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Short-Lived Minus-Strand Polymerase for Semliki Forest Virus

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Semliki Forest virus (SFV)-infected BHK-21, Vero, and HeLa cells incorporated [^{3}H]uridine into 42S and 26S plus-strand RNA and into viral minus-strand RNA (complementary to the 42S virion RNA) early in the infectious cycle. Between 3 and 4 h postinfection, the synthesis of minus-strand RNA ceased in these cultures, although the synthesis of plus-strand RNA continued at a maximal rate. At the time of cessation of minus-strand RNA synthesis, two changes in the pattern of viral protein synthesis were detected: a decrease in the translation of nonstructural proteins and an increase in the translation of the viral structural proteins. Addition of cycloheximide and puromycin to cultures of SFV-infected BHK cells actively synthesizing both viral plus- and minus-strand RNA resulted within 15 to 30 min in the selective shutoff of minus-strand RNA synthesis. Removal of the cycloheximide-containing medium led to the resumption of minus-strand synthesis and to an increased rate of viral RNA synthesis. We conclude that the minus-strand polymerase regulates the rate of SFV plus-strand RNA synthesis by determining the number of minus-strand templates and that the synthesis of the minus-strand templates is regulated at the level of translation by a mechanism which utilizes one or more short-lived polymerase proteins.

Semliki Forest virus (SFV) is an alphavirus and contains as its genome a single strand of RNA of plus polarity having a molecular weight of 4×10^6 to 4.5×10^6 and a sedimentation coefficient of 42S (8, 12). The replication of alphaviruses has recently been reviewed (9, 18, 29). In order for SFV to replicate, the parental 42S plus-strand RNA must be transcribed into a complementary minus strand that in turn serves as a template for the synthesis of both additional 42S plus-strand RNA and subgenomic 26S mRNA. The 42S plus-strand RNA serves as a template for the synthesis of nonstructural viral proteins and is packaged into virions. The subgenomic 26S mRNA serves as a template for the translation of the structural viral proteins and is not found in virions.

In animal viruses that contain a plus-strand RNA genome, it is generally assumed that the RNA polymerases responsible for the replication of the viral genome are composed of nonstructural polypeptides because translation must occur before viral RNA synthesis commences and purified virions lack detectable RNA-polymerizing activity. The nonstructural proteins (ns) of SFV that are repeatedly detected in infected cells are three in number, having molecular weights of approximately 70,000 (ns70), 86,000 (ns86), and 72,000 (ns72), all of which are initially translated from the 42S plus-strand RNA as one polyprotein (9, 29). The products of the alphavirus RNA polymerase are three distinct RNA species: the 42S minus-strand RNA and the plus-strand 42S and 26S RNA. The SFV minus-strand RNA, unlike the plus-strand 42S or 26S RNA, does not accumulate as single-stranded RNA; rather, it is detected only as part of the replicative intermediates (RIs) (26, 27). Apparently, immediately after a minus strand is synthesized, it is used as a template for plus-strand synthesis.

Recently, the nature of the alphavirus polymerase responsible for the synthesis of SFV RNA 4 to 8 h postinfection (p.i.) was partially elucidated. Three laboratories have data which indicate that the viral ns70 is the major polypeptide associated with membrane-bound replication complexes transcribing 42S and 26S RNA (4, 19; P. J. Gomatos, unpublished data). Two of these laboratories also consistently found smaller amounts of ns86 and ns72 in such complexes (19; Gomatos, unpublished data). The overall aim of our studies is to characterize the SFV polymerase responsible for the synthesis of minus-strand RNA. The results presented in this paper indicate that at relatively early times in the infectious cycle, the synthesis of SFV minus-strand RNA ceases while the synthesis of plus strand 42S and 26S RNA continues at a maximal rate; this was found to be true for SFV replicating in three different cell types: BHK-21, Vero, and HeLa. Whereas Bruton and Kennedy (2) previ-

ously reported a similar observation for SFV-infected BHK-21 cells, we also found that cycloheximide and puromycin treatment of SFV-infected cells which were in the process of transcription of both plus- and minus-strand RNA also resulted in the selective shutoff of minus-strand transcription, leaving untouched the ongoing plus-strand RNA synthesis. Although previous investigators have considered the alphavirus RNA polymerase to be one species of enzyme, our results suggest that there may actually exist two forms of the SFV RNA polymerase: one form responsible for the synthesis of minus-strand RNA which is detected only early in the infectious cycle and which requires continuous protein synthesis for activity; and a second, more stable polymerase which is responsible for the synthesis of 42S and 26S plus-strand RNA throughout the infectious cycle.

MATERIALS AND METHODS

Cell culture. BHK-21 cells, a continuous cell line derived from baby hamster kidney cells, were grown in plastic petri dishes in Dulbecco-modified Eagle minimal essential medium (DMEM) supplemented with 5% fetal bovine serum. HeLa (CCL-2) and Vero (CCL-81) cells were obtained from the American Type Culture Collection and were grown in DMEM supplemented with 5% fetal bovine serum.

Virus. Wild-type SFV, obtained from the second passage of SFV-infected mouse brain suspension in BHK cells, was used in these experiments. Growth and purification of virus and determination of its infectivity by plaque assay were described previously (22).

Preparation of infected-cell extracts. BHK monolayers were infected with wild-type SFV at a multiplicity of infection (MOI) of 100 as previously described (22), except that actinomycin D (AMD) at a concentration of 1 μg/ml was present. The time of addition of virus is designated zero time. HeLa and Vero monolayers were similarly infected with wild-type SFV except that AMD was added 30 min before labeling of the infected HeLa monolayers because of the sensitivity of HeLa cells to the cytotoxicity of AMD (24).

SFV RNA was labeled with [5,6-^{3}H]uridine at a final concentration of 250 μCi/ml. For the protein synthesis inhibition studies, cycloheximide or puromycin at a final concentration of 50 μg/ml was added to DMEM containing 0.2% bovine serum albumin (BSA) and 1 μg of AMD per ml. [^{3}H]uridine was added to a fresh portion of this medium for analysis of RNA synthesized in the presence of each drug. Cycloheximide inhibition was reversed by removal of the drug-containing medium followed by four successive washes with 37°C DMEM and further incubation in DMEM containing BSA and AMD.

