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Effect of Actinomycin D and Cycloheximide on Replication of Sindbis Virus in *Aedes albopictus* (Mosquito) Cells

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Production of Sindbis virus in the presence of transcription and translation inhibitors was examined in three *Aedes albopictus* cell lines. Addition of cycloheximide to heat-resistant Sindbis virus (SVHR)-infected mosquito cells arrested viral RNA synthesis completely, in contrast to the effects of this drug on virus-infected vertebrate cells. Production of mature virus by both SVHR (a variant commonly used as a wild-type virus) and SB^{amr} (a mutant which is resistant to the effects of 18 h of pretreatment of vertebrate cells with actinomycin D) in mosquito u4.4, C6-36, and C7-10 cells was inhibited by 2 h of pretreatment with actinomycin D. Pretreatment with this drug for 2 h slightly enhances virus production in vertebrate cells. Treatment of mosquito cells with actinomycin D resulted in shutoff of SVHR RNA synthesis. The mutant SB^{amr} was able to overcome the effects of actinomycin D on viral RNA synthesis and produced both 26S and 49S RNAs, even though no viral structural proteins or mature particles were produced in the presence of the drug. This result suggests that, in the presence of actinomycin, the nonstructural genes of SB^{amr} are translated sufficiently to allow for RNA synthesis but that 26S RNA may not be translated to an extent that allows significant virus production. These data demonstrate that host components are involved in at least two distinct steps in the production of Sindbis virus in mosquito cells: (i) production of viral RNA and (ii) synthesis of viral structural polypeptides.

The alphavirus Sindbis virus replicates efficiently in both vertebrate and invertebrate cells; however, differences are evident in the maturation routes of the virus, the host response to infection, and the involvement of the host in replication of the viral genome (7). In vertebrate cells, viral envelope proteins are glycosylated and fatty acid acylated during transport through the rough endoplasmic reticulum and the Golgi apparatus to their destination in the plasma membrane (17, 29–31). Capsid protein and the 49S viral genome combine to form the viral nucleocapsid in the cytoplasm of the cell (12). The nucleocapsid migrates to the plasma membrane and attaches to the envelope proteins, and the virus matures by envelopment in the modified plasma membrane (1, 6). In invertebrate cells, three pathways of virus maturation have been observed by electron microscopy. One of these (34) involves formation of the viral nucleocapsid free in the cytoplasm of the cell and envelopment in the host cell plasma membrane in a process similar to that described in vertebrate hosts. In other mosquito cells, virus maturation takes place in membrane- and ribosome-rich cytoplasmic vesicles. These virus factories contain both naked and membrane-bound viral nucleocapsids (16). In these cells, the nucleocapsid is apparently assembled within the cytoplasmic vesicles and matures by budding at intravesicular membranes, and mature virus is released upon fusion of the vesicular membrane with the plasma membrane of the host insect cell. A third pathway (22, 32) is an apparent mixture of the first two. Nucleocapsids are found in the cytoplasm and, in this instance, mature virions are observed within cytoplasmic vacuoles. It has been suggested that the nucleocapsid buds into the vesicle to mature. Release of the virus would then occur by an exocytic process, as described above (7). The compartmentalization of events related to virus maturation in invertebrate cells may explain why temperature-sensitive mutants, which readily complement in the vertebrate system, fail to do so in the invertebrate system (23). It has also been postulated that isolation of the virus within cytoplasmic vesicles protects host mosquito cells from cytopathic effects of the viral infection (16, 23, 24). Cells in which the viral nucleocapsids are observed in the cytoplasm are also those which display cytopathic effects (34; M. E. Knipfer, unpublished data).

Upon infection of vertebrate cells with Sindbis virus, host protein synthesis ceases and a strong cytopathic effect develops (35). Virus replication in vertebrates is, therefore, not dependent upon constant host macromolecular synthesis. This is supported by the observations that neither enucleation (14) nor 90 min of incubation in actinomycin D before infection (28) affects Sindbis virus production in vertebrate cells. The importance of relatively stable host functions in the replication of the Sindbis virus genome has been demonstrated in the vertebrate system (3, 4). When the incubation period of vertebrate cells in actinomycin D is extended to 6, 12, or 18 h preinfection, inhibition of Sindbis (but not vesicular stomatitis) virus production occurs, and it increases with the longer incubation periods (3). These extended incubations in actinomycin D have been shown to affect virus production in the vertebrate system by inhibiting the synthesis of negative-strand RNA and imply that some host-encoded component is essential for formation of double-stranded RNA (4). An examination of the ability of Sindbis virus temperature-sensitive mutants to establish homologous interference by Adams and Brown (2) suggested that interaction of a host component with the nonstructural proteins of the virus is essential for viral RNA synthesis; however, the nature of this host component has not been determined. When cycloheximide is added to infected cells after the synthesis of nonstructural proteins and the negative-strand RNA, the synthesis of viral RNA in the vertebrate system is not substantially affected (27), implying that the host component in vertebrate cells is relatively stable.

