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In Vivo Transcription and Protein Synthesis Capabilities of Bunyaviruses: Wild-Type Snowshoe Hare Virus and Its Temperature-Sensitive Group I, Group II, and Group I/II Mutants

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The in vivo primary and secondary transcription capabilities of wild-type snowshoe hare (SSH) virus and certain of its temperature-sensitive (*ts*) mutants have been analyzed. The results obtained agree with in vitro studies (Bouloy et al., C.R. Acad. Sci. Paris **280**:213-215, 1975; M. Bouloy and C. Hannoun, Virology **69**:258-264, 1976; M. Ranki and R. Pettersson, J. Virol. **16**:1420-1425, 1975) which have shown that bunyaviruses are negative-stranded RNA viruses with a virion RNA-directed RNA polymerase. The in vivo transcription studies have demonstrated that in the presence of protein synthesis inhibitors (puromycin or cycloheximide) SSH virus can synthesize viral complementary RNA (primary transcription) throughout the infection cycle. The increased levels of viral complementary RNA obtained in the absence of protein synthesis inhibitors (secondary transcription) were not markedly reduced if cells were pretreated with actinomycin D (5 μg/ml), α-amanitin (25 μg/ml), or rifampin (100 μg/ml), although progeny virus yields were reduced by up to 80% in the actinomycin D- and rifampin-treated cells. The in vivo transcription capabilities of SSH group I *ts* mutants at temperatures which were nonpermissive (40°C) for virus replication gave values comparable to those obtained at permissive temperatures (33°C). The SSH group I mutants appear, therefore, to be RNA-positive mutant types. When compared with their transcription capabilities at 33°C, the in vivo transcription abilities of four SSH group II *ts* mutants (and one double group I/II *ts* mutant) were found to be more impaired at 40°C than those of the SSH group I *ts* mutants or wild-type SSH virus at 40°C, although the viral complementary RNA synthetic capabilities of these group II (and group I/II) mutants at 40°C were significantly higher than their primary transcription capabilities (as measured at 33°C in the presence of puromycin or cycloheximide). It was concluded, therefore, that these SSH group II (and double group I/II) *ts* mutants have an intermediate RNA phenotype. Hybridization studies using ^{32}P-labeled individual L, M, and S viral RNA species of SSH virus have demonstrated the presence of viral complementary RNA to all three species in extracts of cells infected with SSH *ts* II-30 and incubated at 33°C (primary and secondary transcription) or 40°C, a nonpermissive temperature for its replication. The results of pulse-labeled in vivo protein analyses indicated that greater quantities of intracellular N protein (coded for by S RNA [J. R. Gentsch and D. H. L. Bishop, J. Virol. **28**:417-419, 1978]) than G1 and G2 polypeptides (coded for by M RNA [J. R. Gentsch and D. H. L. Bishop, J. Virol. **30**:767-776, 1979]) were present in extracts of cells infected with wild-type SSH virus. In extracts of SSH group I, II, or I/II *ts* mutant-infected cells incubated at 33°C, N and G1, and for the group II mutant-infected cells, G2, viral polypeptides were detected, whereas in extracts obtained from group I or II mutant virus-infected cells incubated at 40°C, low levels of N and G1 polypeptides were evident.

Analyses of Lumbo virus, a member of the California encephalitis serogroup of bunyaviruses, and Uukuniemi virus, a possible member of the Bunyaviridae family, have established that these RNA viruses have virion RNA polymerases (2, 4, 22). It has been shown that their

virion polymerases can catalyze the synthesis of viral complementary RNA (vcRNA) in vitro. It has also been demonstrated that Lumbo virus mRNA isolated from cellular polysomes is complementary to its RNA genome (5), suggesting that one function of the virion polymerase is to direct the synthesis of mRNA.

For other negative-stranded RNA viruses, such as the Indiana serotype of the rhabdovirus vesicular stomatitis virus, the virion polymerase has been shown to be capable of directing the in vivo synthesis of vcRNA in cells in which protein synthesis has been inhibited (7, 8, 15, 17, 23). The fact that the viral polymerase, and not a cellular polymerase, is responsible for the in vivo transcription process has been shown by studies involving temperature-sensitive (*ts*) mutants of vesicular stomatitis virus which have thermolabile transcriptase components (23).

In this study we demonstrated the ability of the bunyavirus snowshoe hare (SSH) virus to direct the intracellular synthesis of vcRNA in the presence or the absence of protein synthesis inhibitors (e.g., puromycin or cycloheximide) as well as other antibiotics. The amounts of intracellular L, M, and S vcRNA species and pulse-labeled viral polypeptides were analyzed as a function of the infection time course. In addition, the RNA and protein phenotypes of selected *ts* mutants of SSH virus (group I, group II, and a double group I/II mutant) were analyzed.

MATERIALS AND METHODS

Materials. Actinomycin D was purchased from GIBCO Laboratories, Grand Island, N.Y. Puromycin, cycloheximide, and α-amanitin came from Sigma Chemical Co., St. Louis, Mo. Radioisotopes were purchased from ICN, Irvine, Calif. Rifampin came from Calbiochem, La Jolla, Calif.

Viruses. Wild-type prototype SSH virus and the following *ts* mutants of that virus were used: *ts* I-1, *ts* I-3, *ts* II-18, *ts* II-26, *ts* II-29, *ts* II-30, and the double mutant *ts* I/II-31 (9, 13). For each virus mutant, the efficiency of plating (PFU at 39.8°C ÷ PFU at 33°C) was less than 0.001, and the efficiency of growth (progeny PFU after growth at 39.8°C for 24 h ÷ progeny PFU after growth at 33°C for 36 h) was less than 0.002. Virus PFU were assayed on BHK-21 cell monolayers as described previously (9, 13).

