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Demonstration In Vitro of Temperature-Sensitive Elongation of RNA in Sindbis Virus Mutant *ts6*

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Characterization of conditionally lethal mutants of alphaviruses, Sindbis virus and Semliki Forest virus, has indicated that in almost all the RNA-negative mutants the temperature-sensitive (*ts*) defect prevents the formation of active transcription complexes at nonpermissive temperature (40°C), but such complexes retain activity at 40°C if formed first at permissive temperature (30°C). Our recent results have extended the characterization of one exception to this finding: Sindbis *ts6* transcription complexes, once formed at 30°C, do not function at 40°C. We used an in vitro assay for viral RNA synthesis to determine whether the *ts* defect was the result of dissociation of the complex or of a failure to elongate RNA chains in a stable complex. Our results indicated that the phenotype of *ts6* observed in vivo was retained in vitro. In vivo incorporation into single-stranded 49S and 26S RNA was inhibited simultaneously with its incorporation into replicative intermediates upon shifting *ts6*-infected cells to 40°C, which was compatible with a defect in elongation. Complexes formed at 30°C and inactivated in vivo by shifting to 40°C were reactivated by incubation in vitro at 30°C but not at 40°C. Thus, the transcription complexes were stable. Nascent RNA chains initiated in vivo and pulse-labeled in vitro were chased into single-stranded 49S and 26S RNA only when incubation was at 30°C, indicating that the *ts6* transcription complex was temperature sensitive in elongation. It should be possible to study in vitro other alphavirus RNA-negative mutants that demonstrate a change in viral RNA synthesis after shift to 40°C. These would include *ts* mutants in the synthesis of subgenomic 26S mRNA and of minus-strand RNA.

Alphaviruses are enveloped, plus-strand RNA viruses belonging to the *Togaviridae*. The genome of Sindbis virus (SIN), a prototypic alphavirus, is composed of a single molecule of 49S RNA which has been completely sequenced and found to be 11,703 nucleotides long exclusive of the cap nucleotide and the poly(A) tail (21). After the genome enters the cytoplasm, it must function first as an mRNA. The translation products of the genome are polyproteins yielding four viral nonstructural proteins, nsp1, nsp2, nsp3, and nsp4 (recently reviewed in references 17 and 23). The viral structural proteins are translated also as a polyprotein from a subgenomic mRNA which is 3′ coterminal with the genome RNA and which arises from internal initiation of transcription on the full-length minus-strand RNA.

The nonstructural proteins function as an RNA-dependent RNA polymerase and are responsible for the synthesis of three species of viral RNA: the genome RNA, the full-length minus-strand RNA, and the subgenomic 26S mRNA. The nonstructural proteins probably function also in capping and methylating the genome RNA and the 26S mRNA. After the genome RNA has been translated, it associates with the nonstructural proteins to form a transcription complex and functions as a template for the synthesis of a minus strand. There is no evidence that multiple minus strands are synthesized from each plus-strand template. Rather, once the minus strand is synthesized, it apparently becomes the preferred template of the transcription complex, which then switches to synthesizing multiple plus strands (12).

Evidence that the viral nonstructural proteins are essential components of the viral replicase come from studies demonstrating that virally encoded proteins are required for the synthesis of viral RNA. Temperature-sensitive (*ts*) mutants of SIN that have an RNA-negative phenotype have been isolated; they fail to initiate viral RNA synthesis when cells are infected at the nonpermissive temperature (1, 10, 22). The RNA-negative *ts* mutants of the heat-resistant strain of SIN (SIN HR) have been assigned to four complementation groups (19). The RNA-negative *ts* mutants of SIN HR have *ts* defects that prevent the formation of viral transcription complexes. Although viral RNA synthesis will not initiate at the nonpermissive temperature, it will occur at the nonpermissive temperature if it was initiated at the permissive temperature. The mutant *ts6* is an exception.