In the invertebrate system, host functions in the early stages of virus replication are similarly undefined; however, a requirement for continuing host macromolecular synthesis

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is apparent. Although some viral proteins are produced, enucleated Sindbis virus-infected mosquito cells (cytoplasts) release negligible amounts of virus, in contrast to cytoplasts derived from vertebrate cells (14). *Aedes albopictus* cells treated with actinomycin D for 90 min before infection with Sindbis virus produce processed viral proteins; however, virus production is drastically inhibited (28). Although inhibition of mature Sindbis virus production is observed when actinomycin D is added after infection is established, inhibition is not immediate and a lag period of 90 min is observed (28). Treatment for 1 to 2 h with the drug before infection results in maximal inhibition of virus production (28). These data suggest involvement of a labile host component in the replication of Sindbis virus. The existence of viral proteins in infected cytoplasts and actinomycin D-treated mosquito cells led to the hypothesis that a labile host function was involved in the assembly and release of the virus in insect cells (14, 28). In this study, the involvement of this labile host component in replication of the virus in vertebrate and invertebrate cells infected with Sindbis virus was examined by using actinomycin D, an inhibitor of DNA-directed RNA synthesis, and cycloheximide, an inhibitor of polypeptide synthesis.

MATERIALS AND METHODS

Cells, media, and virus. Three *A. albopictus* (mosquito) cell lines and one vertebrate (baby hamster kidney; BHK-21) cell line were used in this study. Each of the mosquito cell lines was derived from the original larval isolate of Singh (33). The u4.4 cell line was prepared from cells originally provided by Sonya Buckley (Yale Arbovirus Research Unit, New Haven, Conn.). The C6-36 cells derived by Igarashi (19) were provided by K. Ekels (Walter Reed Army Institute, Washington, D.C.). The C7-10 cells were provided by V. Stollar (Rutgers Medical School, New Brunswick, N.J.). These cells were derived from LT-C7 cells in V. Stollar's laboratory (26, 34). We have previously shown that these three clones of *A. albopictus* cells differ dramatically in their response to virus infection (cytopathic effect), ability to express homologous interference, and ability to produce and respond to antiviral factor (10, 11). Mosquito cells were grown in monolayers in Eagle minimal essential medium (13), pH 7.2 to 7.4, supplemented with 10% fetal bovine serum, 5% tryptose phosphate broth, and 2 mM glutamine. BHK-21 cell culture has been previously described (23, 28). In all experiments, cells were incubated at 28°C.

Heat-resistant Sindbis virus (SVHR; 8) was used as the wild-type virus. A Sindbis virus mutant whose growth is resistant to actinomycin D treatment of BHK-21 cells (3) was provided by R. E. Johnston (North Carolina State University, Raleigh). Both virus stocks were propagated as previously described (3, 23).

Drug treatments of vertebrate and invertebrate cells. In all experiments involving actinomycin D treatment, monolayers of cells were incubated in medium containing 4 μg of actinomycin D per ml for 2 h before infection and incubated in the same medium after adsorption of the virus. This concentration was determined to effectively inhibit both incorporation of [^{3}H]uridine into trichloroacetic acid-insoluble material and SVHR production in vertebrate and invertebrate cells in experiments using concentrations ranging from 0.1 to 8 μg/ml (data not shown).

Cycloheximide was used at a concentration of 75 μg/ml in all experiments described, but the time of drug addition to cell monolayers varied as described elsewhere in the text.

Analysis of viral RNA. Total viral RNA synthesis was examined by the cytoplasmic dot hybridization method of White and Bancroft (36). Mosquito and BHK-21 cell monolayers were treated with actinomycin D, cycloheximide, or a combination of the drugs, as described elsewhere in the text. Equal numbers of cells were then lysed with 1% Nonidet P-40 in TE buffer (10 mM Tris chloride, 1 mM EDTA [pH 7.5 to 8.0]). Nuclei were pelleted, and the supernatant was treated with formaldehyde in a standard saline citrate solution (1× SSC; 150 mM sodium chloride plus 15 mM trisodium citrate). The RNA was blotted onto a GeneScreen hybridization transfer membrane (New England Nuclear Corp., Boston, Mass.) with a Schleicher & Schuell (Keene, N.H.) Minifold II apparatus. Blot hybridizations were performed with a nick-translated, ^{32}P-labeled probe made from a cDNA construct, pSin3′, containing a viral 26S RNA sequence (provided by C. M. Rice and J. H. Strauss) (25), and the radioactive probe was then hybridized to the RNA on the filter. The filter was then exposed to Kodak RP X-Omat XAR-5 film (Eastman Kodak Co., Rochester, N.Y.). Relative amounts of RNA were determined by densitometer tracings of the resulting autoradiograph by using exposures in the linear range of the film. Results obtained in each experiment were controlled by probing mock-infected cells.