Virus growth, purification, infection of cells, and measurement of intracellular vcRNA synthesis. To determine the intracellular synthesis of vcRNA induced by wild-type or *ts* mutant SSH viruses, a hybridization procedure was employed which not only measured the intracellular accumulation of virus-specified RNA, but also accounted for the RNA derived from the inoculum viruses and used these values to normalize raw hybridization data into values which took into consideration the variation (between different samples) in amounts of virus absorbed and losses incurred in processing cell extracts (7, 8, 23). The important features of the process were to use high-specific-activity virus labeled by both purine and pyrimidine precursors, depending on the available isotopes, establish high-multiplicity infections with that virus, and use the inoculum cell-associated label, as well as additional viral RNA, to measure the intracellular vcRNA. The raw data were then normalized to a constant recovery of label from infected cells (7, 8, 23). The procedures used to obtain SSH virus labeled with [^{3}H]uridine and [^{3}H]adenosine (wild-type SSH, *ts* I-1, *ts* I-3, *ts* II-18, *ts* II-26, and *ts* II-29, 20 μCi of each precursor per ml), [^{3}H]uridine and [^{3}H]guanosine (*ts* II-30, 20 μCi of each precursor per ml), or [^{3}H]-guanosine (*ts* I/II-31, 20 μCi/ml) involved infecting BHK-21 cells with virus (input multiplicity of infection [MOI] of 0.01) and, after incubation at 33°C for 2 days, harvesting the supernatant fluids and purifying the virus by polyethylene glycol-NaCl precipitation, followed by brief centrifugation in a gradient of sucrose and then agarose column chromatography in Eagle minimum essential medium, as described previously (12, 18–20, 23). The inoculum viruses obtained after chromatography were titrated at 33 and 39.8°C, and 0.1-ml samples of labeled virus (10^4 to 10^5 cpm; MOI, 30 to 1,100 [see below]) were used to infect 25-cm^2 small T flasks containing 3×10^6 confluent BHK-21 cells (7, 8, 23). On occasion, cells were pretreated with cycloheximide (100 μg/ml), puromycin (100 μg/ml), actinomycin D (5 μg/ml), rifampin (100 μg/ml), or α-amanitin (25 μg/ml) 1 h before adsorption and during the adsorption and infection time course. After an absorption period of 30 min at 4°C, the inocula were removed, 5 ml of prewarmed Eagle medium containing 5% (vol/vol) newborn calf serum was added, and the monolayers were incubated either in an incubator at 33°C or in a circulating water bath at 40°C (located in a 37°C room). At 1 h postinfection, the supernatant fluids (containing desorbed virus) were replaced with fresh prewarmed media, and the incubations were continued (7, 8, 23). At the indicated times, the supernatant fluids were recovered and titrated at 33 and 39.8°C, the monolayers were washed with 0.15 M NaCl–0.01 M Tris buffer (pH 7.4), and the cells were recovered and extracted for nucleic acids as described previously (7, 8, 23). RNA was extracted from the recovered inocula as well as from the unused virus. The specific activities of viral RNA were usually of the order of 10^5 to 10^6 cpm per μg of RNA.

The amounts of ^{3}H-labeled viral RNA in cell extracts and their content of vcRNA were determined as described previously (7, 8, 23). In brief, duplicate 1/10 to 1/100 samples of the cell RNA were melted and annealed to completion with 3,000 to 6,000 cpm of ^{3}H-labeled added viral RNA, and, postannealing, the [^{3}H]RNA that was rendered RNase A resistant was determined. The net increases, corrected for RNase-resistant core values of viral RNA annealed to uninfected cell extracts (usually 2 to 4% of the added [^{3}H] RNA) and normalized to a consistent recovery of cell-associated ^{3}H-labeled viral RNA (usually 5,000 cpm) were used to estimate the content of vcRNA present in the total cell nucleic acid extracts. Since the least abundant [^{3}H]RNA species in a virus extract was the S RNA, usually representing 15 to 25% of the single-stranded RNA mass (6, 12, 19), annealing mixtures were designed to produce no more than a 20% net increase in RNase resistance. In most cases the net

increases used to calculate the vcRNA content were between 5 and 12% of the input radioactivity.

The isolation and recovery of individual ^{32}P-labeled L, M, and S RNA species have been described previously (6).

Pulse-labeling of infected or uninfected cells by [^{3}H]leucine. The intracellular polypeptides of infected or uninfected BHK-21 cells were labeled for 1-h periods at various times by changing the infected cell supernatant fluids for Eagle medium containing 1/10 the normal concentration of leucine and added [^{3}H]leucine (final concentration 100 μCi/ml). After labeling, the cells were washed, suspended in 0.2 ml of 0.0625 M Tris-hydrochloride buffer (pH 6.8) containing 5% sodium dodecyl sulfate, 1% β-mercaptoethanol, 10% glycerol, and 0.005% bromophenol blue, and heated at 100°C for 2 min, and approximately 10^5 cpm of acid-insoluble radioactivity were resolved by electrophoresis in a discontinuous 10% polyacrylamide gel and fluorographed as described elsewhere (1).

RESULTS

Synthesis of vcRNA by wild-type SSH virus. The ability of wild-type SSH virus to direct the synthesis of vcRNA in BHK-21 cells was analyzed as a function of the release of progeny virus, the temperature of incubation, and the effect of protein synthesis inhibitors puromycin and cycloheximide. As measured by the difference in virus released from drug-treated and untreated cells (Fig. 1 and 2), progeny virus was detected by 2 to 3 h postinfection and was found to accumulate throughout the next 6 h of the infection time course, as others have shown (14, 16). By comparison with infected cells incubated at 33°C, somewhat lower levels of progeny virus and, at later time points, vcRNA were obtained from infected cells incubated at 40°C, indicating that at 40°C wild-type SSH virus may be near its limit of productively infecting BHK-21 cells. vcRNA was detected in both the puromycin (33 and 40°C)- and the cycloheximide (33°C)-treated and untreated cells (Fig. 1 and 2).

