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Mechanism for Control of Synthesis of Semliki Forest Virus 26S and 42S RNA

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When cells infected with the Semliki Forest virus (SFV) mutant *ts*-4 were shifted to the nonpermissive temperature, synthesis of 26S RNA ceased, whereas synthesis of 42S RNA continued normally. These two single-stranded SFV RNAs are synthesized in two types of replicative intermediate (RI), 26S RNA in RI_b and 42S RNA in RI_a. Cessation of 26S RNA synthesis after shift up in temperature was accompanied by loss of RI_b. When infected cells were shifted back down to 27°C, 26S RNA synthesis resumed, coincident with the reappearance of RI_b. In both types of RI, the 42S minus-strand RNA is template for synthesis of plus-strand RNA. In pulse-chase experiments, we obtained RIs labeled only in their minus-strand RNA, and thus could follow the fate of RIs assembled at 27°C when they were shifted to 39°C. Our results show that, after shift up to 39°C, there was a quantitative conversion of RIs in which 26S RNA had been synthesized to RIs in which 42S RNA was synthesized. This conversion of RI_b to RI_a was reversible, since RIs in which 26S RNA was synthesized reappeared when the infected cultures were shifted back down to 27°C. We propose that, associated with RI_b, in which 26S RNA is synthesized, there is a virus-specific protein that functions to promote initiation of 26S RNA transcription at an internal site on the 42S minus-strand RNA and to block transcription on the minus strand in this region by the SFV RNA polymerase that had bound and was copying the minus-strand RNA from its 3′ end. A ribonuclease-sensitive region would thus result in the sequence adjacent to the one that was complementary to 26S RNA. This virus-specific protein is not a component of the SFV RNA polymerase that continues to transcribe 42S RNA, and it is temperature sensitive in *ts*-4 mutant-infected cells. When this virus-specific protein is not present on RIs, the SFV polymerase transcribes the whole 42S minus-strand RNA and yields 42S plus-strand RNA.

Two major RNAs of positive polarity are synthesized in alphavirus-infected cells: 42S RNA, which has a molecular weight of 4×10^6 to 4.5×10^6 (5, 7, 12, 20), and 26S RNA, which has a molecular weight of 1.6×10^6 (7, 9, 12, 20, 23). The 26S RNA is identical to the 3′-terminal third of the 42S plus-strand RNA (10, 25), and both the 42S and 26S RNAs contain polyadenylate at their 3′ terminus (3, 4, 6, 16). Both the 42S RNA and the 26S RNA are transcribed from a 42S minus-strand RNA (2, 13, 19, 21). The transcription of the 42S RNA starts at the 3′ terminus of the minus strand, whereas the transcription of the 26S RNA may start at a point 8,000 nucleotides from the 3′-terminal end of the 42S minus strand. The transcription of both RNAs most likely terminates at the 5′ end of the 42S minus-strand RNA, since the polyadenylate sequence in both can be directly transcribed from a polyuridylate sequence present at the 5′ terminus of the 42S minus strand (16).

Analysis of the double-stranded cores (completely double-stranded replicative forms, or RFs) derived by RNase digestion of replicative intermediates (RIs) isolated from alphavirus-infected cells has revealed three size classes of RF with molecular weights of 8.8×10^6 (RFI), 5.6×10^6 (RFII), and 2.9×10^6 (RFIII) (21). There are two different types of RI, RI_a and RI_b, distinguishable by their response to RNase (21). RI_a, in which 42S RNA is synthesized, is resistant to cleavage by RNase and yields RFI. RI_b, in which 26S RNA is synthesized, is cleaved by RNase to yield RFII and RFIII. The RFIII is the double-stranded form of 26S RNA, and RFII represents the double-stranded form of the remaining two-thirds of the total 42S RNA sequence (21).

When protein synthesis is inhibited 90 min

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after infection, synthesis of 26S RNA ceases, but that of 42S RNA continues (17). A similar selective cessation of 26S RNA synthesis occurs when cells infected with some Sindbis virus RNA-negative mutants are shifted to nonpermissive temperature (1, 17, 19, 24). Together, these two findings suggest that there is, in addition to the alphavirus RNA polymerase, a virus-specific protein that is responsible for the regulation of 26S RNA synthesis. To elucidate the mechanism regulating 26S RNA synthesis, we initiated studies with one of the Semliki Forest virus (SFV) RNA-negative mutants, *ts*-4, which, when shifted to nonpermissive temperature, is defective in synthesis of 26S RNA but not of 42S RNA (15). This suggests that the *ts*-4 mutant has a temperature-sensitive defect in a viral protein that regulates 26S RNA synthesis. Our results indicate that the function that this temperature-sensitive protein supplies, when active and bound to an RI, is to convert RI_a to RI_b.

MATERIALS AND METHODS

Cell culture. BHK-21 cells, a continuous cell line derived from baby hamster kidney cells, were grown in plastic petri dishes (100 mm) or in polystyrene tissue culture flasks (Corning Glass Works, Inc., Corning, N.Y.) in Eagle minimal essential medium (MEM) supplemented with 5% fetal bovine serum.

