Requirement for Host Transcription in the Replication of Sindbis Virus†

RALPH S. BARIC,‡ LISA J. CARLIN, AND ROBERT E. JOHNSTON*

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27650

Received 19 July 1982/Accepted 12 October 1982

Host cell involvement in Sindbis virus (SB) replication was examined in cells which had been treated with either actinomycin D (AMD) or α -amanitin (α -A). Treatment with these inhibitors of host transcription before infection reduced the ability of cells to support SB growth by 1 to 2 orders of magnitude, while having little or no effect on the replication of vesicular stomatitis virus. SB replication was sensitive to α -A in wild-type Chinese hamster ovary (CHO) cells but was resistant to α -A in CHOama-1 cells, a line which contains an α -A-resistant RNA polymerase II. A mutant of SB, SB^{amr}, was isolated by mutagenesis followed by selection in cells which had been treated with AMD. SB^{amr} grew normally not only in cells treated with AMD but also in α -A-treated cells. Our results suggest (i) that the synthesis of cellular mRNA (and presumably protein) is required for replication of SB, (ii) that prior treatment with either drug affects the same aspect of SB replication, and (iii) that mutations in the SB genome allow the virus to overcome the effect of inhibitors of host transcription.

The replication of a virus in a particular host cell depends upon the ability of the virus to utilize key constituents of the cell's synthetic machinery. These host cell contributions to virus growth range from the translation of viral messages by cellular ribosomes to the direct participation of specific cellular components in viral transcriptase and replicase enzymes. The foremost example of the latter type of virus-host interaction among RNA viruses is the replicase of bacteriophage Q β (2). Considerable evidence suggesting the direct participation of host constituents in viral synthetic processes also has been reported for RNA-containing animal viruses, such as foot-and-mouth disease virus (1), encephalomyocarditis virus (8), poliovirus (6), influenza virus (3, 10, 15, 16), and vesicular stomatitis virus (VSV) (9, 20, 28).

Several observations indicate that the type and physiological state of the host cell profoundly influence alphavirus replication. Sindbis virus (SB) and Semliki Forest virus produce an acute cytolytic infection in vertebrate cells, whereas cells of invertebrate origin become persistently infected and do not exhibit a cytopathic effect (7, 12, 18, 22). However, mutants of Singh's *Aedes albopictus* cells have been isolated in which a cytopathic effect is evident after SB infection

(11, 23). Tooker and Kennedy (29) have isolated a number of *A. albopictus* cell clones which they classified as high or low yielders of Semliki Forest virus. The restriction of virus replication in the low-yielder clones seemed to occur at the level of nonstructural protein or minus-strand RNA synthesis. A requirement for a host component present in the high-yielding clones but absent or at reduced concentration in the low-yielding clones could account for these differences. In addition to a role in RNA synthesis, a host factor(s) may be involved later in the alphavirus replication cycle in *A. albopictus* cells, as Scheefers-Borchel et al. (25) have shown that SB maturation is inhibited in *A. albopictus* cells treated with actinomycin D (AMD).

Alphavirus replication in vertebrate cells also may be dependent upon the specific participation of host components: a host protein was associated with a partially purified preparation of the Semliki Forest virus polymerase (5), and Ulmanen et al. (30) have suggested the possible participation of host proteins in the formation of intracellular viral ribonucleoprotein. More recently, Mento and Siminovich (17) have selected a line of SB-resistant Chinese hamster ovary (CHO) cells, and Kowal and Stollar (14) have isolated two host-dependent, temperature-sensitive mutants of SB.

To assess the involvement of cellular factors in the lytic replication of SB, we have reexamined the effect of inhibitors of host transcription on virus growth. Our results suggest (i) that the

† Paper no. 8433 of the journal series of the North Carolina Agricultural Research Service, Raleigh, NC 27650.

‡ Present address: Department of Neurology, University of Southern California School of Medicine, Los Angeles, CA 90033.

synthesis of cellular mRNA (and presumably protein) is required for replication of SB, (ii) that prior treatment with either AMD or α -amanitin (α -A) affects the same aspect of SB replication, and (iii) that mutations in the SB genome allow the virus to overcome the effect of inhibitors of host transcription.

