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Role for nsP2 Proteins in the Cessation of Alphavirus Minus-Strand Synthesis by Host Cells

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In order to establish nonlytic persistent infections (PI) of BHK cells, replicons derived from Sindbis (SIN) and Semliki Forest (SFV) viruses have mutations in nsP2. Five different nsP2 PI replicons were compared to wild-type (wt) SIN, SFV, and wt nsPs SIN replicons. Replicon PI BHK21 cells had viral RNA synthesis rates that were less than 5% of those of the wt virus and ~10% or less of those of SIN wt replicon-infected cells, and, in contrast to wt virus and replicons containing wt nsP2, all showed a phenotype of continuous minus-strand synthesis and of unstable, mature replication/transcription complexes (RC+) that are active in plus-strand synthesis. Minus-strand synthesis and incorporation of [^{3}H]uridine into replicative intermediates differed among PI replicons, depending on the location of the mutation in nsP2. Minus-strand synthesis by PI cells appeared normal; it was dependent on continuous P123 and P1234 polyprotein synthesis and ceased when protein synthesis was inhibited. The failure by the PI replicons to shut off minus-strand synthesis was not due to some defect in the PI cells but rather was due to the loss of some function in the mutated nsP2. This was demonstrated by showing that superinfection of PI cells with wt SFV triggered the shutdown of minus-strand synthesis, which we believe is a host response to infection with alphaviruses. Together, the results indicate alphavirus nsP2 functions to engage the host response to infection and activate a switch from the early-to-late phase. The loss of this function leads to continuous viral minus-strand synthesis and the production of unstable RC+.

The alphaviruses Sindbis (SIN) and Semliki Forest (SFV) are plus-stranded RNA viruses whose greater than 40S (11.7 kb) genomes encode four nonstructural proteins (nsP1-4), numbered according to their gene order (reviewed in reference 55), that are the essential components of the viral replicases and transcriptase. They are synthesized initially as polyproteins P1234 and P123, the former by readthrough of an opal termination codon between the nsP3 and nsP4 genes (54). The replicases involved in minus-strand (P123, P23) and genome (P23 or fully cleaved nsPs) synthesis contain uncleaved nsP2-containing polyproteins (32, 33, 52, 59). The transcriptase makes a subgenomic 26S mRNA that encodes the viral structural proteins. Capping of viral genome and 26S plus strands employs methyltransferase and guanylyltransferase activities present within the nsP1 protein (2–4, 23, 35, 51, 58) and 5′-triphosphatase activity resident in the N domain of nsP2 (57). The nsP1 protein also is involved in the initiation of minus-strand synthesis (21, 50, 53, 60) and interacts with nsP4 (12, 53), which is the RNA-dependent RNA polymerase and also affects host cell-dependent replication (11, 33). The N half to two-thirds of the nsP3 phosphoprotein is conserved among alphaviruses and provides essential functions for minus-strand and 26S mRNA syntheses (5, 26, 29, 30, 38, 59); it also includes a conserved macrohistone 2A-like sequence predicted to be an ADP ribose-1 phosphoesterase (5, 26). In several SIN mutants, the loss of nsP3 phosphorylation led to loss of minus-strand synthesis (5, 29).

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As illustrated in Fig. 1, the N-terminal domain of nsP2 expresses nucleoside triphosphatase (NTPase) and helicase activities (19, 26, 40), in addition to the RNA-dependent 5′-triphosphatase. The NTPase conserved motifs I (GSGKS) and II (DEAF) function in NTP binding and begin at residues 189 and 250, respectively. They and downstream conserved motifs III through VI are predicted to share homology with motifs in superfamily 1 helicases (26, 28). The C-terminal domain expresses a papain-like thiol protease that is responsible for processing P1234 and whose catalytic dyad comprises C481 and H558 (reviewed in reference 55). This region also functions in the internal initiation of 26S mRNA synthesis (55, 56) and in translocation to the nucleus (NTS) and nucleolus (NoTS) (27, 37).

The nsP2 protein also appears to play a role in the host's response to infection. Normally, cells are killed by wild-type (wt) virus infection, but mutant forms of nsP2 enabled the establishment of persistent alphavirus or replicon infections (16, 39). Three sets of nsP2 lesions were identified in discrete and similar regions of both SIN and SFV nsP2 (Fig. 1), and the positions of these sets of changes are intriguing. The first set, S1 and 2A, falls near or in the 5′-triphosphatase region in the N domain of nsP2 (57). The alphavirus 5′-triphosphatase resembles other viral 5′-triphosphatases in having an associated NTPase activity but differs in being a divalent cation-dependent enzyme that is activated by both Mg^{2+} and Mn^{2+}, a property it shares with the flavivirus West Nile NS3 enzyme (reference 57 and references therein). For SFV, both 5′-triphosphatase and NTPase activities require the K residue at position 192 within motif I (Fig. 1). A tunnellike structure consisting of an eight-stranded antiparallel β-barrel into which the 5′ end of the RNA extends, with clusters of conserved E

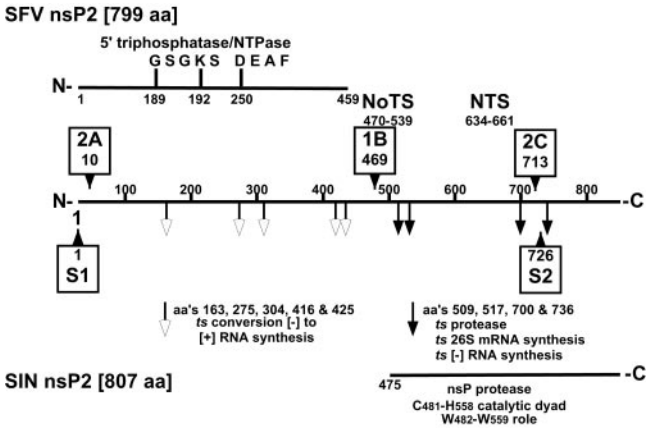

FIG. 1. Schematic of alphavirus nsP2. The N domain (amino acids 1 to 459) encodes an RNA 5′-triphosphatase, NTPase, and helicase; the C domain (amino acids 475 to 799/807) encodes a papain-like protease, functions in internal initiation of 26S mRNA synthesis and in translocation of nsP2 to the nucleus (NTS) and nucleolus (NoTS), and shares homology with 2′-0-methyltransferases (2′-*O*-MT). The *ts* mutants of SIN that map to nsP2 are indicated by downward arrows. The locations of the predicted amino acid substitutions conferring PI phenotype on two SIN mutants (S1, S2) and three SFV mutants (1B, 2A, 2C) are indicated.

residues implicated in metal binding (22, 31, 36), is predicted from the known structure of Cet1, the 5′-triphosphatase of *Saccharomyces cerevisiae* (34). The causal lesions in several temperature-sensitive (*ts*) RNA-negative mutants of SIN map to the N domain of nsP2 and overlap the 5′-triphosphatase and NTPase regions (6, 20, 42, 44, 45). Based on their defective synthesis of plus strands but not minus strands at 40°C, we called them conversion-defective mutants (Fig. 1) (6). Defects in capping of plus strands (5′-triphosphatase) or in other initiation events (unwinding of RNA structures) could explain their inability to convert nsPs to efficient, plus-strand synthesizing enzymes. Such defects did not involve a failure in P23 cleavage, which is required for switching the viral polymerase activity from minus-strand synthesis to plus-strand synthesis (32, 33, 52, 59). Some substitutions in this region of nsP2 (E118 to K, E131 to K, E155 to A, or E212 to G) rescued the growth in *Aedes* cells of SIN mutants that possessed alterations of nucleotides in the sequence of their 5′ nontranslated region 51-nucleotide conserved sequence element (13), suggesting they may also affect triphosphatase requirements.

The second set of nsP2 substitutions, S2 and 2C, are in the C terminus of nsP2, near the C end of the protease domain. They are also near two residues (amino acids 700 and 736) in SIN nsP2 whose alteration led to both the protease activity and the synthesis of 26S mRNA becoming temperature sensitive. In addition to these temperature-sensitive phenotypes, nsP2 mutants with alterations at residues 700, 517, and 522 were able to reactivate minus-strand synthesis at 40°C after it had ceased at 30°C (42, 45). The residue 726 mutant, S2, has also been obtained from persistent infection (PI) cultures independently by others (7, 16, 18) and as variants encoding different amino acids at this position (1). The C domain also contains sequences conserved in 2′-*O*-methyltransferases (2′-*O*-MT) (9), although the alphavirus sequence that begins at nsP2 residue 603 is predicted to be enzymatically inactive (A. Gorbalenya, personal communication). The third set of nsP2 changes, 1B, is a lesion in the interdomain region of nsP2 that lies adjacent to a sequence implicated in nucleolar (NoTS) localization (Fig. 1) (41).