Virus. Wild-type SFV, obtained from the second passage of SFV-infected mouse brain suspension (16) in BHK cells, was used in these experiments. Growth and purification of virus and determination of its infectivity by plaque assay were described previously (8). The temperature-sensitive *ts*-4 mutant of SFV, obtained after treatment of wild-type virus with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (11), was passaged once in BHK cells (multiplicity of infection, 0.01) at 27°C, and the virus was harvested after 48 to 72 h. The medium was clarified at 8,000 rpm for 20 min and used as stock virus. The average titer of the stock *ts*-4 mutant virus was 2×10^8 to 3×10^8 PFU/ml when assayed at 27°C and 2×10^4 PFU/ml when assayed at 39°C. This stock virus was used in all experiments in this paper.

Preparation of infected-cell extracts. BHK monolayers were infected with *ts*-4 mutant or wild-type SFV as previously described (16), except that the multiplicity of infection was 40, actinomycin D (AMD) was at a concentration of 1 μg/ml, and adsorption at 27°C was for 2 h. SFV RNAs were labeled with [5,6-^{3}H]uridine at a final concentration of 40 to 100 μCi/ml.

At the times indicated for harvest, the cells were washed with ice-cold phosphate-buffered saline and with RS buffer (0.01 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4]). When the RNAs in whole cells were to be analyzed directly on 15 to 30% sucrose gradients in TNE buffer (0.01 M Tris-hydrochloride [pH 7.4], 0.1 M NaCl, and 0.001 M EDTA), a small volume of RS, warmed to 60°C and containing 2% sodium dodecyl sulfate (SDS), was added, the lysed cells were scraped from the dish, and the cellular DNA was sheared by passing the extracts one or two times through a 26-gauge needle.

When larger amounts of the viral RNAs were needed for analysis, a cytoplasmic extract was prepared. The RS-washed cells were scraped from flasks into cold RS buffer and allowed to swell at 0°C for 15 min before disruption with 25 to 30 strokes in a Dounce homogenizer. The homogenate was centrifuged at $250 \times g$ for 5 min and the supernatant fluid, the cytoplasmic extract, was adjusted to 0.5% SDS. The cytoplasmic RNAs either were extracted with phenol-chloroform (16) or, where indicated, were adjusted to TNE, layered directly onto 15 to 30% sucrose gradients in TNE buffer, and sedimented in an SW27 rotor at $87,000 \times g$ for 15 h at 18°C. Fractions of 1 ml were collected from below, and the acid-insoluble radioactivity in each was determined by counting a portion in a toluene-based scintillation fluid.

Pulse-chase in vivo. The pulse-chase procedure of Scholtissek (18) as modified by Wertz (26) was used. BHK monolayers were washed with MEM at 27°C and then were incubated at 27°C for 1 h in MEM containing 0.2% bovine plasma albumin, 1 μg of AMD per ml, and 20 mM glucosamine. Before addition of cells, the pH of the MEM containing glucosamine was adjusted to 7.2 with NaOH. After 1 h, the monolayers were washed three times with MEM at 27°C before addition of MEM containing 1 μg of AMD per ml, [5,6-^{3}H]uridine at 10 to 100 μCi/ml, and SFV at approximately 40 PFU/cell. The cultures were incubated at 27°C for 2 or 4 h as indicated. After the pulse, the monolayers were washed three times with MEM at 27°C, and MEM containing 0.2% bovine plasma albumin, 1 μg of AMD per ml, 20 mM glucosamine, and 100 μg of unlabeled uridine per ml was added for the times indicated. The presence of glucosamine during the first 10 h of infection resulted in a 50% decrease in final yield of virus at 48 h.

For shift-up experiments, all procedures were carried out in a 37°C room with medium at 39°C. The cells were washed two or three times with MEM before addition either of MEM containing bovine plasma albumin, AMD, glucosamine, and excess unlabeled uridine or of this medium lacking glucosamine. The cultures were put for the time specified into a 39°C incubator. For shift-down experiments, the cultures at 39°C were washed with medium at 27°C before incubation at 27°C. Preparation of cell extracts was as described above.

Isolation of the RNase-resistant cores of SFV RIs. The double-stranded SFV RNAs were obtained either from material sedimenting from 20S to 35S in sucrose gradients of SDS-treated whole cells or from cytoplasmic extracts treated with phenol-chloroform. After ethanol precipitation at −20°C for 18 h in the presence of 0.1 volume of 4 M LiCl, the RNAs were dissolved in 1 ml of 0.5% SDS buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4], 0.02 M EDTA, and 0.5% SDS) and chromatographed at room temperature through a column (70 by 1.5 cm) of Sepharose 2B in 0.5% SDS buffer. Fractions of 0.5 to 1 ml were collected, and the double-stranded RFs and RIs elut-

ing in the void volume were pooled and concentrated by ethanol precipitation. The double-stranded RNAs were suspended in digestion buffer (0.15 M NaCl, 0.02 M Tris-hydrochloride [pH 7.4]) containing pancreatic RNase at a concentration of 0.02 μg of RNase per 15 to 30 μg of RNA, and were then incubated at room temperature for 15 min. SDS was added to a final concentration of 2%, and the digested sample was overlaid onto a 15 to 30% sucrose gradient in TNE containing 0.2% SDS. Centrifugation was at 154,000 × *g* for 16 h in an SW41 rotor. Fractions of 0.2 ml were collected. The three double-stranded cores were identified by counting a portion in a toluene-based scintillation fluid containing Triton X-100, and the appropriate RNA fractions were collected by ethanol precipitation in the presence of 10 to 50 μg of carrier yeast RNA.