MATERIALS AND METHODS

Virus and cell culture. SB strain AR339 (our wild-type strain) was originally isolated by Taylor et al. (27) and was obtained from H. R. Bose, University of Texas. The Indiana strain of VSV was supplied by Gail Wertz, University of North Carolina. Stocks of both viruses were grown on monolayers of BHK cells in Eagle minimal essential medium (MEM; GIBCO Laboratories) supplemented with 10% donor calf serum (Flow Laboratories, Inc.), 10% tryptose phosphate broth (Difco Laboratories), and 50 U of penicillin (Sigma Chemical Co.) and 50 μ g of streptomycin (Sigma) per ml. BHK cells were purchased from the American Type Culture Collection (ATCC) in passage 52, and only cells from passages 52 through 64 were used in experiments. The VSW cell line (ATCC CCL129) was isolated by Zeigel and Clark (32) from Russell's viper. This line was chosen for use in these experiments because it was highly unlikely that our virus strains had ever been passaged in these cells and thus had ever been adapted to them. CHO and CHOama-1 cells (13) were the kind gift of C. J. Ingles, University of Toronto. BHK and the CHO cell lines were maintained at 37°C in MEM containing 10% donor calf serum and 10% tryptose phosphate broth but no antibiotics. The VSW cell line was grown at 29°C in MEM supplemented with 10% donor calf serum. Experiments were performed at 37°C with all cell lines.

Chemicals. AMD and α -A were purchased from Sigma Chemical Co. and used at 2 μ g/ml and 5 to 10 μ g/ml, respectively. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (K & K Laboratories, Inc.) was used for mutagenesis.

Virus replication in the presence of inhibitors of host transcription. Replicate cultures of BHK or VSW cells were seeded at 1×10^6 to 2×10^6 cells per 60-mm culture dish; the two CHO cell lines were seeded at 5×10^5 cells per plate. After seeding at these densities, the cultures were still subconfluent at the time of infection. Treatment with either AMD (2 μ g/ml) or α -A (5 μ g/ml in CHO cells or 10 μ g/ml in BHK cells) was begun at 4 to 12 h after seeding. In BHK cells, inhibition of cellular RNA synthesis required a longer treatment with a higher concentration of α -A than it did in CHO cells (data not shown). At intervals after addition of the drug, the culture fluids were removed, and the cells were infected with 5 to 10 PFU of SB or VSV per cell. After 1 h for virus adsorption, the inocula were removed, the cultures were washed with phosphate-buffered saline, and the preinfection treatment medium was returned to the infected monolayers. Virus yields were determined by plaque assay. Maximum titers were obtained at 12 h postinfection for VSV and at 18 h for SB in BHK and CHO cells; in VSW cells, maximum titers were obtained at 18 h for both VSV and SB. Cell number and viability at the

time of infection were monitored by a hemacytometer count and exclusion of erythrocin B.

Isolation of SB mutants capable of growth in AMD-treated cells. A stock of SB was mutagenized by incubation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (250 μ g/ml in phosphate-buffered saline) for 1 h at 37°C, conditions which reduced the infectious titer by 95%. After treatment, the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was removed by centrifuging the mutagenized stock through a 5% sucrose cushion (wt/wt in 0.05 M Tris [pH 7.2]–0.1 M NaCl–0.001 M EDTA) for 2 h at 27,000 rpm in an AH627 rotor (Sorvall). The virus pellet was suspended in MEM and used to infect BHK cells which had been treated with 2 μ g of AMD per ml for 18 h before infection. After four passages in AMD-treated cells, several isolates were obtained by plaque purification.