RNA was extracted from mosquito monolayers by using a modified urea-sodium dodecyl sulfate (SDS) extraction method (9, 18) and blotted from 0.8% formaldehyde agarose (21) onto nitrocellulose (Schleicher & Schuell) or Biotrans (ICN Pharmaceuticals, Inc., Irvine, Calif.) and examined by using Northern (RNA) blots, as previously described (21). [^{32}P]UTP-labeled RNA markers were transcribed in vitro by using linear DNA templates (pSP76; a gift from D. A. Melton and Shelly Berger, Harvard University, Cambridge, Mass.) containing polyomavirus inserts under the conditions given by Promega Biotech. Each of the three species was synthesized by using the random primed DNA synthesis system of Feinberg and Vogelstein (15) with linear pSin3′ and hexanucleotide random primers (pdN6; Pharmacia, Inc., Piscataway, N.J.). Blots were hybridized at 55°C for 22 h and exposed to X-ray film.

Polyacrylamide gel electrophoresis of polypeptides. To determine the effect of actinomycin D on protein synthesis, SVHR-infected mosquito or BHK-21 cells (untreated and actinomycin D treated) were incubated in medium containing 100 μCi of [^{35}S]methionine per ml for 3 h before harvest at 6 h postinfection (for trichloroacetic acid precipitations or gel electrophoresis followed by fluorography).

Samples of cell lysates were precipitated with 2.5 volumes of ice-cold 95% ethanol overnight at −20°C, suspended in sample buffer (2% SDS, 1.5% dithiothreitol, 10% glycerol, 0.24 M Tris chloride [pH 6.8]), heated to 100°C for 3 min, and resolved in a 10.8% polyacrylamide gel containing SDS by the method of Laemmli (20). After electrophoresis, the gel was impregnated with diphenyloxazole (5), dried under vacuum at 60°C, and exposed to X-ray film.

RESULTS

Production of SVHR in vertebrate and invertebrate cells in the presence of actinomycin D and cycloheximide. SVHR production was examined to determine whether obvious differences in sensitivity to actinomycin D exist in the three aedine cell lines, u4.4, C6-36, and C7-10. Monolayers of each cell line were incubated in medium containing 4 μg of actinomycin D per ml for 2 h and infected with SVHR at a

TABLE 1. SVHR production in cells treated with actinomycin D and cycloheximide[a]

Drug treatment	Virus PFU/ml (%) on:			
	u4.4	C6-36	C7-10	BHK-21
None	3.20×10^8	1.95×10^9	1.12×10^9	1.65×10^9
A[b]	2.00×10^7 (6.3)	2.60×10^7 (1.3)	8.15×10^7 (7.2)	1.95×10^9 (118)
None	3.30×10^8	2.05×10^9	2.90×10^9	1.65×10^9
CX[c]	2.15×10^7 (6.5)	4.15×10^7 (2.0)	5.00×10^7 (1.7)	1.35×10^8 (8.2)

[a] Cell monolayers were infected with SVHR at a multiplicity of 100 PFU per cell, and virus production was assayed at 24 h postinfection.
[b] A, Addition of 4 μg of actinomycin D per ml to monolayers at 2 h preinfection.
[c] CX, Addition of 75 μg of cycloheximide per ml to monolayers at 4 h postinfection.

multiplicity of infection (MOI) of 100 PFU per cell. Virus production in the u4.4, C6-36, and C7-10 cell lines assayed at 24 h postinfection was inhibited by actinomycin D treatment (Table 1). In contrast, SVHR production in BHK-21 cells was increased slightly or not affected by the brief incubation in the drug (Table 1).

To determine the effect of cycloheximide on SVHR production in the four cell lines, monolayers of mosquito and BHK-21 cells were infected with SVHR (MOI, 100 PFU per cell), and at 4 h postinfection 75 μg of cycloheximide per ml was added. Virus production assayed at 24 h postinfection was inhibited by addition of the drug to all cell lines (Table 1). Therefore, virus production in all three invertebrate cell lines was sensitive to actinomycin D and cycloheximide treatments; however, mature virus production in BHK-21 cells under these experimental conditions was inhibited only by cycloheximide.

