15 of the A complemention group continued to synthesize minus-strand RNA for an additional 3 h after the shift to 39°C (Fig. 3). Similar results have been obtained for cells infected with SIN *ts*18 of the G complementation group and for SIN *ts*24 of the A complementation group (data not shown). Unlike the results obtained for SIN HR, *ts*15, *ts*18, and *ts*24, only a small proportion

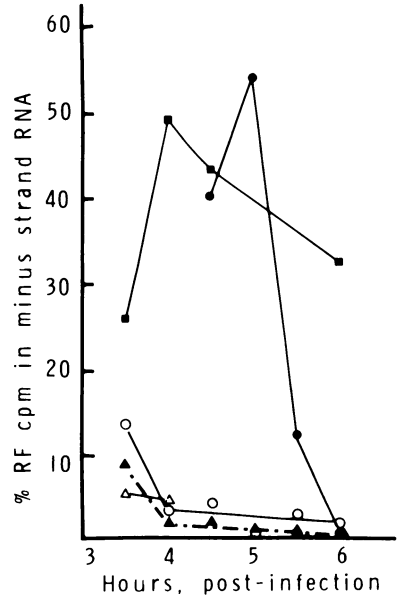

FIG. 3. *Minus-strand RNA synthesis in infected CEF cultures shifted to 39°C. RF RNA was isolated from extracts of the cultures shown in Fig. 2 by CF-11 cellulose chromatography. The percentage of [^{3}H]-uridine incorporated into RF RNA that was in minus strands was determined by hybridization as described in the text. RF RNA was isolated from cultures infected with: ●, SIN HR; ▲, ts4; △, ts6; ○, ts11; ■, ts15.*

of labeled RF RNA obtained from *ts*4-, *ts*6-, and *ts*11-infected cells after the shift to 39°C was in minus strands (Fig. 3). Thus, minus-strand synthesis ceased in *ts*4-, *ts*6-, and *ts*11-infected cells soon after the shift to the nonpermissive temperature.

Analysis of plus-strand RNA synthesis in *ts*11-infected cells. We focused our studies on SIN HR *ts*11, the sole member of the B complementation group, that is, the only mutant in which minus-strand synthesis appears to be selectively temperature sensitive. Although minus-strand synthesis failed to continue at 39°C in either *ts*4- or *ts*6-infected cells (Fig. 3), the *ts* defect does not appear to be restricted to minus-strand synthesis, but also directly affects plus-strand RNA synthesis. In either *ts*4- or *ts*6-infected cells, the synthesis of 42S and 26S plus-strand RNAs is reduced after the shift to 39°C at late times in the infectious cycle when minus-strand transcription has already ceased (16). This is in contrast to *ts*11-infected cells, in which 42S and 26S plus-strand syntheses continue after the shift to 39°C at late times in the infectious cycle (16). We analyzed the pattern and the amounts of 42S and 26S plus-strand RNAs synthesized in *ts*11-infected cells after the shift to 39°C at a time (3 h p.i.) in the infectious cycle when minus-strand synthesis was just beginning. Cultures of CEF cells were infected at 28°C with *ts*11 and were either maintained at 28°C (Fig. 4A) or shifted at 3 h p.i. to 39°C (Fig. 4B) and labeled with [^{3}H]uridine. At both temperatures, full-length 42S and 26S plus-strand RNAs were synthesized. The cultures that had been shifted to 39°C incorporated 18% as much [^{3}H]uridine into 42S and 26S RNAs as did the culture maintained at 28°C, but an amount equal to that obtained in a culture maintained at 28°C but labeled at 3 h p.i. (data not shown). Furthermore, the ratio of labeled 26S RNA to 42S RNA did not change significantly with a shift in temperature; at 28°C the ratio was 1.7, and at 39°C the ratio was 1.2. Therefore, plus-strand RNA continued to be synthesized at 39°C in *ts*11-infected cells, although minus-strand transcription ceased when the cultures were shifted to the nonpermissive temperature at 3 h p.i.