Hybridization of core RNA with unlabeled 42S virion RNA. Unlabeled 42S virion RNA was obtained as previously described (16). The annealing procedure of Roy and Bishop (14) was used. The double-stranded cores, 1 μg/ml or less in 1 mM EDTA, were heat denatured in a water bath at 100°C for 2 min and then quickly cooled to 0°C. After denaturation, as little as 1 to 3% of [^{3}H]uridine-labeled *ts*-4 mutant or wild-type SFV double-stranded cores remained acid insoluble after RNase treatment. When [^{3}H]uridine and [^{3}H]adenosine were both used to label wild-type cores, 10% of the labeled RNA after denaturation was resistant to treatment with RNase. Equal portions containing 0.01 to 0.1 μg of denatured double-stranded RNAs were transferred to tubes containing different amounts of unlabeled 42S virion RNA in 1 mM EDTA. The salt concentration was adjusted to 0.4 M NaCl in a final volume of 1 ml before transfer to a water bath at 70°C. We have determined that the rate of annealing of 42S RNA to denatured RFs or RIs was maximal from 15 to 30 min at 70°C and thereafter it decreased. After 30 min at 70°C, an increase in the percentage of self-annealing occurred. Therefore, annealing was for 30 min at 70°C, followed by 30 min at room temperature. To one-half of the sample was added an equal volume of a solution containing 0.3 M NaCl, 5 μg of pancreatic RNase, and 120 U of RNase T1, and the mixture was incubated at 37°C for 30 min. The untreated and RNase-treated portions were precipitated with trichloroacetic acid, and the acid-insoluble radioactivity was determined.

Materials. D-Glucosamine-hydrochloride and RNase T1 were purchased from Calbiochem, La Jolla, Calif. [5,6-^{3}H]uridine (26 Ci/mmol) and [2,8-^{3}H]adenosine (32 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass. RNase A, crystallized once, and unlabeled uridine were from P. L. Biochemicals, Milwaukee, Wis. Sepharose 2B was purchased from Pharmacia, Uppsala, Sweden. All other materials were obtained from previously described sources (16).

RESULTS

Virus-specific RNAs synthesized at 27 and 39°C. The SFV mutant *ts*-4 was grown in BHK cells at the permissive temperature (27°C) in the presence of AMD, and the viral RNAs synthesized between 7 and 8 h after infection were labeled with [^{3}H]uridine. The RNAs present in infected BHK cytoplasmic extracts were treated with SDS and analyzed on 15 to 30% sucrose gradients. Both 42S and 26S SFV RNAs were synthesized (Fig. 1A). The ratio of 42S:26S RNA synthesized in *ts*-4 mutant-infected cells

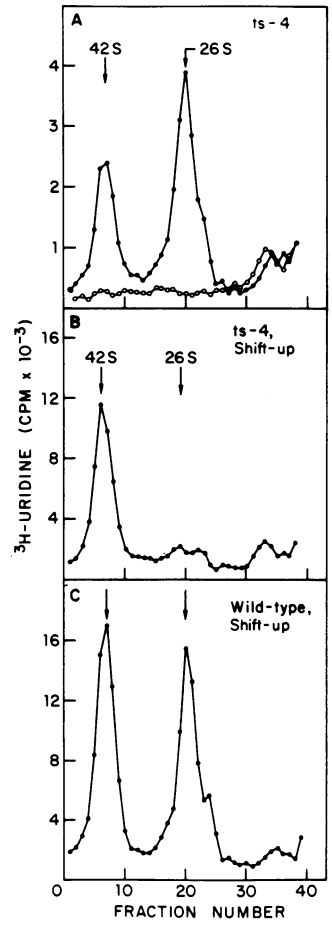

FIG. 1. *Sedimentation on sucrose gradients of ts-4 mutant and wild-type SFV RNAs. BHK cells, infected with ts-4 mutant or wild-type virus in 100-mm petri dishes, were labeled with [^{3}H]uridine (10 μCi/ml) from 7 to 8 h after infection. The viral RNAs, extracted by treatment of the monolayers with 2% SDS, were sedimented in an SW27 rotor at 87,000 × g for 15 h at 18°C through linear 15 to 30% sucrose gradients. Fractions of 1 ml were collected from below, and acid-insoluble radioactivity was determined by counting a portion in a toluene-based scintillation fluid. Radioactivity in (A), (B), and (C) is expressed as counts per minute per 10^6 cells. (A) ts-4 mutant-infected cells maintained at 27°C (●) or at 39°C (○) from the beginning of infection. (B) ts-4 mutant-infected cells maintained at 27°C for 6 h after infection before shift up to 39°C for 2 h. (C) Wild-type virus-infected cells maintained at 27°C for 6 h after infection before shift up to 39°C for 2 h.*

at 27°C was the same as that in similarly treated wild-type SFV-infected cultures. Little or no SFV RNA was synthesized when the *ts*-4 mutant-infected cultures were maintained at the nonpermissive temperature (39°C) from the beginning of infection; thus, the *ts*-4 SFV mutant is phenotypically a temperature-sensitive, RNA-negative mutant (11), as has been discussed recently (15).