We undertook a comparative analysis of five nsP2 mutant replicons to further probe the mechanism(s) responsible for their persistence in BHK21 cells. Persistence implied the survival of the host cell and the continual or continuous viral RNA synthesis to make replicon genomes and the subgenomic mRNA encoding the required drug resistance. Therefore, we considered two alternative mechanisms. In the first model, the presence of mutant nsP2 proteins allows a new wave of minus-strand synthesis to occur with each cell passage or cell division, and then its cessation occurs normally several hours later. In the second model, viral minus-strand synthesis is continuous, i.e., it fails to cease. An earlier study (39) verified the presence and steady-state levels of minus and plus strands but did not monitor rates of minus-strand synthesis over time and therefore could not distinguish between the two possibilities. In vertebrate cells such as BHK21, alphavirus infection is lytic, viral minus-strand synthesis stops selectively 4 to 6 h after infection (43), and, once made, minus strands exist as long-lived templates engaged in plus-strand synthesis by a stable replication/transcription complex (RC+). An exception to this pattern was found with cells deficient in the latent host cell endonuclease RNase L. In alphavirus-infected *Aedes* cells that naturally lack RNase L and in infected mouse embryo fibroblasts (MEF) deficient in RNase L (knockout MEF), viral minus-strand synthesis remained continuous, i.e., it did not shut off, and infected cells did not switch to the late phase (48). Moreover, in cells lacking RNase L, mature viral RC+-making genomes and 26S mRNA were not stable and lost activity with time after inhibiting protein synthesis (48).

The studies reported here found that BHK21 cultures persistently infected with replicons (PI cultures) producing mutated nsP2 exhibited a phenotype similar to that of RNase L-deficient cells infected with wt SIN or wt SFV: minus-strand synthesis was continuous, and unstable viral replication complexes were produced. Finding the same phenotype suggested that the two proteins function in similar events. For example, wt nsP2 proteins could be involved in inducing host responses that act through RNase L, either directly or indirectly, to block the formation of replication/transcription complexes. This would mean that, normally, in wt alphavirus-infected cells, functions associated with wt nsP2 proteins modulate the host response(s) to infection, which in turn leads to the loss of minus-strand synthesis but the production of stable RC+.

MATERIALS AND METHODS

Cells and virus. Chicken embryo fibroblast cells were prepared from 10-day-old embryos from the eggs of leukosis-free (SPF-COFAL/Marek-negative) flocks (Spafas, Roanoke, IL). BHK-21 cells (ATCC CCL 10) were obtained from the American Type Culture Collection (Manassas, VA). The growth medium for both cells was Dulbecco's modified Eagle minimum essential medium (DMEM) supplemented with penicillin (50 U/ml), streptomycin (100 U/ml), tryptose phosphate broth (5%, vol/vol), and fetal bovine serum (6%, vol/vol).

The wild-type viruses, the heat-resistant (HR) strain of SIN, and wt SFV were grown as described previously (5, 46). SIN nsP4 mutant R183S virus was described previously (11). Virus stocks used in this study were obtained by plaque purification and propagated at a low multiplicity of infection (MOI) of 0.1 to 1 PFU/cell.

The alphavirus replicon variants capable of establishing PI have been described previously (39). All the replicons contained a neomycin phosphotransferase under the control of the subgenomic promoter. The cloned SIN and SFV variants were used to derive drug-resistant cell lines (PI cultures) as described previously (39) and grown in G418-containing growth medium. The wt replicon genomes that were packaged into viral particles following their expression in cell lines producing the SIN structural proteins were also assayed (16, 17). We used the wt SIN replicon/GFP (no. 510; titer of 3.8×10^9 infectious units/ml), provided as packaged replicon stock, which was a generous gift of Ilya Frolov (University of Texas Medical Center, Galveston, TX).

Infection and RNA labeling. BHK21 cells were infected with SIN or SFV at an MOI of 100 PFU/cell. Cells in 35- or 60-mm-diameter petri dishes were labeled with 1 ml of 5′-[^{3}H]uridine (50 μCi/ml unless otherwise indicated) in DMEM containing 20 μg/ml of actinomycin D, 5% fetal bovine serum, and 20 mM HEPES (pH 7.4). At the end of the labeling period, cells were washed twice with ice-cold phosphate-buffered saline and lysed with 5% lithium dodecyl sulfate in LET buffer (0.1 M LiCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4) containing proteinase K, and the DNA was sheared with a 27-gauge needle as described previously (6). [^{3}H]uridine incorporation into viral RNA was determined by precipitation with 10% trichloroacetic acid. The precipitates from triplicate samples of 5×10^4 cells were collected on glass fiber filters, heat dried, and immersed in toluene containing OmniFluor (Perkin Elmer, Boston, MA). The radioactivity was determined with a Beckman LS 3801 (Fremont, CA). The relative amount of genome RNA to subgenomic 26S mRNA synthesis was determined after electrophoresis of infected-cell extracts on agarose gels (6).

Isolation of SIN replicative-form (RF) RNA and kinetics of minus-strand RNA synthesis. Minus-strand RNA synthesis was determined as described by Dé et al. (6). The cells were pulse-labeled for 1 h or continuously labeled for 1 to 7 or 8 h postinfection (p.i.) with [^{3}H]uridine in the presence of 20 μg/ml of actinomycin D to suppress host cell DNA-dependent RNA synthesis. The RNA was obtained by low-pH phenol and chloroform/isoamyl alcohol (95:5) extraction and ethanol precipitation. RF RNA was obtained by digestion with RNase A (0.1 μg/ml) and chromatography on CF-11 cellulose (Whatman, Clifton, NJ) as described previously (15). Minus-strand RNA synthesis was measured by RNase-protection assay, which determines the amount of heat-denatured [^{3}H]uridine-labeled RF RNA that was protected from RNase digestion (with 5 μg of RNase A/ml) by hybridization to an excess (about 100-fold) of unlabeled 49S plus-strand RNA (42). In this assay, the results are expressed as the percentage of the [^{3}H]uridine incorporated into the minus-strand component of purified, RNase-resistant RNA cores of viral replicative structures, i.e., RF RNA. If 40 to 50% of the total incorporation in the RF RNA is found in minus strands, it means that 80 to 100% of minus strands that were used as templates during the pulse period had been made during the pulse period. This can be found when minus-strand synthesis is measured early in an infection, when viral RNA synthesis increases exponentially (47). A value of 5% means that only 10% of the templates were newly made during the pulse period and the remaining 90% of the templates were made earlier, and therefore unlabeled; together they were the templates for the 95% of the incorporated [^{3}H]uridine that was recovered in nascent plus strands.

Electrophoretic analysis of replicon and viral RNA. For analysis of overall viral RNA, the proteinase K-treated extracts of 5×10^4 cells that had been labeled with [^{3}H]uridine were subjected to gel electrophoresis on 0.8% agarose gels in TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) buffer containing 0.2% sodium dodecyl sulfate. The gels were dehydrated with methanol and treated in 1% 2,5-diphenyloxazole (PPO) in methanol, followed by hydration to precipitate the PPO in the gel, which was then dried under vacuum and exposed to film at −80°C. Gel slices corresponding to the viral species were excised and counted by liquid scintillation spectroscopy as described above.

