RNA Synthesis of Vesicular Stomatitis Virus

X. Transcription and Replication by Defective Interfering Particles

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In cells coinfecting by standard vesicular stomatitis virus (VSV) and defective interfering (DI) T particles, small RNA consisting of 46 nucleotides was synthesized in molar excess over other VSV-specific RNAs. Although its rate of synthesis increased over time, small RNA accumulated linearly, suggesting that the molecule is unstable. In contrast, replication of the genome RNA of DI T particles was relatively constant after 3 h of infection, resulting in the intracellular accumulation of stable genomic and antigenomic RNA of DI T particles. Coinfection of cells with DI T particles and selected temperature-sensitive mutants from all five complementation groups of VSV indicated that the replication of DI genomes was controlled separately from the synthesis of small RNA. Also, when viral RNA replication was inhibited by cycloheximide, small RNA continued to be synthesized as long as there were enough templates present. These results indicate that small RNA is synthesized by the enzyme(s) involved in VSV transcription and that its dependence on RNA replication is due to the requirement for template amplification.

Vesicular stomatitis virus (VSV) is a large, enveloped RNA-containing virus. Its strategy of nucleic acid synthesis involves two different but interdependent processes: (i) transcription of the genome RNA into multiple small complementary mRNA's (11, 22) and (ii) replication of the genome RNA via an equal-sized complementary RNA. This complementary RNA, in turn, acts as template for the synthesis of progeny genome RNA (21, 31). These two synthetic processes not only are defined by the analysis of their products but are also partially dissociated by the use of conditional lethal mutants which are temperature sensitive (ts) for either transcription or replication (25, 27, 33). Also, inhibitors of protein synthesis, such as cycloheximide, will prevent RNA replication without affecting transcription (24, 25, 35).

When the proteins involved in VSV RNA synthesis are analyzed, three of the five virus-specific proteins appear to be necessary for VSV transcription. The nucleocapsid (N) protein in association with the genome RNA functions as template. The largest of the structural proteins (L protein) is an RNA-dependent RNA polymerase which apparently binds to the VSV template via a phosphorylated ligand, the NS protein (8). The L and NS proteins appear to form a core enzyme which, in association with a putative host factor(s) or another, as yet unidentified viral product(s), replicates the genome of VSV (25). In addition to the involvement of

these three virus-specific proteins, the membrane-associated matrix (M) protein and the glycoprotein attenuate the transcriptive process (3, 6).

Recently a small RNA, lacking polyadenylate [poly(A)] at the 3' end and containing (p)ppAp at the 5' end, was found by direct radiolabeling with ³²P of cells coinfecting with standard VSV and defective interfering (DI) particles (28). Identical small RNAs of 45 to 47 nucleotides are made in vitro by DI particles in a polymerase assay (7, 29, 30). Standard VSV also synthesizes in vitro a plus-strand leader RNA of similar size and lacking poly(A) but different in sequence from the small RNAs associated with DI particles (4, 5). In cells infected with only standard VSV, it has been difficult to detect any plus-strand leader RNA or small RNAs, also called minus-strand leader RNA, unless high-specific radioactive probes are used (19). When cells are infected singly with standard VSV, plus-strand leader RNA together with minus-strand leader RNA accumulate to 1% or less of the total amount of small RNAs found in cells coinfecting with standard and DI particles (19, 28; unpublished data).

Although the bulk of intracellular RNA synthesis specific to DI particles is known to be under replicative and not transcriptive control (23), it is not clear whether this small RNA is the product of abortive replication (7) or a necessary leader RNA synthesized before transcrip-

tion (2). To determine whether this small RNA is synthesized under transcriptive or replicative control, small-RNA synthesis was examined in cells coinfecting with DI particles and VSV ts mutants from each of the five complementation groups. The effect of cycloheximide on small-RNA synthesis was also examined. These results indicate that the regulation of small-RNA synthesis differs from that of genome replication, suggesting that it is synthesized by transcriptive enzymes; however, it requires the amplification or replication of the genome of DI particles so that enough templates are present to permit the detection of small RNA.

