Reduced Synthesis of Sindbis Virus Negative Strand RNA in Cultures Treated with Host Transcription Inhibitors†

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Host cell involvement in Sindbis virus (SB) RNA synthesis was examined in cells which had been treated before infection with actinomycin D or α -amanitin (α -A). Overall synthesis of SB RNA was reduced significantly in CHO cells treated for 18 h before infection with α -A. However, SB RNA was produced at near normal levels in CHOama-1 cells, a line which contains an α -A-resistant RNA polymerase II. In BHK or CHO cells infected with SB^{amr}, a mutant which replicates normally in cells pretreated with either actinomycin D or α -A, viral RNA synthesis was not decreased. The levels of negative strand RNA and of replicative forms I, II, and III in SB-infected cells were progressively reduced with increasing times of pretreatment with host transcription inhibitors, indicating fewer functional replicative intermediates in treated cells. Replicative events after replicative intermediate formation also were inhibited but only to the extent predicted by the reduction in replicative intermediates. Similarly, events preceding negative strand synthesis, adsorption, penetration, uncoating, and translation of nonstructural proteins, apparently were not impeded in treated cells. Therefore, our results are consistent with the involvement of a host component after translation of the nonstructural proteins but before or during the synthesis of SB negative strand RNA.

Sindbis virus (SB) is the prototype of the alphavirus genus (1), a group of small, enveloped viruses containing a single strand of positive polarity RNA (14, 20, 32, 35). One of the more distinctive features of this group of arthropod-borne viruses is their ability to initiate persistent infection in cells of the mosquito vector, both in culture and in vivo, although replicating lytically in cells of vertebrate origin (9, 12, 22, 24). Clearly, the host exerts a powerful influence on the course of alphavirus infection, and this observation in itself constitutes suggestive evidence of a direct host involvement in viral replicative events. In addition, mutant lines of *Aedes albopictus* cells have been isolated which allow lytic SB replication (11, 25, 34). Erwin and Brown (10) have shown that virus growth is restricted in enucleated *A. albopictus* cells and that SB maturation in *A. albopictus* cells is sensitive to actinomycin D (AMD) (29). In the vertebrate system, several results also suggest the participation of host factors in SB growth:

Mento and Siminovitch (19) have isolated Chinese hamster ovary (CHO) mutants which restrict SB replication; two host-dependent temperature-sensitive mutants of SB have been described elsewhere (16); and Clewley and Kennedy (7) found a host protein which copurified with viral polymerase activity from Semliki Forest virus-infected cells, although a requirement for this protein for polymerase function was not demonstrated.

In a previous report (2), we demonstrated that SB replication is dependent on host mRNA transcription and presumably on the synthesis of a particular cell-specified protein. In the present study, we examined the effects of inhibition of host transcription on specific aspects of the SB growth cycle in an effort to determine which viral replicative event(s) requires the putative host factor.

MATERIALS AND METHODS

Virus and cell culture. SB AR339 was supplied by H. R. Bose, University of Texas at Austin. A mutant of SB, designated SB^{amr}, was isolated in this laboratory after mutagenesis and selection for the ability to grow to normal titers in cells treated with 2 μ g of AMD per ml for 18 h before infection (2). Stocks of both viruses were grown on monolayers of baby hamster kidney (BHK) cells in Eagle minimal essential medium

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(GIBCO Laboratories) supplemented with 10% donor calf serum (Flow Laboratories), 10% tryptose phosphate broth (Difco Laboratories), 50 U of penicillin (Sigma Chemical Co.) per ml, and 50 μ g of streptomycin (Sigma) per ml. BHK cells were purchased from the American Type Culture Collection in passage 52, and were used from passages 52 through 64. CHO and CHOama-1 cell lines (13) were the kind gift of C. J. Ingles, University of Toronto. BHK cells and the CHO lines were maintained at 37°C in minimal essential medium containing 10% donor calf serum and 10% tryptose phosphate broth, but no antibiotics.