RESULTS

Kinetics of plus- and minus-strand syntheses in cells harboring wt SIN virus or PI replicons. The kinetics of wt alphavirus plus- and minus-strand syntheses differ (reviewed in references 43 and 55). Briefly, plus-strand SIN and SFV genomes and 26S mRNA accumulate as single-stranded RNA throughout infection and, during their syntheses, are found also as incomplete (nascent) strands within the RNA structures used by the viral polymerases that are called replicative intermediates (RI or native RF; for simplicity, these will be called RIs). Since single-stranded plus strands account for 95 to 98% of the RNA made in an alphavirus-infected cell, the incorporation of [^{3}H]uridine in the presence of actinomycin D into acid-insoluble RNA is taken as a measure of plus-strand RNA synthesis. In contrast to plus strands, which are synthesized throughout infection, minus strands are made only early during infection by cells expressing RNase L and are found exclusively within RIs, either as templates making genomes and 26S mRNA or as nascent strands associated with their plus-strand template, the genome. Detecting minus-strand synthesis requires purifying the RIs from the excess of plus strands. This is facilitated by the generation from RIs of RNase-resistant, double-stranded RNA during deproteinization of infected-cell lysates. Purified double-stranded "RF cores" (see Materials and Methods) of the RIs are analyzed in RNase protection assays that measure the amount of [^{3}H]uridine incorporated into the minus strands of the RF cores. This value indicates when minus strands are made and what fraction of the total minus strands are newly made, because only those minus strands made during the labeling period will have incorporated [^{3}H]uridine. Early after infection, exponentially increasing (10-fold or more per hour) numbers of RIs yield RF cores that are labeled fully in both their minus- and plus-strand components, i.e., ~50% of total RF core radioactivity is in plus strands and ~50% is in minus strands, reflecting the relative proportion of minus and plus strands in RF. A value of 50% of the RF cpm is the maximum possible as labeled minus strands, because RIs also are engaged continuously in plus-strand synthesis. With alphaviruses, minus-strand synthesis fails to continue after the early period due to the activation of some unknown process that blocks minus-strand synthesis (5, 43, 48, 55). The cessation process leads to a steady decline in the amount of labeled minus strands in the RF cores. For example, finding 30% of the total radiolabel in RF cores was in minus strands is relative to a maximum value of 50% when all minus strands are labeled, and thus indicates that 60% of templates active in plus-strand synthesis were made during the labeling period and 40% had been made before the labeling started. This analysis is illustrated in Fig. 2, which presents theoretical structures at early and late times. Minus-strand synthesis stops by 6 h p.i. at 37°C in SIN virus-infected cells (Fig. 3C); after this time, [^{3}H]uridine is incorporated at constant, maximal rates into the RIs because the number of minus-strand templates does not increase and only plus strands are labeled with [^{3}H]uridine.

We first characterized the kinetics of plus- and minus-strand synthesis by wt SIN HR virus and by wt SIN replicons. We infected BHK21 cells with packaged wt SIN replicons and found they resembled wt SIN HR virus infection in their patterns of incorporation of [^{3}H]uridine into plus strands (Fig. 3A), RF cores (Fig. 3C), and minus strands (Fig. 3E). Both SIN virus and wt SIN replicon-infected BHK21 cells showed the expected exponential increase in plus-strand RNA synthesis early in infection, converting to a linear and constant, maximal rate by 3 to 4 h p.i. (Fig. 3A). The wt SIN replicon-infected cells produced about half as many plus strands per hour and had about half the rate of incorporation of [^{3}H]uridine into RF cores (RIs) as wt SIN virus. Thus, for wt replicons as for wt SIN virus, the number of active RIs corresponded to the rate of plus-strand synthesis. Cells infected with wt SIN replicons also showed an exponential burst of minus-strand synthesis similar

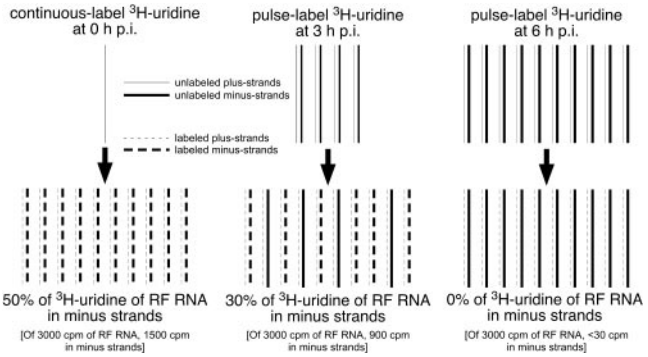

FIG. 2. During alphavirus (SIN or SFV) infection, minus-strand templates are synthesized until 4 to 5 h p.i. in most vertebrate cells, and then their synthesis stops and only plus strands are made. If [^{3}H]uridine is added at the beginning of infection (continuous label), as shown in the left panel, all viral minus and plus strands will be labeled, and 50% of the cpm in RF RNA is found in minus strands. If the [^{3}H]uridine is added early but after 40% of the minus strands have been made, only newly synthesized minus strands are labeled. In this example (middle panel), 30% of the cpm in RF RNA will be found in minus strands. If [^{3}H]uridine is added after 5 h p.i., when there is no longer any minus-strand synthesis occurring, only the plus strands of the RF RNA are labeled, and <1% of the cpm in RF RNA is found in minus strands. Thus, by determining the percentage of the [^{3}H]uridine-labeled RF RNA that is in minus strands, the relative rate of minus-strand synthesis compared to plus-strand synthesis can be determined. A value of 50% means that 100% of the minus-strand templates were made during the pulse-label, and a value of 30% means that 60% of the minus-strand templates were synthesized during the pulse-label and 40% were made earlier.

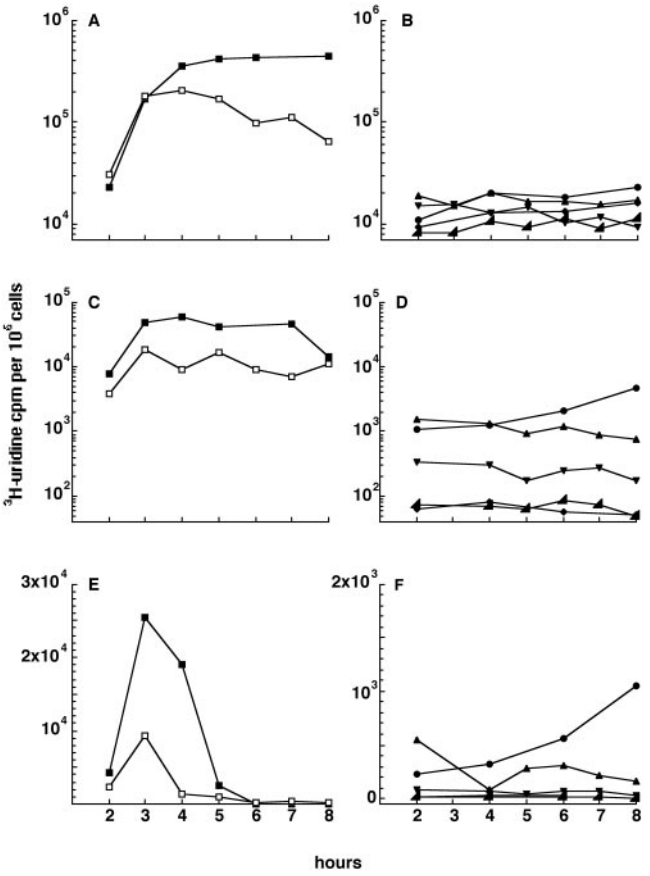

FIG. 3. RNA synthesis by five nsP2 mutant replicons compared to wt SIN virus and wt replicons. BHK21 cells were infected at 37°C either with wt SIN (■) virus at an MOI of 100 or with wt SIN replicons (□) at an MOI of 35 at time 0 (panels A, C, and E). The PI cells (panels B, D, and F) were SFV 1B (▲), SIN S1 (●), SIN S2 (◆), SFV 2A (▼), and SFV 2C (◢). Infected and PI cultures were labeled at 37°C for 1-h pulse periods with 200 μCi/ml of [^{3}H]uridine in the presence of 20 μg/ml of actinomycin D. The cpm per 10^5 cells is shown. Panels A and B show total acid-precipitable incorporation of [^{3}H]uridine (plus-strand RNA). Panels C and D show RF cores. The nuclease-resistant cores of the viral and replicon RIs were isolated as described in Materials and Methods. Panels E and F show minus-strand RNA. PI replicons 1B and S1 had the highest incorporation in minus strands per hour, which was ~5-fold higher than that of 2A and 10- to 20-fold higher than that of S2 and 2C.

to that with wt virus, albeit reduced in amount, and this burst was followed by a similar rapid decline and then overall cessation (Fig. 3E) of minus-strand synthesis. The expression of heterologous proteins (i.e., green fluorescent protein) by the wt replicons did not appear to influence replication kinetics. We conclude that infection by wt SIN replicons was sufficient to induce cessation of minus-strand synthesis, as seen in wt virus infected cells, and that the synthesis of the viral structural proteins was not involved in the process of shutting off minus-strand synthesis. Therefore, the cessation of minus-strand synthesis was not caused by the removal of their templates, i.e., genomes, by encapsidation. Viral mutants that are defective in encapsidation, e.g., SFV *ts*3 at 40°C, also ceased minus-strand synthesis normally (our unpublished data).

