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The Effect of Loss of Regulation of Minus-Strand RNA Synthesis on Sindbis Virus Replication

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During the replication cycle of Sindbis virus minus-strand synthesis stops normally at the time that plus-strand synthesis reaches a maximum rate. We have isolated and characterized revertants of *ts*24, a member of the A complementation group of Sindbis HR mutants, that we had demonstrated previously to have a temperature-sensitive defect in the regulation of minus-strand synthesis. These revertants of *ts*24 replicated efficiently at 40° but nevertheless retained the temperature sensitive defect in the regulation of minus-strand synthesis: they continued to synthesize minus strands late in the replication cycle at 40° but not at 30° and in the presence or absence of protein synthesis. Although failure to regulate the synthesis of minus strands resulted in continuous minus-strand synthesis and in the accumulation of minus strands, the rate of plus-strand synthesis was not increased concertedly. Minus strands synthesized after the rate of plus-strand synthesis had become constant were demonstrated to be utilized as templates for 26 S mRNA synthesis. Thus, the change from an increasing to a constant rate of plus-strand synthesis during the alphavirus replication cycle cannot be governed solely by the number of minus strands that accumulate in infected cells. We present a model for the preferential utilization of minus strands as a mechanism for the cessation of minus-strand synthesis that occurs normally during alphavirus replication.

INTRODUCTION

Sindbis virus (SIN) is an enveloped, plus-strand RNA virus which replicates in a wide variety of vertebrate and invertebrate cells (Kennedy, 1980; Strauss and Strauss, 1983). Replication of SIN virus requires the 49 S genome RNA to serve initially as a messenger RNA for the translation of viral encoded nonstructural proteins which function as components of a viral RNA-dependent RNA polymerase. The RNA polymerase transcribes the parental 49 S plus-strand RNA into a complementary 49 S minus-strand which is recognized in turn as a template by the RNA polymerase for the synthesis of additional plus strands. In addition to serving as a template for 49 S plus-strand RNA, the minus strand also serves as a template for the synthesis via internal initiation of a subgenomic plus-strand RNA that sediments at 26 S and is 3′-coterminal with the 49 S plus strand and that is translated into the viral structural proteins.

Whereas the synthesis of plus strands once started continues throughout the replication cycle and does not require continued protein synthesis (Wengler and Wengler, 1975), the synthesis of minus strands is limited to the early phase of the replication cycle and requires *de novo* protein synthesis (Sawicki and Sawicki, 1980). Our studies (Sawicki *et al.*, 1981a) demonstrated that minus-strand RNA synthesis required a viral encoded function that was temperature sensitive in *ts*11, the sole member of the B complementation group of the heat-resistant strain (HR) of SIN. In contrast to *ts*11, *ts*24 of the A complementation group was demonstrated to have a temperature-sensitive (*ts*) defect in the shutoff of minus-strand RNA synthesis (Sawicki *et al.*, 1981b). This was the phenotype that we predicted for a mutant which was defective in the regulation of minus-strand

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RNA synthesis and suggested that a viral encoded polypeptide functioned to shut off the synthesis of minus strands. Because we could turn back on minus-strand synthesis in *ts*24 infected cells by shifting to the nonpermissive temperature late in the replication cycle after minus-strand RNA synthesis had shut off and in the absence of new protein synthesis, we concluded that proteolytic cleavage of a viral polypeptide was not the mechanism responsible for inactivating minus-strand RNA synthesis. Recent studies (Sawicki and Sawicki, 1985) have demonstrated that *ts*17 and *ts*133, but not *ts*4, *ts*14, *ts*15, *ts*16, *ts*19, *ts*21, and *ts*138, of the A complementation group of SIN HR possessed temperature-sensitive defects in the regulation of minus-strand RNA synthesis similar to *ts*24.

Here we report on the ability of revertants of *ts*24, *ts*17, and *ts*133 to regulate minus-strand RNA synthesis. We determined the fate of minus strands synthesized in excess of normal levels and the effect on the SIN HR replication cycle of continued minus-strand RNA synthesis. We tested our model (Sawicki and Sawicki, 1980) that the number of minus-strand templates determines the rate of plus-strand RNA synthesis.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblast (CEF) cells were prepared from 10-day-old embryos from the eggs of leukosis-free (SPF-COFAL/Marek-negative) flocks (Spafas, Roanoke, Ill.) and were grown as described previously (Sawicki *et al.*, 1981b).

The heat-resistant strain of Sindbis virus (SIN HR) and the RNA-negative temperature-sensitive mutants of the A complementation group of SIN HR have been described previously (Burge and Pfefferkorn, 1966; Strauss *et al.*, 1976; Sawicki and Sawicki, 1985). The parental SIN HR used in these studies was what we call the Ohio strain (Sawicki and Sawicki, 1985). The isolation of revertant viruses of *ts*24, *ts*17, and *ts*133 of the A complementation group and their partial characterization has also been reported previously (Sawicki and Sawicki, 1985). The second stock of *ts*24R1 (6×10^9 PFU/ml) used in these studies was prepared by infecting CEF cells at a m.o.i. of less than 1 PFU/cell with virus from the first stock of *ts*24R1, without any additional plaque purification.