Effects of actinomycin D and cycloheximide on SVHR RNA synthesis. Actinomycin D effectively inhibited SVHR production in all three mosquito cell lines. An attempt was made to determine the effects of the drug on viral RNA synthesis by comparing the incorporation of [^{3}H]uridine into trichloroacetic acid-insoluble material in untreated uninfected and infected monolayers with that of drug-treated cultures. Although actinomycin D effectively inhibited incorporation of labeled precursor into RNA, no substantial difference between uninfected and infected monolayers was observed. Therefore, an alternative method was used to determine the effect of incubation in actinomycin D on viral RNA synthesis in SVHR-infected mosquito cells; cytoplasmic RNA hybridization (slot blot) analyses were performed. Monolayers of u4.4 cells were either incubated in medium containing 4 μg of actinomycin D per ml for 2 h before infection or incubated in drug-free medium. The cells were infected with SVHR at an MOI of 100 PFU per cell and incubated at 28°C. At 3 and 7 h postinfection, actinomycin D-treated and untreated monolayers were lysed, bound to filter paper, and probed for viral RNA content (Fig. 1A). At 7 h postinfection, viral RNA levels in drug-treated cells were 53% of control levels. To determine whether this inhibition of viral RNA synthesis occurred in all three mosquito cell lines, monolayers of u4.4, C6-36, and C7-10 cells were incubated either in medium containing actinomycin D for 2 h preinfection or in drug-free medium, infected with SVHR (MOI, 100 PFU per cell), and harvested at 7 h postinfection. Slot blot analysis revealed inhibition of viral RNA synthesis to 11% of control values by actinomycin D in the u4.4 cell line (Fig. 1B). RNA synthesis continued in C6-36 and C7-10 cells but at reduced levels (68% of control in C6-36 and 31% of control in C7-10 cells).

In tissue-cultured vertebrate cells, it has been demonstrated that addition of cycloheximide to an established Sindbis virus infection does not totally arrest viral RNA synthesis, and it is presumed that synthesis continues utilizing proteins synthesized before addition of the drug (26). The effect of cycloheximide on viral RNA synthesis in invertebrate cells was also examined in experiments similar to the actinomycin D experiments described above. When cycloheximide was added before 3 h postinfection to either vertebrate or invertebrate cells incubated at 28°C, no viral RNA was detected in lysates harvested at 9 h postinfection (data not shown). A reduction in viral RNA synthesis was also observed in cells of both phyla when cycloheximide was added at 4 or 5 h postinfection (data not shown). In fact, viral RNA synthesis in SVHR-infected mosquito cultures was inhibited by addition of cycloheximide at 24 h postinfection (data not shown). Although viral RNA synthesis and infectious virus production (Table 1) were inhibited in vertebrate cells by addition of the drug early in infection, RNA synthesis continued when cycloheximide was added to an established infection. To determine whether RNA synthesis in the invertebrate system would continue in the presence of the drug or be inhibited entirely, monolayers of u4.4 cells were infected with SVHR. Cycloheximide was added at 3 h postinfection or the cells remained free of the drug. Un-

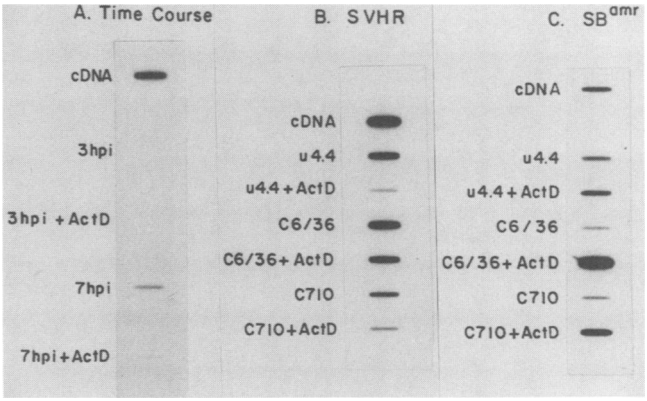

FIG. 1. Effect of actinomycin D on viral RNA synthesis. (A) Monolayers of u4.4 cells were either incubated in medium containing 4 μg of actinomycin D (ActD) per ml from 2 h preinfection until harvest or incubated in drug-free medium. These monolayers were infected with SVHR (MOI, 100 PFU/ml) and incubated at 28°C. Duplicate monolayers (treated and untreated) were harvested at 3 and 7 h postinfection (3 hpi and 7 hpi, respectively). (B) Monolayers of u4.4, C6-36, and C7-10 cells were incubated in either drug-free medium or medium containing 4 μg of actinomycin D per ml from 2 h preinfection until harvest at 7 h postinfection. Cells were infected with SVHR at an MOI of 100 PFU per cell. All incubations were at 28°C. (C) Monolayers of u4.4, C6-36, and C7-10 cells were subjected to the same treatment described for panel B, with the exception that the cells were infected with SB^{amr}. All cell lysates were analyzed by cytoplasmic RNA hybridization with pSin3′ (as described in Materials and Methods).