We next characterized the five PI-replicon cells. The first set of studies used successive 1-h pulse labels to monitor relative transcription rates (Fig. 3). In contrast to wt-infected cells, PI cells showed only a constant, linear rate over time and produced 1 to 5% as much viral RNA each hour as wt SIN, varying little among each other, or by approximately threefold at most (Fig. 3B). All five replicons incorporated [^{3}H]uridine into RIs at essentially constant rates during 1-h pulse-labeling (Fig. 3D), with replicons 1B and S1 showing ~5-fold higher amounts of incorporation per hour than 2A and ~10- to 20-fold higher amounts per hour than S2 and 2C. The results meant that different replicons formed different numbers of RIs. Because the five PI replicons did not differ significantly in overall rates of plus-strand synthesis, finding SIN S2 and SFV 2C had significantly fewer templates (Fig. 3D) suggested that RC+ containing the S2 and 2C mutated forms of nsP2 were more efficient; i.e., fewer minus-strand templates appeared to be required to produce the same number of plus strands. Another difference observed with the PI replicons was their ability to continuously make minus strands over the 8-h period (Fig. 3F). As expected from the low levels of RIs, the five PI cells made small amounts of minus strands. Individual PI replicons differed somewhat in how much minus-strand RNA was made per hour (Fig. 2F), and PI replicons with the highest rates of minus-strand synthesis (SVF1B and SIN S1) also had the most RIs, and those with the lowest rates of minus-strand synthesis (SFV 2A and 2C and SIN S2) had the least RIs. Thus, unlike wt SIN, wt SFV, or wt SIN replicons, minus-strand synthesis with the PI replicons was continuous and failed to shut off, and new RI and/or native RF was formed continuously.

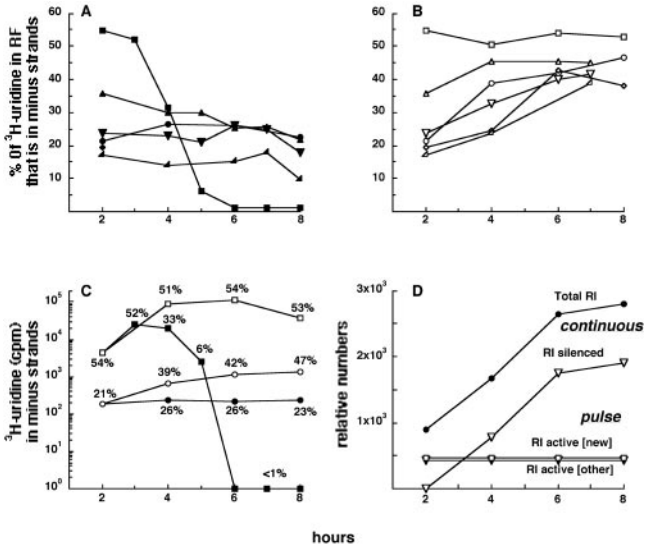

FIG. 4. Synthesis and accumulation of minus strands by PI replicons relative to wt SIN. The incorporation of [^{3}H]uridine into viral minus strands from the samples shown in Fig. 2 is expressed as a percentage of the total incorporation in RF core RNA that was in minus strands. The cells were labeled with 200 μCi/ml of [^{3}H]uridine in the presence of 20 μg/ml of actinomycin D for 1-h pulse periods (panel A) or continuously from 1 to 7 or 8 h p.i. (panel B). Panel C depicts the incorporation of [^{3}H]uridine into minus-strand RNA by SIN S1 PI cells during pulse-labeling (●) or continuous labeling (○) and by wt SIN in pulse-labeling (■) and continuous labeling (□). The percentage of the total incorporation in RF core RNA that was in minus strands at each time is also given. Panel D shows the relative number of RIs in the pulse-labeled, active fraction versus the continuously labeled, total RI fraction. The data for this panel are from Table 1. The total (continuously labeled) RIs minus the active RIs (the sum of those with labeled and unlabeled templates) each hour yields the relative number of inactive (silenced) RIs accumulating in PI cells. (●), S1 total RIs, continuously labeled; (△), S1 silenced RIs (total RI cpm minus the cpm in pulse-labeled [active] RIs); (□), S1 pulse-labeled RIs with radiolabeled minus strands; (▼), S1 pulse-labeled RIs with unlabeled minus strands.

This was novel and intriguing because it meant either that not all minus strands were being utilized as templates or that the normally long-lived RC+ were short-lived in PI cells. To address this issue we determined the fraction of the total RIs that were made each hour by comparing the percentage of the [^{3}H]uridine in RF cores that was in minus strands each hour following 1-h pulse-labels and also over time during a 7-h continuous-labeling period. This allowed us to follow the fate of minus strands over time and observe whether they accumulated in RIs or were rapidly turned over and degraded. Results from pulse-labeling (Fig. 4A) indicated the level of minus-strand synthesis by the PI cells was considerable, as it represented 15% to 30% of the total radiolabeled RF core RNA each hour. This meant an additional 30% to 60% new RIs were being formed each hour by PI cultures that nevertheless incorporated [^{3}H]uridine into RIs at a constant level (Fig. 3D). With continuous labeling, the total amount of radiolabeled RIs (RF cores) increased with time for all five PI cell cultures; wt SIN infected cells showed zero, or no more than ~15%, increases after 4 h p.i. (data not shown). For S1, the total RF core cpm increased 3.4-fold between 1 and 7 h of labeling, and ~2-fold between 2 and 7 h (Table 1). An increase of 3.4-fold would mean only one-third of RIs made over the 7-h period (2,802 RF cpm; Table 1) were active the last hour (1,005 cpm were in labeled RF in a pulse given 7 to 8 h p.i.; Table 1). Second, in addition to an increase in the amount of radiolabeled RIs, the fraction of the RI population that was made during the labeling period also increased with time of labeling (Fig. 4B). Because 90 to 94% of the radiolabeled RIs isolated after 6 or 7 h of [^{3}H]uridine had radiolabeled minus strands, most of the original RI population that would have had unlabeled minus-strand templates had been replaced. The five PI replicons differed slightly in rates of RI turnover/replacement: 1B had 90% or more new RIs within a 3-h period; S1 and 2A required about 5 h, and 2C and S2 required 6 h of labeling (Fig. 4B). This was in contrast to wt SIN, where during continuous labeling ~50% of the radiolabeled RF core RNA was in minus strands at any time after adding the label (Fig. 4B and C), which is consistent with the minus strands being made early and functioning as templates in stable RC+ throughout infection. The data presented in Table 1 and Fig. 4C and D further illustrate the concept that there is a population of inactive RIs accumulating in S1 replicon PI cells. Because, on average, 24% of the total radiolabeled RF core RNA was in minus strands during 1-h pulse-labels, ~50% of the RIs engaged in plus-strand synthesis were newly made that hour and ~50% were made earlier. In contrast, after a continuous 7-h labeling period, the amount of [^{3}H]uridine in RF cores increased sixfold, consistent with their accumulation over time. The original S1 RI population with unlabeled templates now represented only ~6% of the recovered RI population (Table 1). The apparent loss of the original RI population could reflect their dilution by newly made ones (expansion of the RI pool) as well as their degradation. Synthesis of 50% new RIs per hour would lead to an expected dilution of 4.5-fold over the 7-h period, and cannot alone explain the observed low value of ~6%. Thus, it is possible some older, silenced RIs also underwent degradation. Such results were unexpected. This meant the normal stabilization of RC+ seen in wt SIN or wt SFV was not occurring in PI cells with replicons expressing mutant nsP2 proteins.

TABLE 1. Kinetics of labeling of RIs and minus strands by the S1 PI replicon

Labeling method[a]	Labeling time (h)[a]	S1 RF core, cpm/10^5 cells	% RF cpm in minus strands	% of total minus-strand templates	
				New (labeled)	Old (unlabeled)
Pulse	1–2	829	21	42	58
	3–4	893	26	52	48
	5–6	857	26	52	48
	7–8	1,005	23	46	54
Accumulation	1–2	829	21	42	58
	1–4	1,664	39	78	22
	1–6	2,641	42	84	16
	1–8	2,802	47	94	6

[a] Times listed for the persistently infected S1 replicon cells are relative to infections of duplicate cultures with SIN HR (p.i.). Pulse labeling used 1-h pulses of [^{3}H]uridine (200 μCi/ml) in the presence of 20 μg/ml of actinomycin D and harvest of the culture at the end of the pulse period. Accumulation or continuous labeling involved the addition of the radiolabeled uridine-containing medium to all cultures at 1 h p.i. and the harvest of individual cultures at the times indicated.