Infection and RNA labeling. Monolayers of CEF cells in plastic petri dishes, usually 60 mm, were infected with a m.o.i. of 100 with either SIN HR, one of the mutants, or one of the revertants. The infection protocol was as described (Sawicki and Sawicki, 1985). Actinomycin D (usually at 2 μg/ml) was included in all media, and was the generous gift of Merck, Sharp, and Dohme (Rahway, N.J.). Cycloheximide (at 100 μg/ml) was from Boehringer-Mannheim (Indianapolis, Ind.). Radiolabeling of RNA was with [5,6-^{3}H]uridine (38 Ci/ml; ICN, Irvine, Calif.) at a final concentration of 200 μCi/ml of Dulbecco's modified Eagle's essential medium containing 2 mg/ml BSA and 22 m*M* HEPES, pH 7.4 (1.5 ml/60-mm petri dish) unless indicated.

Isolation of Sindbis RF RNA and detection of minus-strand RNA synthesis. The procedures used for the isolation of the double-stranded core (RF RNA) of the replicative intermediates and for the determination of minus-strand RNA synthesis by hybridization of heat denatured RF RNA with unlabeled, purified 49 S plus-strand RNA were as described (Sawicki *et al.*, 1981b).

Preparation of labeled SIN polypeptides. The infection, labeling, and preparation of cell extracts for polyacrylamide gel electrophoresis were as described (Sawicki and Sawicki, 1985). The samples were run on 8–12% linear gradient acrylamide gels in Laemmli buffer (Laemmli, 1970) and were processed for fluorography. Scanning of the fluorograms was with a Shimadzu CS-930 dual wavelength scanner (Shimadzu Corporation, Japan).

RESULTS

We have already reported (Sawicki and Sawicki, 1985) that revertants of *ts*24, *ts*17, and *ts*133 lost their RNA-negative phenotype and the temperature-sensitive defect in cleavage of the viral ns230 polyprotein. The revertant of *ts*133 retained the *ts* defect

in 26 S mRNA synthesis. We wanted to know if these revertants had lost or retained the temperature-sensitive defect in the regulation of minus-strand RNA synthesis. Table 1 presents the results of experiments designed to determine if *ts*17, *ts*24, or *ts*133 or their revertants would cause minus-strand RNA to be synthesized late in the infectious cycle in the presence of cycloheximide at 30° or after shift to 40°. None of the infected cultures pulse labeled late in infection at 30° synthesized significant amounts of minus-strand RNA in the presence of cycloheximide. Cultures infected with *ts*17, *ts*24, or *ts*133 or with the revertants of *ts*24, *ts*24R1, and *ts*24R2, synthesized minus-strand RNA in the presence of cycloheximide after they were shifted to 40° late in infection. The level of minus-strand RNA synthesis increased 4- to 15-fold after the shift to 40°. However, neither the revertant of *ts*17 nor the revertant of *ts*133 retained this *ts* defect in the regulation of minus-strand RNA synthesis; rather, they resembled SIN HR and failed to synthesize minus-strand RNA in the presence of cycloheximide after shift to 40°.

TABLE 1

RESUMPTION OF MINUS-STRAND SYNTHESIS IN THE PRESENCE OF CYCLOHEXIMIDE

	Cycloheximide resistant minus-strand RNA synthesis[a]			
	At 30°		At 40°	
	cpm	%[b]	cpm	%[b]
Sindbis HR	21	0.2 (1.4)	14	0.2 (1.8)
*ts*17	0	0 (0)	2919	14.3 (14.5)
*ts*17R	0	0 (0)	0	0 (0.4)
*ts*24	156	0.6 (2.3)	2363	14.9 (18.4)
*ts*24R1	314	1.6 (2.3)	1330	11.5 (18.3)
*ts*24R2	187	0.9 (0.9)	534	3.7 (15.6)
*ts*133	352	3.2 (2.3)	1124	12.3 (14.1)
*ts*133R	0	0 (0)	0	0 (0)

[a] Cultures were infected and incubated at 30° in the absence of actinomycin D until 10 hr p.i. when one set of cultures was shifted to 40°. Cycloheximide and actinomycin D were added to all cultures at 10 hr p.i. and medium containing [^{3}H]uridine, cycloheximide, and actinomycin D was added from 11 to 12 hr p.i. The RFs were isolated, denatured, and annealed in the presence of an excess of unlabeled plus strands as described under Materials and Methods. The RNase-resistant incorporation was the cpm recovered in the annealed aliquot after subtraction of the background (Sawicki *et al.*, 1981b).