RESULTS

Effect of AMD on SB replication. Addition of AMD at or shortly before infection of cells with SB has little or no effect on viral synthetic processes while suppressing host transcription. However, Pfefferkorn and Burge (19) found that longer treatment of chicken embryo cells with AMD resulted in the loss of their ability to support SB growth. Treatment of BHK cells with AMD for increasing periods of time before infection inhibited the replication of SB; inhibition was greater than 90% in cells which had been incubated in the presence of 2 μ g of AMD per ml for 18 h (data not shown). This effect was more apparent in VSW cells, a viper cell line, where SB growth was reduced by almost 2 orders of magnitude (Fig. 1). Two alternative explanations for this result come readily to mind: (i) that inhibition of cellular transcription by AMD leads to the loss of a specific host function required for SB replication or (ii) that the reduction in SB yield simply reflects a progressive loss of cell viability in the presence of AMD. With respect to the latter possibility, we found that cell viability, as measured by dye exclusion, was not affected significantly even after 24 h. Clearly, however, prolonged treatment with AMD could produce profound effects, short of death, on cellular metabolic and structural integrity. To control for these types of effects, we examined the replication of VSV, a negative-strand, RNA-containing, enveloped virus, in AMD-treated cells. Although other cell-specific effects on the replication of VSV have been documented previously (9, 20, 28), we found that the growth of VSV was not affected by AMD pretreatment in VSW cells (Fig. 1) or in BHK cells (data not shown). Therefore, the effect of AMD treatment upon SB replication was not secondary to a generalized disruption of cellular synthetic activities or structural elements which also would have been required for the replication of VSV. Rather, this result was

FIG. 1. Effect of AMD on virus replication in VSW cells. Cultures of VSW cells were treated with 2 μ g of AMD per ml for 2, 6, 12, or 18 h before infection with either VSV or SB. The culture fluids were sampled 18 h postinfection to determine virus yield. Virus growth in AMD-treated cells was compared with virus yields from control cells which received no AMD. Symbols: SB-infected, untreated control (□); SB infected, AMD treated (●); VSV-infected, untreated control (△); VSV infected, AMD treated (○).

consistent with a requirement for a specific host function in SB replication.

Effect of α -A on SB replication. AMD causes a general inhibition of transcription from DNA templates by intercalating into DNA at guanine-cytosine base pairs (21). If the reduction in SB yield from AMD-treated cells resulted from inhibition of mRNA transcription, then SB also should be sensitive to other, more specific inhibitors of cellular mRNA synthesis. One such inhibitor is α -A, a fungal toxin which specifically inhibits transcription of mRNA in eucaryotic cells by selectively binding to the β -subunit of RNA polymerase II (31). As in cells treated for various intervals before infection with AMD, treatment of BHK cells with α -A before infection suppressed SB growth by 2 orders of magnitude relative to the effect of the drug on VSV replication (data not shown).

To rule out the possibility that α -A was acting directly to inhibit some aspect of SB replication, or that some secondary effect of the drug was responsible, we utilized a CHO cell mutant (CHOama-1) which is permeable to α -A but contains an altered RNA polymerase II that neither binds α -A nor is inhibited by the toxin in intact cells or in vitro (13). This allowed us to examine SB growth under conditions where α -A was present but host transcription was normal. Figure 2 shows the results of an experiment in which CHO and CHOama-1 cells were treated with the toxin for increasing times before infection with SB. Compared with the virus yield from cultures of each cell type in the absence of α -A, it is evident that SB replication was sensitive to α -A only in the α -A-sensitive cell line and was resistant to α -A in the line characterized by an α -A-resistant RNA polymerase II. These data strongly suggest that α -A had no direct effect on

FIG. 2. Effect of α -A on SB replication in CHO and CHOama-1 cells. Cultures of CHO or CHOama-1 cells were treated with 5 μ g of α -A per ml for 12, 24, or 36 h before infection with SB. Virus yields were normalized to cell number at the time of infection. Symbols: CHO, untreated (○); CHO, α -A treated (●); CHOama-1, untreated (□); CHOama-1, α -A treated (■).

FIG. 3. Replication of SB and SB^{amr} 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 or SB^{amr} as described in the text. Symbols: SB infected (●); SB^{amr} infected (○).

SB synthetic processes and that synthesis of cellular mRNA (and presumably protein) was required for SB replication.