Infectious center assays. BHK cultures (4×10^5 cells per 35-mm dish), which had been treated with AMD for 0, 6, 12, or 18 h, were infected at a multiplicity of infection (MOI) of 0.5 PFU per cell. After a 1-h adsorption period, the inoculum was discarded, and AMD-containing medium was placed on half of the cultures and sampled for determination of virus yield at 18 h postinfection. The remaining cultures were trypsinized, and the cells were collected by centrifugation. The cells were diluted appropriately and suspended in 25 ml of complete minimal essential medium containing uninfected BHK indicator cells (8×10^4 cells per ml). The resultant mixture was dispensed in 0.2-ml aliquots in 96-well microtest plates (Corning Glass Works). The remaining cell suspension was centrifuged at $100 \times g$ for 10 min to obtain a cell-free supernatant which was found to be free of plaque-forming activity. At 48 h postinfection, the fraction of wells displaying no cytopathic effect, $f(o)$, was determined. The number of infectious centers per well (m) was calculated by assuming a Poissonian distribution, $m = -\ln f(o)$.

Virus infection in the presence of inhibitors of host transcription. Replicate cultures of BHK cells were seeded at 2×10^6 cells per 60-mm culture dish; the two CHO lines were seeded at 5×10^5 cells per plate. At 12 h after seeding, the cells were treated with either AMD (Sigma; 2 μ g/ml) or α -amanitin (α -A) (Sigma; 5 μ g/ml). The culture fluids were removed at intervals after addition of the drug, and the cells were infected with 10 PFU per cell of SB or SB^{amr}. After 1 h for virus adsorption, the inocula were removed, the cultures washed with phosphate-buffered saline, and the preinfection treatment medium was returned to the infected monolayers. Cell number and viability at the time of infection were monitored by hemacytometer count and exclusion of erythrocin B.

Measurement of virus-directed RNA synthesis. Cultures of CHO or CHOama-1 cells were treated for 18 h with 5 μ g of α -A per ml. The medium was removed, and the cultures were infected with SB at an MOI of 10. After a 1-h adsorption, the inocula were removed, and the treatment media were returned to the cultures. The infected cultures were pulse-labeled with 10 μ Ci of [5,6-³H]uridine (Amersham Corp.; 40 Ci/mmol) per ml for 30 min at 6 h postinfection. Control cultures which received no α -A, as well as all α -A-treated cells, were treated with 1 μ g of AMD per ml 1 h before labeling. After the pulse, the monolayers were harvested in 2 ml of TNE (0.01 M Tris [pH 7.2], 0.1 M NaCl, 0.001 M EDTA) containing 1% sodium dodecyl sulfate (SDS), and the trichloroacetic acid (TCA)-precipitable material was collected and counted by liquid scintillation spectroscopy.

In vitro polymerase assay. RNA-directed RNA poly-

merase activity present in cell extracts was assayed as described previously by Clewley and Kennedy (7). Briefly, BHK cultures (ca. 10^7 cells) were lysed at 5 h after infection in Tris- β -mercaptoethanol (10 mM Tris [pH 7.4], 10 mM NaCl, 1.5 mM MgCl₂, 10 mM β -mercaptoethanol) and Dounce homogenized. After centrifugation at $100 \times g$ for 10 min, 100 μ l of the resulting supernatant was added to 250 μ l of reaction mixture containing phosphoenolpyruvate (5 μ mol), pyruvate kinase (20 μ g), ATP, UTP, and CTP (50 nmol of each), GTP (0.7 nmol), dithiothreitol (1.5 μ mol), dextran sulfate 500 (3.5 μ g), AMD (1 μ g), KCl (4 μ mol), and 3 μ Ci of [8-³H]GTP (13 Ci/mmol; ICN Pharmaceuticals) in Tris-magnesium buffer (0.1 M Tris [pH 8.0], 0.01 M MgCl₂). The reaction was terminated after 30 min of incubation at 37°C by the addition of TCA to a final concentration of 5%. In experiments in which two extracts were mixed, 50 μ l of each supernatant was used.