[b] The percentage of the labeled viral RF RNA that was in minus-strand RNA. The values outside the parentheses represent the results from one experiment which determined the ability to synthesize minus-strand RNA by all the viruses listed; the values within the parentheses are averages of two to seven experiments. A value of zero indicates that the RNase-resistant RNA after annealing was equal to or less than the RNase-resistance in either the heated and fast cooled or the self-annealed control. (Sawicki *et al.*, 1981b).

We then asked if the kinetics of plus-strand and minus-strand RNA synthesis in cells infected with the revertants of *ts*24 differed from that in cells infected with *ts*24 or SIN HR. Infected cultures were maintained at 30° or 40° from the beginning of infection and were pulse labeled with [^{3}H]uridine for 30-min periods. At 30°, cells infected with SIN HR, *ts*24, and the two independently isolated revertants, *ts*24R1 and *ts*24R2, showed similar patterns of RNA synthesis (Fig. 1A). There was a period when the rate of viral RNA synthesis increased followed by a period of a relatively constant rate of viral RNA synthesis. The increase in the labeling of RF RNA (the double-stranded core of the viral replicative intermediates) paralleled the overall increase in the rate of RNA synthesis. Minus-strand synthesis was readily detectable early in infection but was shut off late in infection. When the infection was at 40°, essentially no or very little viral RNA synthesis was detectable in *ts*24 infected cells (data not shown). However, cultures infected with SIN HR or *ts*24R1 or *ts*24R2 showed a similar pattern of viral RNA synthesis at 40° compared to 30°. The rate of plus-strand RNA and RF RNA synthesis increased early in infection and changed to a relatively constant rate late in infection (Fig. 1B). However, whereas the synthesis of minus-strand RNA had ceased by 4–5 hr p.i. in SIN HR infected cells, minus-strand synthesis continued at

a high rate in cells infected with *ts*24R1 and *ts*24R2. Although minus-strand RNA synthesis continued at a relatively constant rate late in the replication cycle of *ts*24R1 and *ts*24R2, the rate of labeling of RF RNA and the rate of viral plus-strand synthesis remained at a constant rate (Fig. 1B). The failure of the rate of plus-strand transcription to increase might result if the new minus-strands were unstable or did not function as templates in plus-strand synthesis. If they were unstable, minus-strands would not accumulate in infected cells; if they did not function as templates, minus-strands would not be found in replicative intermediates active in viral plus-strand synthesis.

To determine whether or not minus strands continued to accumulate in *ts*24R1 infected cells, cells were infected with SIN HR or *ts*24R1 and labeled continuously with [^{3}H]uridine beginning at 1 hr p.i. and harvested at various times thereafter (Fig. 2). As shown in Fig. 2A, there was a 30- to 60-fold increase in the incorporation of [^{3}H]uridine into viral RNA during the 7-hr labeling period. In both SIN HR and *ts*24R1 infected cells, the incorporation was linear with time although the rate of incorpora-

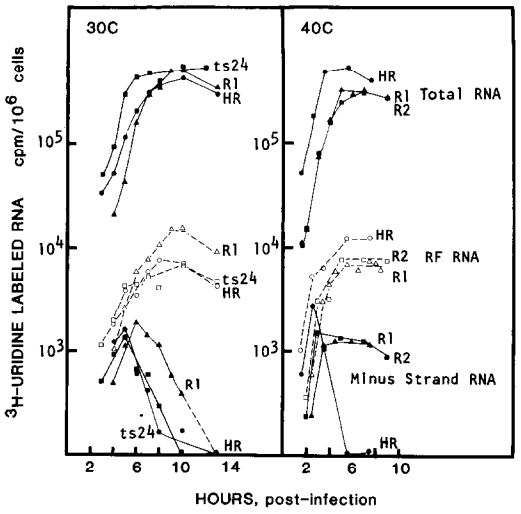

FIG. 1. Kinetics of RNA synthesis at 30 and at 40°. Cultures of CEF cells in 60-mm petri dishes were infected with SIN HR (HR), *ts*24, *ts*24R1, or *ts*24R2 in the presence of actinomycin D at 30° or at 40° as indicated. Medium containing [^{3}H]uridine and actinomycin D was added for 30-min periods beginning at 2 hr p.i. at 30° or at 1.5 hr p.i. at 40°, and the cultures were harvested at the end of the pulse period. The total acid-insoluble radioactivity in viral plus-strand RNA, RF RNA, and minus-strand RNA was determined and is expressed per 10^6 cells.