MATERIALS AND METHODS

Cells and viruses. BHK cells were grown as suspension cultures in Joklik modified Eagle minimal essential medium supplemented with 5% fetal calf serum. Cloned and sucrose gradient-purified VSV of the Indiana serotype, San Juan strain (VSI ts⁺SJ), was used (34). Temperature-sensitive mutants of VSV (tsG114, tsG21, tsG22, tsG31, tsG41, and tsO45) were obtained from C. Pringle (Institute of Virology, Glasgow). Standard VSV from these temperature-sensitive mutants was used after cloning and sucrose gradient purification. The VSI ts⁺SJ DI T (5' 9%) particles have been characterized in detail (12; A. S. Huang, D. D. Rao, and G. Lanman, *Ann. N.Y. Acad. Sci.*, in press). The DI T particles were purified by two rounds of sucrose-gradient centrifugation. Their effective multiplicity was determined as described by Huang and Manders (13).

Infection of cells. Samples of BHK cells were first concentrated to 4×10^7 cells/ml. Purified virus at a multiplicity of 20 was attached to cells at 4°C for 30 min. When cells were coinfecting with standard virus and DI particles, a multiplicity of 20 each was used. After attachment, cells were washed once with organically buffered phosphate-free medium (10) and then resuspended at 4×10^6 cells/ml at 34°C in the same medium supplemented with 2% dialyzed fetal calf serum and actinomycin D at 5 µg/ml. At the indicated times for each experiment, ³²P at 0.1 mCi/ml was added to the cell cultures.

Extraction of cytoplasmic RNA. Infected cells were harvested, washed three times with phosphate-buffered saline, and treated with Nonidet P-40 as described previously (13). The cytoplasm was separated and total RNA was obtained by repeated phenol-chloroform extractions (10). The RNA was precipitated with ethanol, pelleted by centrifugation, and dried in vacuo. Then the RNA was suspended in sterile double-distilled water. The volume was adjusted to contain RNA from 4×10^7 cells in 50 µl.

Polyacrylamide and agarose gel analyses of RNA. Equal portions of these RNA samples were analyzed on two different slab gels by electrophoresis. To analyze large RNA of greater than 200 nucleotides, a 1.5% agarose gel containing urea-citrate buffer was used (10, 18). To separate small RNA, a 10% polyacrylamide gel in Tris borate-EDTA buffer, pH 8.3, was used (20). After electrophoresis, gels were fixed with

methanol-acetic acid (30%:10%) (1), dried, and exposed to X-ray film. Each set of gels for an experiment was exposed to film for the same period of time so that quantitative comparisons within an experiment were valid. In addition, to quantitate the amount of RNA in the individual bands, the bands were excised from the gel and their radioactivity was determined by Cerenkov counting.

Materials. Carrier-free ³²P-orthophosphoric acid was purchased from New England Nuclear Corp., Boston, Mass. Actinomycin D was a kind gift from Merck Sharp & Dohme, Rahway, N.J. Cycloheximide was purchased from Sigma Chemical Co., St. Louis, Mo. Nonidet P-40 was obtained from Particle Data Laboratories, Ltd., Elmhurst, Ill. Acrylamide, *N,N,N',N'*-tetramethylethylenediamine, *N,N'*-methylenebisacrylamide, and X-Omatic regular intensifying screens were all purchased from Eastman Kodak Co., Rochester, N.Y. Urea (ultrapure) was purchased from Schwarz/Mann, Orangeburg, N.Y. DuPont Cronex-4 X-ray films were purchased from E.M. Parker X-Ray, Brookline, Mass. Agarose was purchased from Marine Colloids, Rockland, Maine.

RESULTS

Linkage between the synthesis of small RNA and the replication of the genome of DI particles. In cells coinfecting with standard VSV and its DI particle, primary transcription (all five mRNA species) is programed by the input standard virus. All the other RNA species detectable in these cells are specific to DI particles. These species include RNA equal in size to the genome of the DI particle, a complete complement of this RNA, and a small RNA of 46 nucleotides (28; Huang et al., in press). Previous studies showed that the small RNA is easily detected only when the genome of DI particles is amplified through intracellular replication (28). To determine if this linkage to RNA replication is absolute, conditions were sought to partially reduce the amount of replication of the genome of DI particles and to see the effects of this reduction on small-RNA synthesis.