Extraction of viral RNA. Cultures of BHK cells were treated with AMD (2 μ g/ml) and infected as previously described. The infected cultures were labeled with 100 μ Ci of [5,6-³H]uridine per ml at the times indicated in the text. Intracellular RNA was extracted by harvesting the cells in a solution containing equal volumes of 1% SDS in TNE and double-distilled phenol (Mallinckrodt). The mixture was vortexed for 5 min and centrifuged at $1,150 \times g$ to facilitate phase separation. The aqueous phase was repeatedly extracted with an equal volume of phenol-chloroform-isoamyl alcohol (1:1:0.04) until the miniscus was free of visible debris. The RNA-containing aqueous fraction was precipitated overnight at -20°C by the addition of 2 to 3 volumes of 95% ethanol.

Gradient analysis of intracellular RNA. The precipitated RNA was pelleted by centrifugation at $12,000 \times g$ for 15 min, dried under nitrogen, and suspended in 125 μ l of 1% SDS in TNE. A 25- μ l sample was gently layered on a 15 to 30% sucrose gradient (wt/wt in TNE containing 0.2% SDS) and centrifuged in a TV865 vertical rotor (Du Pont Co.) at 40,000 rpm for 44 min at 19°C. A 50- μ l sample of each fraction was analyzed for TCA-insoluble radioactivity.

The remainder of the RNA preparation was precipitated in ethanol at -20°C overnight and collected by centrifugation at $12,000 \times g$ for 15 min. The RNA was dried under nitrogen and suspended in 1 ml of TMN (0.01 M Tris [pH 7.2], 0.1 M NaCl, 0.01 M MgCl₂, 0.001 M EDTA). The sample was digested for 25 min at 37°C with RNase A (Sigma), at a ratio of 0.015 μ g of RNase per μ g of RNA, and DNase 1 (Worthington Diagnostics), at 10 μ g/ml, to digest single-stranded RNA and convert replicative intermediates (RIs) to replicative forms (RFs) (30). The reaction was stopped by the addition of 1 ml of TNE-1% SDS followed by phenol extraction. After precipitation in ethanol, the RNase-digested RNA sample was collected by centrifugation, dried, resuspended in 75 μ l of TNE, gently layered on a 15 to 30% sucrose gradient (wt/wt in TNE-0.2% SDS), and centrifuged in an SW41 rotor (Beckman Instruments, Inc.) for 10.5 h at 39,000 rpm. Fractions of six drops were collected directly onto filter paper, TCA precipitated, and counted.

Structural protein synthesis in treated cells. BHK cells were treated with 2 μ g of AMD per ml at different times before infection with SB at an MOI of 10. The cultures were labeled with 100 μ Ci of [4,5-³H]leucine

FIG. 1. Effect of AMD on virus yield and infectious centers. BHK cultures were treated with $2 \mu\text{g}$ of AMD per ml for the times indicated and infected at a low MOI as described in the text. From untreated cultures, the yield of SB was 6.5×10^7 PFU/ml, and the number of infectious centers was 5.3×10^4 per culture. Symbols: ●, Virus yield; ○, infectious centers.

(Schwarz/Mann; 50 Ci/mmol) per ml from 5 to 9 h postinfection. The radioactive medium was removed, and the cultures were washed twice with ice-cold phosphate-buffered saline. The cells were collected in $100 \mu\text{l}$ of a solution containing 1% Nonidet P-40 (Bethesda Research Laboratories) and 1 mM MgCl_2 , placed on ice for 5 min, and centrifuged at $1,100 \times g$ to remove the nuclei. To the supernatant was added $50 \mu\text{l}$ of dissociation solution consisting of 3.5 M β -mercaptoethanol, 6% SDS, and 30% glycerol. The sample was subjected to freeze-thawing three times, boiled, and electrophoresed (21). The gels were dried and fluorographed (4) on Kodak SB-5 film.

RESULTS

Attachment, penetration, and uncoating. The replication of SB in several different cell types is suppressed when the cultures are treated with inhibitors of host transcription before infection (2). Since this result was independent of any generalized debilitation of the treated cells and of any secondary toxic effects of the inhibitors, it suggests that a specific host factor may be required at some point in the SB replicative cycle. To determine whether this putative host factor was necessary for SB to attach, penetrate, or uncoat in treated cultures, we examined the

sensitivity of infectious center formation to increasing times of treatment with AMD before infection. At an MOI of less than 1, viral attachment, penetration, and uncoating are all or none with respect to the formation of an infectious center. Therefore, inhibition of one of these early events in AMD-treated cells infected at a low MOI would result in a decrease in the number of infectious centers as well as a decline in virus yield. However, under conditions of AMD pretreatment which reduced the overall yield of SB by over 90%, the number of infectious centers was largely unaffected (Fig. 1). Each infected cell produced sufficient progeny virus to register as an infectious center even after AMD pretreatment. These data demonstrated that the early all or none events in virus replication must have occurred in treated cultures and that the block in virus replication was subsequent to uncoating.