The initial synthesis of vcRNA by negative-stranded viruses in infected cells is termed primary transcription and is believed to involve the input virion transcriptase, although whether host cell enzymes or factors promote the process is not known. The SSH vcRNA syntheses obtained in the presence of puromycin or cycloheximide are compatible with a primary transcription process directed by a virion polymerase. The increased levels of SSH vcRNA syntheses obtained in the absence of puromycin (or cycloheximide) indicated that virus-induced proteins were involved in the amplified levels of RNA

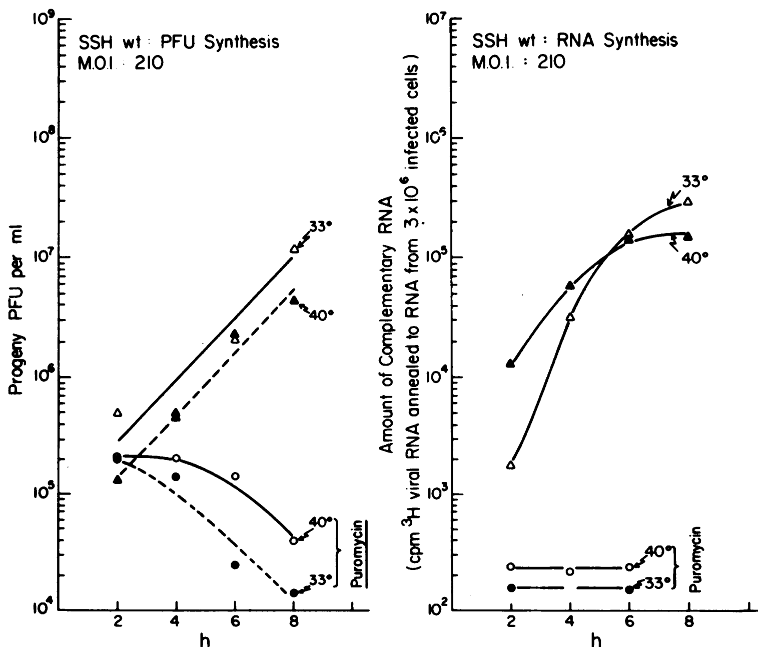

FIG. 1. *Synthesis of vcRNA and release of progeny virus from BHK-21 cells infected with wild-type (wt) SSH virus. Partially purified ^{3}H-labeled wild-type SSH virus (MOI = 210) was used to infect 3 × 10^6 confluent BHK-21 cells; after a 30-min absorption at 4°C, the inoculum was removed, the infected cells were incubated at 33 or 40°C in the presence or absence of 100 µg of puromycin per ml, and the release of progeny virus and intracellular accumulation of vcRNA were determined as a function of the infection time course (as described in the text and references 7, 8, and 23).*

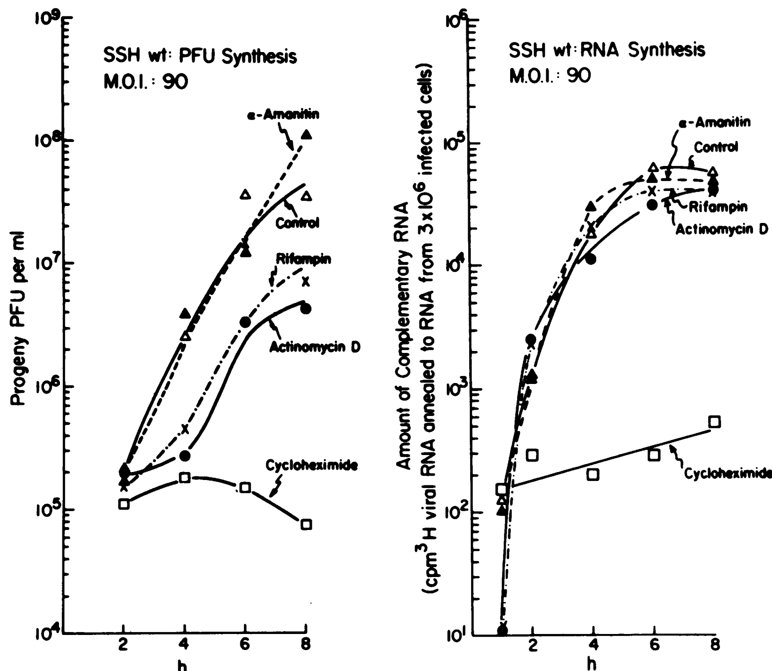

FIG. 2. *Effect of cycloheximide, α-amanitin, rifampin, and actinomycin D upon the release of SSH virus and intracellular accumulation of vcRNA. Confluent BHK-21 cells were infected with ^3H-labeled wild-type (wt) SSH virus (MOI = 90), and the release of progeny virus and accumulation of intracellular vcRNA were determined as a function of the infection time course as described previously (7, 8, 23). In addition to untreated cells, monolayers were pretreated and incubated in the presence of either cycloheximide (100 μg/ml), actinomycin D (5 μg/ml), rifampin (100 μg/ml), or α-amanitin (25 μg/ml).*

transcription obtained in productively infected cells (Fig. 1 and 2). For cells initially infected at MOI values of 90 to 210, these increased levels (secondary transcription) gave at least 50 times more SSH vcRNA by 8 h postinfection (Fig. 1 and 2).

To determine whether the secondary transcription rates of SSH vcRNA synthesis were virus-coded functions or host cell-induced functions, the effects of two drugs known to inhibit host cell DNA-directed RNA synthesis (actinomycin D and α-amanitin) upon the SSH vcRNA synthetic rates were investigated. Under conditions in which actinomycin D pretreatment reduced uninfected host cell RNA synthesis by 90%, as measured by the reduced accumulation of [^{3}H]uridine into acid-insoluble product during a 1-h pulse ([^{3}H]uridine used at 10 μCi/ml), and totally blocked influenza WSN virus replication in chicken embryo fibroblasts (data not shown), the 4- to 8-h accumulation of SSH vcRNA was inhibited no more than 40%, although progeny virus release was curtailed by 90% (Fig. 2). By comparison, the effect of α-amanitin treatment on either progeny virus release or SSH vcRNA synthesis at a concentration which blocked influenza WSN virus replication in chicken embryo cells by 95% (data not shown) was minor (Fig. 2). For both drugs, the levels of vcRNA synthesis and progeny virus release were substantially above the levels obtained when an inhibitor of protein synthesis (cycloheximide) was employed (Fig. 2). Therefore, the results indicated that transcription of SSH vcRNA was predominantly related to virus-coded functions. For the actinomycin D-treated cells, the reduction in the release of infectious virus particles and reduced vcRNA synthesis (by comparison with untreated cells) may mean that the drug directly or indirectly inhibits viral (or host-related) functions which affect the efficiency of virus replication.

When the effect of rifampin upon SSH vcRNA synthesis and progeny virus release was analyzed, little inhibition of vcRNA synthesis was observed, even though for most of the time points there was a significant (up to 80%) reduction in progeny virus release (Fig. 2).