treated and cycloheximide-treated monolayers were harvested at 3 and 7 h postinfection. Treatment of mosquito cells with cycloheximide at 3 h postinfection resulted in no increase in the amount of viral RNA in the cells during the next 4 h (Fig. 2A). The amount of viral RNA in cycloheximide-treated cells at 7 h was identical to the amount in control cells at 3 h and was 5% of the control value at 7 h. Cycloheximide inhibition of Sindbis virus-specific RNA production occurred in all three mosquito cell lines (Fig. 2B). Viral RNA levels were as follows: u4.4 cells, 5% of non-drug-treated control; C6-36, 7.9% of control; C7-10, 11% of control. Two possible explanations exist for the cessation of viral RNA synthesis in cycloheximide-treated cells. It is possible that this arrest was due to inhibition of nonstructural protein synthesis required for continued production of minus-strand RNA in infected mosquito cells. Alternatively, this may be indicative of rapid turnover of the host component involved in viral RNA production, as indicated by the experiments with actinomycin D. Cycloheximide treatment of vertebrate cells at 3 h postinfection (as in the mosquito cell experiments described above) caused RNA synthesis to cease only when the cells were subjected to actinomycin D treatment before infection (data not shown). Thus, a combination of drugs was required to cause cessation of viral RNA synthesis in vertebrate cells, whereas cycloheximide alone was sufficient to generate this effect in invertebrate cells. It is not clear how a combination of these inhibitors is capable of terminating viral RNA synthesis in BHK-21 cells when neither drug alone accomplishes this. This may, however, simply result from the combined toxic effects of two potent chemical agents.

Effect of actinomycin D on SVHR protein production. Scheefers-Borchel et al. (28) found that viral proteins were produced and processed in Sindbis virus-infected u4.4 cells treated with actinomycin D before infection. In that study, proteins labeled with [^{35}S]methionine were examined by polyacrylamide gel electrophoresis by loading of equal amounts of incorporated radioactivity per lane. These researchers further demonstrated that the precursor envelope protein PE_2 was cleaved to E_2 in drug-treated cells. To compare the effects of actinomycin D on Sindbis viral protein synthesis and processing in the three mosquito cell lines (u4.4, C6-36, and C7-10), proteins in monolayers of actinomycin D-treated and untreated, infected mosquito cells were labeled by 3 h of incubation in 100 μCi of [^{35}S]methionine per ml added at 3 h postinfection with Sindbis virus. Cells were harvested and lysed at 6 h postinfection. For Fig. 3A, equal amounts of radioactivity from lysates prepared from untreated (lane 2) and actinomycin D-treated (lane 3) C6-36 cells were loaded per lane and electrophoresed as described in Materials and Methods. Viral structural proteins (PE_2, E_1, E_2, and C) were present in the drug-treated C6-36 cell lysate. A similar profile was observed for u4.4 and C7-10 cells (data not shown). In all cases, substantial but incomplete inhibition of host protein synthesis occurred, as indicated by the presence of radioactively labeled proteins other than virally encoded ones.

When equal volumes of these cell lysates were loaded per lane (Fig. 3B), inhibition of total protein synthesis was demonstrated and dramatic inhibition of viral protein synthesis was apparent. C6-36 cells consistently produced more viral proteins in the presence of actinomycin D than did the

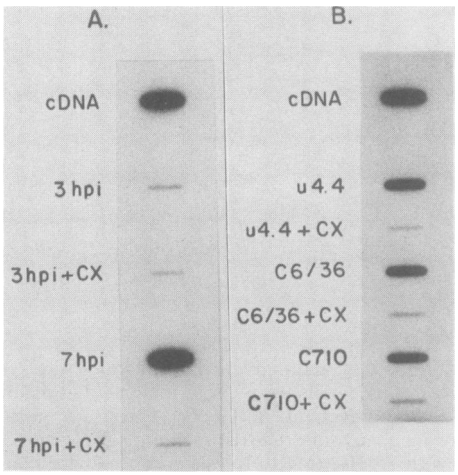

FIG. 2. Effect of cycloheximide (CX) on viral RNA synthesis. (A) SVHR-infected u4.4 monolayers were either treated with 75 μg of cycloheximide per ml at 3 h postinfection or incubated in drug-free medium until harvest. Duplicate monolayers (cycloheximide treated and untreated) were harvested at 3 and 7 h postinfection (3 hpi and 7 hpi, respectively). Cell lysates were examined in a cytoplasmic RNA hybridization assay as described in Materials and Methods. (B) SVHR-infected u4.4, C6-36, and C7-10 cells were either incubated in drug-free medium or treated with cycloheximide at 4 h postinfection. Cell lysates were prepared at 7 h postinfection and examined in slot blot analyses as described in the text.

