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Sindbis Virus nsP1 Functions in Negative-Strand RNA Synthesis

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A mutation at nucleotide 1101 of Sindbis virus *ts*11 nsP1 caused temperature-sensitive negative-strand synthesis and suppressed the 24R phenotype, which is caused by a mutation in nsP4. Nonstructural proteins synthesized and accumulated by *ts*11 at 40°C did not cause the reactivation of negative-strand synthesis upon return to 30°C and did not prevent the formation of new replication complexes at 30°C.

Alphaviruses are enveloped positive-strand RNA viruses that replicate in both vertebrate and invertebrate cells and that cause the synthesis of genome-length negative strands which serve as templates for the synthesis of both genome RNA and subgenomic mRNA. During an alphavirus infection, negative-strand synthesis occurs only at early times and requires simultaneous viral protein synthesis, whereas positive-strand synthesis, once started, is stable and continues even if protein synthesis is inhibited. Figure 1 shows that negative strands were detectable in Sindbis virus (SIN HR)-infected cells at 2 h postinfection (p.i.), increased in amount by 4 h p.i., and remained constant thereafter. These results are in agreement with those of our previously published study (11–13, 15) in which the incorporation rate of radiolabeled precursors in vivo into negative-strand RNA was measured. Quantification of the dot-blots indicated that SIN HR-infected cells contained a maximum of 0.05 pg of replicative intermediate/replicative form (RI/RF) RNA, or approximately 4,000 molecules of negative strands per infected cell, which is about 5% of the viral RNA. We also determined (data not shown) the number of negative strands in cells infected with Semliki Forest virus, another alphavirus. RI RNA, which was purified by Sepharose 2B chromatography, was obtained from cells that had been labeled for 48 h with $^{32}P_i$ and continued to be labeled after infection with Semliki Forest virus, was denatured and hybridized to an excess of unlabeled genome RNA. Based on the calculated specific activity of the RNA, the number of negative strands increased to 4,000 to 7,000 molecules of negative-strand RNA per cell at 4 h p.i. and remained constant thereafter. At 8 h p.i. there were 160,000 molecules of 49S genome RNA per cell, which agreed with the value of 130,000 to 160,000 obtained by Tuomi et al. (16).

We have characterized two temperature-sensitive (*ts*) mutants of SIN HR that showed opposite phenotypes. One, *ts*24, showed that negative-strand synthesis could be turned on at 40°C after it had shut off normally at 30°C (14, 15). Recently, we mapped the mutation responsible for this phenotype to nsP4 (10). The other mutant was *ts*11 of the B complementation group, which we showed had a selective *ts* defect in negative-strand synthesis (13). A mutation in *ts*11 was mapped recently (3) to nucleotide (nt) 1101, which is within the coding region for nsP1. When *ts*11-infected cells were shifted to 40°C before the maximal number of negative-strand templates had accumulated, negative-strand synthesis ceased almost immediately after the shift. A revertant of *ts*11 was not *ts* for growth, RNA synthesis, or negative-strand synthesis. We sequenced the genomic RNA of the *ts*11 used in our earlier studies (13) and of the revertant of *ts*11 (SIN 11R) isolated in our laboratory. The efficiency with which SIN 11R was isolated was consistent with reversion of a single base (efficiency of plaque formation of 10^{-4} to 10^{-5} at 40°C relative to 30°C). The identical base change was present at nt 1101 (a G to A transition, predicting an amino acid change at position 348 in nsP1 of alanine to threonine) in the genome of our *ts*11, and SIN 11R had restored the parental nucleotide at position 1101. Our results therefore confirmed the *ts*11 mapping study of Hahn et al. (3).

Although a *ts* defect in negative-strand synthesis would confer temperature sensitivity to RNA synthesis and virus production, it was important to demonstrate directly that the mutation at nt 1101 was sufficient and necessary to make negative-strand synthesis by Sindbis virus *ts*, a result which was not reported by Hahn et al. (3). Figure 2 shows that negative-strand synthesis was indeed *ts* for virus produced from RNA transcribed from the hybrid Toto1101:*ts*11A1 cDNA, an infectious cDNA clone of the parental SIN HR that differs from the parental sequence only by the base change at nt 1101 (3). Since the temperature sensitivity of negative-strand synthesis by Toto1101:*ts*11A1 virus was identical to that of *ts*11, the change at nt 1101 was necessary and sufficient to make negative-strand synthesis by Sindbis virus *ts*.

