JOURNAL OF VIROLOGY, June 1993, p. 3605–3610
0022-538X/93/063605-06$02.00/0

Vol. 67, No. 6

A Second Nonstructural Protein Functions in the Regulation of Alphavirus Negative-Strand RNA Synthesis

DOROTHEA L. SAWICKI* AND STANLEY G. SAWICKI

Department of Microbiology, Medical College of Ohio, P.O. Box 10008, Toledo, Ohio 43699-0008

Received 20 October 1992/Accepted 24 February 1993

Previous studies (D. L. Sawicki, D. B. Barkhimer, S. G. Sawicki, C. M. Rice, and S. Schlesinger, Virology 174:43–52, 1990) identified a temperature-sensitive (*ts*) defect in Sindbis virus nonstructural protein 4 (nsP4) that reactivated negative-strand synthesis after its normal cessation at the end of the early phase of replication. We now report identification of two different *ts* alterations in nsP2 of Ala-517 to Thr in *ts*17 or Asn-700 to Lys in *ts*133 that also reactivated negative-strand synthesis. These same mutations caused severely reduced protease processing by nsP2 and recognition of the internal promoter for subgenomic mRNA synthesis and were responsible for the conditional lethality and RNA negativity of these mutants. Reactivation of negative-strand synthesis by mutations in nsP2 resembled that in nsP4: it was a reversible property of stable replication complexes and did not require continuation of viral protein synthesis. Recombinant viruses expressing both mutant nsP2 and nsP4 reactivated negative-strand synthesis more efficiently than did either mutant protein alone, consistent with the hypothesis that both nsP2 and nsP4 participate in template recognition. We propose that these alterations cause nsP2 and nsP4 to switch from their normal preference to recognize negative strands as templates to recognize positive strands and thereby mimic the initial formation of a replication complex.

Four members of the Sindbis superfamily (1, 7, 8), Sindbis virus (SIN) and Semliki Forest virus of the *Alphavirus* genus of the *Togaviridae* family, tobacco mosaic virus of the *Tobamoviridae* family, and brome mosaic virus of the *Bromoviridae* family, selectively cease negative-strand RNA synthesis at the end of the early phase of the replication cycle (15, 19, 27, 30). For alphaviruses, inhibition of translation during the early phase inhibits negative-strand synthesis and limits positive-strand transcription to the rate existing at the time translation was inhibited (27, 30). During the late phase, when negative-strand synthesis is no longer detectable, inhibition of translation has no effect on transcription (27, 30). Thus, both the positive- and negative-strand polymerases are composed of viral nonstructural proteins (nsPs) synthesized early in infection, but only the negative-strand polymerase is unstable and needs to be continuously produced. SIN nsPs, numbered according to their gene order, are translated initially as polyproteins nsP123 and nsP1234, the latter arising from read-through of an opal codon at the end of the nsP3 open reading frame, before cleavage by the nsP2 protease to yield nsP1, nsP2, nsP3, and nsP4 or the fusion protein nsP34 (3, 4, 12, 13, 22, 34, 35, 38).

The mechanism responsible for cessation of negative-strand synthesis is unknown. The existence of SIN temperature-sensitive (*ts*) mutant *ts*24 and its revertants (e.g., 24R1), which were capable of turning back on negative-strand synthesis at 40°C late in infection either in the absence of new protein synthesis (28, 31, 32) or in the presence of wild-type nsPs (25), suggested that cessation did not involve an irreversible loss of negative-strand polymerases shortly after their synthesis or a *trans*-active viral protein that turned off negative-strand synthesis. Although the mutation responsible for *ts*24 conditional lethality mapped to nsP2 (10), the mutation conferring *ts* reactivation of negative-strand synthesis was in nsP4, which contains a conserved XGDD replicase sequence (14, 16, 21, 37) and functions in template recognition and elongation (25). Two other A complementation group mutants, *ts*17 and *ts*133, exhibited a similar *ts* reactivation of negative-strand synthesis (28) that, however, was not retained by their respective revertants.

Analysis of nonstructural polyprotein cleavage patterns led de Groot and colleagues (3, 11) and Strauss and Strauss (38) to suggest that the accumulation of nsP2 proteases would lead to cessation of negative-strand synthesis because polyprotein processing would be altered late in infection and prevent production of nsP4 but, in turn, favor production of nsP34. Our results (28) with *ts*17 were interpreted to support this model because loss of 26S mRNA synthesis and reactivation of negative-strand synthesis after a shift to 40°C would occur from nsP34 cleavage and release of nsP4. We set out to test directly their model with use of *ts*17 and *ts*133 by mapping the responsible mutations and determining whether an unstable nsP, such as nsP34, played a role in reactivation.