At the times indicated for harvest, the cells were washed with ice-cold phosphate-buffered saline, a small volume of ET buffer (0.01 M EDTA–0.01 M Tris-hydrochloride, pH 7.4) 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 four times through a 27-gauge needle. For analysis of SFV RNA, the total cell extract was layered directly onto 15 to 30% sucrose gradients in NET buffer (0.1 M NaCl–0.01 M EDTA–0.01 M Tris-hydrochloride, pH 7.4) containing 0.2% SDS and sedimented in an SW27 rotor at 95,400 × *g* for 14.5 h at 20°C. Fractions of 1 ml were collected by using a peristaltic pump, and the radioactivity in each was determined by counting a portion in a toluene-based scintillation fluid containing Triton X-100.

Labeling with [^{35}S]methionine was carried out in methionine-free DMEM, using 50 μCi/ml per 60-mm petri plate. At the times indicated in individual experiments, infected BHK cells were exposed for 30 min to 335 mM NaCl (16, 21) in methionine-free DMEM containing 0.2% BSA and 1 μg of AMD per ml. This medium was removed, and new medium containing [^{35}S]methionine and 0.1 M sucrose was added for the times indicated, followed by the addition of DMEM containing 20-fold the normal concentration of methionine. After a 90-min chase period, the cell monolayers were washed several times with ice-cold phosphate-buffered saline and harvested as described above.

Isolation of RF RNA, the RNase-resistant cores of SFV RIs. The intracellular double-stranded SFV RNA (RIs) was obtained from material sedimenting at the 35 to 20S region of sucrose gradients of SDS-treated whole-cell extracts. After ethanol precipitation at −20°C for 18 h, the 35 to 20S RNA was dissolved in 0.5 ml of digestion buffer (0.15 M NaCl–0.02 M Tris-hydrochloride, pH 7.4); 25 ng of pancreatic RNase was added; and the sample was incubated at room temperature for 15 min. SDS was added to a final concentration of 2%. The digested sample was overlaid onto a 15 to 30% sucrose gradient in NET buffer containing 0.2% SDS. Centrifugation was at 154,000 × *g* for 16 h in an SW40 rotor. Fractions of 0.5 ml were collected; the fractions containing the RF RNA, the RNase-resistant cores of the RIs which sedimented at 20 to 15S, were pooled, and the RNA was ethanol precipitated in the presence of 25 μg of carrier rat liver RNA.

Hybridization of RF RNA with unlabeled 42S virion RNA. The procedures followed were as described previously (23). Briefly, the RF RNA that was obtained from one 100-mm petri plate (approximately 7×10^6 cells) was dissolved in 0.2 ml of 1 mM EDTA, pH 7.4. One-quarter of the dissolved RF RNA was diluted to 3.0 ml with 1 mM EDTA at 100°C, heated at 100°C for 2 min, quickly cooled to 0°C, and divided into three equal portions. One portion of the RF RNA served as a control for the fraction of the RNA remaining double stranded after heating and quick cooling. The second and third portions were subjected to hybridization conditions (0.4 M NaCl at 68 to 70°C for 30 min followed by 30 min at room temperature), one in the absence and the other in the presence of 10 μg of unlabeled virion RNA. To one-half of each sample was added an equal volume of a solution containing 0.3 M NaCl, 0.03 M sodium citrate, and pancreatic RNase (10 μg/ml), 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. The heated and quick-cooled portion and the portion hybridized in the absence of any unlabeled virion RNA gave essentially equal RNase resistance of 1 to 12%. This value was subtracted from fractions of RNase-resistant radioactivity obtained from the corresponding portion hybridized in the presence of an excess of unlabeled virion RNA. This RNase-resistant radioactivity was taken as the relative amount of labeled minus-strand RNA in the RF RNA, since use of RNase-derived RF RNA results in the loss of some nascent minus-strand RNA.

SDS-polyacrylamide gel electrophoresis. Polyacrylamide slab gels with 8% separation gel and 5% spacer gel were prepared according to the method of Laemmli (11). The gels were fixed in 20% trichloroacetic acid for 60 min before treatment with dimethyl sulfoxide and 2,5-diphenyloxazole (PPO) (1).

Materials. [5,6-^{3}H]uridine (41 Ci/mmol) was purchased from Amersham Radiochemicals (Arlington Heights, Ill.) and New England Nuclear Corp. (Boston, Mass.). [^{35}S]methionine (600 Ci/mmol) was obtained from New England Nuclear Corp. Cycloheximide was purchased from Calbiochem (San Diego, Calif.) and puromycin was purchased from Boehringer Mannheim Corp. (Indianapolis, Ind.). All other materials were obtained from previously described sources (22, 23).

RESULTS

Time course of SFV minus-strand RNA synthesis. Functional minus-strand SFV RNA polymerase was detected by assaying for the synthesis of SFV minus-strand RNA in BHK cells infected with SFV (100 PFU/cell) in the presence of 1 μg of AMD per ml. Replicate cultures were labeled with [^{3}H]uridine for 30 min at intervals starting 1.5 h p.i. At the end of the pulse period, the cells were solubilized with 2% SDS in ET buffer and centrifuged on 15 to 30% sucrose gradients; the RIs sedimenting from 35 to 20S were collected, treated with pancreatic RNase, and again subjected to rate zonal centrifugation on 15 to 30% sucrose gradients. The resulting double-stranded cores (RF RNA) sedimenting at 20 to 15S were used in the annealing reactions instead of the intact RIs to eliminate any possible interference by the vast excess of nascent, labeled plus-strand RNA chains in the RIs. Figure 1 shows the amount of [^{3}H]uridine incorporated into RF RNA obtained from a portion (one-seventh) of each sample. Labeled RF RNA was readily detected 1.5 to 2 h p.i. Between 1.5 and 3 h there was a threefold increase in RF RNA synthesized in each 30-min period. At about 3 to 3.5 h p.i., the synthesis of RF RNA became constant. The synthesis of single-stranded SFV RNA also increased between 1.5 and 3 h p.i. and became constant at 3.5 h p.i. (data not shown).