RNA phenotype of selected SSH *ts* mutants. The group I *ts* mutants of SSH virus have been shown from heterologous recombination analyses to have defects in their M RNA species, whereas the SSH group II mutants have defects in their L RNA species (13). Polypeptide analy-

ses correlated to RNA genotype analyses of wild-type and recombinant viruses derived from certain SSH and La Crosse *ts* mutant coinfections have established that the SSH (or La Crosse) viral S RNA codes for the nucleocapsid protein N (10, 12), whereas the viral M RNA codes for the two viral glycoproteins, G1 and G2 (11). Whether the viral L RNA codes for the L polypeptide or other proteins or both remains to be determined. Since the apparent sum molecular weight of SSH (or La Crosse) G1 and G2 proteins (1.6×10^5) is almost equivalent to the coding capacity of their respective viral M RNA species (M RNA molecular weight, 1.9×10^6 [6, 11, 12, 18, 19]), it is likely that these two proteins are the only polypeptides coded for by M RNA. On this basis, it is probable that SSH group I *ts* mutants at nonpermissive temperatures produce defective glycoprotein.

The RNA phenotypes of selected SSH group I and group II *ts* mutants were investigated at both permissive (33°C) and nonpermissive (40°C) temperatures for *ts* mutant virus growth. When the progeny virus from SSH *ts* I-3-infected cells (MOI = 30) were assayed at 33°C, it was found that more virus was released from the cells incubated at 33°C at 4 to 8 h postinfection than from cells incubated at 33°C in the presence of cycloheximide or from cells incubated at 40°C in the absence of the drug (Fig. 3). Assays at 40°C of the progeny virus from all three infections indicated that, as expected, they were temperature sensitive (data not shown). The lower yields of virus from the 33°C incubations at 6 to 8 h postinfection, when compared with those obtained for the wild-type virus infections (Fig. 1 and 2), probably reflect the lower MOI used with the *ts* I-3 mutant (MOI = 30). When compared with the primary transcription levels of vcRNA obtained in SSH *ts* I-3-infected cells incubated at 33°C in the presence of cycloheximide, it was found that from both the mutant virus-infected cells incubated at 40°C and those incubated at 33°C, substantially higher levels of vcRNA were obtained (Fig. 3). The levels of vcRNA synthesis obtained at 40°C represented 95 to 60% those obtained at 33°C, depending on the time point (Fig. 3). Results similar to those described above have also been obtained with SSH *ts* I-1.

When the vcRNA synthesis induced by wild-type SSH virus at 33°C was compared with that obtained at 40°C, it was found that there was slightly less vcRNA produced at the higher temperature than at 33°C (Fig. 1). It was therefore concluded from the SSH *ts* I-3 analyses that the RNA phenotype of this mutant could be categorized as positive.

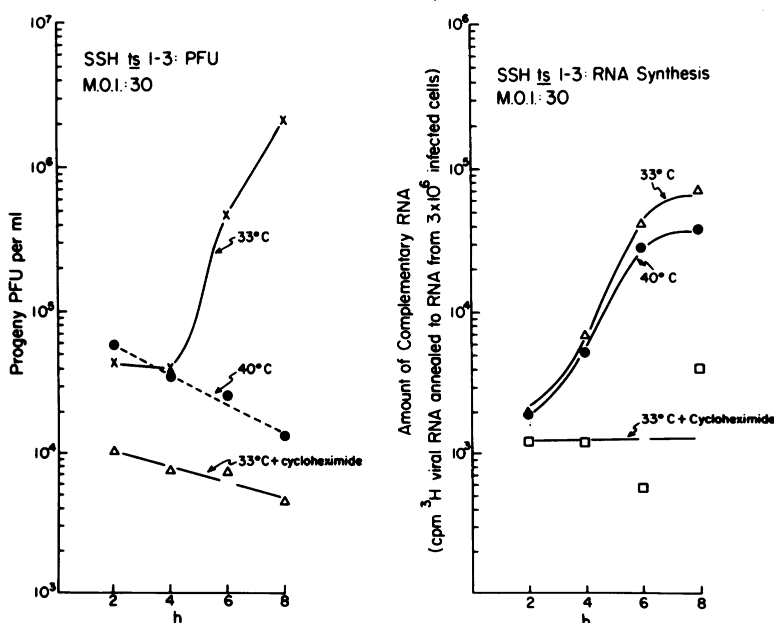

FIG. 3. *Release of progeny virus and intracellular accumulation of vcRNA from BHK-21 cells infected with SSH ts I-3. Partially purified ^{3}H-labeled SSH ts I-3 (MOI = 30) was used to infect BHK-21 cells (in the presence or absence of 100 μg of cycloheximide per ml), and the release of progeny virus (assayed at 33°C) and intracellular accumulation of vcRNA were determined as a function of the temperature of incubation of the infected cells (33 or 40°C), as well as of the infection time course (7, 8, 23). Both the inoculum and progeny viruses, when assayed at 39.8°C, gave PFU titers of less than 0.1% of those obtained at 33°C.*

Analyses similar to those described above were also undertaken with four SSH group II mutants, *ts* II-18, *ts* II-26, *ts* II-29, and *ts* II-30. The results obtained with SSH *ts* II-30 (MOI = 680) are shown in Fig. 4. Little, if any, progeny PFU were detected (other than levels which could be attributed to desorbed virus) from the infected cell monolayers incubated at 40°C (or 33°C in the presence of puromycin). It should be noted parenthetically that the levels of desorbed virus obtained in the *ts* II-30 experiments were greater than those obtained in the SSH *ts* I-3 experiment shown in Fig. 3, presumably because the MOI used for the SSH *ts* II-30 infection was more than 30-fold higher. The levels of progeny virus released from the *ts* II-30 infections incubated at 33°C (Fig. 4) were also more than those obtained from the *ts* I-3 (Fig. 3) or wild-type virus infections (Fig. 1 and 2), presumably also because the *ts* II-30 infections were initiated at a higher MOI. The progeny from the *ts* II-30 infections were shown to be temperature sensitive, as judged from their inability to form plaques at 39.8°C (data not shown).