Effect of AMD on structural protein synthesis. Viral structural protein synthesis was examined in BHK cells treated with AMD for 0, 6, 12, 18, or 24 h before infection with SB and labeled with $[^3\text{H}]$ leucine from 5 to 9 h postinfection. Under these conditions, the structural proteins PE_2 , E_1 , and capsid were observed in control cultures (Fig. 2). However, with increasing times of AMD treatment, the amounts of structural protein synthesized progressively decreased. The

FIG. 2. Structural protein synthesis as a function of AMD treatment. Cultures of BHK cells were treated with AMD for various lengths of time before infection with SB. The cultures were labeled 5 to 9 h postinfection with $100 \mu\text{Ci}$ of $[^3\text{H}]$ leucine per ml. The samples were analyzed on 10.5% polyacrylamide gels. Lane 1, Mock infected, no AMD; lane 2, SB infected, no AMD; lane 3, mock infected, 6-h AMD treatment; lane 4, SB infected, 6-h AMD treatment; lane 5, mock infected, 12-h AMD treatment; lane 6, SB infected, 12-h AMD treatment; lane 7, mock infected, 18-h AMD treatment; lane 8, SB infected, 18-h AMD treatment; lane 9, mock infected, 24-h AMD treatment; lane 10, SB infected, 24-h AMD treatment.

TABLE 1. Sensitivity of viral RNA synthesis to α -A^a

Treatment	TCA-precipitable cpm ($\times 10^3$) (fraction of control) in:	
	CHO cells	CHOama-1 cells
None	72.0	17.5
α -A	0.4 (0.006)	9.0 (0.51)

^a Cultures of CHO or CHOama-1 cells were treated with α -A (5 μ g/ml) for 18 h before infection with SB at an MOI of 10. All cultures were treated with 1 μ g of AMD per ml 1 h before labeling with 10 μ Ci of [³H]uridine per ml. The cultures were labeled for 30 min at 6 h postinfection.

reduced amount of structural protein produced in treated cultures probably reflected the smaller quantities of 26S mRNA available for translation (see below) rather than a block at the level of protein synthesis, per se.

Viral RNA synthesis. SB RNA synthesis was examined in CHO and CHOama-1 cells which had been treated with α -A before infection. We demonstrated previously (2) that the replication of SB is sensitive to α -A pretreatment in wild-type CHO cells but not in CHOama-1 cells, a line characterized by an α -A-resistant RNA polymerase II (13). CHO and CHOama-1 cultures were treated with 5 μ g of α -A per ml for 18 h before infection with SB. Both α -A-treated cultures and untreated controls were exposed to 1 μ g of AMD per ml at 5 h postinfection, to inhibit cellular RNA synthesis completely, and pulse labeled for 30 min with [³H]uridine beginning at 6 h. Incorporation of [³H]uridine into TCA-precipitable material was severely suppressed only in α -A-treated CHO cells but not in the α -A-resistant CHOama-1 line (Table 1). A similar decrease in [³H]uridine incorporation was observed in infected CHO cells pretreated with AMD for 18 h and in infected BHK cells pretreated with either α -A or AMD (data not shown).

RNA polymerase activity in cell extracts. Viral RNA polymerase activity was not detectable in infected BHK cells which had been pretreated with AMD (Table 2). After discounting background incorporation in extracts of mock-infected cells, the level of activity in AMD-pretreated cell extracts was less than 3% of that evident in untreated controls. Moreover, extracts of mock-infected cells did not reconstitute polymerase activity in the treated extracts. Although this polymerase assay measures only run-off synthesis and does not reflect accurately the rate of RNA polymerization, these results are consistent with those shown in Table 1.