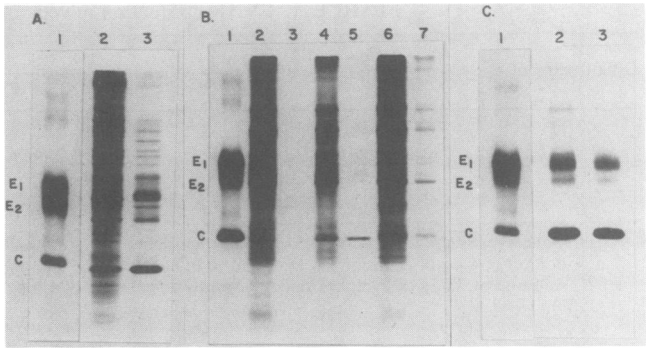

FIG. 3. Effect of actinomycin D on SVHR protein production. (A) Monolayers of C6-36 cells were incubated in either drug-free medium or medium containing 4 μg of actinomycin D per ml from 2 h preinfection until harvest. Cells were infected with SVHR at an MOI of 100 PFU per cell. [^{35}S]methionine (100 μCi/ml) was added to the medium at 3 h postinfection. Cells were lysed at 6 h postinfection, and protein samples were prepared for SDS-polyacrylamide gel electrophoresis. Samples of cell lysates containing approximately equal amounts of radioactivity were subjected to electrophoresis. The gel was then dried and analyzed by autoradiography. Lanes: 1, marker virus protein labeled with ^{35}S; 2, untreated C6-36 cell lysate; 3, actinomycin D-treated C6-36 cell lysate. (B) Monolayers of u4.4, C6-36, and C7-10 cells were subjected to the experimental design described for panel A. Equal volumes of cell lysates derived from equal numbers of cells at 6 h postinfection were loaded per lane. Lanes: 1, marker viral protein labeled with ^{35}S; 2 and 3, lysates of u4.4 cells; 4 and 5, lysates of C6-36 cells; 6 and 7, C7-10 cell lysates. Cell lysates examined in lanes 2, 4, and 6 were obtained from drug-free cells, and those in lanes 3, 5, and 7 were obtained from actinomycin D-treated cells. (C) Monolayers of SVHR-infected BHK-21 cells incubated in actinomycin D-containing medium or drug-free medium from 2 h preinfection until harvest (6 h postinfection) were analyzed by SDS-polyacrylamide gel electrophoresis as described for panel B. Equal volumes of cell lysates were used. Lanes: 1, marker virus labeled with ^{35}S; 2, untreated BHK-21 cell lysate; 3, actinomycin D-treated BHK-21 cell lysate. For all gels, the positions of BHK-21 cell-grown SVHR structural proteins (E_1, E_2, and C) are indicated.

other cell lines. Longer exposure of this gel revealed the presence of PE_2, E_1, and E_2 in addition to the visible capsid protein band (data not shown); however, discrete bands within lanes containing proteins of untreated cultures (Fig. 3B, lanes 2, 4, and 6) were not discernible. When the proteins produced by Sindbis virus-infected BHK-21 cells treated with actinomycin D were examined in a similar manner, this inhibition of viral protein synthesis was not observed (Fig. 3C).

Although the amount of viral protein synthesis was greatly reduced in mosquito cells treated with actinomycin D, the proteins synthesized were apparently normal. These proteins were processed (Fig. 3A) and packaged into viral nucleocapsids and matured into infectious virus particles (data not shown).

Production of a Sindbis virus mutant (SB^{amr}) in mosquito cells in the presence of actinomycin D. Johnston and coworkers recently selected a Sindbis virus mutant (SB^{amr}) in BHK cells which were treated with actinomycin D for 18 h before infection (3). SB^{amr} bypasses the block in replication caused by treatment of vertebrate cells with either actinomycin D or α-amanitin, and normal levels of virus are produced. We used this mutant in an experiment to determine whether the genetic change selected for in the vertebrate host would bypass the actinomycin D-induced block in the invertebrate.

To determine the effect of actinomycin D on production of SB^{amr} in the three mosquito cell lines, monolayers of these cells were incubated in medium containing actinomycin D for 2 h and infected with either SVHR (as a control) or SB^{amr} at an MOI of 100 PFU per cell, and virus production was assayed at 28 h postinfection. Production of both SVHR and SB^{amr} was inhibited by the 2-h actinomycin D treatment in all three mosquito cell lines (Table 2). However, the extent of inhibition of SB^{amr} production by actinomycin D was consistently slightly less than the inhibition of SVHR production in drug-treated cells. SB^{amr} production in the C6-36 cell line was the least sensitive to actinomycin D treatment.

Effect of actinomycin D on viral RNA synthesis in SB^{amr}-infected mosquito cells. In vertebrate cultures, SB^{amr} bypasses the actinomycin D-induced block in virus replication by overcoming a requirement (or reducing the stringency) for host participation in the generation of negative strands of the viral RNA (4). In contrast, SB^{amr} production in invertebrate cells was inhibited by treatment with actinomycin D. Two obvious explanations for this exist. Possibly, the mutation(s) in SB^{amr} which permits viral RNA replication without normal host participation is insufficient to allow replication in the invertebrate system (thus, SB^{amr} RNA synthesis would still be inhibited by the drug). Alternatively, SB^{amr} RNA synthesis occurs in the presence of the drug in mosquito cells, but a block in virus maturation (similar to that proposed by Scheefers-Borchel et al. [28]) at a later step is also produced by the drug treatment.

TABLE 2. SB^{amr} production in cells treated with actinomycin D[a]

Cell line	Actinomycin D treatment	Virus PFU/ml (% control)[b]
u4.4	−	1.9×10^8
	+	2.8×10^7 (12.8)
C6-36	−	1.1×10^9
	+	3.0×10^8 (25.7)
C7-10	−	8.5×10^8
	+	1.1×10^8 (13.1)

[a] Cell monolayers were infected with SB^{amr} at a multiplicity of 100 PFU per cell, and virus production was assayed at 27 h postinfection.