In support of the conclusion that the higher levels of primary transcription obtained with *ts* II-30, when compared with those obtained with *ts* I-3 (Fig. 3) or wild-type virus (Fig. 2), were probably due to the higher MOI used for *ts* II-30, it has been shown with the negative-stranded rhabdovirus vesicular stomatitis virus, Indiana serotype, that primary transcription rates are multiplicity dependent, at least up to MOI values of 500 to 1,000 (8).

By comparison with the primary transcription rates developed in SSH *ts* II-30-infected cells incubated at 33°C in the presence of puromycin (Fig. 4), substantially higher levels of vcRNA were produced in the mutant virus-infected cells incubated at 33 or 40°C in the absence of the drug. For both the drug-inhibited and untreated *ts* II-30-infected cells incubated at 33°C, the levels of vcRNA detected at all time points examined were higher than the corresponding values obtained with SSH *ts* I-3 (Fig. 3) or wild-type SSH virus (Fig. 1 and 2), presumably also representing the differences in MOI used in the different experiments.

The vcRNA levels realized by SSH *ts* II-30 infections incubated at 40°C represented between 50 and 30% of the amounts obtained at 33°C (Fig. 4). However, since these levels were more than 10-fold higher than those obtained through primary transcription, it was concluded that the phenotype of the mutant could not be described as strictly RNA negative, although it

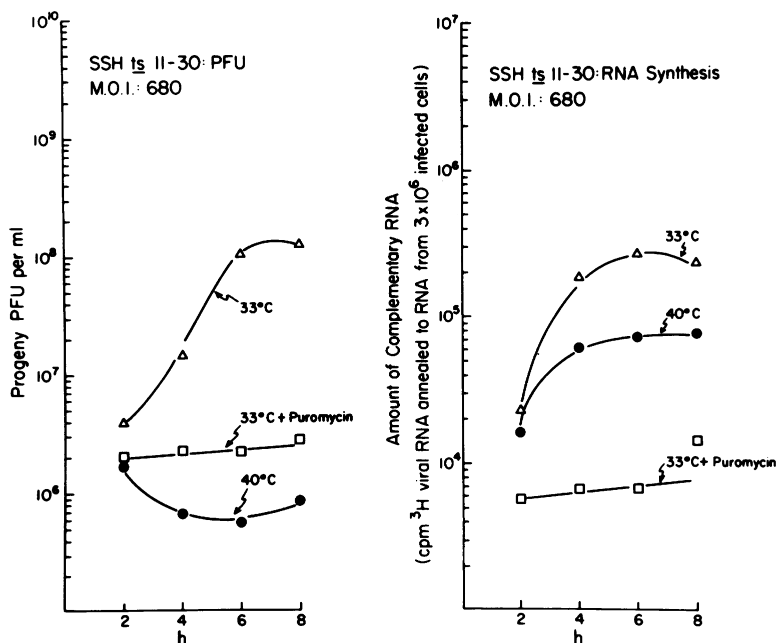

FIG. 4. *Release of progeny virus and intracellular accumulation of vcRNA from BHK-21 cells infected with SSH ts II-30. Partially purified ^3H-labeled SSH ts II-30 (MOI = 680) was used to infect confluent BHK-21 cells (in the presence or absence of 100 μg of puromycin per ml), and the release of progeny virus (assayed at 33°C) and intracellular accumulation of vcRNA were determined as a function of the temperature of incubation (33 or 40°C) and the infection time course (7, 8, 23). Both the inoculum and progeny viruses, when assayed at 39.8°C, gave PFU titers of less than 0.1% of those obtained at 33°C.*

could also not be categorized as completely RNA positive. When similar experiments were undertaken with the three other SSH group II *ts* mutants (*ts* II-18, *ts* II-26, and *ts* II-29), similar results were obtained: all three mutant viruses developed at least 10 times more vcRNA at nonpermissive temperatures than that obtained at 33°C in the presence of a protein synthesis inhibitor.

RNA phenotype of a double group I/II SSH *ts* mutant. In unpublished experiments, a *ts* mutant of SSH (*ts* 31) has been categorized as a double *ts* mutant on the basis of (i) its inability to recombine with selected SSH group I or II *ts* mutants and (ii) the observation that a backcross of SSH *ts* 31 with wild-type SSH virus leads to the high-frequency recovery of both group I and group II *ts* mutants (as well as wild-type virus and *ts* mutants with the genetic characteristics of *ts* 31).

The RNA phenotype of the SSH group I/II *ts* mutant was analyzed at both 33°C (in the presence or absence of puromycin) and 40°C. Figure 5 shows the results for infections initiated at an MOI of 200 or, for the 40°C infections, both 200 and 1,100. Although, as expected, higher levels of desorbed virus were obtained from the 40°C infections initiated at an MOI of 1,100 (by comparison with those initiated at an MOI of 200), the levels of vcRNA synthesized at 40°C were not significantly greater for the higher MOI and, if anything, were somewhat lower at the 6- and 8-h time points. However, for both MOIs, the vcRNA levels obtained at 40°C were substantially higher than those obtained by primary transcription at 33°C (Fig. 5). For this reason, the phenotype of the group I/II mutant, like that of SSH *ts* II mutants, was categorized as intermediate in character.

Amounts of L, M, and S vcRNA species induced in SSH ts II-30 virus-infected cells. The fact that the RNA phenotype of *ts* II-30 was intermediate in character raised the question of whether vcRNA representing all three viral RNA species was present. To obtain an answer to this question, the relative amounts of L, M, and S vcRNA species induced in SSH *ts* II-30 virus-infected cells were analyzed as a function of an infection time course by annealing an excess of the individual ^{32}P-labeled viral RNA species to the RNA recovered from SSH *ts* II-30-infected cells and determining the quantity of ^{32}P that was rendered RNase resistant. Figure 6 shows the amounts of vcRNA species induced in SSH *ts* II-30-infected BHK-21 cells (incubated at 33, 40, or 33°C in the presence of puromycin