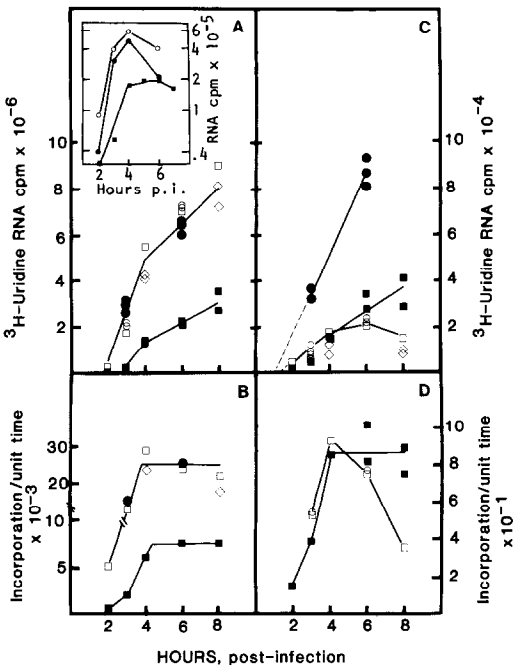

FIG. 2. Accumulation of *ts*24R1 and SIN HR plus-strand RNA and minus-strand RNA in infected cells. Cultures of CEF cells in 60-mm petri dishes were infected at 40° in the presence of actinomycin D with a m.o.i. of 100 of either SIN HR (empty symbols) or *ts*24R1 (filled symbols). The two stocks of *ts*24R1 tested are described below. Different experiments are identified by different symbols; in one experiment, virus was allowed to absorb to cells at 30° for 1 hr, and then the cultures were shifted to 40° and incubated at 40° (circles). At 1 hr p.i., the inoculum was removed and medium containing [^{3}H]uridine and actinomycin D was added to all cultures. At the times indicated, cultures from each set were harvested. The acid-insoluble radioactivity in total viral RNA (A) and in minus-strand RNA (C) was determined. The incorporation per unit time in total viral RNA (B) or minus-strand RNA (D) was obtained by dividing the incorporation at each time point by the number of minutes of the labeling period. Inset: Cultures in 35-mm petri dishes were infected as above and were pulse labeled with [^{3}H]uridine for 30-min periods between 1.5 and 6 hr p.i. The acid-insoluble radioactivity in total viral RNA is shown for cells infected with SIN HR (○), *ts*24R1, 6 × 10^9 PFU/ml (●), or *ts*24R1, 7 × 10^8 PFU/ml (■).

tion of [^{3}H]uridine between 1 and 4 hr p.i. was slightly greater than between 4 and 8 hr p.i. We tested two stocks of *ts*24R1: One stock (6×10^9 PFU/ml) replicated to the same extent as SIN HR; another stock (7×10^8 PFU/ml) gave a total incorporation of [^{3}H]uridine in infected cells that was about 40% of that in SIN HR infected cells. The insert in Fig. 2A shows that in all cultures viral RNA synthesis reached a maximum rate at about the same time after infection. And, if the amount of [^{3}H]uridine that accumulated at any time is divided by the elapsed time of labeling (Fig. 2B), then the incorporation of [^{3}H]uridine into viral RNA per unit time reached a maximum at the same time, 4–5 hr p.i., and remained constant in both SIN HR and *ts*24R1 infected cells. Because the maximum level of viral RNA synthesis attained by one stock of *ts*24R1 was only half of that attained by SIN HR or the second stock of *ts*24R1, we would argue that the leveling in the rate of viral RNA synthesis was not due to the depletion of some cellular component. In SIN HR infected cells, [^{3}H]uridine accumulated into minus-strand RNA until about 4 hr p.i. (Fig. 2C). After this time there was no further accumulation of label into minus strands. In contrast to SIN HR, cells infected with either stock of *ts*24R1 continued to accumulate [^{3}H]uridine into minus strands until at least 8 hr p.i. (Fig. 2C). The level of accumulation of minus strands in cells infected with the two stocks of *ts*24R1 was proportional to the overall incorporation in total viral RNA observed for these stocks. Figure 2D shows the result of dividing the amount of [^{3}H]uridine that accumulated into minus strands by the elapsed time of labeling. In SIN HR infected cells the [^{3}H]uridine incorporated per unit time into minus strands began to decrease about 5 hr p.i., whereas in *ts*24R1 infected cells the [^{3}H]uridine incorporated into minus strands per unit time did not decrease but remained constant between 4 and 8 hr p.i. The higher titer stock of *ts*24R1 gave a similar result, but the incorporation per unit time in minus-strand RNA was 2.5 times higher than that obtained for the lower titer stock (data not shown). We conclude from this data that minus strands continued to accumulate in *ts*24R1 infected cells, in contrast to SIN HR infected cells in which minus strands did not continue to accumulate because their synthesis ceased at about 4 hr p.i.