Previous studies (1, 7, 14) have shown that *ts6* is an RNA-negative mutant of SIN HR belonging to the F complementation group. Not only did viral RNA synthesis fail to occur when the infection was initiated at the nonpermissive temperature, but viral RNA synthesis also ceased after *ts6*-infected cells were shifted up to the nonpermissive temperature. Since both plus- and minus-strand RNA syntheses were inhibited after shifting up to the nonpermissive temperature (14), it was concluded that *ts6* had a *ts* defect in the core polymerase activity responsible for both plus- and minus-strand RNA syntheses (5). After *ts6* RNA synthesis was inhibited by shifting up, it resumed if *ts6*-infected cells were then returned to the permissive temperature (7). No new protein synthesis was required to resume plus-strand RNA synthesis. Therefore, unlike the transcription complex formed by most of the other RNA-negative mutants of SIN HR, the *ts6* transcription complex, once formed at the permissive temperature, will not function at the nonpermissive temperature. We used an in vitro assay for viral RNA synthesis to find out whether the *ts6* transcription complex dissociated from the minus-strand template after shifting to the nonpermissive temperature and whether, if it remained associated with the minus-strand template, it failed to elongate nascent RNA chains at the nonpermissive temperature.

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MATERIALS AND METHODS

Virus and cell cultures. Baby hamster kidney (BHK-21) cells and chicken embryo fibroblast cells were grown in Dulbecco modified Eagle minimum essential medium supplemented with 5% fetal or newborn calf serum and 5% tryptose phosphate broth as described previously (16). The heat-resistant strain of SIN (SIN HR) and the RNA-negative *ts* mutant of the F complementation group of SIN HR, *ts*6, have been described previously (1, 16).

Infection and preparation of RF RNA. Monolayers of BHK or chicken embryo fibroblast cells in plastic petri dishes were infected with a multiplicity of infection of 100 with either SIN HR or *ts*6 at 30°C as described elsewhere (16). At the end of a 1-h adsorption period, the monolayers were rinsed with 30°C medium and incubated as indicated in the text. The RNase-resistant, double-stranded cores of the viral replicative intermediates (RIs), the RF RNA, were obtained after RNase A treatment as described previously (16). The three RF species were separated by centrifugation on 15 to 30% sucrose gradients at 30,000 rpm for 16 h in an SW40 rotor, fractions of 0.15 ml were collected, and the acid-precipitable radioactivity was determined.

In vivo RNA synthesis. BHK cells (3.4×10^6 cells per 35-mm-diameter petri dish) were infected with 100 PFU of SIN HR or *ts*6 per cell. After 11.75 h postinfection (p.i.) at 30°C, one dish of each set was given a 15-min pulse-label with 20 μg of actinomycin D (generous gift of Merck Sharp & Dohme, Rahway, N.J.) and 100 μCi of [5-^{3}H]uridine (16 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) per ml at 30°C. The remaining cultures were shifted to 40°C at 12 h p.i. by rinsing the monolayers with 40°C medium and incubating them at 40°C. At intervals after shift up to 40°C, either beginning immediately at the time of shift up or during the next 2 h, cultures were pulse-labeled for 15-min periods. At the end of the pulse period, radiolabel medium was removed and the monolayers were rinsed with ice-cold phosphate-buffered saline (PBS) and solubilized at 5×10^6 cells per ml with 5% lithium dodecyl sulfate containing 100 μg of proteinase K (Amresco, Solon, Ohio) per ml. For the quantification of in vivo-labeled RNAs, 300-μl samples of the solubilized monolayers were loaded onto 15 to 30% sucrose gradients and spun at 23,700 rpm for 16 h in an SW28.1 rotor at 20°C to separate the viral RNAs. Fractions of 0.5 ml were collected, and each was assayed for total acid-precipitable radiolabeled RNA (50 μl) and for RF RNA (450 μl). The RF RNA was the amount of radiolabeled RNA that remained acid precipitable after digestion with 5 μg of RNase A per ml for 30 min at 37°C.