We asked whether the amino acid substitution in nsP1 of *ts*11 resulted in a *trans*-acting mutation that would affect viral RNA templates having the wild-type nucleotide at position 1101. We choose a Sindbis virus defective interfering (DI) RNA template because DIs are readily and efficiently replicated by helper virus proteins and because their smaller size facilitated the identification of RNA replication. DI RNA was transcribed from an infectious cDNA clone (KDI-25) of a natural Sindbis virus DI that possesses the parental nucleotide at nt 1101 (Fig. 3) but lacks RNA sequences coding for the carboxy-terminal third of nsP1 and the entire sequence for nsP2, nsP3, nsP4, and the structural proteins (7). To prepare a mixed virus stock in which *ts*11 provided all the helper functions necessary for replication and packaging of the KDI-25 RNA, the KDI-25 RNA transcripts were transfected into cells that were infected simultaneously with *ts*11; the resulting virus was passed once to amplify the *ts*11 helper virus and the DI. Cultures of chicken embryo fibroblasts (CEF) that were infected with *ts*11 and KDI-25 were assayed for their ability to replicate both *ts*11 and DI RNAs (Fig. 3). At no time was selective amplification of DI RNA detected, i.e., the ratio of *ts*11 RNA to DI RNA was the same at late times p.i. as at early times. If the nucleotide at nt 1101 was essential for expression of the *ts*11

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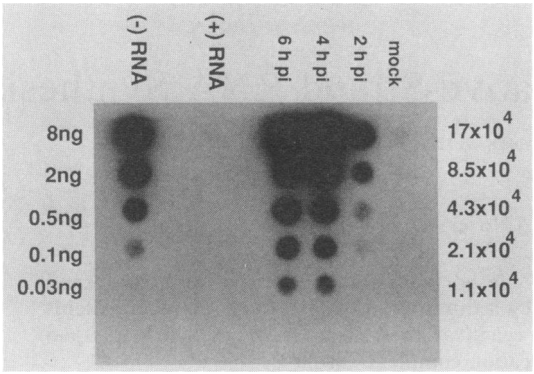

FIG. 1. Quantification of negative-strand RNA in Sindbis virus RI molecules. The viral RIs were isolated from infected cells, and the comparable fraction was isolated from mock-infected cells at the times indicated, as described before (13). After heat denaturation, it was applied to a nylon filter. In addition, dilutions of unlabeled positive-strand and negative-strand in vitro transcripts were applied to the filter to serve as controls and to enable quantification of the bound probes. After irradiation with UV light, the filter was probed with radiolabeled Sindbis virus 26S mRNA riboprobe transcribed in vitro from cDNA.