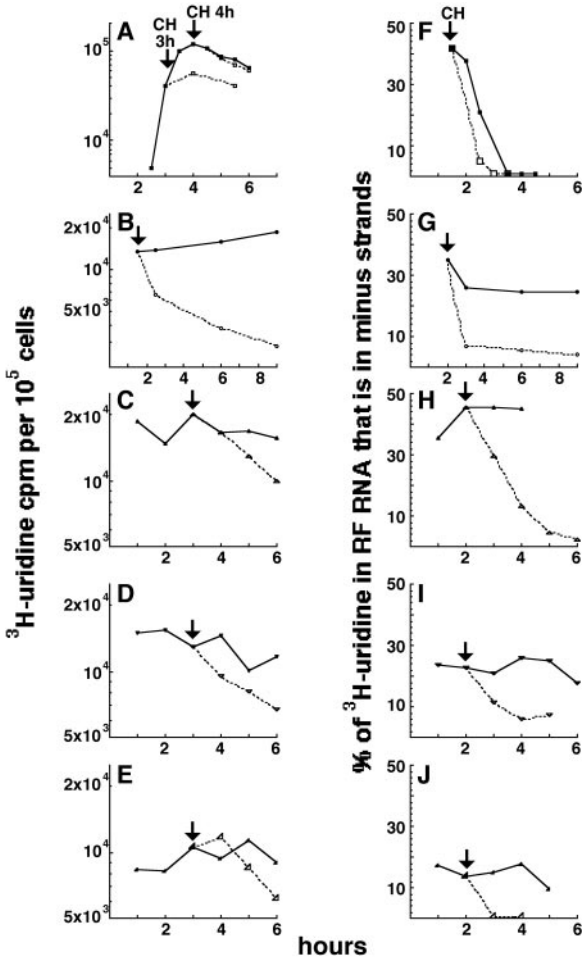

FIG. 5. Effects of inhibiting protein synthesis on PI replicon and wt SIN plus- and minus-strand synthesis. Each of the replicon PI cells and SIN-infected cells was treated with 100 μg/ml of CH or untreated and pulse-labeled for 1-h periods with 200 μCi/ml of [^{3}H]uridine/ml in the presence or absence of CH. Total viral RNA synthesis (panels A to E) and minus-strand synthesis (panels F to J) were determined as described for Fig. 2 and 3. Cultures treated with 100 μg of CH/ml in complete DMEM medium were incubated continuously in CH-containing medium beginning at the time(s) indicated by the arrows, and their incorporation is shown by the dashed lines. The hours indicate the start of the wt SIN infection. A and F, wt SIN; B and G, SIN S1; C and H, SFV 1B; D and E, SFV 2A; E and J, SFV 2C.

Effect of cycloheximide on plus- and minus-strand synthesis in cells harboring SIN and SFV PI replicons. To determine whether the plus-strand activity of the RIs in the replicon-containing PI cells was indeed turning over, i.e., was unstable, we measured plus- and minus-strand synthesis after the addition of the translation inhibitor cycloheximide (CH), which prevents the synthesis of nonstructural (ns) polyproteins and therefore causes minus-strand synthesis (Fig. 5F), but not plus-strand synthesis (Fig. 5A), to stop. When CH was added to wt SIN-infected cells during the early phase (3 h p.i. in Fig. 5A), minus-strand synthesis (Fig. 5F) stopped prematurely and plus-strand synthesis remained at the rate ongoing at the time of CH addition. When added an hour later (4 h p.i. in Fig. 5A), after minus-strand synthesis had already stopped normally and plus-strand synthesis was nearly maximal, it had little effect on plus-strand synthesis. With the PI replicon cells (Fig. 5G, H, I, and J), minus-strand synthesis was inhibited after treatment with CH, as was the case when CH was added to wt SIN infected cells early. Thus, in the PI cultures, minus-strand synthesis remained dependent on a supply of new ns polyproteins, similar to wt virus (46, 47). In contrast to wt virus (Fig. 5A), there was a 13 to 17% loss per hour of plus-strand synthesis in the PI replicon cells after CH addition (Fig. 5B, C, D, and E). After 3 h of drug treatment, plus-strand synthesis was reduced to 50 to 60% of rates ongoing in the absence of the drug and, after 8 h of treatment, to <20% of the rate observed in untreated cells. Thus, CH prevented the formation of new, active complexes, and the activity of the old ones was lost with time. The results corroborated our interpretation of the experiments reported in Fig. 4; mainly, that RC+ with mutated nsP2 became silenced with time and were replaced with new, active ones. This meant that in PI cells, the viral RC+ were targeted for turnover, unlike wt alphavirus-infected BHK21 cells, where they were stable. However, it is possible that the PI cells were a selected subpopulation, not normal BHK cells. The latter possibility has support from Dryga et al. (7), who stated that a selected subpopulation of the original cell population was recovered as the PI cells.

Are PI cells capable of mounting an antiviral host response and shutting off minus-strand synthesis? If the failure to cease PI replicon minus-strand synthesis was due to a change in the host response by cells that had been selected during the establishment of the persistent infection (16, 39), wt virus replication would be affected similarly to nsP2 mutant PI replication. It also would be important to rule out the possibility that the PI cells were unable to enter the late phase of infection because they lacked the ability to induce a full host response to infection that would shut off host translation and/or prevent any new minus-strand replicase from forming. We used wt SFV superinfection and determined the kinetics of wt SFV minus-strand synthesis in each of the PI cultures. As shown in Fig. 6A, all five PI cultures were capable of being superinfected with SFV, and the wt virus produced as much viral plus-strand RNA in PI cells as in control wt BHK21 cells. The superinfecting virus also made minus strands with normal kinetics during the early phase of the superinfection and underwent a normal cessation of its synthesis such that by ~7 h p.i., wt SFV minus strands were no longer made (Fig. 6B). Figure 6C shows the specificity of our RNase protection assay and its ability to distinguish SIN minus strands from SFV minus strands in cells producing both kinds of templates. Even with this specificity, we were not able to separately monitor the fate of PI replicon minus-strand synthesis in the superinfected cultures, due to the overwhelming excess of superinfecting RNA synthesis (50-fold over PI levels). We cannot therefore conclude definitively that the PI replicon minus-strand synthesis was also inhibited in the superinfected cultures. However, the results strongly suggest this occurred, because the levels of overall SFV minus-strand synthesis were below the values seen in the SFV replicon 2A, 1B, and 2C PI cultures that were not superinfected (i.e., average values of 36% [1B], 23% [2A], and 18% [2C]) (data not shown). Therefore, we interpret the results to mean that PI cells were capable of responding fully to alphavirus infection in the presence of wt nsP2 proteins and that BHK21 cells were