Isolation of AMD-resistant mutants. The interaction of viral products with particular host factors or the utilization of host functions in viral synthetic processes is dependent not only upon the availability of such host constituents but also upon the nature of the viral product involved in the interaction. Reasoning that the inhibition of SB replication observed in AMD-treated cells resulted from the depletion of a required host factor, we explored the possibility of obtaining viral mutations which could compensate for the reduced concentration or lack of the putative factor and allow viral replication in AMD-treated cells. An enrichment for such mutants was accomplished by passage of a mutagenized SB stock on BHK cells which had been treated with AMD for 18 h before infection. An AMD-resistant mutant, SB^{amr}, was isolated by plaque purification from the fourth passage in AMD-treated cells. This mutant was much less sensitive to AMD than was wild-type SB in both BHK (Fig. 3) and VSW cells (data not shown).

Treatment with either AMD or α -A reduced the ability of BHK, VSW, and CHO cells to support the replication of SB, even though the mechanisms by which these compounds inhibit cellular RNA synthesis are fundamentally different. It was conceivable, therefore, that the inhi-

bition of SB replication observed in AMD-treated cells was unrelated to the inhibition mediated by α -A. However, the data presented in Fig. 4 demonstrate that SB^{amr}, a mutant selected for its ability to replicate in AMD-treated cells, was also capable of replication in cells treated with α -A. These results indicate that both AMD and α -A acted to cause the depletion of the same required host factor by inhibiting the synthesis of its mRNA, thus rendering treated cells incompetent with respect to the same aspect of SB replication.

DISCUSSION

The results presented in this report strongly suggest that lytic replication of SB is dependent upon transcription of host mRNA and presumably the synthesis of a particular host protein. Because this conclusion is based upon the action of chemical inhibitors, alternative interpretations must be considered. For instance, secondary effects of the drugs, unrelated to transcription, could have caused the inability of treated cells to support SB replication; the drugs could have acted directly upon some viral synthetic activity; or the effect on SB growth could have been due to a loss of cellular metabolic and/or structural elements which would have been required for virus replication in general. The results obtained did not support these alternative explanations. If the inhibition of SB replication

FIG. 4. Replication of SB and SB^{amr} in α -A-treated CHO cells. Cultures of CHO cells were treated with 5 μ g of α -A per ml before infection with SB or SB^{amr}. Culture fluids were harvested at 18 h postinfection. Symbols: SB infected (●); SB^{amr} infected (○).

by AMD were due to some secondary effect of the drug rather than to its effect on transcription, we would not have expected α -A, a much more specific inhibitor of cellular mRNA synthesis, to exert a similar effect. That the two compounds affected the same aspect of SB growth is supported by the finding that enrichment for a viral mutant that replicated in AMD-treated cells simultaneously selected for the ability to replicate in cells treated with α -A. Another alternative, that cellular metabolic and/or structural integrity or both may have been compromised by the drug treatment, cannot explain the continued replication of VSV under conditions which depressed SB growth by 2 orders of magnitude. Also, if the effect on SB resulted from a generalized loss of energy metabolism, membrane structure, or ribosome function, it would seem unusual to be able to isolate viral mutants such as SB^{amr} which could replicate in such debilitated cells.

The most compelling evidence that SB replication requires the synthesis of host mRNA, and by inference, the synthesis of a host protein(s), is the finding that α -A inhibited SB growth in wild-type CHO cells but had no effect in CHOama-1, a mutant cell line containing an α -A-resistant RNA polymerase II. This result rules out the possibilities that (i) secondary toxic effects on the cell or (ii) direct effects of the toxin on viral synthetic functions were responsible for the inhibition. We feel that these data strongly suggest the participation of a specific host factor(s) in SB replication.

The normal cellular role of the host factor is unknown. However, it or a closely related factor must have been conserved through evolution in all of the phylogenetically diverse cell types in which SB growth can occur. Given that selection of SB^{amr} in BHK cells would have produced a mutant which could interact efficiently with the BHK cell factor, the observation that SB^{amr} was also less sensitive to AMD than the wild type in VSW cells indicates that the BHK and VSW cell factors are similar. However, growth of SB^{amr} in VSW cells remained somewhat sensitive to AMD, suggesting that the mutation did not abolish the host requirement entirely.