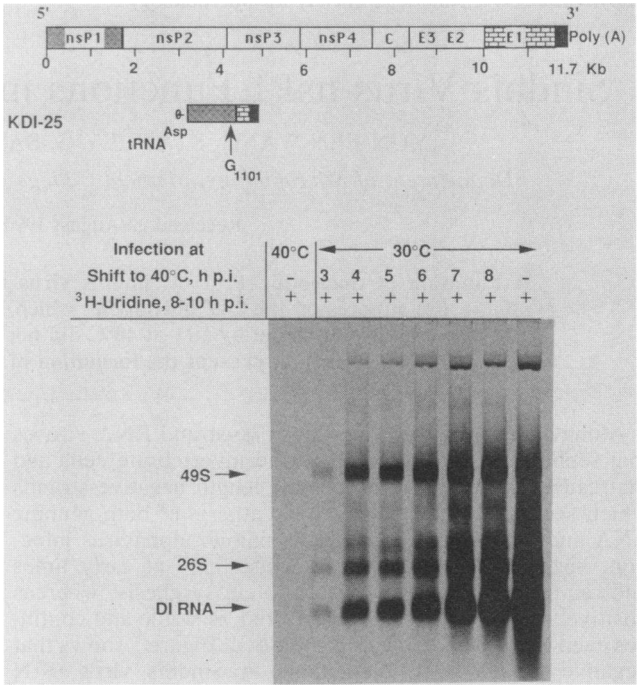

FIG. 3. *ts*11 mutation is *trans* acting and functions at the protein level. Cultures were infected with KDI-25 and *ts*11 and maintained at 30 or 40°C from the time of infection or shifted from 30 to 40°C at 1-h intervals beginning at 3 h p.i. and maintained at 40°C thereafter. All cultures were labeled at 8 to 10 h p.i. with [^{3}H]uridine (50 μCi/ml) in the presence of dactinomycin.

phenotype, DI RNA that contained the wild-type nucleotide at position 1101 would have been amplified by polymerase proteins produced by *ts*11 at 40°C. This was not observed; the pattern of DI RNA synthesis mimicked that seen for *ts*11 RNA synthesis.

We determined by immunoprecipitation with monospecific antibodies to nsP1 through nsP4 (5) that the viral nonstructural proteins (nsPs) were translated, processed proteolytically, and stable at 40°C in *ts*11-infected cells that were shifted from 30 to 40°C at 3 h p.i. Not only were the polyproteins processed, but each of the four nsPs was present in similar amounts in extracts from either *ts*11- or SIN 11R-infected cells (data not shown). These results confirm those published by Hardy et al. (4). Because *ts*11-infected cells that were shifted from 30 to 40°C at 3 h p.i. continued to synthesize and accumulate all four nsPs, *ts* negative-strand synthesis did not result from a defect in the synthesis of nsPs.

Do *ts*11 proteins synthesized at 40°C recover their activity at 30°C? Cells infected with *ts*11 were shifted up to 40°C at 3.5 h p.i., a time when nsPs are synthesized at a maximal rate, and then shifted back down to 30°C an hour later in the presence or absence of protein synthesis inhibitors. Once again we used DI RNA because we assumed that DI RNA would be recognized efficiently by nsPs. Table 1 shows that after return to 30°C, the overall rate of viral RNA synthesis increased, although it did not reach the maximum rate detected in cultures maintained at 30°C. However, when cycloheximide (CHI) or anisomycin (data not shown) was included in the medium when the cultures were shifted down to 30°C, no increase in the rate of RNA synthesis was

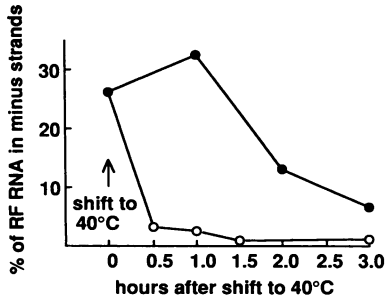

FIG. 2. *ts*11 mutation at nt 1101 is sufficient for *ts* negative-strand synthesis. CEF monolayers in 60-mm petri dishes were infected at a multiplicity of 100 at 30°C with Toto1101:*ts*11A1. Duplicate cultures were shifted to 40°C at 3 h p.i. (○), when the rate of RNA synthesis was about 10% of the maximum rate, or maintained at 30°C (●). The cultures were incubated in medium containing [^{3}H]uridine (200 μCi/ml; ICN, Irvine, Calif.) and 20 μg of dactinomycin per ml for 60-min periods at 30°C or for 30-min periods at 40°C and harvested at the end of the pulse period. The amount of radiolabel in viral negative strands was determined (13).

TABLE 1. Proteins made at 40°C do not function in the recovery of *ts*11 negative-strand synthesis

Incubation temp[a]	CHI added[b]		RNA synthesis[c] (cpm)		Recovery (%)
	3.5 h p.i.	4.5 h p.i.	2.5–3.5 h p.i.	9–10 h p.i.	
Constant			751	19,958 (100%)	
Shifted	Yes	Yes		730	
	No	Yes		892	
	Yes	No		4,843	80
	No	No		6,032 (30%)	100

[a] Incubated at a constant 30°C or begun at 30°C, shifted to 40°C at 3.5 h p.i., and shifted back down to 30°C at 4.5 h p.i.