42 and 26S single-stranded RNA synthesis.

Two major species of positive strand RNA may be isolated from SB-infected cells (33). Full-length 42S RNA serves not only as genome, but also as mRNA for the nonstructural proteins (7, 17, 18) and as template for negative strand synthesis. A subgenomic mRNA (26S RNA) also is synthesized which encodes the structural proteins (6, 23, 31, 36). To examine the effect of host transcription inhibitors on the production of these RNAs, BHK cultures were treated with AMD for 0, 6, 12, or 18 h before infection (Fig. 3). The cultures were labeled with [³H]uridine between 9 and 11 h postinfection, and the RNA was extracted and analyzed on sucrose gradients. Increasing times of AMD treatment reduced the amount of 42 and 26S RNA produced during infection. Although transcription of these viral RNAs was reduced by about 90% in cells pretreated for 18 h, the ratio of 42 to 26S RNA was not altered significantly under any treatment condition. Therefore, AMD treatment did not block virus replication by affecting the ratio of 42 to 26S synthesis, but rather caused a coordinate reduction in the amount of the two species of positive strand produced.

Effect of AMD on RIs. The synthesis of SB positive strand RNA takes place on RIs (30). Two species of RI have been identified in infected cells, RIa and RIb, which produce 42S and 26S RNA, respectively. Upon digestion with RNase, RIa is converted to RFI, the double-stranded equivalent of 42S RNA (molecular weight, 8.8×10^6). RIb is converted to RFII (molecular weight, 5.6×10^6) and RFIII (molecular weight, 2.9×10^6), which is the double-stranded equivalent of 26S RNA. In the following experiments we have compared the levels of RIs in AMD-treated and untreated cells by first

TABLE 2. Effect of AMD pretreatment on SB RNA synthesis in vitro^a

Source of cell extract	Incorporation of [³ H]GTP (cpm)
Mock infected	200
Mock infected, AMD treated	150
SB infected	800
SB infected, AMD treated	180
SB infected, AMD treated, and mock infected	180
SB infected, AMD treated, and SB infected	600

^a BHK cell extracts were prepared from untreated cells which were either infected with SB or mock infected. Extracts also were prepared from SB-infected and mock-infected cells which had been treated with AMD 24 h before infection. The extracts were assayed for in vitro polymerase activity as described in the text.

FIG. 3. Intracellular viral RNA in AMD-treated BHK cells. Cultures of BHK cells were treated with 2 μ g of AMD per ml for 6, 12, or 18 h before infection with SB at an MOI of 10. The cultures were labeled with [3 H]uridine (100 μ Ci/ml) between 9 and 11 h postinfection. Control cultures received 2 μ g of AMD per ml 1 h before labeling. The RNA was extracted with phenol-SDS and analyzed on sucrose gradients. Symbols: $\bullet$, untreated control; $\circ$, 6-h AMD treated; $\bullet$, 12-h AMD treated; $\circ$, 18-h AMD treated.

converting them to the RFs by mild RNase digestion.

The RFs were isolated as described above after pulse-labeling BHK cells with [3 H]uridine from 5 to 7 h postinfection. The cultures had been treated with AMD for 0, 5, or 9 h before infection with SB (Fig. 4, upper panel). Increasing times of AMD treatment reduced the

amounts of RFI, -II, and -III isolated from SB-infected cells. After an 18-h treatment with the drug, we could not detect any RFs in the treated cultures (data not shown). Hybridization of RFs labeled from 0.5 to 8 h postinfection to excess unlabeled positive strand confirmed that the labeling of both positive and negative strand elements of the RFs was reduced (data not shown). RFs were also isolated from cells infected with SB^{amr}, a mutant of SB selected for its ability to replicate normally in cells pretreated with AMD or α -A. In contrast to the result with wild-type infected cells, RF production in SB^{amr}-infected cultures was highly resistant to the effects of AMD treatment (Fig. 4, lower panel). There was no apparent change in the proportion of each RF produced, and the levels of RF production evident in AMD-treated cultures infected with SB^{amr} matched or exceeded those found in untreated controls infected with SB. It is unlikely that the reduction in wild-type RF synthesis was due to a decreased uridine pool in treated cells; TCA-soluble counts were not significantly different in AMD-treated cultures, and SB^{amr} RFs were highly labeled under all conditions. Rather, these results suggest that inhibitors of host transcription blocked SB replication during or before maximal RI production, and indicated that the amount of negative strand RNA available for use as template for positive strand synthesis was reduced in treated cells.