When *ts*-4 mutant-infected cells were maintained at 27°C before shift up to 39°C, and the RNAs synthesized at the nonpermissive temperature were labeled with [^{3}H]uridine, only 42S viral RNA was synthesized (Fig. 1B). After shift up, there was little or no 26S RNA synthesized. The inhibition of the synthesis of 26S RNA was complete by 60 min but was not evident after only 10 min at 39°C. Most, if not all, of the small amount of labeled *ts*-4 mutant RNA that was synthesized after shift up and sedimented from 20S to 35S was found by electrophoresis in 1% agarose gels to be not 26S RNA but RFs and RIs (data not shown). Wild-type SFV continued to synthesize both 42S and 26S RNAs under these shift-up conditions (Fig. 1C), although, as described previously (9, 11), the distribution of 42S:26S RNA was different from that observed at 27°C.

Analysis of the double-stranded cores of *ts*-4 RIs. Since labeled RNA was found in double-stranded RIs and RFs, the failure to detect 26S single-stranded RNA newly synthesized at the nonpermissive temperature could have resulted from a temperature-sensitive defect in the elongation or release of nascent 26S RNA strands from the RI_b. We isolated the double-stranded cores from [^{3}H]uridine-labeled *ts*-4 mutant-infected cells both at permissive temperature and after shift up to nonpermissive temperature, and we further determined whether labeled RNA was present in RFIII. The three double-stranded cores were present in the RNase-treated RIs from *ts*-4 mutant-infected cells maintained at 27°C (Fig. 2A). This was expected, since both 42S and 26S RNAs were synthesized at 27°C. The distribution of label in the three cores was in the same proportion as that in RIs from wild-type-infected cells maintained at 27°C. Figure 2B shows the sucrose gradient profile of the cores present in RNase-treated *ts*-4 mutant RIs that had been obtained from cells infected at 27°C for 6 h, shifted up to 39°C for 1 h, and then labeled with [^{3}H]-uridine from 7 to 8 h. Only RFI was labeled at 39°C; RFII and RFIII were not. In contrast, after a similar experiment with wild-type virus, RIs yielding all three size classes of double-stranded cores contained RNA that had been

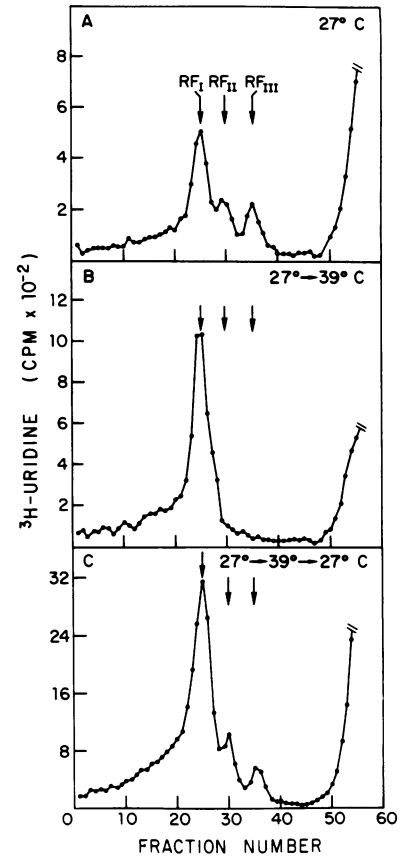

FIG. 2. *Analysis on sucrose gradients of the double-stranded cores obtained after RNase treatment of RIs from ts-4 mutant-infected cells. RNAs, from the 20S to 35S region of the sucrose gradients presented in Fig. 1, and from the same region of sucrose gradients of ts-4 mutant RNAs in cultures shifted up to 39°C at 6 h after infection and then shifted back down to 27°C from 7 to 10 h, were treated for 15 min at 25°C with 0.006 μg of pancreatic RNase, then were centrifuged at 154,000 × g in the SW41 rotor for 16 h at 20°C through linear 15 to 30% sucrose gradients in TNE buffer containing 0.2% SDS. (A) Double-stranded cores from ts-4 mutant RIs in infected cells maintained at 27°C. (B). Double-stranded cores from ts-4 mutant RIs in infected cells shifted up to 39°C for 2 h at 6 h after infection. (C) Double-stranded cores from ts-4 mutant RIs in infected cells shifted up to 39°C from 6 to 7 h and then shifted back to 27°C for an additional 3 h.*

newly synthesized at 39°C. When *ts*-4 mutant-infected cultures that had been shifted up to 39°C were shifted back to 27°C, synthesis of 26S RNA resumed at the lower temperature (15). Coincident with the resumption of 26S RNA synthesis in the shift-up and shift-down experiments, labeled RNA reappeared in RFII and RFIII cores (Fig. 2C).