Previous studies indicated that superinfection of cells at 2 h after standard VSV infection with DI particles reduces the intracellular accumulation of the genomes of DI particles with a concomitant reduction in the ability of DI particles to interfere with the growth of standard VSV (15; Huang et al., in press). To determine how this reduction affected small-RNA synthesis, cells were infected with standard virus and then superinfected with an equal multiplicity of DI particles at 0, 1, 2, and 3 h later. The ³²P-labeled RNA made in cells after they were superinfected was extracted, and equal portions were placed on a 1.5% agarose gel (Fig. 1, top) or on a 10% polyacrylamide gel (Fig. 1, bottom).

Cells that were superinfected with DI particles 3 h after standard VSV infection (Fig. 1d) synthesized RNA species almost identically as previously described for cells infected with standard virus alone (28). The bands marked L, G, N, NS, and M indicate the five VSV mRNA species. Genome-sized RNA containing virion-like negative strands and its full complement are marked by 40S. The origin is indicated by 0. Very faint doublet bands indicated by DI T represent the minus-strand genome of DI particles (top) and its complementary plus-strand RNA (bottom). Other minor bands between the DI T RNA and the L mRNA represent either minor poly(A)-containing species or nonspecifically trapped radioactivity which comigrated with unlabeled 28S rRNA. The lower half of Fig. 1 shows 4S tRNA molecules and small RNA. The band marked small RNA is not homogeneous, and there are minor sequence homologs migrating ahead of the major broad band.

Although the labeling time for ^{32}P varied for each sample in this experiment, comparison in the amount of genome-size RNA of DI particles agreed with previous findings that the synthesis of this RNA was reduced considerably if there was a delay of 2 or more hours in the addition of DI particles to cells after infection with standard VSV (Fig. 1c). The amount of small RNA synthesized correlated with the amount of DI genome replication. Therefore, to maximize small-RNA synthesis, coinfection of cells at the same time was preferable to delayed superinfection with DI particles.

Kinetics of small-RNA synthesis. Because the presence of small RNA appeared to be linked to the replication of the genome of DI particles, a kinetic analysis of its accumulation and rate of synthesis may shed light on the stability of small RNA and its specific template dependence. Therefore, cells coinfecting with standard VSV and DI particles were either exposed to ^{32}P for a total of 9 h or pulse-labeled for 1-h periods at 3, 5, and 7 h postinfection. The virus-specific RNA species were extracted and separated on gels as for Fig. 1. Each of the bands containing RNA was excised, and the radioactivity was determined.

Figure 2 shows both the cumulative 9-h incorporation as well as the rate of incorporation of ^{32}P into four species of RNA in these cells. All of the RNA species accumulated in amount over time, with the minus-strand genome RNA of the DI particle accumulating at the greatest rate and the mRNA for the N protein accumulating at the lowest rate. Radioactivity in the plus-strand RNA complementary to DI genomes accumulated at an identical rate to small RNA

FIG. 1. Effects of superinfection with DI particles on small-RNA synthesis. Four cultures of BHK cells were first infected with standard VSV and then superinfected with DI particles at 0, 1, 2, and 3 h later. Cells superinfected at 0 time were exposed to ^{32}P from 1.5 h postinfection, whereas cells superinfected at 1, 2, and 3 h were exposed to ^{32}P immediately after superinfection. All cells were harvested at 6 h, and equal portions of their radiolabeled RNA were analyzed on 1.5% agarose gels (top) and 10% polyacrylamide gels (bottom). (a) Standard VSV at 0 h and DI particles at 0 h; (b) standard VSV at 0 h and DI particles at 1 h; (c) standard VSV at 0 h and DI particles at 2 h; (d) standard VSV at 0 h and DI particles at 3 h.

FIG. 2. Kinetic analysis of VSV RNA species synthesized by cells coinfecting with standard virus and DI particles. Coinfected BHK cells were divided into two equal samples. One was immediately exposed to ^{32}P , and at hourly intervals thereafter equal portions were removed and processed for the cumulative labeling of RNA. Equal portions were removed from the other half of the infected cells at 3, 5, and 7 h postinfection and pulse-labeled for 1 h each. RNA species were separated by each of the two gel systems, and the radioactivity in each species was quantitated as described in Materials and Methods. Symbols: (○) RNA genome of DI particles; (■) full-length RNA complementary to the genome of DI particles; (□) small RNA; (●) mRNA for the N protein.