Effect of AMD on rates of positive strand RNA synthesis. A decrease in the level of functional negative strand templates might result from (i) a direct effect on negative strand synthesis or (ii) an indirect effect whereby positive strand synthesis was inhibited early in infection preventing the amplification of negative strands to their normal levels. Accordingly, we estimated the rate of 42S positive strand synthesis as a function of the amount of RFI present under the various treatment conditions. BHK cells were treated with AMD for 0, 5, or 9 h before infection. The cultures were labeled with [3 H]uridine from 9 to 11 h postinfection, a time when radioactivity is incorporated exclusively into positive strands (5, 15, 26) and a pulse length sufficient to label all of the positive strand elements of the RFs (30). The amount of label incorporated into RFI and into 42S RNA was determined, and the counts per minute in single-stranded 42S RNA were divided by the label present in RFI. The rate of 42S synthesis was similar in SB- and SB^{amr}-infected control cultures (Table 3) and was, in turn, consistent with previously reported values (30). Although increasing times of AMD treatment reduced the overall amount of positive strand produced (Fig. 3), each functional RI synthesized 42S RNA at least as fast in treated cultures as in untreated controls (Table 3). In

FIG. 4. Comparison of SB and SB^{amr} RFs in AMD-treated cultures. Cultures of BHK cells were treated with AMD (2 μ g/ml) for 5 or 9 h before infection with 10 PFU per cell (SB or SB^{amr}). After adsorption, the medium was replaced, and the cultures were labeled with 100 μ Ci of [³H]uridine per ml from 5 to 7 h postinfection. The double-stranded RNA was extracted and analyzed on sucrose gradients. Upper panel, SB-infected cultures; lower panel, SB^{amr}-infected cultures.

infected cells which had been pretreated for 9 h with AMD, an apparent increase in the relative rate of 42S synthesis was observed consistently. However, this could have been due to inaccuracies in quantitating the small amounts of RFI present under these conditions. Nevertheless, these results demonstrated that the block in SB replication was not at the level of positive strand synthesis per se but that pretreatment of cells

with AMD blocked SB replication at or before the production of negative strand RNA.

DISCUSSION

We have investigated the participation of host factors in SB replicative processes by treating cells before infection with inhibitors of host transcription. Both α -A and AMD suppress SB

TABLE 3. Synthesis of 42S RNA^a

Treatment before infection	42S molecules synthesized/RFI per h	
	SB	SB ^{amr}
None	43	45
AMD (5 h)	46	48
AMD (9 h)	84	49

^a Cultures of BHK cells were treated with AMD (2 µg/ml) for 0, 5, or 9 h before infection with either SB or SB^{amr} at an MOI of 10; all cultures received AMD after infection. The RNA was labeled with 100 µCi of [³H]uridine per ml from 9 to 11 h postinfection, extracted with phenol, and analyzed for single- and double-stranded RNA species on sucrose gradients as described in the text.

growth in a variety of cell types under conditions in which vesicular stomatitis virus replication is not inhibited (2). Moreover, SB replication, as measured by production of progeny virus, is sensitive to α -A in wild-type CHO cells but is not sensitive to the toxin in CHOama-1 cells which possess an α -A-resistant RNA polymerase II. The observed inhibition, therefore, is not due to a generalized deleterious effect on treated cells, a secondary effect unrelated to transcription, or a direct effect of the inhibitors on SB replication. We suggest that host mRNA synthesis is required for SB growth and that the declining ability of treated cells to support SB replication results from the decay of a required host factor or its mRNA, or both.