Analysis of viral RNA synthesis at the permissive temperature. We next determined whether the regulation of minus-strand synthesis in SIN HR *ts*11-infected cells was normal at the permissive temperature. Cultures of CEF cells were infected with *ts*11 at 28°C and were pulsed for 60 min with [^{3}H]uridine at intervals throughout infection (Fig. 5A). An increasing amount of [^{3}H]uridine incorporation into RNA was detected between 2 and 6 h p.i., followed by several hours of a more or less constant rate of

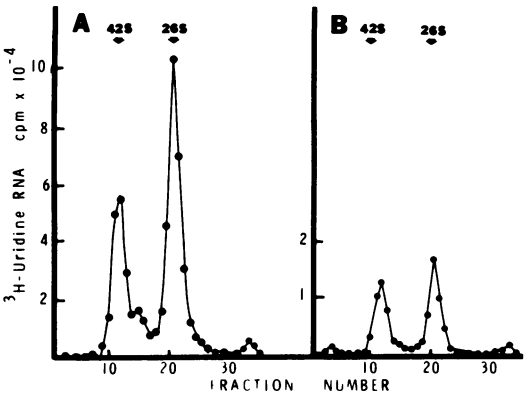

FIG. 4. *Sucrose gradient analysis of RNA synthesized in ts11-infected cultures at the permissive and nonpermissive temperatures. Monolayers of CEF cells were infected with ts11 at 28°C. Certain of these cultures were maintained at 28°C, receiving a 60-min pulse of [^{3}H]uridine at various intervals; other cultures were shifted to 39°C at 3 h p.i. and were maintained at 39°C. Cells were harvested after the pulse into ET buffer containing 2% sodium dodecyl sulfate, and the extracts were layered onto 15 to 30% sucrose gradients in NET buffer (0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-hydrochloride [pH 7.4]) containing 0.2% sodium dodecyl sulfate and sedimented in an SW27.1 rotor at 93,000 × g for 18 h at 20°C. Samples of 0.5 ml were collected, and the radioactivity in each was determined by counting the entire fraction after acid precipitation and collection on a glass fiber filter. (A) ts11-infected culture maintained at 28°C and pulsed with [^{3}H]uridine between 7 and 8 h p.i.; (B) ts11-infected culture shifted to 39°C at 3 h p.i., subsequently maintained at 39°C, and pulsed with [^{3}H]-uridine between 7.5 and 8 h p.i.*

incorporation. RF RNA was isolated and showed a similar pattern of synthesis (Fig. 5B). As shown in Fig. 5C, the period of increasing RNA synthesis was associated with an increase in the incorporation of radiolabel into minus-strand RNA. The insert to Fig. 5C shows the proportion of the total incorporation in RF RNA that was in minus strands. The synthesis of minus-strand RNA occurred at an increasing rate at 28°C until after 4 h p.i., at which time it declined: between 2 and 3 h and 3 and 4 h p.i., about 50% of the radiolabel in RF RNA was in minus strands; by 6 p.i., less than 20% of labeled RNA was in minus strands; and only 3% was found between 6 and 7 h p.i. Thus, at the permissive temperature, the synthesis of minus-strand RNA in *ts*11-infected cells was temporally regulated and was similar to that seen in the parental SIN HR-infected cells (Fig. 1). Since plus-strand RNA continued to be synthesized for at least 5 h after the shift to 39°C (Fig. 4), the previously formed minus strands continued to function as templates for plus-strand synthesis