The mutations in *ts*17 and *ts*133 map to nsP2. Reversion frequencies suggested that a single-point mutation in *ts*17 and one or possibly two mutations in *ts*133 (31) were responsible for *ts* reactivation of negative-strand synthesis. To map the mutation in *ts*17, we used hybrid cDNA clones originally constructed by Hahn et al. (10) (Table 1). They reported that only Toto:*ts*17B- and Toto:*ts*17B1-derived viruses were *ts* for plaque formation, RNA negativity, and nonstructural polyprotein cleavage. We confirmed these results (Table 1; data not shown) and, furthermore, found that only *ts*17, Toto:*ts*17B, and Toto:*ts*17B1 viruses had *ts* 26S mRNA synthesis and reactivated negative-strand synthesis (Table 1). Sequencing of Toto:*ts*17B1 cDNA independently identified a single mutation at nucleotide (nt) 3228 and its true reversion in a *ts*17 revertant genome (data not shown; 10). Our findings thus extend previous analyses by showing that the predicted change of Ala-517 to Thr in nsP2 was responsible for all phenotypes of *ts*17.

By creating similar recombinant clones, the *ts*133 mutation was mapped to the B1 region (Table 1), which contained

* Corresponding author.

TABLE 1. Analysis of *ts*17 and *ts*133 constructs

Parental virus or recombinant clone	Replaced fragment (nt)	Phenotype[a]	% of labeled RF RNA in negative strands[b]
SIN HR		wt	5.8
*ts*17		*ts*	16.1
Toto:*ts*17A[c]	504–2713	wt	4.6
Toto:*ts*17B	2713–5262	*ts*	17.5
Toto:*ts*17B1	2713–4280	*ts*	17.7
Toto:*ts*17C	5262–7334	wt	5.7
*ts*133		*ts*	21.2
Toto:*ts*133B1	2288–4280	*ts*	19.6
Toto:*ts*133CX[d]	2713–3594	wt	5.9
Toto:*ts*133XA[d]	3594–4280	*ts*	14.8

[a] Viruses having a phenotype indicated as wild type (wt) gave similar numbers of plaques at 30°C and 40°C, parental virus levels of overall RNA synthesis when infected cells were maintained at 40°C, and continued synthesis of 26S mRNA after a shift to 40°C. The latter was determined from the molar ratio of incorporation into 49S and 26S RNAs obtained after electrophoresis of cell lysates on agarose gels and cutting out and counting the respective two areas of the gels.

[b] Negative-strand synthesis was determined as described previously (30).

[c] The *ts*17 recombinant clones are those described by Hahn et al. (10).

[d] Toto:*ts*133CX is the recombinant clone containing the *Cla*I (nt 2713)-to-*Xmn*I (nt 3594) fragment from *ts*133 cDNA, and Toto:*ts*133XA is that containing the *Xmn*I-to-*Avr*II (nt 4280) fragment from *ts*133. The clones were constructed as described previously (23, 25) and sequenced (24).

TABLE 2. Reversibility of 26S mRNA synthesis

Incubation conditions	Cycloheximide	49S/26S RNA molar ratio	
		*ts*17	*ts*133
Maintained 30°C	−	0.10	
	+	0.06	0.11
Shifted to 40°C at 6 h p.i.	+	2.73	1.31
Shifted to 40°C at 6 h p.i., returned to 30°C at:			
7 h p.i.	+	0.06	0.14
8 h p.i.	+	0.06	0.16
9 h p.i.	+	0.07	0.15