Several workers have suggested the participation of a host component in alphavirus RNA synthesis based on either biochemical (7) or genetic (16, 34) evidence. Our results with transcription inhibitors support these suggestions. In the present study, overall synthesis of SB RNA was severely suppressed in α -A-treated CHO cells but not in the toxin-resistant CHOama-1 line. Similarly, viral polymerase activity in extracts of treated cells was greatly reduced and could not be reconstituted with extracts from untreated infected or mock-infected cells. These results suggested that the putative host factor was required for SB replication either during or before the synthesis of viral RNA.

The reduction in viral RNA synthesis observed in cells treated with inhibitors of host transcription was the result of a decrease in the number of RIs which were functional under these conditions. The molecular basis for this reduction remains unproven at present. A requirement for host involvement at any one of several points in the replicative cycle could cause such an effect. Specifically, adsorption, penetration, uncoating, translation of parental RNA into nonstructural proteins, synthesis of negative strands, and amplification of negative

strands through positive strand intermediates must all occur normally if appropriate levels of RIs for progeny RNA production are to be attained. Our data suggest that each of these steps, with the exception of negative strand synthesis, did proceed normally even in cells treated with inhibitors of host transcription. It is unlikely that SB growth was restricted at the level of adsorption, penetration, or uncoating as the number of infectious centers formed after a low MOI infection was unaffected by AMD treatment. We also demonstrated that each functional RI produced positive strands at a normal rate. This finding implies that the reduced levels of functional RIs could not be explained by a failure in positive strand synthesis which caused a lack of negative strand amplification through positive strand intermediates. Furthermore, those nonstructural proteins required for positive strand synthesis must have been present and functioning normally in treated cells. As all of the nonstructural proteins, including those required for negative strand synthesis, are synthesized as polyprotein precursors (8, 17, 18), it seems improbable that translation of the nonstructural proteins was the point at which replication was inhibited. However, we cannot rule out a differential effect on the processing of nonstructural proteins involved in positive and negative strand synthesis under inhibiting conditions. Therefore, our results are consistent with a requirement for a host factor after translation of the nonstructural proteins but before or during the synthesis of SB negative strand RNA.

We also examined RF levels in cells infected with SB^{amr}, a mutant which grows equally well in untreated cells and in cells treated with AMD or α -A. In control cells, SB^{amr} consistently overproduced RFs by 20 to 50% compared with SB. The effect of AMD pretreatment on SB^{amr} RFs was much less dramatic than on RFs in SB-infected cells (Fig. 4). Although some decrease was observed in cells treated for 9 h before infection, the levels of SB^{amr} RFs remained comparable to those in SB-infected, untreated cells. This suggests that the mutation in SB^{amr} allowed a more efficient interaction between the putative host factor and one of the viral proteins such that SB^{amr} could produce a number of RIs sufficient for normal replication even under conditions in which the concentration of the host factor was reduced. A similar phenotype was observed with a second mutant, SB-RL (unpublished data). SB-RL was selected for efficient growth on BHK cells by serial passage, harvesting at 5.5 h postinfection (3). Compared with SB, SB-RL overproduced RNA and RFs, and its growth and RF production were much less sensitive to AMD pretreatment. It appeared that selection for rapid growth also may have select-

ed for efficient utilization of host factors required for RI formation.

Numerous models could be proposed for the participation of a host subunit in the viral polymerase complex. However, as detailed information is lacking concerning the interaction of viral proteins involved in negative strand synthesis, it seems premature to propose a specific model which accommodates a putative host component in addition to the viral proteins. Studies with the temperature-sensitive RNA⁻ mutants of SB by Sawicki et al. (28) have shown that the members of complementation group F are defective in both positive and negative strand synthesis, implying that the protein product of this cistron is a core component of the polymerase. The host-dependent temperature-sensitive mutants isolated by Kowal and Stollar (16) also are members of the F complementation group, suggesting an interaction between a host factor and the F cistron product. However, such an association cannot be sufficient in itself for negative strand synthesis, as the sole group B mutant, ts-11, is also defective in negative strand synthesis (28), and ts-24, a group A mutant, is involved in the temporal regulation of negative strand production (27). We hope that detailed analysis of SB^{amr} replication will reveal which viral gene(s) interacts with the host component to promote negative strand synthesis. However, a complete understanding of SB negative strand metabolism must await the construction of a system capable of reproducing these synthetic and regulatory activities *in vitro*.

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