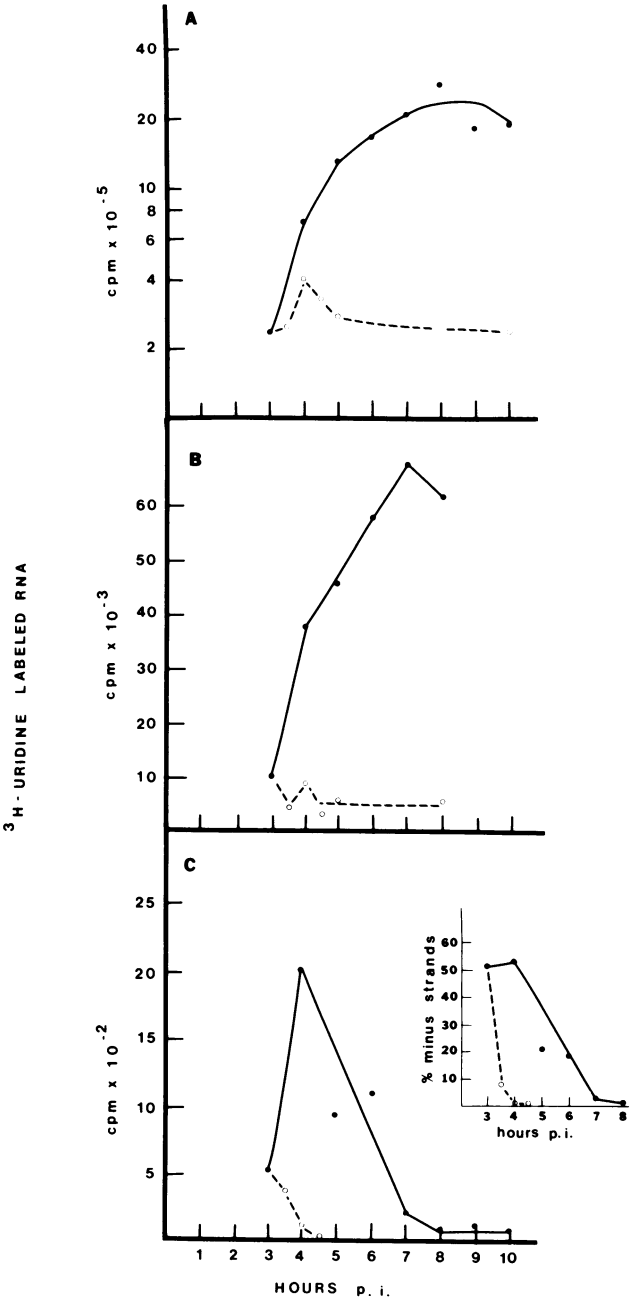

FIG. 5. *Rate of [³H]uridine incorporation into SIN ts11 RNA at 28°C and after the shift to 39°C. CEF monolayers were infected with ts11 (MOI, 50) and maintained at 28°C. Beginning at 2 h p.i., a pulse of [³H]uridine was given for 60-min periods at 28°C. Duplicate cultures were shifted to 39°C at 3 h p.i. and were pulsed with [³H]uridine for 30-min periods beginning at the time of the shift. (A) Total incorporation into viral ts11 RNA; (B) total incorporation into RF RNA that was isolated from the extract of each of the samples shown in (A); (C) total incorporation into ts11 minus-strand RNA. RF RNA was denatured and allowed to reanneal in the presence of an excess of unlabeled 42S virion RNA as described in the text. The insert shows the same results as those shown in (C) expressed as a percentage of the total labeled RF RNA that is in minus-strand RNA. Symbols: ●, cultures maintained at 28°C; ○, cultures shifted to 39°C at 3 h p.i. and maintained at 39°C.*

after *ts*11-infected cells were shifted from 28 to 39°C at 3 h p.i. For a direct comparison with events at 28°C, duplicate cultures were shifted to 39°C at 3 h p.i. and similarly analyzed. An initial two- to threefold rise in plus-strand synthesis was followed by a slow decline, so that 7 h after the shift, 40% of the initial rate was observed (Fig. 5A). A constant amount of incorporation was detected in RF RNA (Fig. 5B), and as seen previously (Fig. 3), minus-strand synthesis ceased about 60 min after the shift to 39°C (Fig. 5C).