The fraction of [^{3}H]uridine incorporated into minus strands of the RF RNA was determined by hybridization of denatured RF RNA with unlabeled 42S virion RNA. Incorporation of [^{3}H]uridine into minus-strand RNA reached a maximum at 3 h p.i., after which time it declined (Fig. 1). After 3.5 h, essentially no minus-strand synthesis was detectable, although plus-strand RNA continued to be synthesized at a maximal rate.

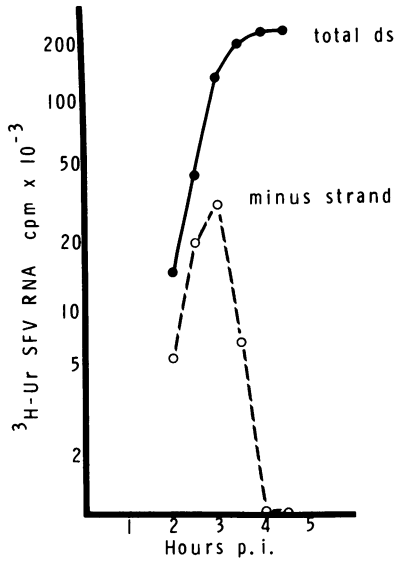

FIG. 1. *Time course of SFV minus-strand RNA synthesis. BHK-21 cells in 100-mm petri plates were infected with SFV (MOI of 100) in the presence of AMD. Starting at 1.5 h p.i., [^{3}H]uridine (250 μCi/ml) was added for sequential 30-min periods, followed by preparation of infected-cell extracts as described in the text. In all figures, the amount of RNA synthesized during a pulse is indicated at the end of the pulse period. [^{3}H]uridine-labeled SFV RNA which sedimented in the 35 to 20S region of 15 to 30% sucrose gradients was pooled and treated with pancreatic RNase, and the RF RNA was resedimented on a second sucrose gradient. An equal portion (one-seventh) of the labeled RF RNA (ds RNA) was analyzed for its [^{3}H]uridine incorporation (●), and, after heat denaturation and hybridization in the presence of excess unlabeled 42S virion RNA, for the amount of [^{3}H]uridine incorporated into minus-strand RNA (○). The [^{3}H]uridine-labeled RF RNA which specifically hybridized to unlabeled virion RNA was considered minus-strand RNA (see text).*

We investigated the time course of SFV minus-strand RNA transcription in two other cell types. Monolayers of Vero cells and HeLa cells were infected with SFV at an MOI of 100, the viral RNA was labeled with [^{3}H]uridine in the presence of AMD for sequential 30-min periods, and the RF RNA was isolated and analyzed as described above for the BHK cell extracts (Ta-

ble 1). Both cell types produced viral RNA, although HeLa cells incorporated only about one-fourth as much [^{3}H]uridine into viral RNA as did either Vero or BHK cells. The synthesis of SFV minus-strand RNA declined 3 to 4 h p.i. in each of these cell types: Vero cells exhibited a similar if not identical time course as the BHK-21 cell cultures; in HeLa cells, the inhibition was not as abrupt.

If SFV minus-strand RNA were synthesized but rapidly degraded after 3 h p.i., we might not have detected synthesis of new minus-strand RNA after 3 h, using 30-min labeling periods. Therefore, we determined the amount of incorporation of [^{3}H]uridine into nascent minus-strand RNA, using shorter pulse-labeling times. A 5-min pulse was sufficient to label minus-strand RNA when the pulse was given at 2 h p.i. (Fig. 2). This resulted in the vast majority of the labeled RNA sedimenting as RIs and only very small amounts of labeled RNA sedimenting as completed 42S and 26S species (data not shown). Pulse times of 10 to 30 min at 2 h p.i. resulted in maximal levels of labeled minus strands in the RF RNA. However, during a 5- to 30-min pulse of [^{3}H]uridine given to infected BHK cell cultures at 3 h p.i., little or no synthesis of minus-strand RNA was detected (Fig. 2). We conclude from these results that transcription of minus-strand RNA, and thus the activity of the polymerase synthesizing the minus strands, ceases after 3 h. Furthermore, since the rate of [^{3}H]-uridine incorporation into newly synthesized plus-strand RNA remained constant for 1 to 2 h after the cessation of minus-strand synthesis, previously synthesized minus-strand RNA must continue to function as template for plus-strand synthesis and not be subject to degradation after 3 h.

TABLE 1. *Time course of SFV minus-strand synthesis in three different cell types*[a]

[^{3}H]uridine time of addition (h p.i.)	% of RF RNA in minus-strand RNA		
	BHK-21 cells	Vero cells	HeLa cells
2–2.5	46	44	45
2.5–3	24	28	41
3–3.5	4	6	38
3.5–4	0	5	26
4.5–5	0	1	13
5.5–6		0	8
6.5–7			6
7.5–8			6
8.5–9			4

[a] Labeled viral RNA in total cell extracts harvested at the end of the pulse period was centrifuged on 15 to 30% sucrose gradients; RNA sedimenting in the 35 to 20S region was collected by ethanol precipitation; and the double-stranded RF RNA was prepared as described in the text. Between 1,000 and 50,000 cpm (approximately 0.03 μg) of RF RNA was used in each annealing reaction with 10 μg of unlabeled 42S virion RNA. Hybridization of SFV RF RNA isolated from infected BHK-21 cell extracts, which had been labeled with [^{3}H]uridine from 0 to 4 h p.i., gave values ranging from 38 to 47% of the total labeled RNA in minus-strand RNA.