The precise point in the replication of SB at which a host factor may function has yet to be determined, although several observations suggest the possibility of a host involvement in viral RNA synthesis. Kowal and Stollar (14) have isolated two host-dependent, temperature-sensitive mutants of SB which failed to complement *ts6*, an RNA⁻ mutant isolated by Burge and Pfefferkorn (4) and assigned to the F complementation group by Strauss et al. (26). The product of the F cistron is important in the synthesis of both positive- and negative-strand

RNA (24), and the host dependency of these newly isolated mutants may reflect a cell influence upon F cistron function. A host polypeptide is associated with partially purified preparations of the Semliki Forest virus polymerase (5), although a requirement for this protein for polymerase activity has not been demonstrated. We presently are approaching this question by investigating SB and SB^{amr} replication in the presence of inhibitors of host transcription. In cells treated with AMD before infection, synthesis of both 42 and 26S positive strand was inhibited coordinately, and the levels of all three RFs were reduced (unpublished observations); the synthesis of SB^{amr} RNA continued at high levels even after prolonged treatment with the drug. Therefore, the results presented here strongly indicate a direct host involvement in SB growth and, considered with the work of others and our preliminary data, suggest that this involvement is at the level of viral RNA synthesis.

ACKNOWLEDGMENTS

This research was supported by the North Carolina Agricultural Research Service, project 03554, and by Public Health Service grant AI 15196 from the National Institute of Allergy and Infectious Diseases. R.S.B. was the recipient of an Agricultural Foundation Pre-Doctoral Assistantship.

We wish to thank C. J. Ingles for the gift of the CHOama-1 cell line.

LITERATURE CITED

1. Black, D. N., and F. Brown. 1969. Effect of actinomycin D and guanidine on the formation of a ribonucleic acid polymerase induced by foot-and-mouth disease virus and on the replication of virus and viral ribonucleic acid. *Biochem. J.* 112:317-323.
2. Blumenthal, T., and G. G. Carmichael. 1979. RNA replication: function and structure of Q β replicase. *Annu. Rev. Biochem.* 48:525-548.
3. Bouloy, M., S. J. Plotch, and R. M. Krug. 1978. Globin mRNAs are primers for the transcription of influenza viral RNA *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 75:4886-4890.
4. Burge, B. W., and E. R. Pfefferkorn. 1966. Isolation and characterization of conditional lethal mutants of Sindbis virus. *Virology* 30:204-213.
5. Clewley, J. P., and S. I. T. Kennedy. 1976. Purification and polypeptide composition of Semliki Forest virus RNA polymerase. *J. Gen. Virol.* 32:395-411.
6. Dasgupta, A., P. Zabel, and D. Baltimore. 1980. Dependence of the activity of the poliovirus replicase on a host cell protein. *Cell* 19:423-429.
7. Davey, M. W., and L. Dalgarno. 1974. Semliki Forest virus replication in cultured *Aedes albopictus* cells: studies on the establishment of persistence. *J. Gen. Virol.* 24:453-463.
8. Dmitrieva, T. M., M. V. Scheglova, and V. I. Agol. 1979. Inhibition of activity of encephalomyocarditis virus-induced RNA polymerase by antibodies against cellular components. *Virology* 92:271-277.
9. Hamilton, D. H., R. W. Moyer, and S. A. Moyer. 1980. Characterization of the non-permissive infection of rabbit cornea cells by vesicular stomatitis virus. *J. Gen. Virol.* 49:273-287.
10. Herz, C., E. Stavnezer, and R. M. Krug. 1981. Influenza virus, an RNA virus synthesizes its messenger RNA in the nucleus of infected cells. *Cell* 26:391-400.