Temperature-dependent conversion of *ts*-4 RIs. The results described above could be explained in two alternate ways: either (i) when shifted to 39°C, RIs yielding RFII plus RFIII were present but no transcription occurred on them, or (ii) RI_b was converted to RI_a and thus only RFI was obtained after RNase treatment. The distinction between these two types of RI, both of which contain a 42S minus-strand RNA as template, rests solely on the presence in the RIs in which 26S RNA is synthesized of a small region that remained sensitive to RNase after deproteinization of the RIs. If this region were altered, after the shift up in temperature, so that it no longer was sensitive to RNase, the RI_b previously yielding RFII plus RFIII would be resistant to cleavage and would be indistinguishable from RI_a yielding RFI. This would imply that a conversion between the two types of RI was possible and was under the control of a virus-specific protein.

Both these possibilities could be tested by following the fate at 39°C of RIs that had been assembled at 27°C. Pulse-chase experiments were necessary in which the labeled precursor was incorporated only during the pulse period and in which synthesis and processing of RNAs during the chase period proceeded normally. The two types of RI could be directly compared if they contained radioactive label only in their 42S minus-strand RNA. We used pulse-chase techniques previously described (18, 26), and verified that labeled precursor was not incorporated during the chase.

Monolayers of BHK cells were pretreated with 20 mM glucosamine and 1 μg of AMD per ml for 1 h before infection at 27°C with *ts*-4 mutant virus. [^{3}H]uridine was present from 0 to 2 h. Thereafter, the labeled medium was removed and replaced with growth medium at 27°C containing 20 mM glucosamine and 100 μg of unlabeled uridine per ml. After 2 h, one set of cultures was put into new medium containing glucosamine and uridine, and the second set was changed to medium containing 100 μg of unlabeled uridine per ml only. At the times indicated, the infected cells were lysed by the addition of 2% SDS, and the labeled RNAs were directly analyzed on linear 15 to 30% sucrose gradients. The total *ts*-4 mutant RNAs sedimenting from 15S to 45S that were synthesized under the two conditions of chase was determined. To correct for any variation in recovery of RNAs, the total amount of labeled *ts*-4 mutant RNA in each sample was normalized to a constant optical density for 28S rRNA. Only when maintained in glucosamine and uridine was there no increase in total labeled *ts*-4 mutant RNA during the chase (Fig. 3). This was true whether the cultures were maintained at 27°C and shifted up to 39°C, or shifted up to 39°C and shifted back down to 27°C. In contrast, the cells maintained in excess unlabeled uridine only showed a steady increase, after a lag period of 2 h, in the amount of newly synthesized RNA that was labeled.

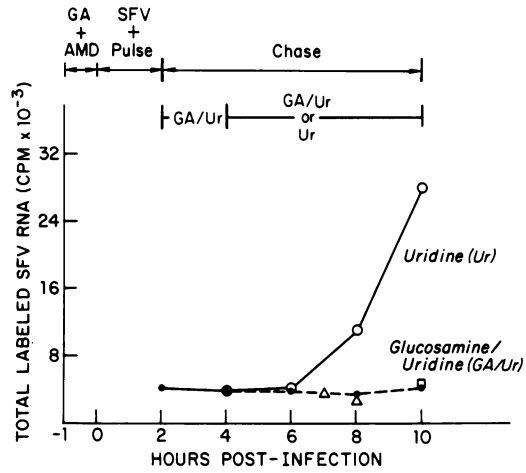

FIG. 3. *Total amount of labeled ts-4 mutant RNA newly synthesized under two conditions of chase. Monolayers of BHK cells in 100-mm petri dishes were pretreated with 20 mM glucosamine and 1 μg of AMD per ml for 1 h at 27°C. Fresh medium containing [^{3}H]uridine (10 μCi/ml) and ts-4 mutant virus (40 PFU/cell) was added, and the cells were incubated for 2 h at 27°C. At 2 h after infection, the monolayers were washed, and all received medium containing 20 mM glucosamine and 100 μg of unlabeled uridine per ml. At 4 h after infection, the infected monolayers were again washed and divided into two sets for further incubation. One set received medium containing glucosamine and 100 μg of unlabeled uridine per ml, and the second set received medium containing 100 μg of uridine per ml. AMD was present at 1 μg/ml throughout the infectious cycle. At the times indicated, SDS was added to the washed cells to a concentration of 2%, and the RNAs were sedimented on linear 15 to 30% sucrose gradients in TNE as described in Fig. 1. The total amount of labeled ts-4 mutant RNA expressed on the ordinate is the labeled, acid-insoluble RNA sedimenting from 15S to 45S, normalized so that each gradient contained the same amount of 28S ribosomal RNA as determined by optical density at 260 nm. (○) Cultures chased after 4 h at 27°C by the addition of medium containing 100 μg of uridine per ml. Cultures chased after 4 h at 27°C by the addition of medium containing 20 mM glucosamine and 100 μg of uridine per ml: (●) when maintained at 27°C; (△) when shifted to 39°C at 6 h for 1 or 2 additional h; and (□) when shifted up to 39°C for 1 h at 6 h after infection, then shifted back down to 27°C for an additional 3 h.*