We next determined if the minus strands that accumulated in *ts*24R1 infected cells after the rate of viral RNA synthesis had became constant were utilized as templates for plus-strand RNA synthesis. The 49 S minus-strand RNA would be expected to be synthesized by a replicative structure with the same relative resistance to ribonuclease as replicative intermediates (RIs) engaged in 49 S plus-strand RNA synthesis, i.e., a 22 S RFI-like molecule would be expected to result from limited RNase digestion (Simmons and Strauss, 1972; Sawicki and Gomatos, 1976). Therefore, the presence of labeled minus-strand RNA associated with RFI molecules would reflect nascent or newly completed minus-strand RNA, as well as minus strands that had been synthesized during the labeling period and functioned as templates for 49 S plus-strand synthesis. The presence of newly synthesized minus strands in RFIII molecules would specifically indicate that the newly synthesized minus strands functioned as templates in plus-strand synthesis because RFII and RFIII are derived from RIs, the so-called RI_b, which are active in 26 S plus-strand RNA synthesis (Simmons and Strauss, 1972; Sawicki *et al.*, 1978). RFIII is separable from RFI molecules by velocity sedimentation. Cells infected with *ts*24R1 were labeled for 2-hr periods early or late in infection. RFI, RFII, and RFIII were generated from the viral RIs by limited digestion with RNase and were separated by centrifugation on sucrose gradients. As was expected, newly synthesized minus strands were present in viral RFs isolated from infected cells early after infection (Table 2). More significantly, all three RFs isolated from infected cells that had been labeled from 6 to 8 hr p.i. also contained newly synthesized minus-strand RNA, although the newly synthesized minus strands appeared to accumulate preferentially in RFI molecules. Therefore, minus strands synthesized in excessive numbers in *ts*24R1 infected cells

continued to accumulate in viral RIs, and at least some of these minus strands entered transcription complexes and served as templates for 26 S mRNA, and presumably also 49 S plus-strand RNA, synthesis.

Because minus strands were utilized as templates for plus-strand synthesis after the rate of plus-strand synthesis had become constant, we wanted to know whether a transcription complex needed to be newly formed in order for newly synthesized minus strands to be utilized as templates or whether newly synthesized minus strands would replace old minus strands as templates for transcription. Late in infection viral RNA synthesis continued in the absence of protein synthesis. The rate of viral RNA synthesis became constant at about 4 hr p.i. and the addition of cycloheximide at 4 hr p.i. resulted in the same rate of viral RNA synthesis as in the absence of cycloheximide (data not shown). In *ts*24R1 infected cells the minus strands synthesized in the absence of protein synthesis between 4 and 6 hr p.i. did not accumulate as single-stranded 49 S molecules: no labeled minus strands could be detected in the 49 S region of a sucrose gradient (data not shown). However, labeled minus strands that were synthesized in the presence of cycloheximide were found in RF cores of the viral RIs (Fig. 3). Labeled minus strands were detected in RFI and RFIII as early as 30 min after the addition of [^{3}H]uridine to *ts*24R1 infected cells that had been either treated or not treated with cycloheximide at 4 hr p.i., and the accumulation of [^{3}H]uridine into minus strands was proportional to the length of the labeling period. More [^{3}H]uridine-labeled minus strands accumulated in RFI than in RFIII in both cycloheximide and untreated cultures. The rate of accumulation of labeled minus-strand RNA in RFI and RFIII cores in the presence of cycloheximide was reduced by 40 and 17%, respectively, compared to that detected in the absence of cycloheximide. Therefore, the accumulation of newly synthesized minus strands was affected to a slight degree by the presence of the drug. These results are in marked contrast to cells infected with SIN HR: almost no [^{3}H]uridine was incorporated into minus strands after 4 hr p.i. at

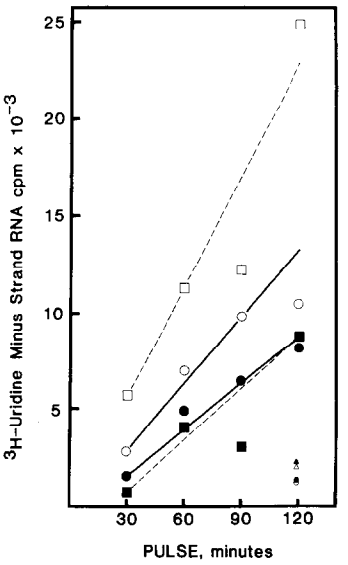

FIG. 3. Accumulation of newly synthesized minus-strand RNA at 40° in cells infected with *ts*24R1 or SIN HR. RFI and RFIII were obtained from *ts*24R1 or SIN HR infected cells that had been labeled beginning at 4 hr p.i. for 30–120 min with [^{3}H]uridine in the presence or absence of cycloheximide. *ts*24R1 in the absence (RFI, □; RFIII, ■) or in the presence of cycloheximide (RFI, ○; RFIII, ●). SIN HR in the absence (RFI, △; RFIII, ▲) or in the presence of cycloheximide (RFI, ○; RFIII, ●).