[b] The control was calculated by dividing the titer of the virus produced by drug-free cells with that obtained with drug-treated cells and multiplying by 100.

To determine the effect of actinomycin D treatment on SB^{amr} RNA synthesis in mosquito cells, monolayers of u4.4, C6-36, and C7-10 cells were incubated in drug-free medium or in medium containing 4 μg of actinomycin D per ml for 2 h and infected with SB^{amr} at an MOI of 100 PFU per cell. Virus-specific RNA synthesis was examined at 7 h postinfection by slot blot analyses as described previously. In each cell line, actinomycin D treatment of SB^{amr}-infected cells resulted in enhanced viral RNA synthesis (Fig. 1C). Viral RNA synthesis was 1.5 times control levels in drug-treated u4.4 cells and was 6 times control levels in C7-10 cells. The stimulating effect of actinomycin D on SB^{amr} RNA synthesis was most dramatic in C6-36 cells, in which concentrations of viral RNA were 20-fold higher than control. This increased level of viral RNA synthesis was not accompanied by an increase in mature virus production (Table 2).

Effect of actinomycin D on SB^{amr} protein production. In the mosquito cell lines used in these studies, SB^{amr} bypassed the actinomycin D-induced block in viral RNA replication but failed to produce infectious virus at levels characteristic of untreated cells. Thus, an examination of the sensitivity of subsequent steps in virus maturation to the actinomycin D treatment was possible. An analysis of viral protein production in mosquito cells infected with SB^{amr} in the presence of actinomycin D (similar to the experiment described for SVHR-infected cells) was performed. Untreated and actinomycin D-treated monolayers of u4.4, C6-36, and C7-10 cells were infected with SB^{amr} at an MOI of 100 PFU per cell. At 3 h postinfection, 100 μCi of [^{35}S]methionine per ml was added to cultures for 3 h of incubation at 28°C. The cells were then harvested and lysed, and incorporation of label was determined. Equal volumes of these cell lysates were examined by SDS-polyacrylamide gel electrophoresis followed by fluorography, as described in Materials and Methods (Fig. 4). A net inhibition of both host and viral protein syntheses occurred in cells treated with actinomycin D. However, as with SVHR-infected mosquito cells, the small amount of viral proteins produced was processed normally. Therefore, actinomycin D treatment of mosquito cells 2 h preinfection inhibited at least two independent stages in the Sindbis virus replication cycle: synthesis of viral RNA and generation of viral polypeptides.

RNA species synthesized in SB^{amr}-infected actinomycin D-treated mosquito cells. An increase in SB^{amr} viral RNA synthesis in the presence of actinomycin D without an increase in virus production or protein synthesis could result from an ability of the SB^{amr} mutant to synthesize only 49S RNA in the presence of the drug. Failure to synthesize 26S RNA would result in an accompanying failure to produce structural proteins and virus particles. To determine what RNA species were produced in u4.4 cells infected with SB^{amr} in the presence or absence of actinomycin D, cells were infected with the mutant in the presence or absence of 4 μg of the drug per ml and lysed at 7 h postinfection. RNA was extracted from the lysates and run on formaldehyde-agarose gels, as described in Materials and Methods. The gels were probed with a ^{32}P-labeled Sindbis virus-specific cDNA probe prepared as described in Materials and Methods. The results of this experiment (Fig. 5) revealed that 26S RNA was produced by SB^{amr} in the presence of actinomycin D. Densitometer tracings of these blots revealed that the

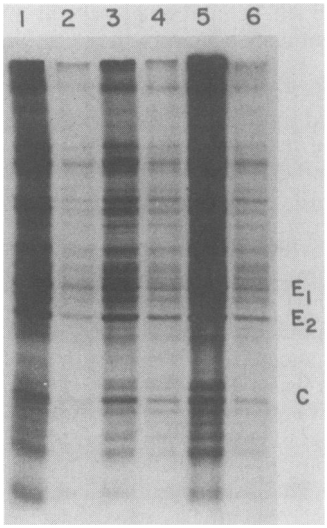

FIG. 4. Effect of actinomycin D on SB^{amr} protein production. Monolayers of u4.4, C6-36, and C7-10 cells infected with SB^{amr} were treated as described in the legend to Fig. 3. Equal volumes of cell lysates containing equal numbers of cells were examined by SDS-polyacrylamide gel electrophoresis and autoradiography. Lanes: 1, u4.4 cell lysate; 2, actinomycin D-treated u4.4 cell lysate; 3, C6-36 cell lysate; 4, actinomycin D-treated C6-36 lysate; 5, C7-10 cell lysate; 6, actinomycin D-treated C7-10 lysate. The positions of mosquito cell-grown Sindbis virus structural proteins are indicated.

ratio of 26S RNA to 49S RNA was the same in the presence or absence of the drug.