[b] CHI used at 100 μg/ml.

[c] Average acid-insoluble [^{3}H]uridine cpm in viral RNA per 50,000 cells.

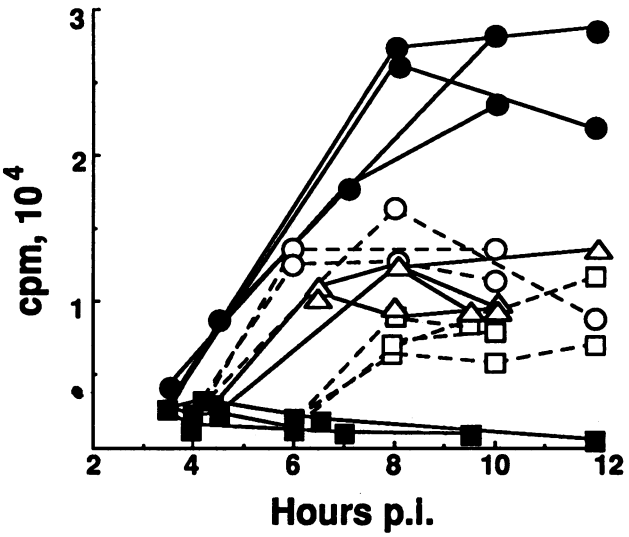

FIG. 4. Rate of RNA synthesis increased in *ts*11-infected cells that were shifted down to 30°C after being shifted up to 40°C early in infection. Infected cultures were shifted up to 40°C at 3.5 h p.i. and maintained at 40°C for either 30 (○), 60 (△), or 150 (□) min before returning to 30°C. Duplicate cultures were either maintained at 30°C from the beginning of infection (●) or shifted to 40°C at 3.5 h p.i. and maintained at 40°C thereafter (■). The rate of RNA synthesis was determined by giving pulses of [^{3}H]uridine (in the presence of 20 μg of dactinomycin per ml) at the respective temperatures, harvesting the cultures at the end of the pulse period, and measuring the acid-insoluble incorporation in a fraction (equivalent to 5×10^4 cells) of the lysates.

observed. When CHI was added at the time of shift up to 40°C and removed at the time of shift down to 30°C, the rate of RNA synthesis increased to 80% of the rate observed in cultures shifted in the absence of CHI. Therefore, *ts*11 nsPs synthesized at 40°C did not function at 30°C to raise the rate of viral RNA synthesis by increasing the number of negative-strand templates and replication complexes. Furthermore, shifting to 40°C was more effective at inhibiting the rise in the rate of positive-strand synthesis than translation inhibition at 30°C with either anisomycin or CHI (data not shown). Because anisomycin inhibited translation to greater than 99% almost immediately, the twofold rise observed after addition of translation inhibitors (11) was not the result of continued synthesis of nsPs at 30°C but of utilization of newly made proteins. In contrast, shifting to 40°C caused nascent nsP1 to immediately lose negative-strand-synthetic activity, which suggested that nsP1 functions in promoter recognition and initiation of negative-strand synthesis or in elongation of negative strands. In any case, the mutation in *ts*11 does not affect positive-strand synthesis by a stable replication complex.

Does the accumulation of nsPs cause inactivation of negative-strand polymerase activity? The rate of viral RNA synthesis increased to 30 to 60% of maximum in cells that were infected with *ts*11 and DI, shifted up to the nonpermissive temperature at 3.5 h p.i., and returned to the permissive temperature 30, 60, or 150 min later (Fig. 4). Similar results were found with cells infected only with *ts*11 (data not shown) (15). The accumulation of nsPs, which must have included the nsP2 protease (data not shown) (1, 2, 6), did not prevent the subsequent increase in the number of negative strands and in the overall rate of RNA synthesis at the permissive temperature.