Resumption of minus-strand synthesis. Since raising the temperature to 39°C resulted in the cessation of minus-strand synthesis in *ts*11-infected cells, we next asked whether minus-strand synthesis would resume after a return to the permissive temperature. The requirement for translation of new viral proteins for the resumption of minus-strand transcription upon a shift down from 39 to 28°C was investigated to distinguish between the reactivation of previously formed proteins and the requirement for newly synthesized ones. Cultures that were shifted up to 39°C at 3 h p.i. were returned to 28°C 1 h later and incubated in the presence or absence of cycloheximide (100 μg/ml). Viral RNA synthesized in these cultures was labeled during 60-min pulse periods with [^{3}H]uridine, and the amounts of labeled viral RNA and RF RNA and the proportion of labeled RF RNA in minus strands were determined. A return to the permissive temperature in the absence of cycloheximide led to the resumption of an increasing rate of RNA synthesis that eventually reached about 60% of that in infected cultures that were maintained at 28°C (Fig. 6). However, a return to 28°C in the presence of cycloheximide led to only a small increase over the rate of RNA synthesis occurring at 39°C. After the return to 28°C, the amount of labeled RF RNA in the untreated cultures increased, but essentially no increase with time in the amount of labeled RF RNA occurred in cultures treated with cycloheximide (Fig. 6B). Corresponding to an increase in synthesis of total RNA and RF RNA after the return to 28°C, there was a resumption of minus-strand synthesis (Fig. 6C). A similar resumption of minus-strand transcription after the shift back down to 28°C did not occur in the presence of cycloheximide. Active minus-strand polymerase must have been present in all of these cultures at the time of the shift to the nonpermissive temperature, since minus strands were accumulating at 3 h p.i. at 28°C. Since minus-strand polymerase activity in SIN HR-infected cells was normally short-lived, as was demonstrated by its rapid disappearance after the inhibition of

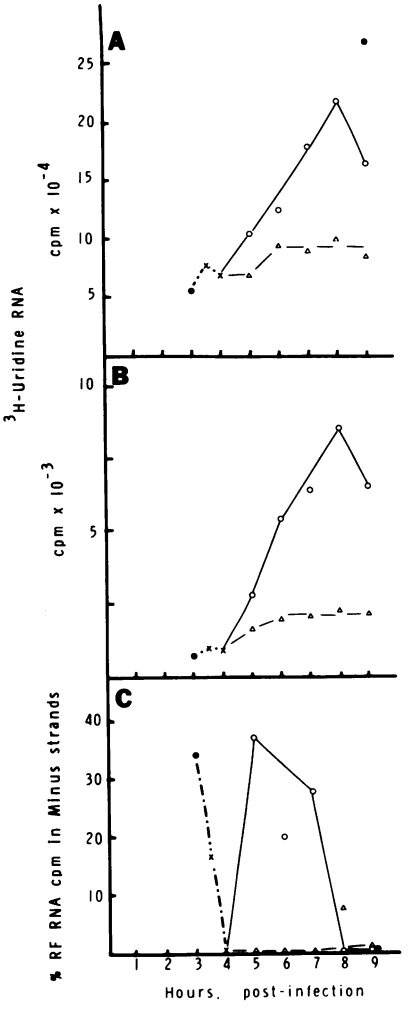

FIG. 6. *Effect of cycloheximide addition on the temperature-dependent resumption of minus-strand synthesis. ts*11-*infected cultures were shifted to 39°C at 3 h p.i., and RNA synthesis was monitored by the addition of [^{3}H]uridine for 30-min pulses at 39°C (×). Two sets of these cultures were returned to 28°C at 4 h p.i. One set was incubated continuously in the presence of 100 μg of cycloheximide per ml (△); the second set was not further treated and served as the 28°C control (○). Incorporation of radioactivity into the acid-insoluble form by these cultures was determined after the cultures were given 60-min pulses of [^{3}H]uridine beginning from the time of the return to 28°C. In addition, several cultures which were maintained at 28°C throughout the experiment (●) were pulsed for 60-min periods with [^{3}H]uridine between 2 and 3 h p.i. and between 8 and 9 h p.i. to monitor the amount of RNA synthesis occurring in infected cultures not subjected to these temperature shifts. (A) Total incorporation into viral RNA; (B) incorporation into RF RNA; (C) percentage of total labeled RF RNA in minus strands.*