Since the amount of labeled RNA at the end of the pulse and after the 8-h chase remained

constant when glucosamine and uridine were present, we determined the effect of this treatment on ongoing RNA synthesis. To obtain RIs labeled only in their minus strands, it was necessary that RNA transcription continue during the chase period to allow replacement in RIs of pulse-labeled nascent plus strands with unlabeled nascent plus strands. We analyzed the distribution of the pulse-labeled *ts*-4 mutant RNAs synthesized from 0 to 2 h after infection and compared this with the distribution of these pulse-labeled RNAs after 6 h of chase in the presence of glucosamine and uridine. At the end of the 2-h pulse, the distribution of the labeled RNA resembled that of the double-stranded RIs, and there was little, if any, labeled 42S single-stranded RNA (Fig. 4A). After the 6-h chase (Fig. 4B), the total amount of labeled RNA had not increased, but labeled 42S and 26S plus-strand RNAs were completed and released from the RIs. Thus, in the presence of glucosamine and uridine, RNA transcription had continued during the chase, and labeled nascent RNA chains initiated during the 2-h pulse were released as single-stranded, full-sized RNAs.

To confirm that only the minus strands synthesized during the pulse would be labeled in these RIs at the end of the chase, we isolated the double-stranded RNAs from *ts*-4 mutant-infected cells on Sepharose 2B columns. The RFs and RIs eluted in the void volume during chromatography, and the single-stranded RNAs were retarded. The double-stranded RNAs were concentrated by ethanol precipitation and treated with pancreatic RNase, and the double-stranded cores were separated on sucrose gradients. The RFs were denatured and annealed in the presence of unlabeled 42S virion RNA, and the percentage of the [^{3}H]uridine-labeled RNA that had been converted to RNase resistance by hybridization with unlabeled 42S plus-strand RNA was determined. At the end of the pulse period, the pulse label was in both plus and minus strands of RIs isolated from wild-type virus-infected cells maintained at 37°C (Fig. 5A). At this time, about 40% of the labeled RNA in cores from these RIs was in minus-strand RNA.

After a chase period of 1 h at 27°C, from 36 to 42% of the labeled RNA in RIs from *ts*-4 mutant-infected cells maintained at 27°C was also in minus-strand RNA (Table 1). After 2 h, 64 to 72% was in minus-strand RNA, and after 3 h over 80% of the pulse label was in minus-strand RNA (Table 1 and Fig. 5B). A similar chase of nascent plus strands out of the RIs was found when cultures were shifted up to 39°C (Fig. 5C). Thus, it is clear that, at the end of a

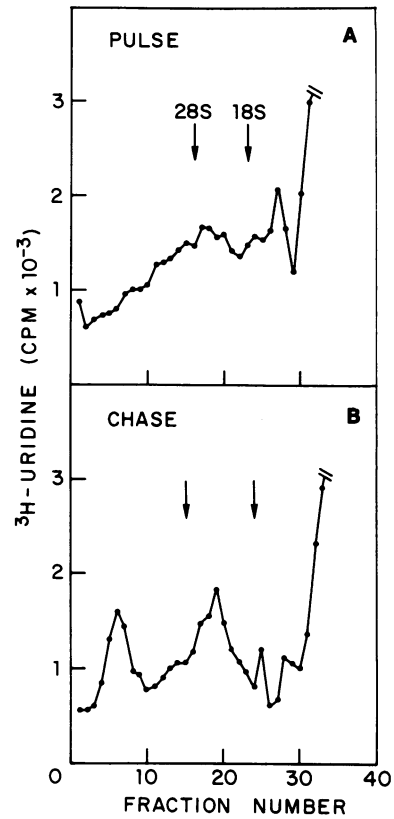

FIG. 4. *Sedimentation on sucrose gradients of ts-4 mutant RNAs at the end of the 2-h pulse at 27°C (A) and after a 6-h chase period at 27°C (B). Monolayers of BHK cells infected with ts-4 mutant virus were labeled with [^{3}H]uridine (10 μCi/ml) from 0 to 2 h after infection. The monolayers were pretreated before infection with glucosamine and AMD and, after the 2-h virus adsorption and labeling period, were treated with glucosamine, unlabeled uridine, and AMD, as outlined in Fig. 3. At the end of the pulse or chase period, SDS at a final concentration of 2% was added to the monolayers, and the labeled RNAs were sedimented in a SW27 rotor at 87,000 × g for 15 h at 18°C through 15 to 30% linear sucrose gradients in TNE buffer. Fractions were collected from below, and a portion was analyzed for labeled, acid-insoluble RNA in a toluene-based scintillation fluid.*

chase period of 3 h or more, most of the nascent, labeled plus-strand RNAs had been chased out of the *ts*-4 mutant RIs, and that the majority, if not all, of the labeled RNA in the cores was in minus-strand RNA.