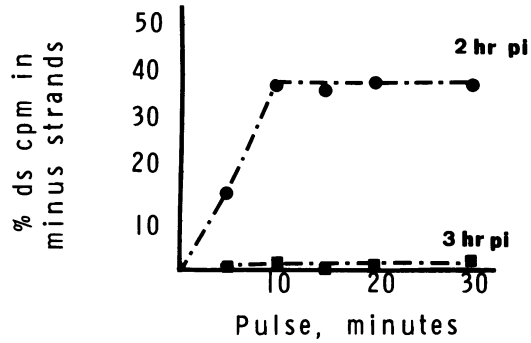

FIG. 2. *Presence of nascent SFV minus-strand RNA in RF RNA. SFV-infected BHK-21 cell cultures were exposed at 2 or 3 h p.i. to [^{3}H]uridine (250 μCi/ml) for periods of 5 to 30 min, after which time they were harvested as described in the text. The RF RNA was obtained and, after heat denaturation, the amount of labeled RF RNA in minus-strand RNA was determined by hybridization with excess unlabeled virion RNA (see Fig. 1). Symbols: ●, Minus-strand RNA in RF RNA obtained from cultures pulsed at 2 h p.i.; ■, minus-strand RNA in RF RNA obtained from cultures pulsed at 3 h p.i.*

Pattern of virus-specific proteins. We next determined whether there was a change in the pattern of viral protein synthesis at the time of minus-strand RNA inhibition. At different times after infection with SFV, BHK cells were labeled with [^{35}S]methionine after treatment of the cultures for 30 min with hypertonic medium to reduce the background of host protein synthesis. Figure 3 represents a fluorogram of the viral proteins synthesized during 30-min intervals between 1 and 4.5 h after infection. The synthesis of ns70 and ns86 was detectable beginning at 1.5 h and was maximal between 2.5 and 3 h; thereafter the synthesis of ns70 and ns86 declined precipitously. On the other hand, the synthesis of the structural proteins, capsid and the two envelope proteins (E1 and E2), was first observed at 2 h and only became maximal at 3 to 3.5 h p.i. The structural proteins became the predominant proteins synthesized by infected cells at 3.5 h. Thus, the time at which minus-strand synthesis ceased was nearly the same time that the infected cell began to synthesize almost exclusively structural proteins.

Effect of protein synthesis inhibition on

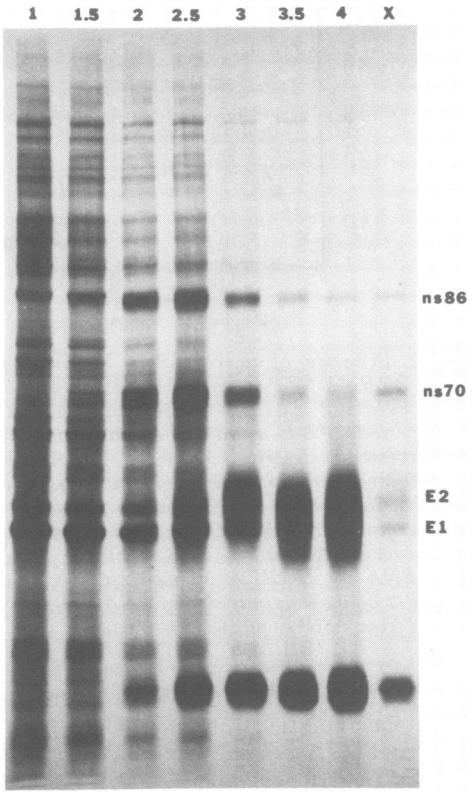

FIG. 3. *SFV viral proteins synthesized at various times after infection. BHK-21 cell cultures in 60-mm petri plates were infected with SFV at an MOI of 100. The times indicated represent the time of addition of medium containing 0.335 M NaCl (16, 21) to the individual cultures. Starting at 1 h p.i., the cultures were exposed to the hypertonic medium for 30 min, followed by a 30-min pulse of [^{35}S]methionine in medium containing 0.1 M sucrose and a 90-min chase period. Whole cells were collected into ET buffer containing 2% SDS, and portions containing equal amounts of total cell protein were electrophoresed on an 8% polyacrylamide slab gel as described in the text. At 3 h p.i., a duplicate culture was pulsed with [^{35}S]methionine for 7 min after synchronization of translation by hypertonic treatment and chased for 90 min in order to label specifically the N-terminal two polypeptides translated from the 42S plus-strand RNA, ns70 and ns86 (lane X). The portions applied to the gel contained approximately the following cpm: (1 h) 200,000; (1.5 h) 160,000; (2 h) 120,000; (2.5 h) 130,000; (3 h) 90,000; (3.5 h) 90,000; (4 h) 120,000. The exposure time was 20 h.*

minus-strand synthesis. We then asked whether the cessation of synthesis of minus-strand RNA would occur when protein synthesis was inhibited before the time that the infected cells began to synthesize predominantly viral structural proteins. Replicate cultures of BHK cells were infected with SFV. At 0.5-h intervals beginning at 1.5 h p.i., cycloheximide was added to a final concentration of 50 μg/ml. Thirty minutes after the cycloheximide was added, half of the cultures were labeled for 30 min with [^{3}H]uridine. The other half were labeled for 30 min with [^{3}H]uridine at 4.5 h after infection. Replicate cultures of SFV-infected cells that received no cycloheximide also were labeled for 30 min with [^{3}H]uridine at 0.5-h intervals beginning 1.5 h p.i. Between 1.5 and 2.5 h p.i., there was an exponential rise in the rate of RNA synthesis in SFV-infected cultures (Fig. 4). The addition of cycloheximide blocked the increase in the rate of SFV RNA synthesis. Addition of cycloheximide to infected cultures after 3 h p.i. had no effect on the rate of RNA synthesis. RF RNA was isolated from cells that either were treated with cycloheximide at 1 or 1.5 h p.i. or were