(Fig. 2a). When the counts per minute was corrected for the molar yield of the RNA species, there were many more molecules of small RNA, greater than 50-fold, compared with any of the larger RNA species.

Because the genome-size RNA of DI particles increased at approximately linear rates, their accumulation would be expected to be due to constant rates of synthesis. From 3 to 7 h postinfection, this was indeed so, to a first approximation, for all of the three large RNA species (Fig. 2b). Surprisingly, the rate of synthesis of small RNA increased during the infection. These results indicate that the DI genome RNA, its complement, and mRNA were relatively stable species, synthesized at a constant rate after 3 h and accumulating linearly. The small RNA,

however, was being degraded even though it accumulated in time. The results on the two genome-size DI RNA species agree with previous studies on the rate of synthesis of nucleocapsids of standard VSV (14, 31). The rate of mRNA synthesis is limited and constant in these coinfecting cells because transcription is dependent on input standard virus genomes and these are not amplified in coinfecting cells. Control studies indicated that during such 1-h pulse-labeling procedures, most of the RNA synthesized remained intracellular.

Effects of RNA⁺ ts mutants on the synthesis of DI particle-specific RNA. Although synthesis of the small RNA was dependent on the replication of the genome of DI particles, its kinetics of synthesis suggested that it was not under the same control mechanism as RNA replication. To determine whether small RNA was synthesized under replicative or transcriptional controls, temperature-sensitive mutants of VSV which have lesions affecting RNA synthesis, rather than the usual wild-type standard virus, were used as helper viruses.

The first mutants used for coinfection in these studies were tsG31 (complementation group III) and tsO45 (complementation group IV). Both complementation groups are RNA⁺ and contain lesions affecting maturation (27). Surprisingly, they also affect RNA synthesis by increasing transcription at the nonpermissive temperature (3, 6).

When tsG31 was used as the helper virus, the replication of the genome of the DI particle was virtually equivalent at both permissive and nonpermissive temperatures (Fig. 3c,d, top). Small RNA, on the other hand, when compared with the permissive temperature, was increased in amount at the nonpermissive temperature (Fig. 3c,d, bottom). Control cells infected with tsG31 alone showed the expected increase of transcription at the nonpermissive temperature (Fig. 3a,b). Also, small RNA was not detected in significant amounts in tsG31-infected cells in the absence of DI particles; what radioactivity is seen in the bottom half of Fig. 3b was due to spillover of mRNA resulting from increased transcription.

When tsO45 was used as the helper virus in coinfecting cells, similar results were obtained. The increase in small RNA was approximately four to eight times at the nonpermissive compared with the permissive temperature when this RNA⁺ mutant was used as helper virus in the coinfecting cells (Fig. 3g,h). The accumulation of mRNA in these mutant-infected cells was increased by two- to fivefold at the nonpermissive temperature, whereas genome replication

was either unaffected or reduced (Fig. 3e,f).

Effects of RNA⁻ mutants on the synthesis of DI particle-specific RNA. Because the syn-

thesis and accumulation of small RNA were apparently increased by mutants which caused an increase in transcription but not RNA replication, tsG114, a ts mutant of complementation group I and negative for transcription at the nonpermissive temperature (32), was used as helper virus to see if this would result in a direct inhibition of the synthesis of small RNA.

Figure 4 shows the RNA patterns obtained

FIG. 3. Effects of *tsG31* and *tsO45* on small-RNA synthesis. Samples of BHK cells were infected with *tsG31* or *tsO45* alone or coinfecting with each of the *ts* mutants and DI particles. These were incubated at 31°C, and another identical set of samples was incubated at 38°C. ³²P was added at 1 h postinfection. All samples were exposed to ³²P between 1 and 5 h and then harvested, and the radiolabeled RNA was analyzed on a 1.5% agarose gel (top) and a 10% polyacrylamide gel (bottom). (a) *tsG31*, 31°C; (b) *tsG31*, 38°C; (c) *tsG31* plus DI particles, 31°C; (d) *tsG31* plus DI particles, 38°C; (e) *tsO45*, 31°C; (f) *tsO45*, 38°C; (g) *tsO45* plus DI particles, 31°C; (h) *tsO45* plus DI particles, 38°C.