Preparation of the P15 fraction. Monolayers of BHK cells were infected with SIN HR or *ts*6 and incubated at 30°C for 12 h. Four 15-cm petri dish cultures (9×10^7 cells per dish) were then shifted up to 40°C and incubated at 40°C for an additional 2 h before harvest. These are referred to as $ts6^{40°C}$ P15 extracts. Additional sets of four cultures that had been infected with either SIN HR or *ts*6 and maintained at 30°C were harvested at 12 h p.i. The preparation of the 15,000 × *g* pellet fraction of infected cells was as described previously (3, 11, 24), with the following modifications. All steps were performed at 0 to 4°C. At the time of harvest, infected cells were washed with 0°C PBS without Ca^{2+} and Mg^{2+} (PBS-Ca,Mg), scraped into PBS-Ca,Mg, and pelleted at 900 × *g* in 50-ml conical centrifuge tubes for 5 min at 4°C. The cells were suspended in hypotonic RS buffer (10 mM Tris hydrochloride, pH 7.8; 10 mM NaCl), incubated on ice for 15 min, and lysed by 20 strokes with a Dounce homogenizer. The nuclei were removed by pelleting at 750 × *g* for 5 min at 4°C. The postnuclear supernatant was centrifuged at 15,000 × *g* in a J2-21 centrifuge (Beckman Instruments, Inc., Fullerton, Calif.) by using a JS13.1 rotor for 20 min at 4°C. The pelleted material (P15 fraction) was suspended in storage buffer (10 mM Tris hydrochloride, pH 7.8; 10 mM NaCl; 15% glycerol) at 4 to 8 mg of protein per ml and stored in aliquots at −84°C.

In vitro transcription. Equal volumes of P15 and a 2× reaction mixture (100 mM Tris hydrochloride, pH 7.8; 100 mM KCl; 7 mM $MgCl_2$; 20 mM dithiothreitol [Sigma Chemical Co., St. Louis, Mo.]; 20 μg of actinomycin D per ml; 10 mM creatine phosphate and 50 μg of creatine phosphokinase [Calbiochem, San Diego, Calif.] per ml; 4 mM ATP, GTP, and UTP and 0.2 to 0.4 mM CTP [Sigma]; 3 mCi of [α-^{32}P]CTP [ICN Radiochemicals, Irvine, Calif.] per ml; and 400 to 800 U of RNasin [Promega Biotec, Madison, Wis.] per ml) were incubated at the temperatures and times indicated elsewhere in the text. Reactions were terminated by the addition of 5% lithium dodecyl sulfate containing 100 μg of proteinase K per ml. Incorporation of radiolabel into acid-precipitable products was monitored by trichloroacetic acid precipitation of portions of the reaction samples after termination. Portions were also analyzed directly or after phenol and chloroform extractions and ethanol precipitation by separation on 1% agarose-TBE (89 mM Tris hydrochloride, pH 8.0; 89 mM boric acid; 2 mM EDTA) gels or on 1% agarose gels containing 2.2 M formaldehyde in MOPS (morpholinepropanesulfonic acid) buffer (20 mM MOPS, pH 7.0 [Sigma]; 5 mM sodium acetate; 1 mM EDTA) and were visualized by autoradiography of the dried gels.

RESULTS

In vivo characterization of *ts*6. The synthesis of viral RNA cannot be detected in cells that are infected with *ts*6 and maintained at 40°C; when cells are infected with *ts*6 and maintained at 30°C until viral RNA synthesis reaches a maximum rate and then shifted up to 40°C, the rate of viral RNA synthesis falls dramatically. Figure 1 demonstrates the rapidity at which the drop in the rate of viral RNA synthesis occurs after shift-up. BHK cells were infected with SIN HR or with *ts*6 and maintained at 30°C until 12 h p.i., when they were shifted up to 40°C. The infected cells were pulse-labeled with [^{3}H]uridine for 15 min at various intervals after shift-up. During the first 15 min after shift-up, cells infected with *ts*6 incorporated 50% less [^{3}H]uridine into viral RNA than did *ts*6-infected cells incubated at 30°C. The rate of incorporation of [^{3}H]uridine in *ts*6-infected cells dropped quickly to less than 20% of the 30°C rate and less than 10% of the rate observed in shifted-up SIN HR-infected cells. Decreased incorporation of [^{3}H]uridine occurred in 49S, 26S, and RF RNA, the RNase-resistant core of the RIs. Because the rate of incorporation of [^{3}H]uridine into 49S and 26S RNA decreased as quickly as the rate of incorporation into RIs, we concluded from this experiment that a *ts* defect in *ts*6 affected viral RNA synthesis in vivo either by slowing the rate of elongation of RNA chains or by causing the viral transcription complex to dissociate at 40°C.