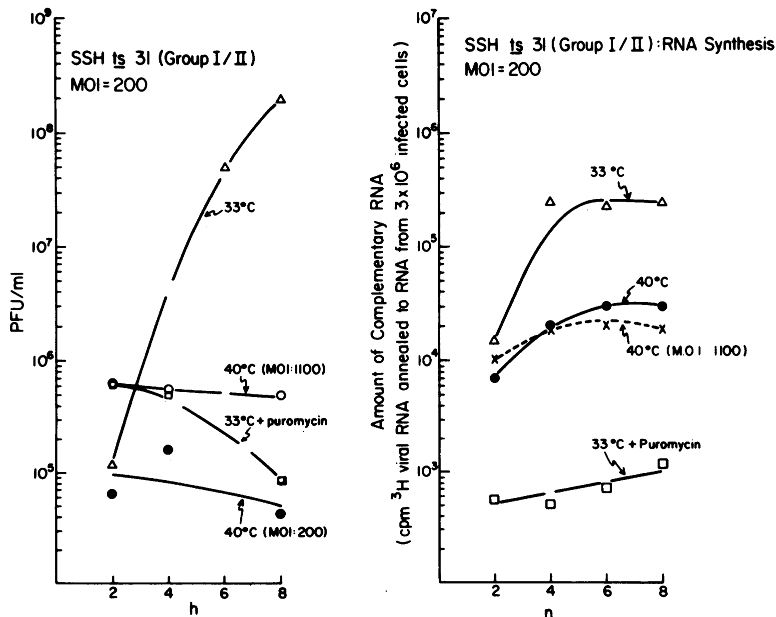

FIG. 5. *Release of progeny virus and intracellular accumulation of vcRNA from BHK-21 cells infected with a double SSH mutant ts I/II-31. Partially purified ^3H-labeled SSH ts I/II-31 (MOI = 200) was used to infect confluent BHK-21 cells (in the presence or absence of 100 μg of puromycin per ml), and the release of progeny virus (assayed at 33°C) and intracellular accumulation of vcRNA were determined as a function of the temperature of incubation (33 or 40°C) and the infection time course (7, 8, 23). An additional set of BHK-21 cell monolayers were infected at a MOI of 1,100 and incubated at 40°C. Both the inoculum and progeny viruses, when assayed at 39.8°C, gave PFU titers of less than 0.1% of those obtained at 33°C.*

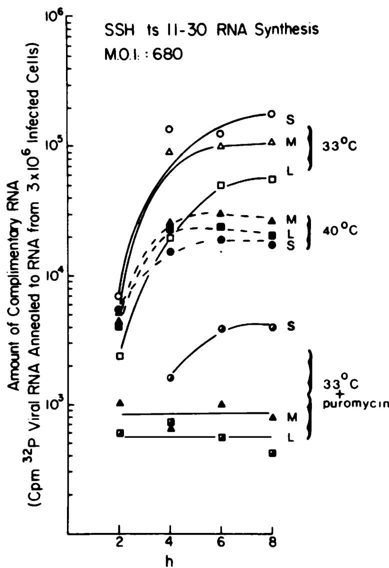

FIG. 6. *Accumulation of SSH L, M, and S vcRNA in BHK-21 cells infected with SSH ts II-30. Individual* ^{32}*P-labeled SSH L, M, and S RNA species were obtained as described previously (6) and used to determine the corresponding vcRNA species in SSH ts II-30-infected cells treated (33° C) or untreated with puromycin (33 and 40° C) as described in the legend to Fig. 4.*

[Fig. 4]). It was observed that vcRNA equivalent to all three viral RNA species was present in the extracts obtained from both the control 33°C (treated or untreated with puromycin) and the nonpermissive 40°C infected cells. The total quantities of vcRNA from the cells incubated at the two temperatures were comparable to the results shown in Fig. 4 with ^{3}H-labeled total viral RNA. On the basis that the sizes of the S and M viral RNA species of SSH virus are one-sixth and two-thirds, respectively, that of the L RNA species (6), it was evident that there were more S than M vcRNA molecules and more M than L vcRNA molecules in the infected cells incubated at 33°C.

Protein synthesis in BHK-21 cells infected with wild-type and mutant SSH viruses at permissive and nonpermissive temperatures. The fact that vcRNA was detected in SSH group I and II *ts* mutant-infected cells incubated at 40°C raises the question of whether under these conditions these mutants also induced the synthesis of virus-specific polypeptides. To investigate this question, we compared the intracellular proteins induced by the mutant viruses at nonpermissive temperatures with those obtained for the wild-type virus or for the mutant viruses grown at permissive temperatures. Wild-type SSH virus-infected cells incubated for various times at 33 or 40°C were pulse-labeled for 1 h with [^{3}H]leucine, and the labeled intracellular proteins, together with marker quantities of purified viral polypeptides, were recovered and resolved by polyacrylamide gel electrophoresis. The results (data not shown) demonstrated that, as others have shown (21), virus-induced polypeptides were the principal species labeled during the pulses, with more N polypeptide labeled than other viral polypeptides.

For SSH *ts* II-29 and *ts* II-30 mutant virus-infected cells incubated at 33°C and pulse-labeled at 6 h postinfection, viral N, G1, and G2 polypeptides, as well as several bands of host cell polypeptides, were identified in the cell extracts (Fig. 7). It could not be concluded that the lack of inhibition of host cell polypeptide synthesis was characteristic of these mutant viruses since a parallel wild-type SSH virus 33°C infection, initiated at a multiplicity similar to that used in the previous experiment, also did not inhibit host cell protein synthesis (Fig. 7). In cell extracts obtained from a wild-type SSH virus infection incubated for 6 h at 40°C, all three viral polypeptides were detected, albeit when compared with the 33°C infection, relatively less N than G1. For the 40°C group II *ts* mutant-infected cells, G1 and very few N polypeptides were observed in the cell extracts. There was significantly less label incorporated into viral polypeptides than in the extracts obtained from the 33°C infected cells. Although no G2 polypeptides were detected in any of the 40°C mutant virus-infected cell extracts, it is not certain whether this was due to its total absence or to the fact that overall there were lower amounts of viral polypeptides synthesized and that the G2 polypeptides eluded detection.

Thus, the results obtained with the SSH group II *ts* mutant-infected cells indicated that some viral polypeptides were synthesized in the mutant virus-infected cells incubated at 40°C. The lower amounts are compatible with the studies which indicated that these mutants synthesized less vcRNA at nonpermissive temperatures.