FIG. 4. Effects of *tsG114* on small-RNA synthesis. Samples of BHK cells were infected with *tsG114* alone or coinfecting with *tsG114* and DI particles. Their labeling conditions and subsequent RNA analysis were as described for Fig. 3. (a) *tsG114*, 31°C; (b) *tsG114*, 38°C; (c) *tsG114* plus DI particles, 31°C; (d) *tsG114* plus DI particles, 38°C.

from cells singly infected with tsG114 and coinfecting with the mutant and DI particles. The singly infected cells showed the expected inhibition of all RNA synthesis at the nonpermissive temperature (Fig. 4b). In the coinfecting cells, replication of the genome of DI particles was greatly reduced but detectable at the nonpermissive temperature (Fig. 4d), presumably due to complementation of the polymerases between the incoming DI particles and tsG114. The lack of detectable small RNA at the nonpermissive temperature in this experiment could be due to the transcriptive lesion in tsG114 or to the lack of template amplification which is known to require prior transcription and protein synthesis (13, 25).

Another RNA⁻ mutant is tsG41 (complementation group IV), which is defective in RNA replication but not transcription at the nonpermissive temperature (25, 33). To test the effect of this mutant on small-RNA synthesis by DI particles, cells were coinfecting and the RNA species were labeled either continuously from 1 h postinfection to 5 h at each of the two temperatures or labeled between 3 and 5 h postinfection at 38°C after incubation at 31°C for 2.5 h postinfection.

Figure 5 shows the RNA pattern obtained from cells infected with standard VSV alone or coinfecting with standard and DI particles. Continuous labeling at 31°C resulted in the expected pattern of normal VSV RNA synthesis (slot a). In this experiment, some NS and M mRNA's were electrophoresed out of the bottom of the gel (Fig. 5, top). With DI particles and tsG41 there was inhibition of 40S RNA synthesis as well as secondary transcription (slot b). At 38°C, continuous labeling of cells infected by standard tsG41 alone showed the ts lesion where 40S RNA synthesis was reduced by 80 to 85% (slot c). Surprisingly, the genome of DI particles was not affected in coinfecting cells under these same nonpermissive conditions. Small RNA, however, was increased by as much as threefold (slot d). When cells were first incubated at the permissive temperature and then shifted to the nonpermissive temperature and labeled, 40S RNA synthesis by tsG41 was reduced by 90% when compared with cells labeled for the same period but incubated at 31°C (slots e and g). In coinfecting cells, the temperature shift again did not appreciably affect the replication of the genome of DI particles; however, small-RNA synthesis was increased at 38°C (slots f and h). As a control for the shift-up experiments, RNA from coinfecting cells incubated at 38°C throughout, but labeled only between 3 and 5 h postinfection, is shown in slot i.

These results with tsG41 confirm previous

observations (23) on the relative resistance of the replication of the genome of DI particles to the lesion in tsG41. This lesion has since been mapped to the gene coding for the N protein and not to genes corresponding to direct polymerase functions (9, 17). This resistance may indicate that the genomes of DI particles were more efficient in binding to N protein than those of standard virus or that the input nucleocapsids of DI particles, which are derived from wild-type VSV, are better templates for replication than those of tsG41 virions.

Despite the lack of inhibition of RNA replication of DI particles in the presence of tsG41, it can be concluded that the increase in small-RNA synthesis at the nonpermissive conditions reflects a control in small RNA that differed from that on the replication of standard RNA as well as DI genomic RNA. How a lesion in the N protein can result in increased synthesis of small RNA will be discussed.

Effects of complementation group II mutants on small-RNA synthesis. Group II mutants are both RNA⁺ and RNA⁻ (27). To determine their effects on small RNA, cells were infected at 31 or 38°C and continuously labeled with ³²P. RNA obtained from cells infected with tsG21 alone showed an overall fourfold increase of RNA synthesis at the nonpermissive temperature (Fig. 6a,b). When cells were coinfecting, the same increase, about fivefold, was seen at the nonpermissive temperature for all the RNAs specific to DI particles (Fig. 6c,d).