We determined whether the rapid drop in the rate of viral RNA synthesis that occurred after *ts*6-infected cells were shifted up was accompanied by the dissociation of viral transcription complexes. Dissociation of viral transcription complexes would result in the minus-strand templates being released as RNase-sensitive single-stranded RNA or as full-length double-stranded replicative-form RNA (RF I RNA). On the other hand, if the rapid decrease in the rate of

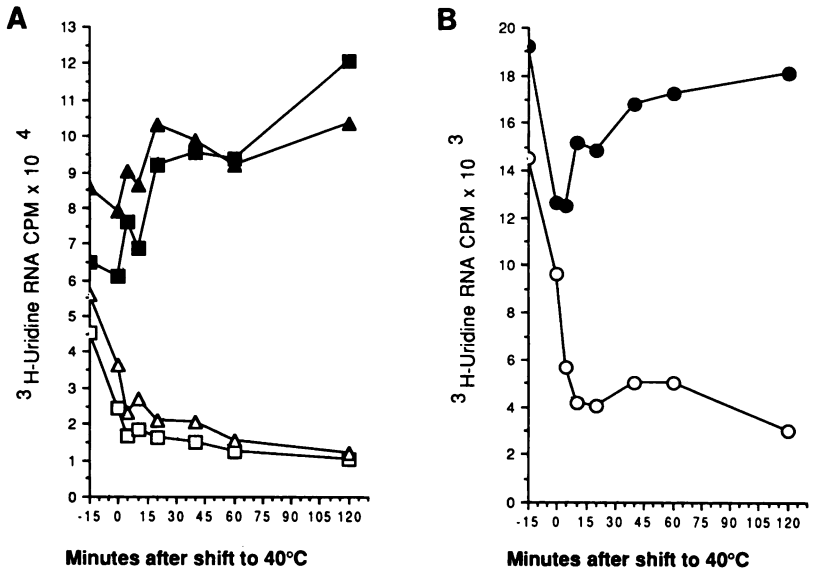

FIG. 1. SIN in vivo RNA synthesis. SIN HR- or *ts*6-infected BHK cells were pulse-labeled for 15 min at 30°C at 11.75 h p.i. (−15 min). Duplicate cultures were shifted to 40°C at 12 h p.i. and pulse-labeled in the presence of actinomycin D (20 μg/ml) at 40°C for 15-min periods from 0 to 2 h after shift. Viral RNA was separated by centrifugation on 15 to 30% sucrose gradients, and the amounts of radiolabeled 49S, 26S, and RF RNAs were determined as described in Materials and Methods. (A) SIN HR 49S RNA (■) and 26S RNA (▲) and SIN *ts*6 49S RNA (□) and 26S RNA (△); (B) RF RNA from SIN HR-infected (●) or *ts*6-infected (○) cells.