protein synthesis (Fig. 1), the failure to demonstrate a resumption of minus-strand RNA synthesis in SIN HR *ts*11-infected cells that had been shifted to 39°C at 3 h p.i. and then returned to 28°C 1 h later in the presence of cycloheximide could have resulted from either the rapid degradation or the rapid functional inactivation at 39°C of a viral polypeptide required for minus-strand RNA synthesis. In either case, the minus-strand polymerase activity that reappeared in SIN HR *ts*11-infected cells upon the return to 28°C required the synthesis of proteins.

Cultures shifted to 39°C at 3 h p.i. were returned to 28°C at various times (Fig. 7). In all cases, there was an immediate increase in the [^{3}H]uridine incorporation as compared with that detected in infected cultures not returned to 28°C. Increasing the time at the nonpermissive temperature before the return to the permissive temperature decreased the extent to which total RNA synthesis recovered, but, nevertheless, minus-strand synthesis resumed in *ts*11-infected cultures if the cultures were shifted back to 28°C.

DISCUSSION

Previously, we have shown that minus-strand transcription in Semliki Forest virus-infected cells is shut off selectively when protein synthesis is inhibited with puromycin or cycloheximide (25). The data presented in this study extended this observation to include SIN and demonstrated that when SIN HR *ts*11-infected cells were shifted to the nonpermissive temperature during the early phase of the viral replication cycle, when the rate of minus-strand RNA synthesis was increasing, minus-strand transcription ceased selectively. Temperature sensitivity of a component of the viral RNA-dependent RNA polymerase that is required for minus-strand transcription would account for the RNA$^-$ phenotype of SIN mutant *ts*11. The products of at least four cistrons (complementation groups A, B, F, and G) of SIN HR are required for viral RNA synthesis (5, 6, 22, 29). SIN HR *ts*11 is the only member of the B complementation group (22, 28, 29). The results of our studies on *ts* mutants of SIN HR suggest that the product of the B cistron of SIN HR is a polypeptide that functions in minus-strand transcription and demonstrated that thermal inactivation of minus-strand transcription occurred in *ts*11-infected cells without inactivation of plus-strand transcription.

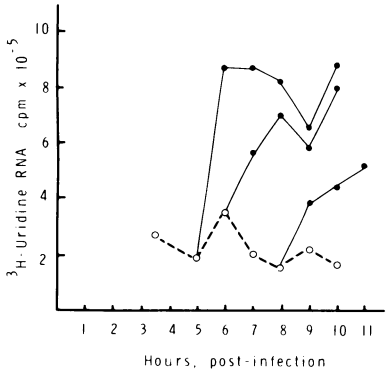

FIG. 7. *Temperature-dependent increase in ts11 RNA synthesis. CEF monolayers infected with ts11 were shifted to 39°C at 3 h p.i. At the indicated times, certain of these cultures were returned to 28°C and labeled with [^{3}H]uridine at 28°C for 60-min periods; cultures maintained at 39°C were pulsed for 30-min periods. Cultures were harvested into ET buffer with 2% sodium dodecyl sulfate immediately after the pulse, and the amount of incorporation into the acid-insoluble form was determined. Symbols: ○, 39°C; ●, 28°C.*