base changes at nt 2605 (A → T), 3579 (A → G), 3779 (C → G), and 3897 (C → G) from the Toto1101 sequence. A revertant of *ts*133 (28, 31) retained the changes at nt 2605 and 3897 but reverted nt 3579 to the parental base and restored Asn-700 by changing the G at nt 3779 to a U instead of the parental C. Only recombinant virus containing the sequence from nt 3594 to 4280 reproduced the phenotypes of *ts*133 (Table 1). We conclude that in *ts*133, a single mutation at nt 3779, predicting a change of Asn-700 to Lys in nsP2, was necessary and sufficient for its *ts* phenotypes, and unlike the case for *ts*24, conditionally lethal mutations led to *ts* reactivation of negative-strand synthesis by *ts*17 and *ts*133. Because the *ts*133 (28) and *ts*24 (10, 28) mutations had reversion frequencies (10^{-7} to 10^{-8}) lower than expected for a single mutation in an RNA genome, sequences surrounding these nucleotides may influence reversion to the wild-type sequence. As shown in Fig. 1, the region of nsP2 containing the *ts*133 mutation is relatively highly conserved among alphaviruses and near the mutation in *ts*24, which also makes the protease *ts*. It contains a pattern of leucine residues that, while not heptadic, is present in the otherwise nonconserved rubella virus sequence (5) and may have a structural role in hydrophobic interactions. The *ts*17 change in nsP2 is not in a highly conserved region (10), and thus critical residues or overall structure may be important.

Reversal of *ts* RNA phenotypes is independent of new protein synthesis. If expression of the two phenotypes were coupled to nsP34 cleavage at 40°C, restoration of the 30°C patterns of high 26S mRNA synthesis and shutdown of negative-strand synthesis would require translation of new nsP34 proteins at 30°C. Therefore, we determined whether *ts*17- and *ts*133-infected cells shifted to 40°C late in infection continued negative-strand synthesis and reduced mRNA synthesis after a return to 30°C in the absence of new protein synthesis. This approach permitted us to assess the activities of viral proteins synthesized prior to the shift, since previous studies indicated that these proteins reactivated negative-strand synthesis (28, 31). The assay was performed as follows. Cycloheximide (100 μg/ml) was added beginning at 6 h postinfection (p.i.) when appropriate. Labeling was from 9.5 to 10.5 h p.i. by refeeding the cultures with Dulbecco modified Eagle medium (DMEM) containing 200 μCi of [^{3}H]uridine per ml and 20 μg of actinomycin D per ml in the presence or absence of cycloheximide and at the respective temperatures. Shifting to 40°C or back to 30°C was accomplished by two washes with DMEM at the respective temperature before refeeding with complete DMEM. For both *ts*17 and *ts*133, a shift to 40°C led to decreased 26S mRNA synthesis and increased molar ratios of 49S to 26S RNAs (Table 2). Upon a return to 30°C in the absence of protein synthesis, restoration of 26S mRNA synthesis and of normal 30°C molar ratios was observed. Reversibility was not de-

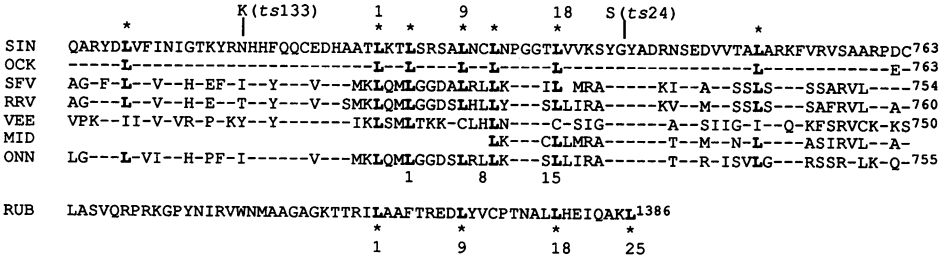

FIG. 1. Comparison of alphavirus and rubella virus amino acid sequences in a region of nsP2 encompassing the *ts*133 and *ts*24 mutations. Certain leucine residues are in bold, and patterns of leucine residues are indicated by numbers that refer to their relative spacing; a three-heptad repeat was noted in the O'Nyong-nyong sequence. Sequence data are from Strauss et al. (37) for SIN, Shirako et al. (33) for Ockelbo virus (OCK), Takkinen (39) for Semliki Forest virus (SFV), Faragher et al. (6) for Ross River virus (RRV), Kinney et al. (18) for Venezuelan equine encephalitis virus (VEE), Hahn et al. (10) for Middleburg virus (MID), Strauss et al. (36) for O'Nyong-nyong virus (ONN), and Dominguez et al. (5) for rubella virus (RUB).