incorporation of [^{3}H]uridine resulted from the temperature sensitivity of the elongation reaction, the minus-strand templates would remain associated with transcription complexes, and RIs isolated from these transcription complexes would generate RF II and RF III after cleavage by RNase. RF II and RF III are derived from RIs that are engaged actively in 26S mRNA synthesis. Figure 2 shows the results of an experiment designed to determine which of these two possibilities occurred in *ts*6-infected cells. Chicken embryo fibroblast cells were infected with *ts*6 or with SIN HR and maintained at 30°C until 8 h p.i. The cells were labeled from 3 to 8 h p.i. with [^{3}H]uridine and then rinsed and refed with medium containing unlabeled uridine. One set of infected cells was then shifted up and incubated for 2 h at 40°C. The RNase-resistant RF RNA was isolated and size fractionated on sucrose gradients. Figure 2 demonstrates that minus strands were being used as templates for the synthesis of 26S mRNA at 30°C in *ts*6-infected cells: after limited treatment with RNase, the RIs were cleaved to form 22S RF I RNA (the double-stranded form of the genome length minus strands), 18S RF II RNA (the double-stranded form of the 3′ two-thirds of the minus-strand RNA), and 15S RF III RNA (the double-stranded form of the portion of the minus-strand RNA that encodes 26S mRNA). The presence of RF II RNA and RF III RNA indicated that minus strands were engaged actively as templates for 26S mRNA synthesis. In contrast, only RF I RNA was obtained from *ts*6-infected cells that had been shifted up and incubated for 2 h at 40°C. Because at least 75% of the RF RNA was obtained from the shifted-up cells compared with the cells maintained at 30°C, we concluded that the minus strands were not released from replication complexes as single-stranded RNA. However, the failure to generate RF II RNA and RF III RNA indicated that the nuclease-sensitive region of the RIs was protected from RNase attack. Because we could not demonstrate conclusively with in vivo experiments whether it was the elongation reaction that was temperature sensitive or the transcription complex that dissociated at 40°C and released RF I, we used an in vitro method to determine the nature of the temperature sensitivity of *ts*6 transcription complexes.

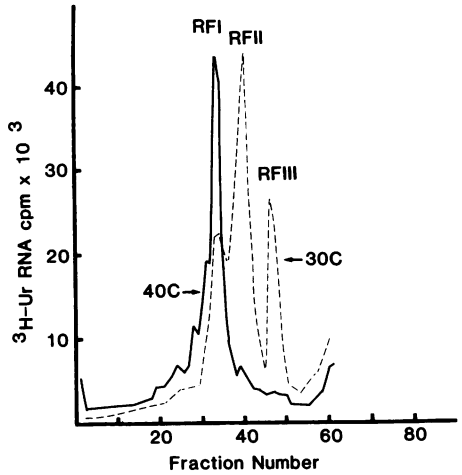

FIG. 2. SIN *ts*6 RF RNA labeled at 30°C was found as RF I molecules after shift to 40°C. SIN *ts*6-infected chicken embryo fibroblast cells were labeled in the presence of actinomycin D with [^{3}H]uridine at 30°C from 3 to 8 h p.i. as described in Materials and Methods. One half of the cultures were harvested at 8 h p.i. at 30°C. The remaining cultures were shifted to 40°C and incubated at 40°C in the presence of an excess of unlabeled uridine for an additional 2 h. The viral RFs were isolated and separated according to size by centrifugation on sucrose gradients as described in Materials and Methods.

In vitro characterization of *ts*6. Transcription complexes active in vitro in viral RNA synthesis have been found in a 15,000 × *g* pellet (P15) fraction of infected cell extracts (2, 3, 11). We determined whether the P15 fraction that was obtained from cells infected with SIN HR or *ts*6 and maintained at 30°C synthesized viral RNA when incubated in vitro at 30 or 40°C. Figure 3 shows an analysis using agarose

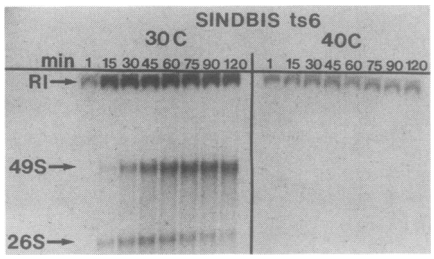

FIG. 3. In vitro transcription of *ts*6 RNA at 30 or 40°C. The P15 fraction from *ts*6-infected cells was incubated in vitro as described in Materials and Methods at 30 or 40°C at 0.5 mg of protein per ml (diluted in storage buffer immediately before incubation) in a final volume of 100 μl. Portions of 10 μl were removed at the indicated times into a solution of 5% lithium dodecyl sulfate containing proteinase K. The viral RNA was separated by electrophoresis on 1% agarose gels in TBE buffer and was visualized by autoradiography with X-ray film (XAR; Eastman Kodak Co., Rochester, N.Y.).