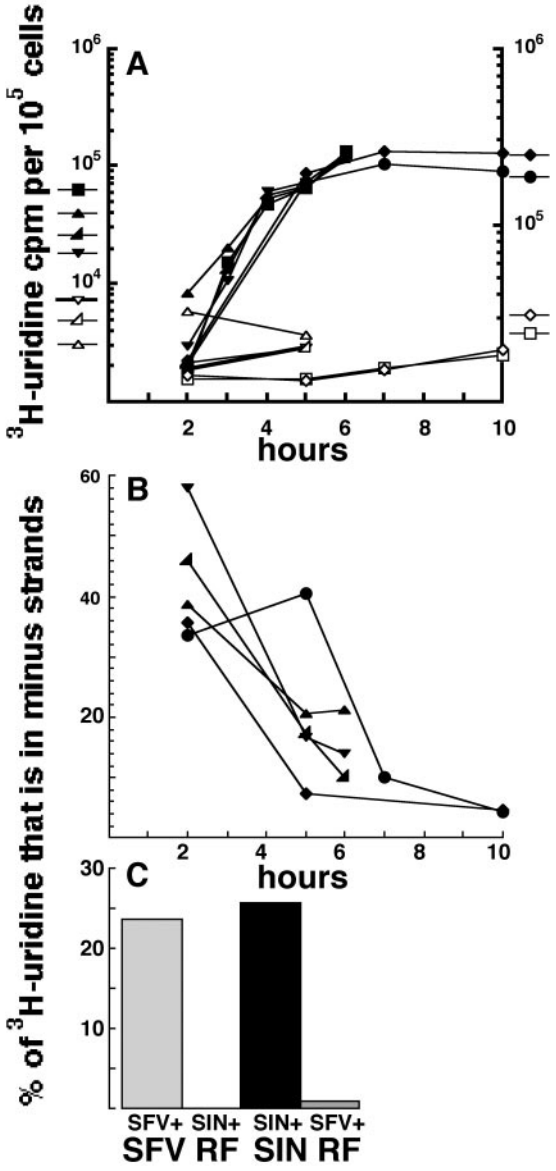

FIG. 6. Superinfection with wt SFV of PI cells activates cessation of minus-strand synthesis. One set of cultures of each of the PI cells was infected with wt SFV (solid symbols) and one set was left uninfected (open symbols). To aid detection of the low levels of S2 RNA, the S1 and S2 cells were pulse-labeled for 1-h periods with 200 μCi/ml of [^{3}H]uridine/ml and are plotted using the right axis, while the infected BHK21 cells and the 1B, 2A, and 2C cultures were labeled with 50 μCi/ml of [^{3}H]uridine/ml and are plotted using the left axis. The PI cells are SIN S1 (●), SIN S2 (◆), SFV 1B (▲), SFV 2A (▼), and SFV 2C (◢). BHK cells infected only with wt SFV are shown as (■). Hours shown are postinfection. (A) Total incorporation (plus-strand synthesis); (B) Minus-strand synthesis (% of the [^{3}H]uridine in RF RNA that was in minus strands); (C) Annealing controls. RF RNA from either wt SIN or wt SFV infected cells that had been labeled continuously with [^{3}H]uridine from 2 to 6 h p.i. was heat-denatured and allowed to anneal in the presence of an excess of unlabeled SIN or SFV genomes (plus strands) from purified virions.

not selected by the replicons to be those incapable of mounting an antiviral response to alphaviruses.

Superinfection is blocked in cells expressing wt nsP2. We next established that the failure to cease PI replicon minus-strand synthesis was not due merely to a low level of viral transcription in the PI cells. The concept that there is a threshold above which viral transcription triggers a host response, but below which it does not, is not supported by the results of Frolova et al. (18) nor by findings using an SIN HR nsP4 mutant R183S, which produces viral RNA at 2 to 5% of wt rates at 40°C and whose infected cells undergo a normal cessation of minus-strand synthesis (11). Using this mutant, we tested whether induction of cessation of minus-strand synthesis by wt nsP2 proteins would occur in the absence of high levels of viral replication and in turn would block minus-strand synthesis and overall RNA synthesis by a superinfecting genome. Mutant R183S virus-infected cells (MOI of 100 PFU/cell) were pulse-labeled at 40°C for 1-h periods between 1 to 9 h p.i. At 4 h p.i., a duplicate set of R183S-infected cultures were superinfected, and cultures of uninfected BHK21 cells were infected, with wt SIN HR (MOI of 100). The results (Fig. 7) demonstrated that at 40°C, while the R183S-infected cultures gave low rates of viral transcription and the wt SIN HR-infected cultures gave high rates of viral transcription, superinfected R183S-infected cultures allowed only minimal, if any, replication of the superinfecting virus. Processing the R183S-infected cultures for superinfection involved a brief period (1 to 2 min) at 25°C and allowed some increase in background RNA synthesis by the R183S in the newly superinfected cells. This twofold increase in level is seen in the 5- to 6-h-p.i. pulse that was slightly higher than in SIN HR singly infected cells, but this level did not increase further over time, i.e., the 6- to 7-, 7- to 8-, and 8- to 9-h pulses. Thus, it appears that SIN HR replication in superinfected R183S cells failed to occur when they were superinfected at the same time that cessation occurred in cells singly infected with wt SIN. This was in contrast to the results shown in Fig. 6A, where PI cells allowed the full replication of superinfecting alphaviruses. The results propose that superinfection exclusion was activated in cells with wt nsP2 proteins but not in cells expressing mutant nsP2 proteins,

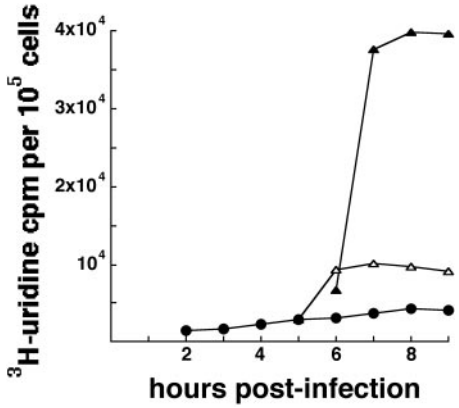

FIG. 7. SIN R183S does not allow superinfection with wt SIN. BHK21 cells were infected at 30°C with SIN R183S (arginine-to-serine substitution in amino acid 183 of nsP4) (●) and shifted to 40°C at 1 h p.i. At 4 h p.i., one set of SIN R183S-infected cells was superinfected at 40°C with wt SIN HR (Δ), and a set of uninfected BHK21 cells were infected with wt SIN HR (▲) at 40°C. The infected cells were pulse-labeled at 40°C for 1-h periods with 50 μCi/ml of [^{3}H]uridine. Total incorporation (plus-strand synthesis) was determined as described in Materials and Methods.

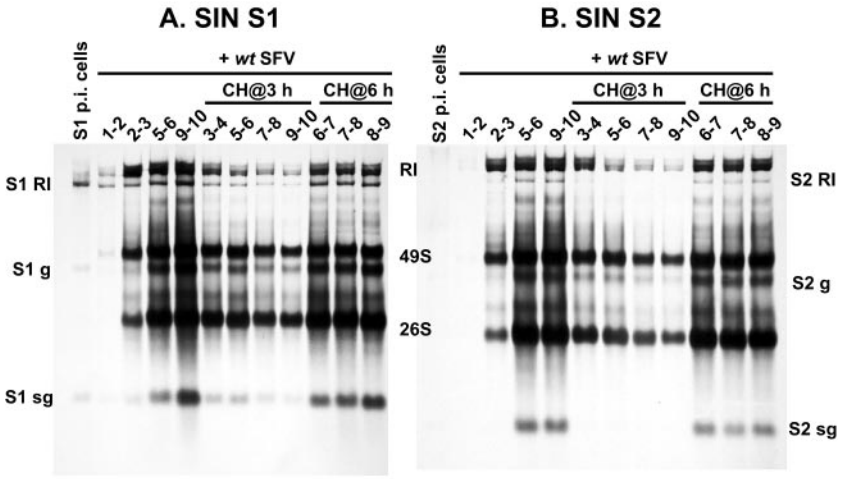

FIG. 8. Analysis by gel electrophoresis of PI replicon RNA synthesis after superinfection with wt SFV. Cultures of SIN S1 (A) and SIN S2 (B) replicon PI cells were or were not superinfected with wt SFV beginning at 0 h. Duplicate sets of superinfected cultures were treated with 100 μg/ml of CH, beginning at either 3 h or 6 h after superinfection. A sample of each extract solubilized with 5% lithium dodecyl sulfate-200 μg/ml proteinase K was analyzed directly by electrophoresis on 0.8% agarose-TBE gels. The gels were processed for fluorography and scans of the resulting fluorographs are shown. Scanning was with the Kodak electrophoresis documentation and analysis system 290 for Macintosh computers (Kodak Corp., Rochester, NY).

and that this phenotypic difference was due to qualitative (loss of function) and not quantitative differences.

Fate of viral RI complexes during superinfection. We determined whether, in PI cells, the superinfecting wt SFV replication complexes were subjected to turnover, similar to the PI replicon complexes, or whether they exhibited a normal stability. S1 and S2 PI cells were infected with wt SFV and some of these cultures were also incubated with CH beginning at 3 h p.i. or at 6 h p.i. The ability to continue transcription in the presence of CH was monitored with 1-h pulse-labels. Both SIN S1 and S2 PI cells gave similar results, with S1 showing higher levels of replicon RNA synthesis (Fig. 8A and B, respectively). Superinfection increased the amounts of replicon RNA synthesis significantly, as reported previously (16), and we also observed the expected increase in the rate of wt SFV RNA synthesis following superinfection. When CH was added at 3 h postsuperinfection, there was a loss over time in the amount of radiolabel being incorporated by both the wt SFV and PI-replicon RI/native RF (the bands at the top of the gel lanes in Fig. 8A and B) compared to that observed at 3 h p.i., the time of treatment; some, but not as much, reduction in synthesis of single-stranded SFV genome and 26S RNA is also seen, and is most evident for loss of S1 subgenomic mRNA synthesis (Fig. 8A). The loss in the transcriptional activity of preformed RI/native RF was not seen when the translation inhibitor was added at 6 h after superinfection (6 h p.i.). In summary, we found, in nsP2 mutant replicon PI cells, CH treatment led to a decline in plus-strand RNA synthesis if added early, but not late, after superinfection. There are two possible explanations for this. First, the mutant, but not the wt, nsP2 RC+ are unstable and turn over. Initially even wt RC+ may be formed using mutant nsP2, but later, when wt nsPs are overproduced, the RC+ would be formed with wt nsP2 and would be stable. The second possibility, which we favor, is that wt nsP2 blocks whatever causes alphavirus RC+ to turn over in PI cells but only after 3 to 6 h following superinfection. RC+ assembled after this time, either by mutant nsP2 or wt nsP2, would be stable. The second possibility suggests that a wt nsP2 function leads to the activation of antiturnover processes that protect the activity of viral RC+ assembled from that time onward.