DISCUSSION

A requirement for continued host cell nuclear function in Sindbis virus replication has been demonstrated in both vertebrate and invertebrate systems. In vertebrate cells, incubation in actinomycin D for 18 h preinfection inhibits RNA replication by affecting negative-strand synthesis (3, 4). It has been suggested that a host component present in a limited amount interacts with the nonstructural proteins of the virus in a replication complex (2). Previous reports have also indicated a requirement for host nuclear functions in virus production in the invertebrate system (14, 28). Enucleation of mosquito cells (14) or treatment of mosquito cells with actinomycin D for 90 min preinfection (28) resulted in reduced levels of mature virus production.

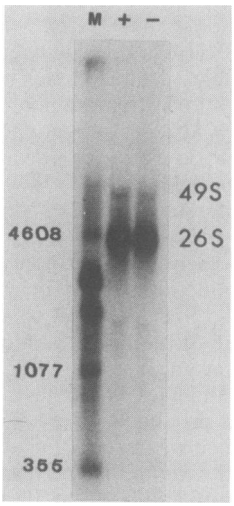

FIG. 5. SB^{amr} RNA synthesized in the presence or absence of actinomycin D. Mosquito cell (C6-36) monolayers were treated with actinomycin D (4 μg/ml) for 2 h, infected with SB^{amr} (MOI, 15), incubated for 7 h at 28°C, and lysed for RNA extraction; control monolayers were not drug treated. RNA extracts were blotted as described in Materials and Methods. Lanes: M, RNA markers (transcripts of 355, 1,077, and 4,608 bases); +, RNA from cells treated with actinomycin D; −, untreated control.

The data presented here support these results. Incubation in actinomycin D for 2 h preinfection inhibited Sindbis virus production in three *A. albopictus* cell lines. Viral RNA was synthesized at reduced levels in the presence of the drug, viral proteins were synthesized at reduced levels but were processed normally, and the limited amounts of viral proteins were incorporated into mature virus. The simple explanation of this is that a reduced message results in reduced protein synthesis. When cycloheximide was added to infected monolayers of mosquito cells after synthesis of nonstructural proteins had begun, viral RNA synthesis was arrested. This finding contrasts with the situation in vertebrate cells, in which viral RNA synthesis continues, utilizing nonstructural proteins synthesized before addition of the drug (27). This implies that a component of the replication complex in invertebrate cells is very labile and that continued synthesis of this protein component must occur for viral RNA replication to take place in the invertebrate system.

Although the Sindbis virus mutant SB^{amr} (selected in vertebrate cells treated with actinomycin D) produces virus efficiently in drug-treated BHK-21 cells (3), SB^{amr} production in mosquito cells treated with actinomycin D preinfection was inhibited to nearly the same extent as SVHR production. In light of these data, it seemed possible that the level at which actinomycin D affects virus replication in the two systems differs. However, SB^{amr} viral RNA synthesis takes place at elevated levels in drug-treated monolayers, and both 26S and 49S RNA species are produced. Therefore, the mutant SB^{amr} can bypass the actinomycin D-induced block in viral RNA production in both vertebrate and invertebrate systems. This observation suggested that actinomycin D treatment inhibits viral RNA synthesis by affecting a host component similar in its function but differing in its stability in the two systems. The observation that SB^{amr} can efficiently produce 26S and 49S viral RNA species implies that translation of the 49S RNA containing information for nonstructural RNA-replicating proteins takes place. The failure to detect significant amounts of viral structural proteins or progeny virions implies that a second actinomycin D-sensitive component functions at the level of 26S RNA protein synthesis and that SB^{amr} does not bypass this block. Neither of these inhibitory effects are seen in vesicular stomatitis virus infection of mosquito cells (14, 28); however, it is difficult to imagine the specifics involved in the differentiation of vesicular stomatitis virus and Sindbis virus protein production. The development of mutants of SB^{amr} which produce virions in the presence of the drug may be useful in determining the further involvement of the host factors in viral 26S RNA translation.

Previous reports have indicated differences among the three mosquito cell lines used in these studies with respect to cytopathic effect, virus production, and establishment of homologous interference and production of and sensitivity to an antiviral agent produced by persistently infected u4.4 cells (7, 10, 11, 34). In this study, additional differences in

the cells were evident. C6-36 cells produced more viral proteins when infected with Sindbis virus in the presence of actinomycin D than did u4.4 and C7-10 cells. Viral RNA synthesis was shown to be the most sensitive to actinomycin D treatment in u4.4 cells. These differences in the cell lines imply that the three cell lines (each derived from the Singh larval isolate) possess genetic alterations due to several years of maintenance in tissue culture. Alternatively, the three cell lines examined here may be derived from cells in the original Singh isolate which are not totipotent but are partially differentiated and destined to become specific cell types within mosquitos.

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