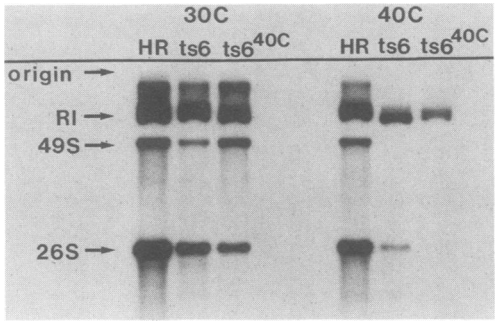

FIG. 4. SIN *ts*6 defect in RNA synthesis reversible in vitro. The P15 fractions from SIN HR and from *ts*6-infected cells maintained at 30°C were prepared at 12 h p.i. The *ts*6$^{40°C}$ P15 fraction was prepared from *ts*6-infected cells maintained at 30°C until 12 h p.i. and then shifted to 40°C and harvested at 14 h p.i. The in vitro transcription reactions, used at 2 to 4 mg of protein per ml in a final volume of 20 μl, were for 15 min at the indicated temperatures and were terminated by solubilization with 5% lithium dodecyl sulfate. After deproteinization with phenol and chloroform, the samples were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde as described in Materials and Methods. The RNA was visualized by autoradiography.

gels of the products of such a reaction. After only 1 min of incubation at 30°C, [^{32}P]CTP was incorporated into RIs. Labeled 26S mRNA was released from the RIs beginning after 4 min of incubation at 30°C, and labeled 49S RNA was released beginning after 10 min and was readily detected at 15 min. The amount of [^{32}P]CTP incorporated into RIs leveled off after about 15 to 30 min, but it continued to increase in the single-stranded products until about 60 min, when it too reached a maximum. The activity at 30°C of the P15 extracts prepared from cells infected with *ts*6 and maintained at 30°C was the same as the activity of the P15 extracts prepared from cells infected with SIN HR and maintained at 30 or 40°C (data not shown). Figure 3 shows the products of an in vitro reaction incubated at 40°C. Reduced incorporation of [^{32}P]CTP into *ts*6 RNA was observed, and most of the [^{32}P]CTP that was incorporated was found in RIs; the rate of incorporation into the RIs was much less than that observed at 30°C, and very little single-strand product was synthesized at 40°C in contrast to what was observed at 30°C. The P15 extracts prepared from SIN HR-infected cells incorporated [^{32}P]CTP into RIs, 26S mRNA, and 49S RNA both at 30 and at 40°C (data not shown, but see Fig. 4 for 15-min incubation). Thus, the transcription complex that was formed in vivo by *ts*6-infected cells at 30°C was temperature sensitive when incubated in vitro at 40°C.

Next we used the in vitro assay to determine whether the viral transcription complexes dissociated in *ts*6-infected cells that were shifted up. If the transcription complex dissociated, then the P15 extracts prepared from shifted-up cells would fail to synthesize viral RNA when incubated in vitro at 30°C. P15 extracts were prepared from SIN HR- or *ts*6-infected cells that were shifted up at 12 h p.i. and incubated at 40°C until 14 h p.i. Under these conditions, the rate of viral RNA synthesis in the *ts*6-infected cells was less than 10% of that in the SIN HR-infected cells (Fig. 1). When the P15 extracts from these cells harvested at 40°C were incubated in vitro at 30 or 40°C, large amounts of [^{32}P]CTP were incorporated at 30°C but not at 40°C into RI, 49S, and 26S RNA by the P15 extracts from *ts*6-infected cells (Fig. 4). At 40°C only the P15 extracts prepared from SIN HR-infected cells incorporated large amounts of [^{32}P]CTP into RI, 49S, and 26S RNA. P15 extracts prepared from *ts*6-infected cells that had been maintained at 30°C throughout infection, or had been shifted up to 40°C late in infection, incorporated in vitro reduced amounts of [^{32}P]CTP at 40°C into RI RNA and even less into 49S and 26S RNA compared with P15 extracts prepared from SIN HR-infected cells.