TABLE 3. Reversibility of negative-strand synthesis

Expt	Incubation conditions	% of labeled RF RNA in negative-strand RNA[a]			
		*ts*17	*ts*133	SIN HR	24R1
1	Maintained at 30°C	1.9	1.8		
	Shifted to 40°C at 6 h p.i.	13.8	13.6		
	Shifted to 40°C at 6 h p.i., returned to 30°C at:				
	7 h p.i.	0.9	3.7		
	8 h p.i.	1.1			
	9 h p.i.	0.9	4.3		
2	Maintained at 30°C	2.0		1.4	4.8
	Shifted to 40°C at 6 h p.i.	11.2		1.8	16.7
	Shifted to 40°C at 6 h p.i., returned to 30°C at 9 h p.i.	2.4		<1.0	3.6

[a] The values shown are averages of multiple hybridizations of RFs from two to five experiments.

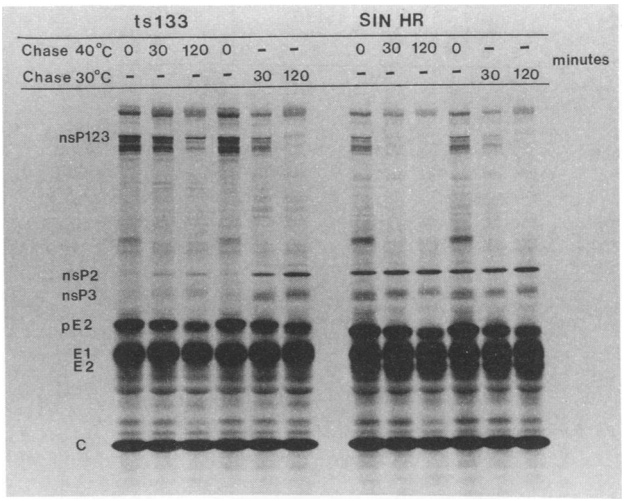

FIG. 2. Assay determining whether polyproteins produced at 40°C by *ts*133-infected cells were cleaved at 30°C but not at 40°C. Chicken embryo fibroblast cells infected with *ts*133 or SIN HR were maintained at 30°C until 6 h p.i., when they were shifted to 40°C, treated with hypertonic salt medium (0.335 M NaCl in DMEM lacking methionine and containing 5% dialyzed fetal bovine serum and 22 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid [HEPES; pH 74]) for 40 min, and given a 30-min pulse of [^{35}S]methionine (50 μCi/ml). Duplicate cultures were chased at either 40 or 30°C for the times indicated. Samples were electrophoresed on 5 to 10% polyacrylamide gels in Laemmli buffer (20) and fluorographed. An autoradiograph of the gel is shown.

pendent on the length of time spent at 40°C, since similar levels were reached after 1, 2, or 3 h of incubation.

To test the reversibility of negative-strand synthesis, cells were infected with each virus at a multiplicity of infection of 100 and were treated and labeled as described above except that cultures in experiment 1 were labeled from 9.5 to 10.5 h p.i. and cultures in experiment 2 were labeled from 9.5 to 11.5 h p.i. The cells were harvested by lysis at 0°C in buffer containing 1% Triton X-100 and scraped from the dish, the nuclei were removed by centrifugation at 1,000 rpm, and the supernatant was adjusted to 1% lithium dodecyl sulfate–100 μg of proteinase K per ml before extraction with phenol and chloroform and ethanol precipitation of the nucleic acids. The viral replicative forms (RFs) were purified, and the amount of labeled minus strands in the RF RNA was determined as described previously (25). Reactivation of *ts*17 and *ts*133 negative-strand synthesis was also temperature dependent and fully reversible in cultures treated with cycloheximide from the time of the shift to 40°C (Table 3). Reversal did not depend on the length of time spent at 40°C before shift-down. Table 3 also shows that this pattern resembled that for the nsP4 mutant 24R1.

Polyprotein processing. Although nsP1234 and nsP123 polyproteins synthesized at 40°C by *ts*133 (Fig. 2) and *ts*17 (data not shown; 11, 28) were not cleaved or poorly cleaved at 40°C compared with parental proteins, processing occurred when the cultures were shifted down and chased at 30°C. Because proteolytic processing of the nsPs was reversible, these mutations most likely affect the activity of nsP2 protease rather than the cleavage sites in the polyprotein. To mimic conditions used to demonstrate reactivation of negative-strand synthesis, we determined whether nonstructural polyproteins synthesized at 30°C were cleaved at 40°C to yield mature nsPs. As shown in Fig. 3A, the nsP2 defects in *ts*17 and *ts*133 allowed efficient processing of polyproteins at 30°C but reduced processing at 40°C. Moreover, immunoprecipitation (Fig. 3B) and quantitation of labeled *ts*17 nsPs found that compared with the 30°C chase period, only 18 to 50% as much nsP34, nsP3, and nsP4 were present after the 40°C chase, while nsP2 levels were unchanged from levels present at the end of the 30°C pulse period. In summary, our analysis did not support the concept (3) that reactivation of negative-strand synthesis resulted from specific cleavage at 40°C of *ts*17 polyproteins.