Examination of the patterns of intracellular polypeptides induced by SSH group I *ts* mutants in infected cells incubated for 6 h at 40°C (or incubated for 5.5 h at 33°C and then transferred to 40°C for 0.5 h and pulse-labeled for 1 h) demonstrated that N and G1 polypeptides were the principal polypeptides labeled at 40°C (data not shown). All three viral polypeptides were found in the group I mutant virus-infected cells incubated at 33°C (data not shown). The intracellular polypeptides obtained from SSH group I/II *ts* mutant virus-infected cells incubated at 40°C, like the group II mutant virus-infected

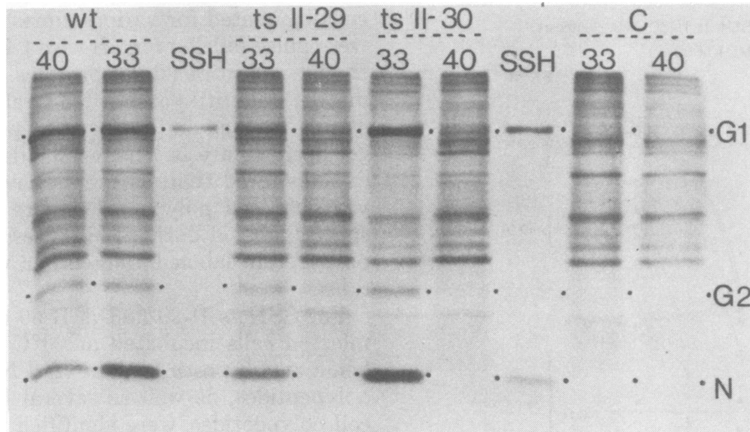

FIG. 7. Induction of viral polypeptides in BHK-21 cells infected with wild-type (wt) SSH and its ts mutants ts II-29 and ts II-30. Monolayers of BHK-21 cells were infected with either wild-type SSH virus (MOI = 200), SSH ts II-29 (MOI = 170), or SSH ts II-30 (MOI = 110), incubated at 33 or 40°C for 6 h, and pulse-labeled for 1 h with [³H]leucine. Uninfected or the infected cell polypeptides were processed (together with marker viral polypeptides) by discontinuous slab gel electrophoresis as described previously (1, 10–12). The positions of the viral G1, G2, and N polypeptides are indicated. Although the G2 polypeptide was often difficult to identify, possibly due to the use of [³H]leucine, which is not an effective precursor of G2 (11), its indicated position is consistent with previous studies (12). In addition, no viral L polypeptide was detected in the virus preparations (due to its low amount [18]) or in the infected cell preparations (21).

cells, were found to contain few viral polypeptides although infected cells grown at 33°C, shifted to 40°C, and then pulse-labeled synthesized both G1 and N polypeptides (data not shown).

DISCUSSION

The in vivo synthesis of vcRNA by wild-type SSH virus was analyzed. vcRNA was detected in wild-type virus-infected cells both when protein synthesis was inhibited (primary transcription) and at amplified levels (secondary transcription) for cells not treated with protein synthesis inhibitors. By comparison with the wild-type virus-infected cells incubated at 40°C (a temperature at which SSH virus is near its limit for developing a productive infection), the RNA phenotypes of SSH group I *ts* mutants (*ts* I-1 and *ts* I-3; M RNA defects [13]) were judged to be like that of the wild-type virus (i.e., RNA positive). The RNA phenotypes of four group II mutants (*ts* II-18, *ts* II-26, *ts* II-29, and *ts* II-30; L RNA defects [13]) were all somewhat inhibited at 40°C in comparison with the wild-type or group I mutant virus infections, and they were therefore judged to have an intermediate RNA phenotype. For the various experiments involving wild-type SSH virus or SSH *ts* mutant infections at permissive temperatures, from the specific activities of the purified viral RNA used to measure the intracellular vcRNA, it was calculated that the range of SSH vcRNA (plateau levels) obtained from 3×10^6 infected BHK-21 cells was of the order of 0.05 to 1 μg.

Since none of the SSH *ts* mutants analyzed was strictly an RNA-negative phenotype, the studies with these mutant viruses did not allow a definitive conclusion to be drawn concerning whether SSH vcRNA synthesis was under the direction of a virus- or host-coded polymerase. However, the demonstration of SSH primary transcription in the presence of protein synthesis inhibitors and the results obtained with actinomycin D are compatible with the in vivo transcription process being directed by a virus-coded polymerase. Even though infectious virus release was partially inhibited when actinomycin D was used (as others have shown [3]), in the presence of the antibiotic, vcRNA synthesis was detected at a level which was significantly greater than that obtained by primary transcription. The reduction in infectious virus release from actinomycin D-treated (and rifampin-treated) cells and somewhat lower vcRNA synthesis in comparison with untreated cells (Fig. 2) may be an indirect effect (for example, cellular toxicity). In conclusion, the in vivo studies reported in this paper agree with the postulate developed from in vitro analyses (2, 4, 22) that bunyaviruses are negative-stranded viruses having a virion polymerase which can direct the synthesis of vcRNA.

The in vivo experiments with the double group I/II *ts* mutant SSH *ts* 31 were performed to determine whether its RNA phenotype was

more restricted than those of the group I and II *ts* mutants. The results obtained indicated that the RNA phenotype of the double mutant was not much more restricted than the group II *ts* mutants.

Hybridization studies with ^{32}P-labeled individual L, M, and S viral RNA species have shown that in extracts of SSH *ts* II-30-infected cells incubated at 33°C (or 40°C), vcRNA species representing all three RNA segments are present at levels which are higher than those produced by primary transcription at 33°C. Particularly at 33°C there appeared to be more molecules of S than M vcRNA species and more molecules of M than L vcRNA species. The fact that at 33°C for either the wild-type or *ts* mutant-infected cells the most abundant pulse-labeled viral protein was the N polypeptide agrees with the observation that S vcRNA is more abundant than the other vcRNA species, indicating that in all probability many of these S vcRNA species are functioning as mRNA species. The observation that in the 40°C group II *ts* mutant-infected cell extracts there were relatively fewer S vcRNA species (in comparison with M and L vcRNA species) may indicate that there are fewer S mRNA species and that this is the reason for the relatively poor pulse-labeling of N polypeptide at 40°C. A detailed examination of the individual vcRNA species of wild-type and other group II *ts* mutant-infected cells at 33 and 40°C will be needed to substantiate this observation and to determine whether low S vcRNA at 40°C is a characteristic of only the *ts* 30 mutant or whether it is characteristic of all of the group II mutants, as well as of group I and wild-type viruses grown at that temperature.