To demonstrate conclusively that the temperature sensitivity of the *ts*6 transcription complex resulted from temperature sensitivity of the elongation reaction, we performed an in vitro pulse-chase experiment (Fig. 5). The P15 extracts of SIN HR- or *ts*6-infected cells were incubated for 1 min at 30°C in the presence of high-specific-activity [^{32}P]CTP, i.e., low CTP concentration, and then chased at 30 or 40°C after the addition of a high concentration of unlabeled CTP. Only the RIs became labeled after a short pulse with a low concentration of high-specific-activity [^{32}P]CTP. The label was chased from the RIs in P15 extracts of *ts*6-infected cells at 30°C but not at 40°C (Fig. 5). On the other hand, the label was chased from the RIs at 30 and 40°C in the P15 extracts from SIN HR-infected cells (Fig. 5B). Therefore, at 40°C the *ts*6 transcription complex was unable to elongate RNA that had been initiated at 30°C.

DISCUSSION

We characterized in vivo the *ts* lesion in the transcription complex formed in cells infected with *ts*6. Keränen and Kääriäinen (7) had demonstrated that the syntheses of 49S and 26S RNA were inhibited equally after shift-up and that plus-strand RNA synthesis resumed after the infected cells were returned to the permissive temperature even in the absence of new protein synthesis. Our results have extended these findings and have demonstrated that [^{3}H]uridine incorporation into 49S and 26S RNA was inhibited simultaneously with its incorporation into RIs upon shifting *ts*6-infected cells to 40°C late in infection. This indicated that there was a *ts* lesion in the elongation reaction. If initiation of viral RNA synthesis had been inhibited, incorporation of [^{3}H]uridine into RIs would have decreased before its incorporation into 49S RNA and 26S mRNA.

Because the P15 fraction prepared from *ts*6-infected cells retained in vitro the in vivo phenotype of *ts*6, we were able to further characterize the *ts* lesion exhibited by the *ts*6 transcription complex. The results from the in vitro experiments substantiated our conclusion based on the in vivo

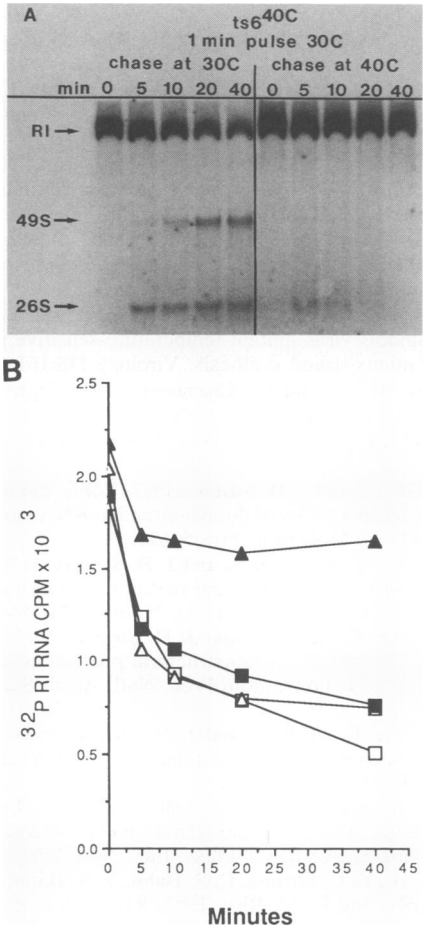

FIG. 5. Indication by in vitro pulse-chase experiments that the *ts* defect in *ts*6 was in elongation of RNA chains. The P15 fractions from SIN HR- or *ts*6-infected cells at 2 to 4 mg of protein per ml in a final volume of 40 μl were pulse-labeled in vitro for 1 min at 30°C in the absence of added unlabeled CTP and were then chased for the times indicated in the presence of 2 mM unlabeled CTP in a final volume of 80 μl at either 30 or 40°C; 15-μl portions were removed at the times indicated. (A) Radiolabeled *ts*6 RNA was separated on 1% agarose gels in TBE buffer and visualized by autoradiography. (B) The amounts of radioactivity in areas of the gels containing the viral RIs were determined as SIN HR RI RNA from reactions chased at 30 (□) or 40°C (■) and *ts*6 RI RNA from reactions chased at 30 (△) or 40°C (▲).