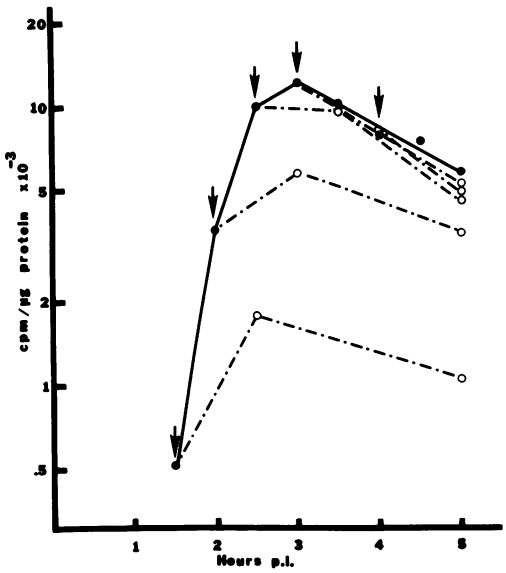

FIG. 4. *Effect of cycloheximide treatment on SFV RNA synthesis. BHK-21 cell cultures in 35-mm petri plates were infected with SFV (MOI of 100). Untreated cultures were labeled in the presence of AMD for 30 min with [^{3}H]uridine (50 μCi/ml) at 30-min intervals between 1 and 5 h p.i. At 0.5-h intervals beginning 1.5 h p.i., replicate cultures were treated with cycloheximide (50 μg/ml). The arrows indicate the time of cycloheximide addition. Half of the cultures were labeled for a 30-min period with [^{3}H]uridine beginning 30 min after addition of the drug. The other half was pulsed with [^{3}H]uridine at 4.5 to 5 h p.i. The infected cells were harvested at the end of the pulse period by solubilization in ET-SDS buffer as described in the text. Portions in duplicate were assayed for acid-insoluble radioactivity and for protein by the method of Lowry et al. (13). Symbols: ●, [^{3}H]uridine SFV RNA in untreated cultures; ○, [^{3}H]uridine SFV RNA in cycloheximide-treated cultures.*

untreated and that were labeled at 6 h p.i. for 1 h with [^{3}H]uridine. At 1 to 1.5 h p.i., there was no detectable synthesis of 26S SFV RNA (data not shown) or of viral structural proteins (Fig. 3). The presence of labeled minus-strand RNA in the RF RNA was determined by hybridization to an excess of unlabeled virion RNA. No labeled minus-strand RNA was detected when the label was added 5 h after cycloheximide (data not shown). We conclude, therefore, that the synthesis of minus-strand, but not plus-strand, RNA is prevented by long-term cycloheximide treatment, and that the accumulation of viral structural proteins is not required to shut off the synthesis of minus-strand RNA.

A possible requirement for continued translation of the viral nonstructural proteins for SFV minus-strand transcription was suggested from these results and from those of Fig. 3 and was investigated further. The effect of cycloheximide on minus-strand synthesis was monitored at short intervals after its addition to the infected monolayers. At 2 h p.i., when transcription of minus-strand RNA was at a maximum, cycloheximide was added to a final concentration of 50 μg/ml. The cultures were pulsed with [^{3}H]uridine for 30-min periods between 2 and 4.5 h p.i. The addition of cycloheximide at 2 h p.i. stopped the increase in viral RNA synthesis (Fig. 5A) and the increase in RF RNA (Fig. 5B). Compared with the increasing amounts of labeled RF RNA isolated from untreated cultures between 2 and 3.5 h p.i., a constant amount of labeled RF RNA was obtained from infected cultures that were treated with cycloheximide at 2 h p.i. and maintained in the presence of cycloheximide.

Hybridization of the labeled RF RNA with an excess of unlabeled virion RNA indicated that minus-strand RNA synthesis rapidly ceased in the presence of cycloheximide (Fig. 5C). When infected cells were labeled at 2 h p.i. with [^{3}H]uridine for 30 min in the presence of cycloheximide, the amount of labeled minus-strand RNA detected in the RF RNA was the same as that found in infected cells that were labeled at 1.5 h p.i. for 30 min in the absence of cycloheximide. When infected cells were treated with cycloheximide at 2 h p.i. and labeled for 30 min with [^{3}H]uridine at 2.5 to 3 or 3 to 3.5 h, essentially no labeled minus-strand RNA was detected in the RF RNA. Sucrose gradient analysis demonstrated that 42S and 26S RNA was synthesized during cycloheximide treatment (data not shown). Therefore, minus-strand, but not plus-strand, RNA synthesis rapidly declined in the presence of cycloheximide.

An identical experiment was carried out, using puromycin to inhibit protein synthesis. At 2 h p.i., medium containing puromycin (50 μg/ml) was added to SFV-infected cultures which were subsequently labeled with [^{3}H]uridine for 30-min intervals over the next 2 to 3 h (Fig. 6A). Compared with the untreated cultures, puromycin addition resulted in a 50% decrease in the amount of SFV RNA synthesized in the first 30 min of drug addition. This was followed by a constant or even decreased level of RNA synthesis for the next 2 h. Similar to cycloheximide treatment, the addition of puromycin stopped the increase in total RF RNA, and the total amount of labeled RF RNA present in puromycin-treated cultures remained relatively constant. Also similar to the effect of cycloheximide, puromycin addition led to the selective and rapid shutoff of minus-strand RNA transcription (Fig. 6B).

A closer examination of minus-strand transcription was undertaken by labeling with [^{3}H]uridine the SFV RNA synthesized in 5-min intervals during the first 30 min of cycloheximide treatment. Cycloheximide was added at 2 h p.i., and the cells were given a 5-min pulse label between 2 and 2.5 h p. i. (Fig. 7A). The rate of SFV transcription increased during the first 15 min of cycloheximide treatment and became constant by 20 min. The amount of labeled minus-strand RNA in isolated RF RNA decreased at a rapid rate for the first 15 min after drug addition, continuing to decrease slowly thereafter (Fig. 7B). Therefore, labeled minus strands detected after cycloheximide addition in Fig. 5C were synthesized mainly during the first 15 min of the 30-min labeling period. Since the synthesis of minus-strand RNA essentially ceased 20 min after the addition of cycloheximide but the synthesis of plus-strand RNA continued at a given rate, the number of minus-strand RNA templates most probably determines the rate of plus-strand RNA synthesis. These results demonstrate that the SFV polymerase responsible for the synthesis of minus strands is short-lived, whereas the SFV polymerase responsible for the synthesis of plus strands is long-lived.