TABLE 2

MINUS STRANDS MADE LATE ARE USED AS TEMPLATES FOR 26 S mRNA[a]

[^{3}H]Uridine (hr p.i.)	Minus-strand RNA (cpm)		
	RFI	RFII	RFIII
2–4	6,976	3451	1712
6–8	10,437	3772	2051

[a] CEF cells in 100-mm petri dishes were infected with *ts*24R1 at 40° in the presence of actinomycin D. Cultures were labeled for the times indicated with medium containing [^{3}H]uridine (200 μCi/ml, 5 ml/culture) and actinomycin D. The viral RFs, the RNase-A resistant core of the RIs, were obtained (Sawicki *et al.*, 1981b), and the presence of labeled minus-strand RNA was determined by hybridization. From 40 to 60% of the total labeled RF RNA annealed to an excess of unlabeled virion plus strands.

40° during a 2-hr labeling period either in the absence or presence of cycloheximide. Therefore, in *ts*24R1 infected cells newly made minus strands replaced at 40° previously synthesized minus strands. This suggested that old minus strands accumulated as RFI structures and was reminiscent of the turnover of poliovirus minus-strand RNA which accumulate as RF structures (Baltimore, 1968). The rate of flow of newly synthesized minus strands into poliovirus RIs was similar to that observed for alphavirus minus strands early in infection (Sawicki and Sawicki, 1980). Figure 4 shows that [^{3}H]uridine normally labeled the minus-strand component of the RF RNA early but not late in alphavirus infected cells. When Semliki Forest virus infected cells were labeled with [^{3}H]uridine beginning at 2 hr p.i. at least 35% of the label in RF RNA was in minus strands within 10 min. However, if [^{3}H]uridine was added at 3 hr p.i. or later, essentially no labeled minus strands were detected in the RF RNA; all of the label was in plus strands. In contrast to SIN HR infected cells, when cells infected with *ts*24R1 were labeled with [^{3}H]uridine at 4 hr p.i. or later, labeled minus strands accumulated in RF RNA. However, instead of taking only 10 min, it took 60–90 min before 35% of the label in RF RNA was in minus strands. Therefore, the minus strands synthesized late in *ts*24R1 infected cells may enter a pool of previously synthesized minus strands before being utilized as templates for plus-strand synthesis or only some of the newly synthesized minus strands are utilized as templates for plus-strand synthesis. Thus, the *ts* defect in *ts*24 affecting minus-strand synthesis may reside in a function normally responsible for the stable association of alphavirus minus strands with an active replication complex.

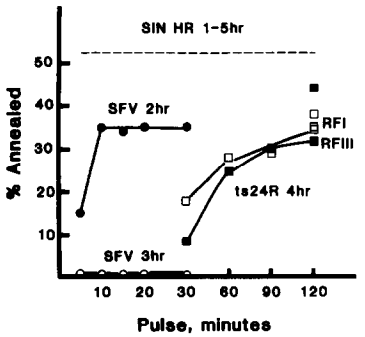

FIG. 4. Accumulation of alphavirus minus strands in RF RNA early and late. The percentage of labeled RFI (□) and RFIII (■) obtained from *ts*24R1 infected cells incubated at 40° and labeled with [^{3}H]uridine beginning at 4 hr p.i. (from Fig. 3) and harvested at 30, 60, 90, and 120 min later or beginning at 6 hr p.i. and harvested after 120 min that was in minus-strand RNA. SFV infected cells were pulse labeled for 5, 10, 15, 20, and 30 min with [^{3}H]uridine beginning at either 2 hr p.i. (●) or at 3 hr p.i. (○) (Sawicki and Sawicki, 1980). The dashed line is the average value (53%, range 40–64%) obtained from 13 determinations of the percentage of label in RF RNA that was in minus-strand RNA when SIN HR infected cells were labeled with [^{3}H]uridine from 1 to 5 hr p.i.

Both *ts*24R1 and *ts*24R2 replicated as efficiently at 40° as at 30° and continued to synthesize minus strands at 40° in the absence of protein synthesis if viral RNA and protein synthesis were allowed to start first before adding cycloheximide. Therefore, using these revertants, we were able to ask directly if there was a pool of viral proteins present early in the alphavirus replication cycle that would assemble into transcription complexes with newly synthesized minus-strand templates. The results of an experiment designed to answer this question is shown in Fig. 5. The addition of cycloheximide to cells infected with *ts*24R1 at half-hour intervals beginning at 1.5 hr p.i. resulted in the failure to attain the level of viral RNA synthesis attained by the untreated infected cells. Under the conditions described in Fig. 5, cycloheximide inhibited [^{14}C]amino acid or [^{35}S]methionine incorporation by 94–97% within 5 min (data not shown). Inhibition of protein synthesis between 1.5 and 2 hr p.i. resulted in the greatest proportional increase (about threefold) in the rate of viral RNA synthesis after stopping protein synthesis with cycloheximide. Thus, early in infection and under conditions of continued minus-strand RNA synthesis but no new viral protein synthesis, there appeared to be a pool of viral proteins sufficient to form additional transcription complexes for only about 20–30 min. Another interpretation