Since the conditions of chase used were effective and met all of our requirements, we examined the fate after shift up to 39°C of the 42S minus-strand template in pulse-labeled RI$_b$ in which 26S RNA was synthesized and that yielded RFII and RFIII after RNase. Cultures

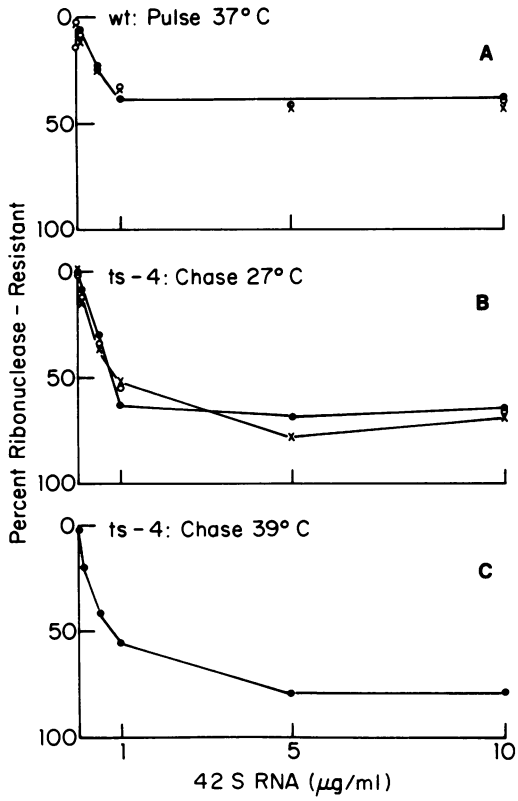

FIG. 5. *Percent of RNase-resistant labeled RNA in the three double-stranded cores of RIs from wild-type and ts-4 mutant virus-infected cells after hybridization with increasing amounts of unlabeled 42S wild-type virion RNA. Cultures were labeled at 27°C from 0 to 4 h after infection rather than from 0 to 2 h, since more labeled RNA was available for analysis after the 4-h labeling period. The RIs and RFs in extracts from infected cells were purified by chromatography through Sepharose 2B as described in the text. The double-stranded RNAs in the void volume were treated with 0.006 μg of pancreatic RNase per 5 to 10 μg of RNA for 15 min at 25°C, then were sedimented in the SW41 rotor at 154,000 × g for 16 h at 20°C. The three cores were separately pooled and concentrated by ethanol precipitation. The RFI, RFII, and RFIII, after denaturation with heat, were annealed with unlabeled 42S virion RNA, and the amount of RNase-resistant RNA was determined as described in the text. (A) Wild-type cores labeled with [^{3}H]uridine and [^{3}H]adenosine (each at 100 μCi/ml) from 0 to 4 h at 37°C, then harvested at 4 h. (B) ts-4 mutant cores from cultures labeled at 27°C with [^{3}H]uridine (100 μCi/ml) from 0 to 4 h, thereafter chased with medium containing 20 mM glucosamine, 100 μg of unlabeled uridine per ml, and 1 μg of AMD per ml at 27°C from 4 to 7 h, at which time the cells were harvested. (C) ts-4 mutant cores from cultures handled identically to those in (B), but shifted up to 39°C for 1 h, 6 h after infection at 27°C. Symbols: ●, RFI; ○, RFII; ×, RFIII.*

of *ts*-4 mutant-infected cells were pulsed with [^{3}H]uridine from 0 to 2 h and chased with glucosamine and excess unlabeled uridine. The labeled RIs sedimenting from 20S to 35S on sucrose gradients were collected from cultures maintained at 27°C, from cultures shifted to 39°C from 6 to 8 h after infection, and from cultures shifted up to 39°C and then shifted back down to 27°C. The total amount of labeled minus-strand RNA remained constant from 8 to 10 h after infection (Table 2). Minus-strand RNAs were distributed in all three cores in approximately equimolar amounts (RFI, 1.00; RFII, 0.95; RFIII, 0.89) in cultures maintained at 27°C, whereas all of the radioactivity in minus strands originally in RFII and RFIII was quantitatively shifted to RFI in cultures shifted at 6 h to 39°C. When cultures shifted to 39°C were subsequently shifted back to 27°C for 3 h, labeled minus strands reappeared in RFII and RFIII in almost the same ratio as when maintained at 27°C throughout infection. There was thus a reversible conversion between the two types of RI, RI_a, in which 42S RNA was synthesized, and RI_b, in which 26S RNA was synthesized.

DISCUSSION

Both the 42S and 26S SFV RNAs are transcribed from a 42S minus-strand RNA, but in two different types of RI, both of which sediment from 20S to 35S (21). These two types of RI are only distinguishable by their response to pancreatic RNase. The results reported in this study with an RNA-negative mutant of SFV indicated that one mechanism for the regulation of 26S and 42S RNA synthesis was through the reversible interconversion of RI_a, in which 42S

TABLE 1. *Percentage of total RFI, RFII, or RFIII in minus strands after chase at 27°C*[a]

Length of chase at 27°C (h)	Amt of labeled minus-strand RNA (%)		
	RFI	RFII	RFIII
1	36	42[b]	
2	72	64	70[b]
3	82	74	83

[a] The pulse and chase conditions and the isolation of the three RFs were identical to those described in Fig. 3. The cores, 0.01 to 0.1 μg, were annealed with 5 or 10 μg of unlabeled 42S plus-strand virion RNA as described in the text, and the amount of minus-strand RNA was taken as the percentage of total labeled RF that remained RNase resistant after annealing.