Recombinants possessing both mutant nsP2 and nsP4. Our data suggested that nsP2 and nsP4 interact functionally, since mutations in the two proteins show similar phenotypes. Furthermore, the conditional lethality of *ts*118 requires mutations in both nsP2 and nsP4 (9). To probe such interactions, we created a recombinant genome that contained both the *ts*17 nsP2 and 24R1 nsP4 lesions and tested them as follows. Infected cultures were shifted to 40°C at 6 h p.i. in the presence of cycloheximide (100 μg/ml) and labeled with [^{3}H]uridine (200 μCi/ml in the presence of 20 μg of actinomycin D per ml) from 6.5 to 8.5 h p.i. Purified RF RNA was denatured and hybridized to an excess of purified SIN virion plus strands as described previously (25). Viruses possessing both mutant nsP2 and nsP4 genes (Toto:*ts*17:24R1) were viable and expressed all of the *ts*17 phenotypes (Table 4). The double mutants also resumed negative-strand synthesis when shifted to 40°C but at higher levels typical of 24R1 viruses which possessed only the mutation in nsP4 (31). In cells infected with *ts*17, 24R1, and Toto:*ts*17:24R1, negative strands synthesized at 40°C were stable and accumulated as double-stranded RNA at a rate that was linear but higher for Toto:*ts*17:24R1 and 24R1 than for *ts*17 (Fig. 4A). For all three viruses, significant fractions of the replicative intermediate and RF populations reactivated negative-strand transcription (Fig. 4B). Consistently, we observed in multiple experiments the accumulation of negative strands by the double mutant Toto:*ts*17:24R1 that was the sum of the amounts accumulated by 24R1 and *ts*17, and we concluded there was an additive effect when replication complexes contained both mutant nsP2 and nsP4 and that the nsP2 defect did not interfere with or alter significantly the expression of the nsP4 defect or vice versa. However, replication complexes possessing mutant nsP4 were more efficient at reactivating negative-strand synthesis than were those containing only mutant nsP2. These results suggest that the nsPs