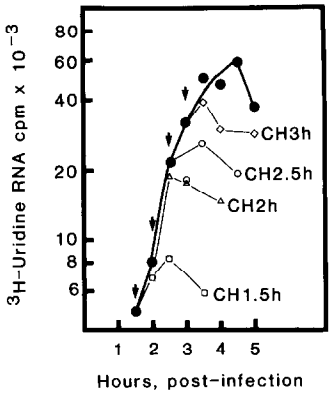

FIG. 5. The effect of cycloheximide addition on the rate of viral RNA synthesis in *ts*24R1 infected cultures at 40°. Duplicate sets of infected cultures in 35-mm petri dishes were treated with cycloheximide (100 μg/ml) at various times after infection at 40°. Cultures were pulse labeled with [^{3}H]uridine for 30-min periods between 1 and 5 hr p.i. in the absence of cycloheximide (filled symbol) or were pulse labeled for 30-min periods in the presence of cycloheximide (empty symbols) beginning at the time of addition of the drug (indicated in the figure) and at two other intervals during the next 2 hr. Acid-insoluble radioactivity in each culture is shown.

is that there is a 15- to 30-min lag between when minus strands associate with a viral polymerase and when they become active as templates for plus-strand synthesis. This was the same result obtained with Semliki Forest virus (Sawicki and Sawicki, 1980) and SIN HR (Sawicki *et al.*, 1981a) which do not continue to synthesize minus strands in the presence of cycloheximide. Therefore, both the continued synthesis of minus strands and of viral proteins are required to increase the rate of viral RNA synthesis early in infection.

Figure 6 presents the results of an analysis of the ability of cells infected with SIN HR or *ts*24R1 to synthesize viral nonstructural proteins at early and late times. The synthesis of viral proteins at 40° was clearly apparent, in the absence of hypertonic treatment, between 1.5 and 2 hr p.i., reaching maximal rates of synthesis at 4.5 to 5 hr p.i. In cells infected with both SIN HR or *ts*24R1, the synthesis of viral nonstructural proteins continued at a high rate late in infection. Furthermore, cells infected with *ts*24R1 synthesized greater amounts of the viral nonstructural proteins than did cells infected with SIN HR. The increased rate of synthesis of nonstructural proteins was related to a high level of 49 S RNA synthesis in the *ts*24R1 infected cells. However, in cells infected with SIN HR and in cells infected with *ts*24R1 the maximum rate of viral RNA synthesis was about the same and occurred at the same time (insert, Fig. 2A). The large molecular weight precursors seen in the *ts*24R1 infected cells were also present, but in reduced amounts, in SIN HR infected cells. A similar increased production of the viral nonstructural proteins was also observed in *ts*24R2 infected cells (Sawicki and Sawicki, 1985).

DISCUSSION

A summary of our analysis of the revertants of the three A complementation group mutants which possessed defects in the regulation of minus-strand synthesis

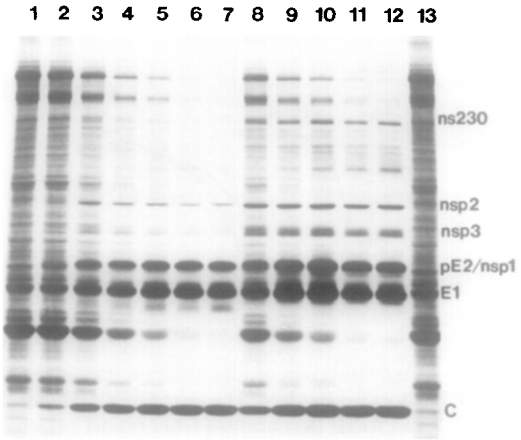

FIG. 6. Protein synthesis in SIN HR and SIN HR *ts*24R1 infected cells. CEF cells were infected with 100 PFU/cell of SIN HR or *ts*24R1 and maintained at 40° in the presence of actinomycin D. The cells were pulse labeled for 30 min with [^{35}S]methionine at 1.5 hr (lane 2), at 2.5 hr (lanes 3 and 8), 3.5 hr (lanes 4 and 9), 4.5 hr (lanes 5 and 10), 5.5 hr (lanes 6 and 11), and 6.5 hr (lanes 7 and 12). Lanes 1 and 13 are of mock-infected cells. Lanes 2–7 are of SIN HR infected cells and lanes 8–12 are of SIN HR *ts*24R1 infected cells. The gel shown is an 8 to 12% Laemmli (1970) gel.

is presented in Table 3. All the revertants lost both the RNA-negative phenotype and the *ts* defect in cleavage of the ns230 polyprotein. The *ts* defects in synthesis of 26 S mRNA and in regulation of minus-strand synthesis were segregated independently from both the RNA-negative phenotype and the *ts* defect in ns230 cleavage (this report; Sawicki and Sawicki, 1985). The *ts*133 revertant retained the *ts* defect in 26 S mRNA synthesis but regained the capacity to regulate minus-strand synthesis at 40°; the revertants of *ts*24 lost the *ts* defect in 26 S mRNA synthesis but retained the *ts* defect in the regulation of minus-strand synthesis. Thus, only in the revertants of *ts*24 was the *ts* defect in the regulation of minus-strand synthesis independent of all other *ts* phenotypes. Also, because the revertants of *ts*24 replicated efficiently at 40°, the inability to regulate alphavirus minus-strand synthesis was not of itself conditionally lethal.