With tsG22, there was an overall inhibition of all RNA synthesis at 38°C (Fig. 6e-h). However, when the radioactivity in the RNA species was quantitated for cells coinfecting by tsG22 and DI particles, there was a 12-fold inhibition of the replication of the genome of DI particles compared with only a 4-fold reduction in the synthesis of small RNA. These results indicate that small RNA was dependent on some replication of the genome of DI particles, but that its synthesis was not directly proportional to them.

Effects of cycloheximide on the synthesis of small RNA. Because the interpretation of the results with RNA⁻ ts mutants was complicated by pleiotropic effects and only partial nonpermissiveness, another method was used to dissociate RNA replication from transcription. Previously, it was shown that VSV RNA replication, but not transcription, was exquisitely sensitive to protein synthesis inhibitors (24, 26, 35). Therefore, cells were coinfecting with standard wild-type VSV and DI particles and allowed to amplify their content of DI genomes for 3 h, and then cycloheximide was added to determine its effects on subsequent RNA replication and the synthesis of small RNA.

FIG. 5. Effects of *tsG41* on small-RNA synthesis. Samples of cells were infected with either *tsG41* alone or *tsG41* with DI particles. One pair of samples was incubated at 31°C, and another pair was incubated at 38°C and labeled with ^{32}P continuously between 1 and 5 h postinfection. A third pair was incubated at 31°C for 2.5 h and then shifted to 38°C and labeled with ^{32}P from 3 to 5 h. As controls, another pair was

When RNA synthesis was examined at 0, 0.5, or 1.0 h after the addition of cycloheximide, there was a complete inhibition of further replication of the genome of DI particles (Fig. 7a-c). In contrast, small RNA continued to be made in an abundant amount in the presence of cycloheximide. For comparison, cells not treated with cycloheximide but labeled identically as cells treated with cycloheximide and exposed to ^{32}P 1 h after the drug showed the continued accumulation of the genomes of DI particles as well as the synthesis of small RNA (Fig. 7d).

We previously reported that without the amplification of genomes, small RNA is not detected in cells infected with DI particles even when the multiplicity is 100 (28; D. D. Rao, unpublished data). Given adequate numbers of templates, i.e., genomes of DI particles or its RNA complement, small RNA is synthesized in the presence of cycloheximide. Therefore, the synthetic machinery for small RNA is the transcriptive one.

DISCUSSION

These results indicate that small RNA, like the *in vitro* leader RNA described by Banerjee and co-workers (2), is synthesized by transcriptive enzymes. Intracellularly, its synthesis is unaffected by cycloheximide and its rate of synthesis is increased by mutants which have lesions in their ability to attenuate transcription. Although the enzymes for RNA replication and transcription are intimately linked in the VSV system, small RNA does not appear to be a product of abortive RNA replication, as had been previously suggested (7).

There are three RNA species which can be potential templates for small-RNA synthesis. These are the genome of DI particles, its complementary RNA, and the 40S RNA complementary to the genome of standard VSV. All three contain virtually identical sequences at the 3' end for the first 46 to 48 nucleotides (16, 16a, 28). However, in cells coinfecting with standard

continuously incubated at 31°C and labeled only between 3 and 5 h. Another control sample infected with *tsG41* and DI particles was incubated continuously at 38°C and labeled only between 3 and 5 h. All cells were harvested at 5 h and analyzed as described for Fig. 3. (a) *tsG41*, 31°C, ^{32}P labeled from 1 to 5 h; (b) *tsG41* plus DI particles, 31°C, ^{32}P labeled from 1 to 5 h; (c) *tsG41*, 38°C, ^{32}P labeled from 1 to 5 h; (d) *tsG41* plus DI particles, 38°C, ^{32}P labeled from 1 to 5 h; (e) *tsG41*, 31 → 38°C, ^{32}P labeled from 3 to 5 h; (f) *tsG41* plus DI particles, 31 → 38°C, ^{32}P labeled from 3 to 5 h; (g) *tsG41*, 31°C, ^{32}P labeled from 3 to 5 h; (h) *tsG41* plus DI particles, 31°C, ^{32}P labeled from 3 to 5 h; (i) *tsG41* plus DI particles, 38°C, ^{32}P labeled from 3 to 5 h.