Reversal of inhibition of minus-strand RNA synthesis. Since our results implicated continued protein synthesis as a requirement for minus-strand transcription, the resumption of translation after removal of the cycloheximide-containing medium and thorough washing of the cell monolayers should in turn be followed by a resumption of minus-strand transcription. When cycloheximide was added at 2 h p.i. and removed 30 or 60 min later and then the newly synthesized RNA was labeled with [^{3}H]uridine, the

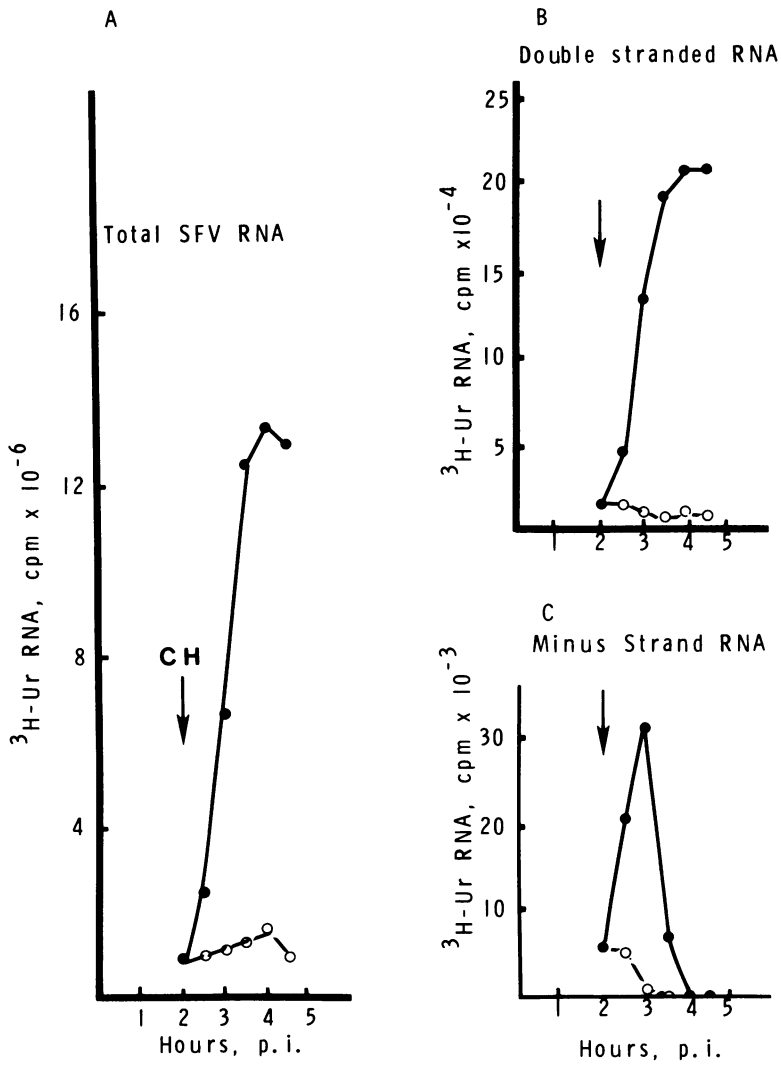

FIG. 5. *Effect of cycloheximide treatment on SFV minus-strand RNA synthesis. Starting 1.5 h p.i., duplicate cultures of SFV-infected BHK-21 cells (100-mm petri plates) were labeled with [^{3}H]uridine (250 μCi/ml) for sequential 30-min periods. One set of cultures was left untreated (●); to the other set, medium containing 50 μg of cycloheximide (CH) per ml was added beginning at 2 h p.i. (○). The cultures were harvested at the end of the pulse period as described in the text. (A) Total acid-insoluble [^{3}H]uridine incorporation into SFV RNA. (B) Total [^{3}H]uridine incorporation into RF RNA obtained from untreated and cycloheximide-treated cultures. The RF RNA was obtained as described in the text. (C) The amount of labeled RF RNA in minus-strand RNA. The RF RNA was denatured and hybridized in the presence of excess unlabeled virion RNA as described in Fig. 1.*

rate of synthesis of total SFV RNA increased after a lag period of approximately 30 min (Fig. 8A). The amount of labeled double-stranded RNA also increased, and was proportional to that observed for single-stranded RNA. Analysis of the polarity of the labeled RNA in the isolated RF RNA confirmed the presence of newly synthesized minus strands in these RIs (Fig. 8B). Transcription of minus-strand RNA began within the first 30 min of drug removal and increased substantially during the next 30 min (drug removed at 3 h) to 60 min (drug removed at 2.5 h). Interestingly, the transcription of minus strands declined between 4 and 4.5 h in

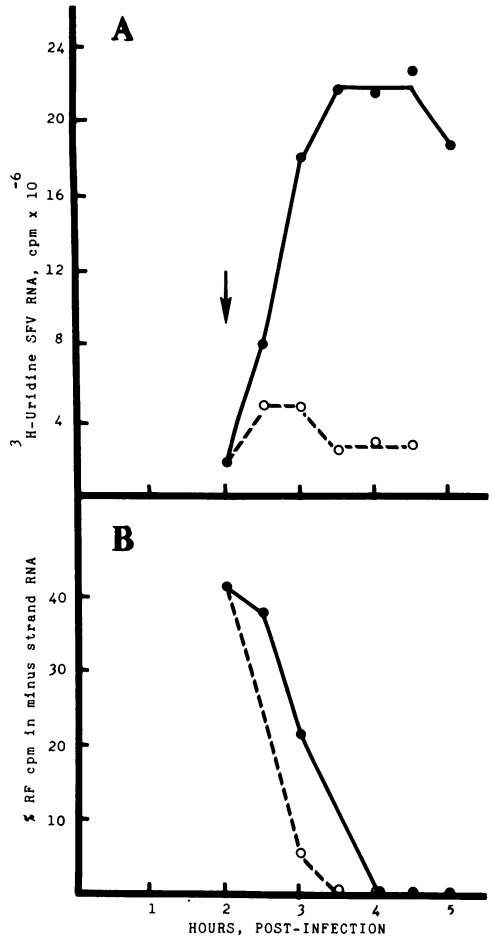

FIG. 6. *Effect of puromycin addition on SFV RNA synthesis. (A) Total [^{3}H]uridine incorporation into SFV single- and double-stranded RNA during 30-min pulse periods from 1.5 to 4.5 h p.i. BHK-21 cell cultures in 100-mm petri plates were infected with SFV at an MOI of 100, labeled with [^{3}H]uridine (250 μCi/ml), and harvested as described in Fig. 5. Medium containing puromycin (50 μg/ml) was added to cultures at 2 h p.i. (arrow). (B) Percentage of total [^{3}H]uridine incorporated into RF RNA that was in minus-strand RNA. The RF RNA was isolated as described in the text, denatured by heating, and hybridized in the presence of excess unlabeled virion RNA. Symbols: ●, [^{3}H]uridine-labeled SFV RNA obtained from untreated cultures; ○, [^{3}H]uridine-labeled SFV RNA obtained from puromycin-treated cultures.*

these cultures, although only 40 to 75% of the amount of total RF RNA present in untreated cultures had been formed by this time.