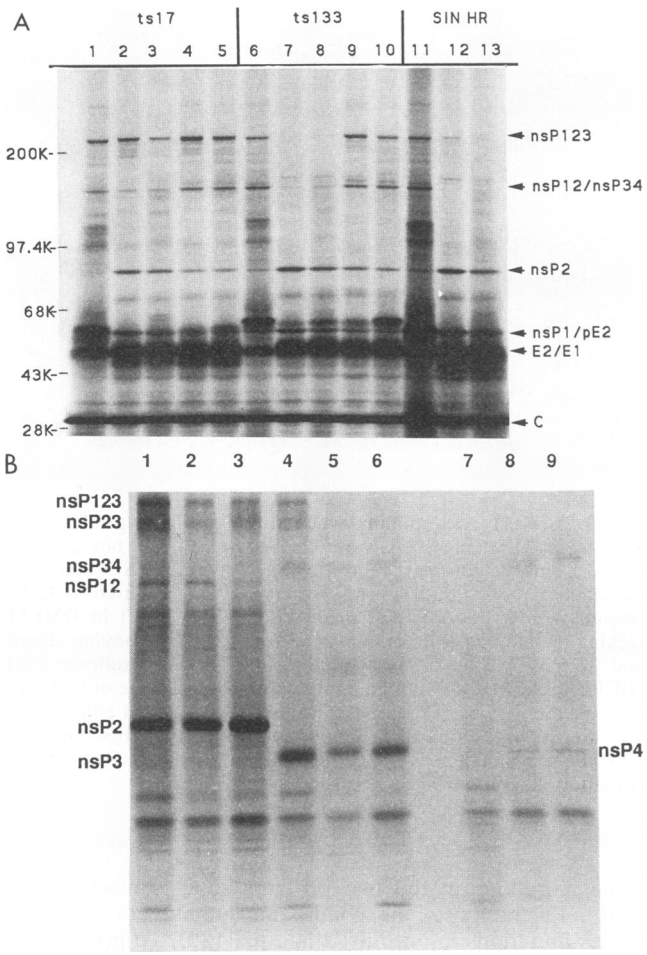

FIG. 3. Assays determining whether polyproteins produced at 30°C by *ts*17- and *ts*133-infected cells were cleaved in the presence or absence of cycloheximide at 30°C but not at 40°C. (A) Chicken embryo fibroblast cells in 35-mm-diameter petri dishes were infected with *ts*17, *ts*133, or SIN HR at a multiplicity of infection of 100 and maintained at 30°C. At 4 h p.i., the cultures were incubated in medium lacking methionine and containing 2 μg of actinomycin D per ml, 5% dialyzed fetal bovine serum, and 22 mM HEPES (pH 7.4) for 2 h and then treated for 90 min with the same medium containing 0.335 M NaCl. Cultures were pulsed with [^{35}S]methionine (50 μCi/ml) for 30 min at 30°C, after which they were either harvested immediately (lanes 1, 6, and 11) or chased for 60 min at 30°C in the absence (lanes 2, 7, and 12) or presence (lanes 3, 8, and 13) of 100 μg of cycloheximide per ml. Duplicate cultures were shifted to 40°C and chased at 40°C for a further 60 min in the absence (lanes 4 and 9) or presence (lanes 5 and 10) of cycloheximide. Samples were electrophoresed together with molecular weight markers on 5 to 10% polyacrylamide gels in Laemmli buffer (20). (B) Immunoprecipitation of *ts*17 nsPs. Cultures of *ts*17-infected cells were treated with hypertonic medium from 5 to 6 h p.i. and then subjected to a 1-h pulse of [^{35}S]methionine (200 μCi/ml) at 30°C. Duplicate cultures were harvested immediately after the pulse (lanes 1, 4, and 7) or were chased in the presence of cycloheximide from 7 to 8 h p.i. at 40°C (lanes 2, 5, and 8) or at 30°C (lanes 3, 6, and 9). Samples were denatured and reacted with nsP2 antibodies (lanes 1 to 3), nsP3 antibodies (lanes 4 to 6), or nsP4 antibodies (lanes 7 to 9) as described previously (2). An autoradiograph of the resulting gel is shown.

retain and express individual functions when assembled together in transcriptionally active complexes (2).

To conclude, we report that defective nsP2 proteins in preformed replication complexes were responsible for loss

TABLE 4. Evidence that recombinant viruses with nsP2 and nsP4 mutations expressed conditionally lethal nsP2 phenotypes but levels of negative-strand synthesis typical of mutant nsP4

Virus[a]	Growth	RNA negative	26 mRNA synthesis	% labeled RF RNA in negative strands (range)[b]
Toto1101	wt[c]	No	wt	4.1 (2–6)
24R1	wt	No	wt	27.6 (19–34)
*ts*17	*ts*	Yes	*ts*	15.9 (12–20)
*ts*17:24R1.1	*ts*	Yes	*ts*	29.2 (17–39)
*ts*17:24R1.2	*ts*	Yes	*ts*	34.1 (32–35)

[a] The *ts*17:24R1.1 and *ts*17:24R1.2 viruses were derived from two independently isolated hybrid clones.
[b] The range of values is from four individual determinations, whose average values are listed.
[c] wt, wild type.