It was concluded from the studies with SSH *ts* II-30 that the defect of the mutant was not an inability to transcribe RNA per se at nonpermissive temperatures. Since the amounts of vcRNA produced at 40°C by SSH *ts* II-30 were less than those developed at permissive temperatures, it is possible that secondary transcription either is inefficient due to impaired de novo-synthesized transcriptases or is reduced due to impaired viral RNA replication at 40°C.

In studies on the induction of viral polypeptides by SSH *ts* mutant and wild-type viruses, it was found that the wild-type virus frequently, but not always, inhibited host cell protein synthesis. The variation in such inhibition may be related to the growth state of the cells used in the different experiments. Although for the SSH *ts* II-29- and *ts* II-30-infected cells, both N and G1 polypeptides were detected in cells incubated at 40°C, the amounts were less than in the corresponding infected cells incubated at 33°C. As discussed above, the lower amounts probably reflect the lower quantities of vcRNA species in the *ts* II-30-infected 40°C cells. Conclusions concerning L and G2 syntheses by SSH mutant virus-infected cells were often difficult to reach due to the problems of consistently observing these polypeptides in virus or infected cell preparations.

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LITERATURE CITED

1. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46:**83–88.
2. **Bouloy, M., F. Colbere, S. Krams-Ozden, P. Vialat, A. C. Garapin, and C. Hannoun.** 1975. Activité RNA polymerasique associée à un Bunyavirus (Lumbo). C.R. Acad. Sci. Paris **280:**213–215.
3. **Bouloy, M., and C. Hannoun.** 1973. Effet de l'actinomycine D sur la multiplication du virus Tahyna. Ann. Microbiol. (Paris) **124b:**547–553.
4. **Bouloy, M., and C. Hannoun.** 1976. Studies on Lumbo virus replication. I. RNA-dependent RNA polymerase associated with virions. Virology **69:**258–264.
5. **Bouloy, M., and C. Hannoun.** 1976. Studies on Lumbo virus replication. II. Properties of viral ribonucleoproteins and characterization of messenger RNAs. Virology **71:**363–370.
6. **Clewley, J., J. Gentsch, and D. H. L. Bishop.** 1977. Three unique viral RNA species of snowshoe hare and La Crosse bunyaviruses. J. Virol. **22:**459–468.
7. **Flamand, A., and D. H. L. Bishop.** 1973. Primary in vivo transcription of vesicular stomatitis virus and temperature-sensitive mutants of five vesicular stomatitis virus complementation groups. J. Virol. **12:**1238–1252.
8. **Flamand, A., and D. H. L. Bishop.** 1974. *In vivo* synthesis of RNA by vesicular stomatitis virus and its mutants. J. Mol. Biol. **87:**31–53.
9. **Gentsch, J., and D. H. L. Bishop.** 1976. Recombination and complementation between temperature-sensitive mutants of the bunyavirus, snowshoe hare virus. J. Virol. **20:**351–354.
10. **Gentsch, J. R., and D. H. L. Bishop.** 1978. Small viral RNA segment of bunyaviruses codes for viral nucleocapsid protein. J. Virol. **28:**417–419.
11. **Gentsch, J. R., and D. H. L. Bishop.** 1979. M viral RNA segment of bunyaviruses codes for two glycoproteins, G1 and G2. J. Virol. **30:**767–776.
12. **Gentsch, J., D. H. L. Bishop, and J. F. Obijeski.** 1977. The virus particle nucleic acids and proteins of four bunyaviruses. J. Gen. Virol. **34:**257–268.
13. **Gentsch, J., L. R. Wynne, J. P. Clewley, R. E. Shope, and D. H. L. Bishop.** 1977. Formation of recombinants between snowshoe hare and La Crosse bunyaviruses. J. Virol. **24:**893–902.
14. **Goldman, N., I. Presser, and T. Sreevalsan.** 1977. California encephalitis virus: some biological and biochemical properties. Virology **76:**352–364.
15. **Huang, A. S., and E. K. Manders.** 1972. Ribonucleic acid synthesis of vesicular stomatitis virus. IV. Transcription by standard virus in the presence of defective interfering particles. J. Virol. **9:**909–916.

16. **Kascsak, R. J., and M. J. Lyons.** 1977. Bunyamwera virus. I. The molecular complexity of the virion RNA. Virology **82**:37-47.
17. **Marcus, P. I., D. L. Engelhardt, J. M. Hunt, and M. M. Sekellick.** 1971. Interferon action: inhibition of vesicular stomatitis virus RNA synthesis induced by virion-bound polymerase. Science **174**:593-598.
18. **Obijeski, J. F., D. H. L. Bishop, F. A. Murphy, and E. L. Palmer.** 1976. Structural proteins of La Crosse virus. J. Virol. **19**:985-997.
19. **Obijeski, J. F., D. H. L. Bishop, E. L. Palmer, and F. A. Murphy.** 1976. Segmented genome and nucleocapsid of La Crosse virus. J. Virol. **20**:664-675.
20. **Obijeski, J. F., A. T. Marchenko, D. H. L. Bishop, B. W. Cann, and F. A. Murphy.** 1974. Comparative electrophoretic analysis of the virus proteins of four rhabdoviruses. J. Gen. Virol. **27**:21-33.
21. **Pennington, T. H., C. R. Pringle, and M. A. McCrae.** 1977. Bunyamwera virus-induced polypeptide synthesis. J. Virol. **24**:397-400.
22. **Ranki, M., and R. Pettersson.** 1975. Uukuniemi virus contains an RNA polymerase. J. Virol. **16**:1420-1425.
23. **Repik, P., A. Flamand, and D. H. L. Bishop.** 1976. Synthesis of RNA by mutants of vesicular stomatitis virus (Indiana serotype) and the ability of wild-type VSV New Jersey to complement the VSV Indiana *ts* G I-114 transcription defect. J. Virol. **20**:157-169.