DISCUSSION

Our results demonstrate that the synthesis of SFV minus-strand RNA, in contrast to the synthesis of plus-strand RNA, is temporally regulated and that the polymerase activity responsible for minus-strand RNA synthesis is short-lived and disappears quickly after inhibition of protein synthesis with cycloheximide or puromycin. Our results strengthen and extend those of Bruton and Kennedy (2), who also demonstrated the temporal regulation of minus-strand RNA synthesis in SFV-infected BHK cells and suggested that the rate of plus-strand RNA synthesis might be correlated with the amount of minus-strand RNA. We observed that the rate of plus-strand RNA synthesis becomes constant when minus-strand RNA synthesis ceases, and that blockage of protein synthesis, which inhibits rapidly the synthesis of minus-strand

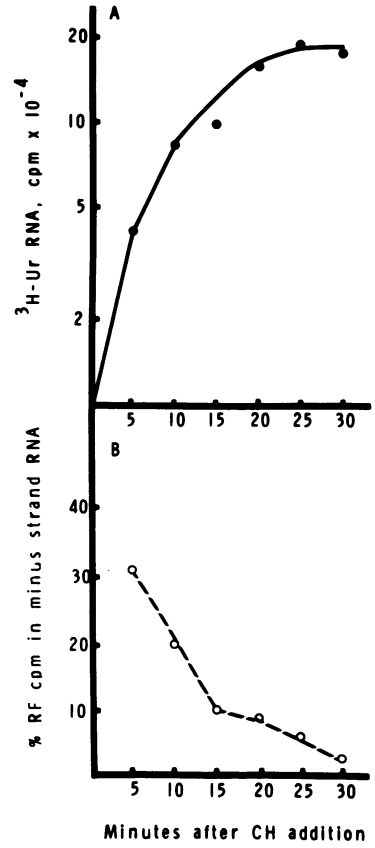

FIG. 7. *Kinetics of minus-strand inhibition by cycloheximide. SFV-infected BHK-21 cell cultures in 100-mm petri plates were treated with cycloheximide (50 μg/ml) at 2 h p.i. and pulsed with [^{3}H]uridine (250 μCi/ml) for sequential 5-min periods between 2 and 2.5 h p.i. (A) Total [^{3}H]uridine incorporated into acid-insoluble SFV single- and double-stranded RNA. (B) Percentage of the total [^{3}H]uridine incorporated into RF RNA in minus-strand RNA, determined as described in Fig. 1.*

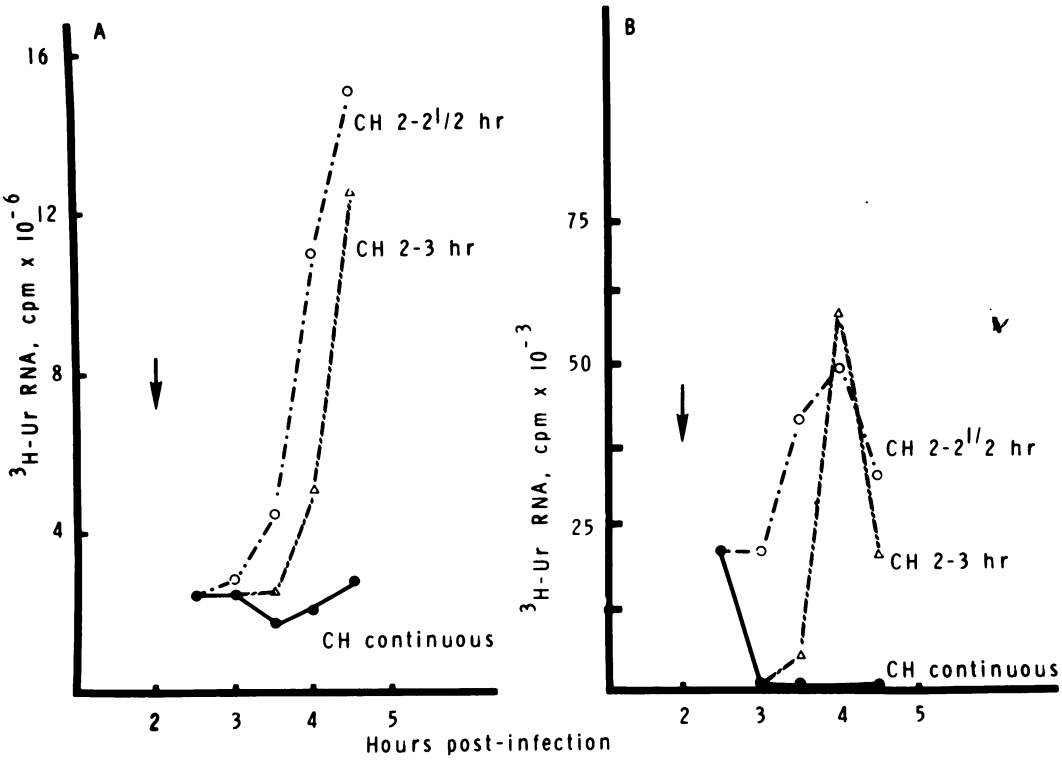

FIG. 8. *Resumption of SFV minus-strand RNA synthesis after removal of cycloheximide. Medium containing cycloheximide (50 μg/ml) was added at 2 h p.i. to all cultures (arrow); in certain cultures, it was removed 30 min (○) or 60 min (△) later (see text). [^{3}H]uridine (250 μCi/ml) was added for 30 min at 0.5-h intervals either in the presence (cultures continuously treated with cycloheximide) or in the absence (cultures from which the drug had previously been removed) of cycloheximide. The cpm shown represents the total incorporation obtained from each 100-mm petri plate. The cultures were harvested at the end of the pulse period. (A) [^{3}H]uridine incorporation into SFV single- and double-stranded RNA. (B) [^{3}H]uridine-labeled RF RNA in minus-strand RNA. Between 2 and 4.5 h p.i., the amount of labeled minus-strand RNA detected in these cultures was as follows: (cycloheximide, 2 to 2.5 h) 625,000 cpm; (cycloheximide, 2 to 3 h) 356,000 cpm; (untreated) 820,000 cpm; (cycloheximide continuous from 2 h) 100,000 cpm.*