Does the *ts*11 defect in nsP1 function only early in infection to inhibit negative-strand synthesis by preventing the formation of new replication complexes and not at late times, after the replication complexes had already formed? A point mutation in nsP4 allows negative-strand synthesis at 40°C late in infection and in the presence of CHI (10). We created the double mutant Toto1101:*ts*11:24R1 (Fig. 5). As

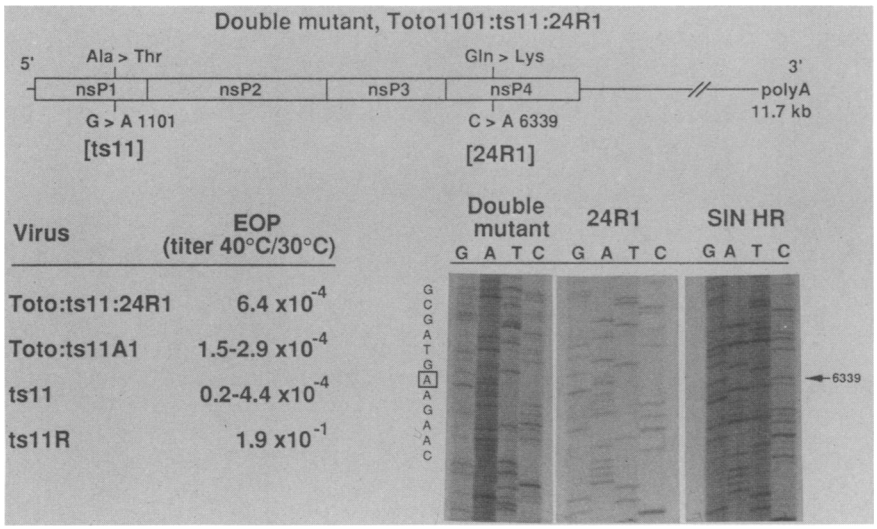

FIG. 5. Mutation in nsP1 at nt 1101 suppressed the mutation in nsP4 at nt 6339 that caused negative-strand synthesis at 40°C in SIN 24R-infected cells. The cDNA clone Toto1101:*ts*11A1 (3) and the cDNA clone Toto1101:24R1B4 (10), which contains the 24R mutation at nt 6339, were cut with *Spe*I (nt 5262) and *Aat*II (nt 7999). The appropriate fragments were purified after electrophoresis in low-melting-temperature agarose gels and ligated to create the double mutant Toto:*ts*11:24R1 containing the *ts*11 mutation at nt 1101 and the 24R mutation at nt 6339 in an otherwise Toto1101 genome. The presence of both mutations was confirmed by sequencing the cDNA (9). Infectious transcripts were obtained (8, 10) and used to transfect CEF monolayers for production of virus. The efficiency of plaquing (EOP) was determined by counting virus on CEF monolayers at 30 or 40°C. The presence of the 24R mutation in nsP4 at nt 6339 (C to A change), which is not a conditionally lethal mutation (14), was confirmed by sequencing the genome RNA obtained from purified virions of the double mutant.

with *ts*11, the double mutant was *ts* for RNA synthesis (data not shown) and for virus production. It had an efficiency of plaque formation in the range of 10^{-4}, consistent with a single point mutation and with the values found for *ts*11 and for Toto1101:*ts*11A1, which contains only the *ts*11 mutation. Sequencing the genome RNA of Toto:*ts*11:24R1 virions confirmed the presence of the nsP4 mutation at nt 6339, a C to A base change (Fig. 5). When CEF were infected with Toto:*ts*11:24R1, shifted to 40°C at 6 h p.i., and labeled with [^{3}H]uridine in the presence of CHI, the double mutant failed to resume negative-strand synthesis; it was the same as the level of negative-strand synthesis found with Toto1101 and *ts*11 (data not shown). The *ts*11 mutant nsP1 suppressed (dominated or complemented) the 24R mutant nsP4 protein, which demonstrated that the mature nsP1 plays a direct and dominant role in negative-strand synthesis.

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