[b] Represents one determination with 10 μg of virion RNA. All other values are the average of from two to four determinations on two different preparations of the individual RFs.

TABLE 2. *Temperature-dependent conversion of ts-4 RIs labeled with [^{3}H]uridine from 0 to 2 h after infection*[a]

Time (h) of infection at:			Radioactivity in cores (cpm)			
27°C	39°C	27°C	Total	RFI	RFII	RFIII
0–8			2,492	1,351	812	329
0–6	6–7		2,473	2,291	179	3
0–6	6–8		2,599	2,533	63	3
0–6	6–7	7–10	2,508	2,166	228	114

[a] The *ts*-4 mutant RIs obtained in pulse-chase experiments as described in Fig. 3 and 4 were pooled, and the cores were isolated.

RNA was synthesized, and RI_b, in which 26S RNA was synthesized. Demonstration of the reversible interconversion of the two types of RI was dependent on the successful preparation of RIs labeled only in their minus strand in pulse-chase experiments. The 42S minus strands synthesized during the 0- to 2-h or 0- to 4-h pulse period at 27°C were still present 6 to 8 h later; thus SFV minus strands, once synthesized, were conserved throughout infection.

The inhibition of 26S RNA synthesis in *ts*-4 mutant-infected cells shifted to 39°C; the concomitant loss of RNase sensitivity of the minus strand in RIs that previously could be cleaved to yield two cores; and the observed quantitative conversion of RI_b (yielding RFII and RFIII) to RI_a (yielding only RFI) suggest to us that a virus-specific protein was bound to RI_b, in which 26S RNA was synthesized, and was responsible for the conversion of RI_a to RI_b. It had been postulated previously that a specific protein was necessary for 26S RNA synthesis, but its function was unknown (18). The function provided by this virus-specific "conversion protein" is different from polymerase activity, since *ts*-4 mutant-infected cultures, when shifted to 39°C, continue to synthesize 42S RNA with essentially the same efficiency as those maintained at the permissive temperature (15).

The virus-specific protein necessary for the synthesis of 26S RNA was temperature sensitive in *ts*-4 mutant-infected cells. It did not function in cells shifted up to 39°C, but did function when infected cells were shifted back down to 27°C. That the protein was nonfunctional but present at 39°C was indicated by the recovery of its function when cells were shifted down in the presence of cycloheximide (15). These results suggest that, when active, this virus-specific protein served to promote internal initiation of synthesis of 26S plus-strand RNA on the minus-strand RNA template. It simultaneously served to block transcription of the 42S minus strand in this region by the SFV RNA polymerase that had bound to and was copying the minus strand from its 3′ end. The blocked sequence on the minus strand between RFII and RFIII remained single-stranded after extraction of the RIs and thus was susceptible to digestion with pancreatic RNase. That initiation of transcription was simultaneously occurring from the 3′ end of the minus strand and also internally near or at the site where the virus-specific conversion protein was bound was suggested (i) by the constant finding, after RNase treatment, of RFII and RFIII in equimolar amounts (our results; 13, 16, 21); (ii) by the slow but steady increase of newly synthesized RNA in isolated RFII at all times during infection (21); and (iii) by the observed reversible, quantitative interconversion of the two types of RI. These three findings indicate that the whole of the 42S minus-strand RNA was available for transcription by the SFV polymerase in RIs in which 26S RNA was being synthesized.

Since no single-stranded, plus-strand RNA copies of the RFII sequence have been detected in infected cells (22), this region of the 42S minus-strand RNA probably does not contain internally a sequence required for termination. In RI_b, nascent plus-strand RNA chains initiated at the 3′ end of the 42S minus strand would be blocked from proceeding beyond the 3′ two-thirds of the minus strand by the virus-specific conversion protein that promotes internal initiation of 26S RNA synthesis. It is possible that the 3′-terminal two-thirds of the minus strand could be fully loaded with SFV RNA polymerase molecules that are unable or only infrequently able to proceed beyond the block toward the 5′ terminus of the minus-strand RNA.

If the model proposed is valid, the virus-specific conversion protein regulates the synthesis of 26S RNA by the conversion of RI_a to RI_b and is not a component of the SFV RNA polymerase. It functions only by binding internally on the 42S minus-strand templates. In *ts*-4 mutant-infected cells, this protein bound at 27°C to an RI, resulting in the synthesis of 26S RNA. When cultures were shifted to 39°C, this bound protein dissociated from the template, and RI_b was converted by the SFV RNA polymerase to RI_a by

read-through of the region recognized by the conversion protein. Synthesis of 42S RNA resulted.

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