RNA but not of plus-strand RNA, also results in a constant rate of RNA synthesis. Polymerase proteins present in SFV-infected cells that have had their protein synthesis arrested continue to function in the synthesis of plus-strand RNA by transcribing the template minus-strand RNA which was formed before protein synthesis inhibition; thus, the number of minus-strand templates appears to control the rate of plus-strand synthesis. Only when minus-strand synthesis was allowed to resume by removal of the cycloheximide did we observe an increase in the rate of plus-strand synthesis.

We conclude from these results that transcription of SFV minus-strand RNA is regulated at the level of translation by a mechanism which utilizes one or more short-lived polymerase proteins that either are not involved in the transcription of plus-strand RNA or are modified by cleavage or other posttranslational modifications to form the stable plus-strand polymerase. Thus, there may exist in SFV-infected cells either two forms of the viral polymerase or two distinct polymerases.

Nothing is known of the location within the SFV-infected cell of minus-strand polymerase or of the replication complex transcribing minus-strand RNA. The recent report of Dasgupta et al. (5) indicates that the presumptive poliovirus minus-strand polymerase (replicase) was detected in a soluble rather than membrane-bound fraction of the infected cell. The SFV plus-strand polymerase is associated with a membrane-bound replication complex (4, 7, 9, 15, 18, 28, 29). Recently, the SFV nonstructural protein ns70 has been shown to be the major viral polypeptide associated with the replication complex (4, 19; Gomatos, unpublished data). In addition,

smaller amounts of ns72 and ns86 were found in this complex (19; Gomatos, unpublished data). The migration of ns70 in polyacrylamide gels is always as a broad band, suggesting that this polypeptide chain may have undergone post-translational modification. Such a modification could account for temporal alteration of template preference of the SFV polymerase. Also, uncleaved or incompletely cleaved precursors of the nonstructural proteins could function in alphavirus minus-strand synthesis. In support of these possibilities is the finding that continued protein synthesis is necessary for the replication of vesicular stomatitis virion RNA (17, 31) and that there is an apparent correlation between the cleavage of certain poliovirus proteins and replicase instability in poliovirus-infected cells (10).

It has been reported previously that the amount but not the ability of the viral polymerase to synthesize plus-strand RNA in alphavirus-infected cells could be varied by the addition of puromycin or cycloheximide at different times early in infection (30). However, by 3 to 4 h p.i., essentially all of the normal amount of viral polymerase responsible for the synthesis of plus-strand RNA had been formed and inhibition of protein synthesis after this time did not affect the amount of RNA synthesized (6, 25, 30). We found, as did others (6, 25, 30), that the SFV plus-strand polymerase formed before the addition of cycloheximide or puromycin remained active for many hours in the absence of continued protein synthesis. Our results indicate that this is not the case for the synthesis of minus-strand RNA. The addition of cycloheximide or puromycin to cultures actively synthesizing both plus- and minus-strand SFV RNA led very quickly to the selective loss of minus-strand transcription. The fact that two protein synthesis inhibitors whose mechanisms of inhibition are different result in the same phenomenon leads us to conclude that this is due to the inhibition of translation. Thus, we argue that it is the synthesis of the minus-strand polymerase that regulates the rate of plus-strand RNA synthesis by determining the number of minus-strand templates formed in the infected cell.

Our characterization of the synthesis of minus-strand RNA suggested that its cessation was correlated with two changes in the pattern of viral protein synthesis: a decrease in the translation of nonstructural proteins and the appearance of large amounts of structural proteins. The nonstructural proteins are translated from the 42S plus-strand RNA as a polyprotein followed by cleavage into the individual proteins, whereas the structural proteins are read from the 26S mRNA, also initially as a polyprotein (2, 9, 18, 29). Therefore, the ability to regulate the production of the nonstructural proteins independently of the structural proteins exists for the alphaviruses but not for other plus-strand RNA viruses such as poliovirus, which produces all of its viral proteins from the same polyprotein (20). The observed instability of the minus-strand polymerase coupled with the decreased translation of all nonstructural proteins could be one explanation for the selective cessation of detectable minus-strand transcription at about 3 to 4 h p.i.

We propose the following scheme for the early events in SFV replication. The SFV parental genome is first translated into the viral nonstructural proteins, some of which function to transcribe this same RNA molecule into complementary minus strands. For this to occur, the minus-strand polymerase must initiate RNA synthesis at the 3′ end of the 42S parental plus strand (22). Because ribosomes bind to the 5′ end of 42S plus-strand RNA and fail to proceed past a termination signal located approximately two-thirds of the distance from the 5′ end (9, 18, 29), the 3′ terminus of the parental plus strand might be readily accessible for recognition by minus-strand polymerase. We have been unable to detect the synthesis of only minus-strand RNA; RIs synthesizing both plus and minus strands were always present early in infection, in approximately a 5:1 ratio (see Fig. 2). At approximately 3.5 h after infection, minus-strand synthesis ceased although plus-strand RNA continued to accumulate: 26S on polysomes and 42S in nucleocapsids. A possible advantage conferred by the cessation of minus-strand RNA synthesis is that of allowing "free" 42S plus strands to accumulate for the formation of nucleocapsids; otherwise, 42S plus strands might continue to be converted into RIs at the expense of progeny virion production.

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