experiments that the transcription complex formed in *ts*6-infected cells was temperature sensitive in the elongation reaction. Furthermore, we demonstrated that the transcription complex formed in *ts*6-infected cells at the permissive temperature and inactivated in vivo by shifting the infected cells to 40°C was reactivated by incubation in vitro at 30°C but not at 40°C. Dilution and pelleting of the P15 membrane fraction would have removed any components of the transcription complex that were released at 40°C. Therefore, either the transcription complex did not dissociate at 40°C or it dissociated at 40°C but remained with the P15 membranes and reassociated with the RIs at 30°C. Our experiments do not distinguish formally between these two possibilities. If dissociated transcription complexes had to reassociate, we would have expected a delay in the restoration of viral RNA synthesis at 30°C by transcription complexes obtained from *ts*6-infected cells that had been shifted to 40°C. Because we did not observe a delay, we favor the interpretation that the transcription complexes did not dissociate at 40°C.

Even though the transcription complexes were stable at 40°C and could be reactivated in vitro at 30°C, only RF I cores were obtained by nuclease treatment of RIs that were originally synthesizing 26S and 49S RNA before shift to 40°C. RNase treatment of the RI_b, the RI which is engaged in the synthesis of 26S mRNA, results in RF II and RF III core structures (13, 18). One explanation for the recovery of only RF I cores is that only 26S mRNA synthesis was temperature sensitive. Since 26S RNA synthesis was not selectively affected by the shift to 40°C, but rather both 49S and 26S plus strands and the 49S minus strands cease to be produced by this mutant at nonpermissive temperature (7, 14), an alternative explanation is required. One possibility is that the junction region just upstream of the initiation site for 26S RNA synthesis was protected from RNase cleavage after incubation at 40°C by a polymerase molecule advancing into this region before being inhibited itself. Another explanation would be that the junction region was sensitive to RNase only during the process of initiating 26S RNA synthesis; slowing the rate of elongation would result in fewer RIs initiating 26S RNA synthesis at any given time and in resistance of this region to cleavage by low concentrations of RNase.

At least one alphavirus RNA polymerase phenotype observed in vivo can be reproduced in vitro. Martin (9) used the postnuclear cytoplasmic pellet fraction to characterize in vitro the RNA polymerase formed by two RNA-negative *ts* mutants of Semliki Forest virus. However, the same activity was observed in vitro at the nonpermissive temperature as at the permissive temperature. We picked *ts*6 because its transcription complex retained temperature sensitivity after its formation. Most other RNA-negative, *ts* mutants of SIN HR failed to assemble the transcription complex at the nonpermissive temperature; however, once the transcription complexes formed at the permissive temperature, they continued to function at the nonpermissive temperature. It should be possible to study in vitro other alphavirus *ts*, RNA-negative mutants that demonstrate a change in viral RNA synthesis after shift to the nonpermissive temperature. This would include mutants that show temperature sensitivity of 26S RNA synthesis and of minus-strand RNA synthesis.

Recently, the nucleotide change responsible for the RNA-negative phenotype of *ts*6 has been mapped to the gene *nsp4* (Y. Hahn et al., presented at the VII International Congress of Virology, 1987). The nsp4 sequence contains short regions of homology with replicase proteins of several other viruses (6). Our results support a model in which nsp4 would be involved in the elongation of RNA chains by the alphavirus transcription complex. It is interesting that nsp4 is underproduced relative to nsp1, nsp2, and nsp3 in SIN- or Semliki Forest virus-infected cells (4, 8, 15). In SIN, but not Semliki Forest virus, an opal termination codon precedes the *nsp4* gene (20, 25), and nsp4 is found predominantly as an nsp3+nsp4 polypeptide (4). Thus, relatively small amounts of nsp4 or nsp3+nsp4 may be required to form a transcription complex. Because the alphavirus transcription complex, once formed, is very stable, nonstructural proteins synthesized after the transcription complexes have formed probably do not participate in viral RNA synthesis. Therefore, the overproduction and disproportionate production of the alphavirus nonstructural proteins may not reflect the composition of the alphavirus transcription complex.

ACKNOWLEDGMENTS

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