DISCUSSION

Our studies found the ability of SIN or SFV replicons to cause a persistent infection involved significant changes to two generally observed features of alphavirus replication. In BHK21 cells, SIN and SFV PI replicons exhibited continuous minus-strand synthesis, which was unlike minus-strand synthesis in wt virus or wt SIN replicon-infected BHK21 cells that shut it off at ~4 h p.i. Also, the activity of RC+ making replicon genomes and subgenomic mRNA was unstable, unlike those assembled in wt SIN or SFV infected cells. Intriguingly, the same two features were observed for wt virus-infected cells lacking RNase L, a host-encoded, latent endonuclease (48). This commonality potentially implicates both viral functions (nsP2) and host functions (RNase L) in events leading to the cessation of minus-strand synthesis and the stabilization of RC+; whether or not the two factors are directly linked is not known.

The different behavior of PI replicons compared to the wt was not due merely to their low levels of viral RNA synthesis, since certain SIN mutants replicating at the same low levels efficiently caused minus-strand synthesis to cease and caused the formation of stable RC+. Also, the ability of SIN to block host translation does not depend on the level of viral RNA synthesis (12, 18). It was not due to a lack of synthesis of structural proteins, since wt SIN replicons exhibited a wt pattern of cessation (Fig. 3). Cessation of minus-strand synthesis also was not influenced by higher amounts of the viral nsPs because, for instance, SFV *ts*1-infected cells shut off minus-strand synthesis at the same time as the wt virus (49), even though they expressed high (twice as much as wt) levels of nsPs and reduced amounts of structural proteins. Also, it was earlier

TABLE 2. RNA synthesis properties of the nsP2 PI mutants

Virus	nsP2 lesion	26 RNA:genome		Minus-strand synthesis		P123/P23 accumulation	% wt RNA synthesis	RC+		ssRNA:RF (cpm)
		Ratio	Fold	Continuous	Relative rate			Turnover	Rate/h	
SIN wt	wt	17:1[a]	1.0	−		−	100%	−	ND[c]	13:1
SIN S1	A1>E	13:1[a]	0.8	+	2×	+	5	+		20:1
SFV 2A	L10>T	2.7:1[a]	1.4	+	2×	+	3	+	17%	40:1
SFV wt	wt	2:1[a]	1.0	−		−		−	ND	
SFV 1B	D469Δ	0.8:1[a]	0.4	+	4×	+	4	+	13%	15:1
SFV 2C	L713>P	5.9:1[a]	3.0	+	1×	−	2	+	13%	100:1
SIN S2	P726>T	21:1[a]	1.2	+	1×	−	2	+		125:1
SIN rep wt	wt	2.7:1[b]	1.0	−			45			10:1
RNase $L^{-/-}$	wt nsP2	wt	1.0	+		ND	10 (CEF)	+	16%	
Aedes C7/10	wt nsP2	ND	ND	+		ND	2	+	22%	

[a] Values are molar ratios, obtained from Perri et al. (39), and represent accumulated RNA from a 7-h continuous-labeling period.
[b] Values are molar ratios and represent the incorporation (averages from 4 to 5 h p.i. and 6 to 7 h p.i. lysates) in 1-h pulse-labeling periods by BHK21 cells infected with packaged wt SIN replicons. The areas of a 0.8% TBE-agarose gel containing the viral RI/native RF, or 49S or 26S mRNA were identified by fluorography and were cut out and their cpm determined by counting in a Beckman LS-335 scintillation counter. For molar ratios, the incorporation value in 49S genome RNA was divided by a factor of 2.8, the size difference between the 49S genome and 26S subgenomic RNA, and this value was divided by the incorporation value in 26S mRNA.
[c] ND, not done.

shown that adding CH to wt-infected cells very early or very late does not change the stability of RC+ nor does temperature shift of other RNA-negative *ts* mutants of SIN or SFV (reviewed in reference 43). In addition to supporting wt nsP2 functions in cessation, such results and the findings in this study argue against models for cessation (24, 55) that invoke altered processing of nsP polyproteins by the nsP2 protease, and instead implicate a host response to infection, with nsP2 functions determining the nature of the response and RNase L playing an essential role, at least in MEF cells (48).

One factor affecting persistence would be host translation, which was inhibited in BHK21 cells either infected with SIN virus or with wt SIN replicons that lack structural genes but are cytopathic for cells (17). The replication of SIN and SFV replicons requires the production of nsPs, which is only possible if the PI cultures remain permissive for replicon RNA synthesis, presumably by retaining host translation and synthesis of host factors so the cells do not die. We found that minus-strand synthesis in replicon PI cells, like wt-infected cells, required production of new ns polyproteins; the addition of CH caused PI minus-strand synthesis to stop (Fig. 5). Therefore, continuous minus-strand synthesis in replicon PI cultures cannot be explained by a long-lived or stable minus-strand activity that might occur due to altered or failed processing of P123 and P23 (*cis*-active effects) or to reactivation of minus-strand synthesis by mature RC+ via template switching (42, 43, 45). Presumably, persistent infection, or the maintenance of replicons, requires continuous minus-strand synthesis because without it, the host response that targets and inactivates previously formed viral templates (RC+) would cure the infection over time, i.e., the two phenotypes are compensatory. Nor was the PI phenotype due to the selection of cell mutants, because PI cells responded to wt viral infection the same as parental BHK21 cells. Superinfection with wt virus induced PI cells to enter the late phase, e.g., activate the cessation of minus-strand synthesis, after a lag or early period that was more than 3 h but less than 6 h in length (Fig. 8). Thus, replicon PI cells switched from the "permissive" state for minus-strand synthesis to the nonpermissive state following wt virus infection. After the induction of the nonpermissive state, RC+ turnover also was prevented. The major difference, although possibly not the only difference, from wt virus or wt replicon-infected cells was that all replicon PI cells expressed mutant forms of the viral nsP2 protein (1, 7, 16, 18, 39). This suggests a loss of wt functions by mutant nsP2 proteins led to a failure of the host to respond fully to infection and to shut off minus-strand synthesis.

Why nsP2 functions? Only nsP2, among the four nsPs, is found as several distinct populations in infected cells. About 50% of the nsP2 is transported to the nucleus and nucleolus, even though a viable nucleus is only required for alphavirus replication in insect cells (10). About 25% of the total nsP2 are components of RC+ that are present in membrane-associated, spherulelike structures that are in the P15 fraction. The stable activity of RC+ in the absence of continued nsP translation argues its nsP2 proteins are stable (permanent) components normally. The remaining 25% of nsP2 is recovered in the S15 fraction, where it was found to reversibly associate with the P15 fraction for 26S mRNA synthesis (56). The different cell distributions support the notion of different functional roles for nsP2, and loss of one or more of these appear to be required for the ability to exist in a PI cell (16, 18).