of subgenomic synthesis and reactivation of negative-strand synthesis by *ts*17 and *ts*133. The *ts*17 and *ts*133 mutations did not affect the putative nsP2 helicase activity because transcription was not inhibited after a shift to 40°C (19). Second, reactivation of negative-strand synthesis, once ceased in infected cells, was induced by alterations in either of two different and stable proteins, nsP2 or nsP4 (25), and not by cleavage of a nsP34 precursor as proposed by deGroot et al. (3). Reactivation of negative-strand synthesis caused neither an increase in the rate of positive-strand synthesis nor formation of additional replication complexes (this study; 25, 31). Therefore, we argue that reactivation must be due to the replacement of the preferred template of the replication complex, the negative strand, with a positive-strand template that in turn is copied into a negative strand. Thus, reactivation results from template switching by previously formed replication complexes. In addition, our results suggest that nsP2 and nsP4 function differently in template recognition and initiation of negative-strand synthesis, since the amount of negative-strand synthesis was greater when replication complexes contained both mutant nsP2 and nsP4. Not all nsP2 mutations conferring *ts* 26S mRNA synthesis reactivated negative-strand synthesis (10, 17, 28). Reversibility of reactivation suggests that these particular nsP2 and nsP4 defects induce changes in protein interactions, possibly with limiting host factors (15, 21, 25, 26), or changes in folding or conformation of the nsPs that modify the structure or affinity of the positive-strand polymerase to resemble the negative-strand polymerase and thus allow recognition of the 3′ promoter on a positive strand. We interpret such findings as supporting our model (25, 29, 31): a replication complex is formed by the association of newly synthesized nsPs with a positive-strand template and synthesizes a negative strand. The newly created negative strand becomes the preferred template of the replication complex. Both nsP2 and nsP4 would function normally to fix the negative strand as the stable template of alphavirus replication complexes. Because both nsPs are essential for negative-strand synthesis, subsequent modifications that restrict their activity to positive-strand synthesis would shorten the functional half-life of the negative-strand polymerase. This, in turn, would convert the replication complex from negative-strand to positive-strand synthesis and thereby lead to overproduction of positive strands relative to negative strands in infected cells and to cessation of negative-strand synthesis if no new replication complexes form. Given their resemblance to SIN, the mechanism for cessation of negative-strand synthesis may be common to other superfamily members. The

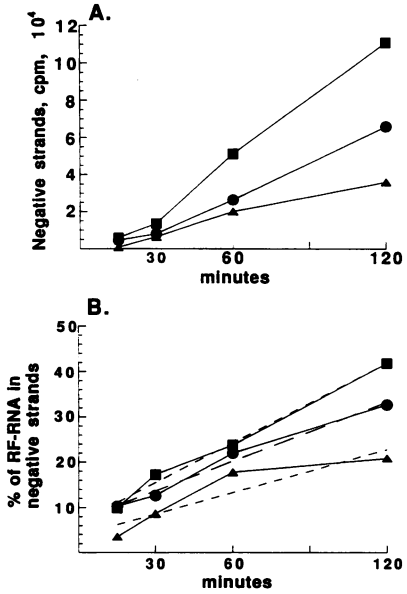

FIG. 4. Accumulation of negative strand RNA at 40°C by *ts*17, *ts*24R1, and the double mutant Toto:*ts*17:R1. Chicken embryo fibroblast cultures in 60-mm-diameter petri dishes were infected at a multiplicity of infection of 100 with *ts*17 (▲), *ts*24R1 (●), or Toto:*ts*17:R1 (■) at 30°C and maintained at 30°C until 6 h p.i., when they were shifted to 40°C and incubated continuously in the presence of 100 μg of cycloheximide per ml. At 6.5 h p.i., the cultures were labeled at 40°C with [^{3}H]uridine (500 μCi/ml, 2 ml per petri dish; ICN, Irvine, Calif.) and were harvested 15, 30, 60, or 120 min later. To measure negative-strand RNA synthesized at 40°C in the presence of cycloheximide, the double-stranded cores (RF RNA) of the viral replicative intermediates were purified by chromatography on CF-11, denatured, and hybridized in the presence of an excess of unlabeled genomic positive strands (25). Typically, 6×10^4 to 8×10^4 cpm in labeled RF RNA per 10^6 infected cells is obtained after a 60-min pulse with 200 μCi of [^{3}H]uridine per ml. The results shown represent the average values from duplicate experiments. (A) Accumulation of incorporation of [^{3}H]uridine in the negative-strand component of RF RNAs; (B) percentage of the total labeled RF RNA that was in labeled negative-strand RNA. Because the replicative intermediates are active also in positive-strand synthesis, a value of 50% of the labeled RF RNA in negative-strand RNA is taken to mean that 100% of RFs contain newly synthesized negative strands.

intriguing aspect of such a mechanism is its stability under normal conditions but its *ts* reversibility in certain SIN nsP2 and nsP4 mutants.

We thank J. H. Strauss for providing *ts*17 recombinant cDNAs and monospecific nsP antibodies, J. Lemm and C. M. Rice for providing the deletion plasmids for subcloning, and I. Dé for performing the immunoprecipitations shown in Fig. 3B.

We acknowledge support from the Public Health Service through NIAID award AI-15123.

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