Minus-strand RNA synthesis was also observed to be temperature sensitive in SIN HR *ts*4 (complementation group A)-infected cells and SIN HR *ts*6 (complementation group F)-infected cells. This observation was consistent with the results reported by Pfefferkorn et al. (21) and Pfefferkorn and Burge (20), who demonstrated that input *ts*4 and *ts*6 viral RNAs are not converted into a nuclease-resistant, double-stranded form at the nonpermissive temperature. However, since we (16; unpublished data) and others (22) have shown that *ts*6 has difficulty synthesizing plus-strand RNA at the nonpermissive temperature, we conclude that the product of the F cistron is required for both plus-strand and minus-strand RNA syntheses. Cells infected with *ts*4 of the A complementation group synthesize viral RNA poorly at both the permissive and nonpermissive temperatures, suggesting a temperature-independent defect in the viral RNA polymerase (16). Another member of the A complementation group, *ts*15, did not stop synthesizing minus-strand RNA upon the shift to the nonpermissive temperature. Most *ts* mutants of the A complementation group show an alteration in the ratio of 26S-to-42S plus-strand synthesis after the shift to the nonpermissive temperature (29), with the exception of *ts*4, which does not significantly change the ratio of 26S-to-42S plus-strand synthesis (16). Therefore, we conclude that only *ts*11 of the B complementation group unequivocally appears to be defective selectively in minus-strand RNA synthesis. A shift of *ts*11-infected cells to the nonpermissive temperature at a late time after infection does not affect plus-strand RNA synthesis (16). When *ts*11-infected cells were shifted to the nonpermissive temperature at

early times in the infectious cycle, plus-strand RNA synthesis continued at the rate that was occurring at the time of the shift.

Our results with SIN HR *ts*11 suggest that the product of the B cistron is a polypeptide which is required selectively for minus-strand RNA synthesis. Because minus-strand, but not plus-strand, RNA synthesis is short-lived and requires continual protein synthesis, it is not possible from our results to determine whether the product of the B cistron is temperature sensitive in *ts*11 or whether the cleavage of the polypeptide encoded by the B cistron is defective at the nonpermissive temperature. It has been suggested (29) from the results reported by Waite (30) that one of the polypeptide intermediates in the processing of the SIN nonstructural proteins accumulates in *ts*11-infected cells at the nonpermissive temperature. The isolation of additional *ts* mutants belonging to the B complementation group which also share the phenotype of SIN HR *ts*11 is necessary to conclusively demonstrate that the B cistron is responsible for minus-strand synthesis.

To date, only three SIN nonstructural proteins have been identified (4, 14). Their gene order has been determined to be 5′-*ns*60-*ns*89-*ns*82-3′ (4). These proteins are translated from the 42S polycistronic mRNA with a single initiation site (1, 4, 15). A recent study by Fuller and Marcus (14) with increasing doses of UV irradiation, which results in the blocking of translation of polypeptides beyond the site of damage on the mRNA, confirmed the gene order of the nonstructural proteins of SIN and also assigned two of these proteins to particular complementation groups; the N′-*ns*60 protein was identified as the product of the G complementation group, and the *ns*89 protein was identified as the product of the A complementation group. The third protein (*ns*82) could not be assigned to either the B or F groups because of the very similar dose of UV irradiation resulting in loss of complementation with mutants of both the F (*ts*110) and the B (*ts*11) groups. The authors considered the possibility that the fourth complementation group represents a noncoding region of the genome RNA and thus functions at the nucleotide level rather than at the polypeptide level. If the B cistron represents such a noncoding region of the genome, its ability to function in minus-strand transcription would not be expected to be sensitive to protein synthesis inhibition. After the return to 28°C of SIN *ts*11-infected cells that were shifted to 39°C early in infection, the synthesis of minus-strand RNA recovered only in the absence of cycloheximide. We would suggest that, if there are only three nonstructural proteins, the *ns*82 protein contains two functionally distinct domains, one of which functions in minus-strand RNA synthesis and thus can be independently mutated, resulting in the B and F complementation groups.