Our characterization of minus-strand synthesis in cells infected with SIN HR *ts*24 (Sawicki *et al.*, 1981b) and in cells infected with revertants of *ts*24 demonstrate that previously formed replication complexes are able to associate with newly synthesized minus strands and synthesize plus strands. Continued synthesis of minus strands must have resulted either from reactivation of inactive replication complexes or from the replication complex exchanging a minus-strand template for a plus-strand template. Our results favor the latter interpretation because the rate of plus-strand synthesis does not increase in *ts*24R1 infected cells even though they accumulated two to four times more minus strands than SIN HR infected cells and continued to synthesize high levels of the nonstructural proteins. Friedman and Grimley (1969), Scheele and Pfefferkorn (1969), Ranki and Kaariainen (1970), Bruton and Kennedy (1975), Wengler and Wengler (1975), and Sawicki and Sawicki (1980) demonstrated that the alphavirus replication complex, once formed, was stable and continued to synthesize plus strands in the absence of new protein synthesis. However, in *ts*24R1 or *ts*24R2 infected cells the continued synthesis and utilization of minus strands late in infection and in the absence of continued protein synthesis demonstrated that newly made minus strands replaced old minus strands. Normally minus strands would not be replaced by newly formed minus strands because minus-strand synthesis ceased late in infection or in the absence of protein synthesis.

Our original model (Sawicki *et al.*, 1981b) for the mechanism by which alphaviruses temporally regulate minus-strand synthesis involved a regulator whose activity re-

TABLE 3

SUMMARY OF TEMPERATURE-SENSITIVE DEFECTS POSSESSED BY *ts*24, *ts*17, AND *ts*133 OF THE A COMPLEMENTATION GROUP AND BY THEIR REVERTANTS

Virus	RNA negativity	Temperature sensitivity of: ns230 cleavage	26 S mRNA synthesis	Cycloheximide-resistant minus-strand synthesis
Sin HR	−	−	−	−
*ts*24	+	+	+	+
*ts*24R1	−	−	−	+
*ts*24R2	−	−	−	+
*ts*17	+	+	+	+
*ts*17R	−	−	−	−
*ts*133	+	+	+	+
*ts*133R	−	−	+	−

sulted in the selective inactivation of replication complexes engaged in minus-strand synthesis and suggested that *ts*24 contained a *ts* mutation in the regulator. Although these studies do not rule out this model, another model which is equally attractive predicts that the preferred template of the viral polymerase is the minus strand. In the preferred template model, the accumulation of minus strands would lead eventually to replication complexes synthesizing exclusively plus strands because new replication complexes stop being formed late in infection or after treatment with cycloheximide. Mutations in *ts*17, *ts*24, *ts*133, and the revertants of *ts*24 allow for the continuation of minus-strand synthesis because at 40°, but not at 30°, the replication complex switches templates from a minus strand to a plus strand. This results in minus-strand synthesis with the newly synthesized minus strand being used preferentially as a template for plus-strand synthesis and the old minus strand becoming inactive. It will be important to determine if the mutations responsible for the *ts* regulation of minus-strand RNA synthesis are located in the viral polymerase (*trans*-acting) or in a *cis*-acting sequence of the viral RNA.

Why did the overproduction of potentially functional minus strands in cells that were also synthesizing nonstructural proteins *not* result in increased rates of viral RNA synthesis? The overall rate of RNA synthesis might not be expected to increase if newly synthesized minus strands merely replaced old minus strands in replication complexes. This would occur if the number of replication complexes became fixed early during infection. Factors in addition to the nonstructural proteins and the number of minus strands appear to limit the rate of transcription late in infection. Host factors have been implicated as playing a role in alphavirus replication (Kowal and Stollar, 1981; Scheefers-Borchel *et al.*, 1981; Baric *et al.*, 1983). However, because we find (Fig. 2A, insert) that different stocks of a virus isolate caused different levels of viral RNA synthesis in the same host cells, we argue that host factors alone cannot be the determinant which sets the level of transcription in alphavirus infected cells. The results of our studies reemphasize the uniqueness of alphavirus replication when compared with that of picornaviruses (Baltimore, 1969) and coronaviruses (Sawicki and Sawicki, 1986), both of which require continued protein synthesis for plus-strand as well as minus-strand RNA synthesis.

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