Certain of the nsP2 PI mutants had subtle effects on RNA synthesis that are likely expressed by the nsP2 population associated with replication complexes (RC+ or RC−). In the replicon PI cells, minus-strand synthesis and its accumulation as RIs varied somewhat, in the order 1B > S1 and 2A (N-domain mutants) > S2 and 2C (C-domain mutants), as summarized in Table 2. Although the C-domain mutants S2 and 2C made the least minus strands, their synthesis of plus strands relative to numbers of minus-strand templates was the highest, and thus, these nsP2 substitutions may have enhanced transcription efficiency in some way. One possibility is enhanced initiation of subgenomic mRNA synthesis, because all known *ts*

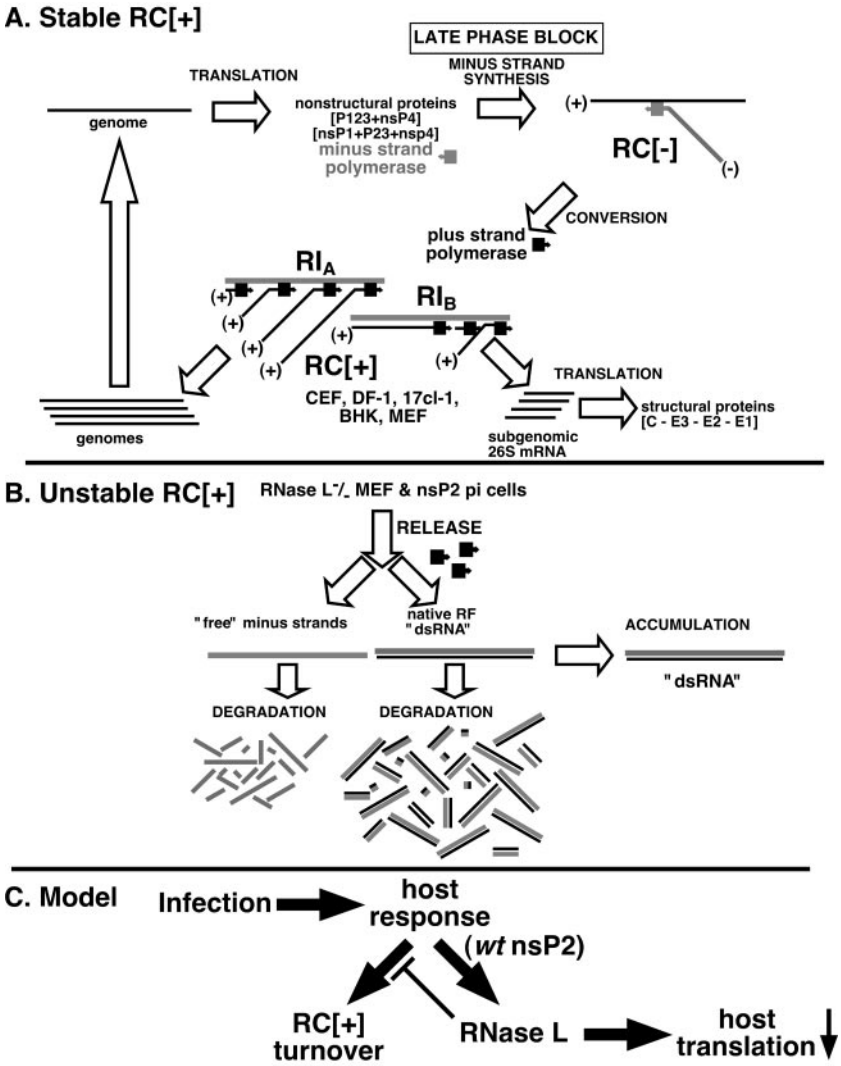

FIG. 9. Model for alphavirus replication/transcription. Translation of the incoming genome produces ns polyproteins P123 and P1234, whose regulated processing by the nsP2 protease produces polymerases active first in minus-strand synthesis (called RC− or $RC_{INITIAL}$), then in both genome plus- and minus-strand synthesis. Cleavage of P23 at the 2/3 site eliminates minus-strand activity and fully activates the plus-strand activity of the replication/transcription complex (RC+ or RC_{STABLE}), especially that of 26S mRNA synthesis. In cells lacking the host latent endonuclease RNase L and in the nsP2 mutant replicon expressing BHK cells, RC+ is transcriptionally short-lived and its templates can be recovered as RF core RNA ("accumulation of double-stranded RNA [dsRNA]"). Our working model is that wt virus infection leads to host responses that act via RNase L dependent pathways to inhibit host translation, thereby limiting essential host factors for the formation of RC−. The RC+ that form under these conditions are stable and turn over only slowly if at all. In the absence of wt nsP2 functions, host responses target and inactivate RC+ but fail to induce a late phase of virus replication (e.g., fail to inhibit host translation), which permits RC−, in addition to RC+, to be continually formed. Thus, nsP2 mutant replicon PI cells allow for continuous minus-strand synthesis.

26S mRNA-defective SIN and SFV mutants have lesions in the C domain of nsP2 (43, 55). The C domain encodes an enzymatically inactive, 2′-*O*-MT-related sequence (A. Gorbalenya, personal communication) compared to plus-strand RNA flaviviruses and coronaviruses that encode an active 2′-*O*-MT (14, 25, 61). In alphaviruses, such a domain may function to compete with cellular homologs or have separate RNA-associated activities mediated by a substrate-binding domain that is conserved in cellular and viral homologs also and that may target rRNA or viral RNA species (A. Gorbalenya, personal communication). Consistent with the absence of active 2′-*O*-MT, the SIN genome and 26S mRNA possess only type 0 cap structures (8). Finding a similar phenotype for nsP2 mutants that map outside of this domain suggests the 2′-*O*-MT-associated function is not responsible (solely) for the PI phenotype or that substitution at distant sites affects this C-domain sequence once it is folded.

We also predict that the shared ability of the PI replicons to establish and maintain a persistent state reflects different and shared functions expressed by the nuclear or cytoplasmic populations of nsP2, and not those associated with RC+. Interestingly, the similar rate of RC+ loss (13 to 17% per hour) by the five PI cell cultures was similar also to the loss in plus-strand synthesis observed in SIN-infected RNase L-deficient MEF

and SIN-infected *Aedes* cells (Table 1) (48), once again highlighting some kind of link between the viral nsP2 and the host RNase L proteins.

A working model for alphavirus replication that incorporates our findings is shown in Fig. 9. Early in wt virus infection (Fig. 9A), nascent P123 and P1234 are synthesized, processed, and assembled into RC− that copy the genome and produce a minus-strand template. The ns proteins remain associated with the new minus strand and undergo further cleavage to form replicases active in 49S plus-strand synthesis while retaining the activity for minus-strand synthesis (nsP1 + P23 + nsP4). Final cleavage of its P23 to nsP2 and nsP3 inactivates minus-strand synthesis and permits the reassembly into RC+ that efficiently synthesizes both genomes and subgenomic mRNA using the minus-strand template. The mature RC+ are long-lived, or stable, and associated with membranes (P15) in wt virus-infected cells and produce viral RNA in the absence of new protein synthesis. Also, nsP2 accumulates in the cytosol (S15) and in the nucleus, and associates with ribosomes (55), which gives it the potential to influence translation and the host response to infection. At about 4 h p.i., depending on the cell type, the host response switches the cell environment to the late phase and inhibits host but not viral translation (49). During the late phase, nascent nsPs continue to be made but do not assemble into an active RC−, and minus-strand templates are not made (late phase block in Fig. 9A). Cells lacking RNase L (48) or PI cells expressing mutant nsP2 proteins (Fig. 9B) did not switch from the early to the late phase, allowed new P123 and P1234 to assemble continuously into RC− synthesized minus-strand templates and utilized them in newly formed RC+ solely for plus-strand synthesis. Recently, we found that, at the time cessation is seen, the host's type 1 interferon feedback loop is in an early, secondary response phase and a specific subset of host mRNA appears to be targeted for decay in normal MEF but not in RNase L-deficient MEF (D. Sawicki, S. Sawicki, D. Leaman, unpublished results). One possible but not exclusive model (Fig. 9C) to put together the nsP2 PI cells and RNase L deficient MEF results with these preliminary findings proposes that the host response to infection causes the early loss of a subset of host mRNAs that produce factor(s) required to activate the viral nsPs to form RC− and thus blocks all further minus-strand synthesis (cessation). Loss of either nsP2 functions or RNase L prevents these events. However, loss of either RNase L or nsP2 functions allowed host responses now to target mature RC+ for turnover. Recovery of RF core-like molecules from PI cells predicts such targeting prevented further initiation on these templates because the last nascent plus strand remained associated with its template (Fig. 9B). We propose wt nsP2 acts via an RNase L-dependent pathway to block specific parts of the host response that would otherwise inactivate RC+ or target it for destruction (Fig. 9C). Future studies will attempt to probe the validity of such a model.

In summary, we suggest that nsP2 is a master regulator of the host response to infection. It controls P123 and P1234 processing and functions in capping and promoter recognition for 26S mRNA and for minus-strand synthesis. It also influences the switch from early to late phase (the host response to infection) and thereby infection outcome (lytic versus persistent). Of interest in future studies will be the elucidation of the RNase L-dependent pathways (Fig. 9C) that appear to play a role, with the viral nsP2, in controlling the host response that influences the degradation of specific host mRNA, the inhibition of host translation, and the protection of viral RC+ from inactivation.

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