That a specific function not needed for viral plus-strand transcription is involved in minus-strand synthesis is not surprising. The initiation of minus-strand synthesis may be unusual in that the 5′-initiating nucleotide may be a pyrimidine: 5′-ppUp and 5′-pUp residues have been detected in a polyuridylate sequence which is located at the 5′ terminus of Semliki Forest virus minus-strand RNA (23). A polyuridylate sequence has been found also in SIN HR minus-strand RNA (12). Although a similar 5′-terminal polyuridylate sequence is present in poliovirus minus-strand RNA (27, 33, 34), its 5′ terminus is covalently coupled to a viral protein (10, 18). Since alphavirus plus-strand RNA synthesis probably initiates with a purine residue (9, 19, 32), an additional function may be required for pyrimidine initiation. Also, the ability to regulate the amount of minus-strand RNA, which is only needed in a template capacity and is stable once synthesized (3, 24–26, 31), confers the added advantage of the efficient utilization of available substrates for single-stranded virion RNA and mRNA formation once sufficient minus-strand templates are formed. Since regulation of the initiation of minus-strand transcription would control directly the number of templates produced, translational control of the protein required for this initiation would be a sensitive regulatory mechanism. The actual process of initiation may require the function of more than a single protein. The role of one viral and several host cell proteins in QB and f2 RNA phage transcription and replication has been recently reviewed (1), and in vitro evidence indicates that several picornaviruses, e.g., poliovirus (7) and encephalomyocarditis virus (8), may utilize a host cell protein in addition to viral proteins for transcription on a plus-strand template, possibly for the initiation of transcription (7). Whether this is true for alphaviruses also remains to be determined.

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LITERATURE CITED

1. **Blumenthal, T., and G. G. Carmichael.** 1979. RNA replication: function and structure of QB-replicase. Annu. Rev. Biochem. **48**:525–548.