11. Igarashi, A. 1978. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to Dengue and Chikungunya viruses. *J. Gen. Virol.* 40:531-544.
12. Igarashi, A., R. Koo, and V. Stollar. 1977. Evolution and properties of *Aedes albopictus* cell cultures persistently infected with Sindbis virus. *Virology* 82:69-83.
13. Ingles, C. J., A. Guialis, J. Lam, and L. Siminovitch. 1976. α -Amanitin resistance of RNA polymerase II in mutant Chinese hamster ovary cell lines. *J. Biol. Chem.* 251:2729-2734.
14. Kowal, K. J., and V. Stollar. 1981. Temperature-sensitive host-dependent mutants of Sindbis virus. *Virology* 114:140-148.
15. Lamb, R. A., and C.-J. Lai. 1980. Sequence of interrupted and uninterrupted mRNAs and cloned full length DNA coding for the two overlapping non-structural proteins of influenza virus. *Cell* 21:475-485.
16. Lamb, R. A., C.-J. Lai, and P. W. Choppin. 1981. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. *Proc. Natl. Acad. Sci. U.S.A.* 78:4170-4174.
17. Mento, S. J., and L. Siminovitch. 1981. Isolation and preliminary characterization of Sindbis virus-resistant Chinese hamster ovary cells. *Virology* 111:320-330.
18. Peleg, J. 1969. Inapparent persistent virus infection in continuously grown *Aedes aegypti* mosquito cells. *J. Gen. Virol.* 5:463-471.
19. Pfefferkorn, E. R., and B. W. Burge. 1967. Genetics and biochemistry of arbovirus temperature-sensitive mutants, p. 403-426. *In* J. S. Colter and W. Paranchych (ed.), *The molecular biology of viruses*. Academic Press, Inc., New York.
20. Pringle, C. R. 1978. The tdCE and hrCE phenotypes: host range mutants of vesicular stomatitis virus in which polymerase function is affected. *Cell* 15:597-606.
21. Reich, E., and I. H. Goldberg. 1964. Actinomycin and nucleic acid function. *Prog. Nucleic Acid Res. Mol. Biol.* 3:183-234.
22. Riedel, B., and D. T. Brown. 1977. Role of extracellular virus in the maintenance of the persistent infection induced in *Aedes albopictus* (mosquito) cells by Sindbis virus. *J. Virol.* 23:554-561.
23. Sarver, N., and V. Stollar. 1977. Sindbis virus-induced cytopathic effect in clones of *Aedes albopictus* (Singh) cells. *Virology* 80:390-400.
24. Sawicki, D. L., S. G. Sawicki, S. Keränen, and L. Kääriäinen. 1981. Specific Sindbis virus-coded function for minus-strand RNA synthesis. *J. Virol.* 39:348-358.
25. Scheefers-Borchel, U., H. Scheefers, J. Edwards, and D. T. Brown. 1981. Sindbis virus maturation in cultured mosquito cells is sensitive to actinomycin D. *Virology* 110:292-301.
26. Strauss, E. G., E. M. Lenches, and J. H. Strauss. 1976. Mutants of Sindbis virus. I. Isolation and partial characterization of 89 new temperature-sensitive mutants. *Virology* 74:154-168.
27. Taylor, R. M., H. S. Hurlbut, T. S. Work, J. R. Kingston, and T. E. Frothingham. 1955. Sindbis virus: a newly recognized arthropod-transmitted virus. *Am. J. Trop. Med. Hyg.* 4:844-862.
28. Thacore, H. R., and J. S. Younger. 1975. Abortive infection of a rabbit cornea cell line by vesicular stomatitis virus: conversion to productive infection by superinfection with vaccinia virus. *J. Virol.* 16:322-329.
29. Tooker, P., and S. I. T. Kennedy. 1981. Semliki Forest virus multiplication in clones of *Aedes albopictus* cells. *J. Virol.* 37:589-600.
30. Ulmanen, I., H. Söderlund, and L. Kääriäinen. 1976. Semliki Forest virus capsid protein associates with the 60S ribosomal subunit in infected cells. *J. Virol.* 20:203-210.
31. Wieland, T., and H. Faulstich. 1978. Amatoxins, phallotoxins, phallolysine and antamanid: the biologically active components of poisonous *Amanita* mushrooms. *Crit. Rev. Biochem.* 5:185-260.
32. Zeigel, R. F., and H. F. Clark. 1969. Electron microscopic observations on a "C"-type virus in cell cultures derived from a tumor-bearing viper. *J. Natl. Cancer Inst.* 43:1097-1102.