FIG. 6. Effects of *tsG21* and *tsG22* on small-RNA synthesis. Samples of BHK cells were infected with either *tsG21* or *tsG22* alone or coinfecting with each of the *ts* mutants and DI particles. Cells were labeled and harvested for their RNA as described in Fig. 3. (a) *tsG21*, 31°C; (b) *tsG21*, 38°C; (c) *tsG21* plus DI particles, 31°C; (d) *tsG21* plus DI particles, 38°C; (e) *tsG22*, 31°C; (f) *tsG22*, 38°C; (g) *tsG22* plus DI particles, 31°C; (h) *tsG22* plus DI particles, 38°C.

VSV and DI particles, the plus-strand 40S RNA is barely detected in these cells and, therefore, is unlikely to act as templates for small-RNA synthesis. Since small RNA appears to be dependent only on the replication of the genome

FIG. 7. Effects of cycloheximide on small-RNA synthesis. Samples of BHK cells were coinfecting with wild-type standard VSV (San Juan strain) and DI particles and incubated at 34°C. At 3 h postinfection, cycloheximide at 100 µg/ml was added to three of four samples. ³²P was added to the cycloheximide-treated cells at 3, 3.5, and 4 h postinfection. The sample without cycloheximide was exposed to ³²P at 4 h postinfection. All samples were harvested at 6 h, and the radiolabeled RNA was analyzed as described for Fig. 3. (a) Cycloheximide treated, ³²P labeled at 3 h; (b) cycloheximide treated, ³²P labeled at 3.5 h; (c) cycloheximide treated, ³²P labeled at 4 h; (d) untreated, ³²P labeled at 4 h.

of DI particles, the genome or its complementary RNA is the likely template.

Despite the large number of molecules of small RNA found, its functional role, if any, remains obscure. Because it is not associated with the N protein in cesium or sucrose gradients (19; D. D. Rao and A. S. Huang, unpublished data), it is possible that increased synthesis of small RNA is dependent on defective or low concentrations of N protein. Our studies with tsG41, where a lesion in the N protein appears to increase small-RNA synthesis without an overall increase in transcription, suggests that the absence of N protein induces the synthesis of small RNA. It is possible that the binding of N protein to nascent RNA chains, such as small RNA, changes RNA synthesis from a transcriptional phase to a replicative phase. This hypothesis has been previously proposed by Leppert et al. (19) and others.

From the kinetic analyses it is now understood why small RNA or leader RNA, which is readily synthesized *in vitro*, has been so difficult to detect in VSV-infected cells. The kinetics of small-RNA synthesis indicate that it is very unstable. Its accumulation in cells is dependent on the amplification of intracellular templates which contribute to the increasing rate of small-RNA synthesis. This marked instability has also been detected *in vitro* (19). Compared with small RNA and leader RNAs, the other VSV-specific RNAs are relatively stable.

The presence of DI particles of VSV offers considerable advantage not only for studying small-RNA synthesis *in vivo* but also for analyzing the mechanism of VSV RNA replication. DI particles successfully compete for the RNA synthetic machinery provided for by the standard helper VSV. Over 50% of the RNA species in cells coinfecting by standard and DI particles of VSV are the genome of DI particles and its complement, whereas in cells infected by standard virus alone, less than 5% of the RNA is found in the genome of standard virus and its complement. Besides the large amount of replicative products, another advantage in studying DI particles is that the two strands involved in the replication of the genome of DI particles can be separated and quantitated (Huang et al., *in press*).

The kinetic analysis of the synthesis of individual RNA species from cells coinfecting with standard VSV and DI particles indicates one important parameter concerning VSV RNA synthesis. The constant rate of genome RNA replication and its linear accumulation after 3 h suggest that the total number of templates par-

ticipating in RNA replication is fairly constant. This number appears to have been determined relatively early during infection. At late times during infection, the majority of the templates accumulating in cells are templates for transcription or small-RNA synthesis. What determines or limits the rate of replication is currently under investigation.

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