2. **Bracha, M., A. Leone, and M. J. Schlesinger.** 1976. Formation of a Sindbis virus nonstructural protein and its relation to 42*S* mRNA function. J. Virol. **20**:612–620.
3. **Bruton, C. J., and S. I. T. Kennedy.** 1975. Semliki Forest virus intracellular RNA: properties of the multistranded RNA species and kinetics of positive and negative strand synthesis. J. Gen. Virol. **32**:413–430.
4. **Brzeski, H., and S. I. T. Kennedy.** 1977. Synthesis of Sindbis virus nonstructural polypeptides in chicken embryo fibroblasts. J. Virol. **22**:420–429.
5. **Burge, B. W., and E. R. Pfefferkorn.** 1966. Isolation and characterization of conditional-lethal mutants of Sindbis virus. Virology **30**:204–213.
6. **Burge, B. W., and E. R. Pfefferkorn.** 1966. Complementation between temperature-sensitive mutants of Sindbis virus. Virology **30**:214–223.
7. **Dasqupta, A., P. Zabel, and D. Baltimore.** 1980. Dependence of the activity of the poliovirus replicase on a host cell protein. Cell **19**:423–429.
8. **Dmitrevia, T. M., M. V. Schleglova, and V. I. Agol.** 1979. Inhibition of activity of encephalomyocarditis virus-induced RNA polymerase by antibodies against cellular components. Virology **92**:271–277.
9. **Dubin, D. T., K. Timko, S. Gilies, and V. Stollar.** 1979. The extreme 5′-terminal sequences of Sindbis virus 26S and 42S RNA. Virology **98**:131–141.
10. **Flanigan, J. B., R. F. Pettersson, V. Ambros, M. U. Hewlett, and D. Baltimore.** 1977. Covalent linkage of a protein to a defined nucleotide sequence at the 5′-terminus of virion and replicative intermediate RNAs of poliovirus. Proc. Natl. Acad. Sci. U.S.A. **74**:961–965.
11. **Franklin, R.** 1966. Purification and properties of the replicative intermediate of the RNA bacteriophage R17. Proc. Natl. Acad. Sci. U.S.A. **55**:1504–1513.
12. **Frey, T. K., and J. H. Strauss.** 1978. Replication of Sindbis virus. VI. Poly(A) and poly(U) in virus-specific RNA species. Virology **86**:494–506.
13. **Friedman, R. M., and P. M. Grimley.** 1969. Inhibition of arbovirus assembly by cycloheximide. J. Virol. **4**: 292–299.
14. **Fuller, F. J., and P. I. Marcus.** 1980. Sindbis virus. I. Gene order of translation *in vivo*. Virology **107**:441–451.
15. **Kääriäinen, L., and H. Soderlund.** 1978. Structure and replication of alphaviruses. Curr. Top. Microbiol. Immunol. **82**:15–69.
16. **Keränen, S., and L. Kääriäinen.** 1979. Functional defects of RNA-negative temperature-sensitive mutants of Sindbis and Semliki Forest viruses. J. Virol. **32**:19–29.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
18. **Nomoto, A., B. Detjen, R. Pozzatti, and E. Wimmer.** 1977. The isolation of the polio genome protein in viral RNAs and its implication for RNA synthesis. Nature (London) **268**:208–213.
19. **Pettersson, R. F., H. Soderlund, and L. Kääriäinen.** 1980. Nucleotide sequence of the 5′-terminal T1-oligonucleotide of Semliki Forest virus 42S and 26S RNAs is different. Eur. J. Biochem. **105**:435–443.
20. **Pfefferkorn, E. R., and B. W. Burge.** 1968. Morphogenic defects in the growth of ts mutants of Sindbis virus. Perspect. Virol. **6**:1–14.
21. **Pfefferkorn, E. R., B. W. Burge, and H. M. Coady.** 1967. Intracellular conversion of the RNA of Sindbis virus into a double-stranded form. Virology **33**:239–249.
22. **Pfefferkorn, E. R., and D. Shapiro.** 1974. Reproduction of togaviruses, p. 171–230. *In* H. Fraenkel-Conrat and R. R. Wagner. (ed.), Comprehensive virology, vol. 2. Plenum Publishing Corp., New York.
23. **Sawicki, D. L., and P. J. Gomatos.** 1976. Replication of Semliki Forest virus: polyadenylate in plus-strand RNA and polyuridylate in minus-strand RNA. J. Virol **20**: 446–464.
24. **Sawicki, D. L., L. Kääriäinen, C., Lambek, and P. J. Gomatos.** 1978. Mechanism for control of synthesis of Semliki Forest virus 26S and 42S RNA. J. Virol. **25**:19–27.
25. **Sawicki, D. L., and S. G. Sawicki.** 1980. Short-lived minus-strand polymerase for Semliki Forest virus. J. Virol. **34**:108–118.
26. **Scheele, C. M., and E. R. Pfefferkorn.** 1969. Inhibition of interjacent ribonucleic acid (26*S*) synthesis in cells infected by Sindbis virus. J. Virol. **4**:117–122.
27. **Spector, D. H., and D. Baltimore.** 1975. Polyadenylic acid on poliovirus RNA. IV. Poly(U) in replicative intermediate and double-stranded RNA. Virology **67**: 498–505.
28. **Strauss, E. G., E. M. Lenches, and J. H. Strauss.** 1976. Mutants of Sindbis virus. I. Isolation and partial characterization of 89 new temperature-sensitive mutants. Virology **71**:154–168.
29. **Strauss, J. H., and E. G. Strauss.** 1980. Mutants of alphaviruses: genetics and physiology, p. 393–425. *In* R. W. Schlesinger (ed.), Togaviruses, biology, structure, replication. Academic Press, Inc., New York.
30. **Waite, M. R. F.** 1973. Protein synthesis directed by an RNA$^-$ temperature-sensitive mutant of Sindbis virus. J. Virol. **11**:198–206.
31. **Wengler, G., and G. Wengler.** 1975. Studies on the synthesis of viral RNA polymerase-template complexes in BHK-21 cells infected with Semliki Forest virus. Virology **66**:322–326.
32. **Wengler, G., G. Wengler, and H. J. Gross.** 1979. Replicative form of Semliki Forest virus RNA contains an unpaired guanosine. Nature (London) **282**:754–756.
33. **Yogo, Y., M. Y. Teng, and E. Wimmer.** 1974. Poly(U) in poliovirus minus-strand RNA is 5′-terminal. Biochem. Biophys. Res. Commun. **61**:1101–1109.
34. **Yogo, Y., and E. Wimmer.** 1973. Poly(A) and poly(U) in poliovirus double-stranded RNA. Nature (London) New Biol. **242**:171–174.