

Ribonucleic Acid Synthesis of Vesicular Stomatitis Virus

IV. Transcription by Standard Virus in the Presence of Defective Interfering Particles

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Exposure of vesicular stomatitis virus-infected Chinese hamster ovary cells to cycloheximide results in the complete transcription of virion ribonucleic acid (RNA) into only 28S and 13 to 15S viral-specific RNA species. These RNA are identical to viral messenger RNA by the following criteria: size, single-strandedness, complementarity to virion RNA, and formation of messenger ribonucleoproteins. This transcription represents the intracellular enzymatic activity of the virion-associated polymerase and is shown to be dependent on input multiplicity. Intracellular transcription differs from *in vitro* polymerase activity in having a temperature optimum of 34 to 37 C and in synthesizing 28S as well as 13 to 15S messenger RNA species. Addition of interfering quantities of defective T particles to these cycloheximide-treated cells, either an hour before or at the same time as standard B particles of vesicular stomatitis virus, does not alter the rate of transcription nor does it change the sucrose gradient pattern of the viral RNA species. From these results it is concluded that the RNA of defective T particles does not serve a transcriptional function and probably interferes through the replicative mechanism for virion RNA synthesis.

Vesicular stomatitis virus (VSV) provides an example of a viral system exhibiting homologous interference mediated by defective particles (6). This interference occurs intracellularly and results in an inhibition of the growth of plaque-forming, standard B particles (2, 5, 9). Interference is detected only if defective particles are added before the first half of the growth cycle of standard virus (10). This suggests that the inhibition occurs at an early step during viral replication. All the virus-specific events, such as ribonucleic acid (RNA) synthesis (20) and protein synthesis (12, 15, 22) occur within the first 3 hr after viral penetration. It is not known which of these functions is primarily inhibited by defective particles. Total viral protein synthesis is reduced when defective particles are added early during the replication of B particles, but the pattern of viral polypeptides is not altered (15, 22). When total viral RNA synthesis is examined, interference by defective particles results in an overall decrease of RNA synthesis and a change in the pattern of RNA species which are synthesized (20).

VSV RNA synthesis can be divided into two distinct kinds: one which results in the synthesis of multiple pieces of messenger RNA complementary to virion RNA (8, 14) and another which results in production of progeny virion RNA. The latter synthesis, called RNA replication as opposed to transcription, is at present not understood in detail. Nevertheless, it is now possible to examine the effect of defective particles on viral RNA synthesis in some detail because intracellular viral transcription by input virions can be studied in the absence of viral replication. The finding of a virion-associated RNA polymerase in VSV (1) suggested that early viral transcription would occur in cells in the absence of any other viral replicative steps if cells were treated at the beginning of infection with an inhibitor of protein synthesis (18, 23). Such a system to study early transcription by VSV has been described (13). The results presented here show the early transcription in VSV-infected Chinese hamster ovary (CHO) cells catalyzed by the virion polymerase and the effect of defective interfering particles on this transcription.

MATERIALS AND METHODS

Viruses and cells. Growth of the Indiana serotype of VSV in CHO cells has been previously described (20). Purified preparations of standard B particles of VSV completely free of defective virions were obtained by clonal isolation (21) and found to contain 4×10^9 to 8×10^9 plaque-forming-units (PFU)/ml by assay on CHO cells (21). One preparation of defective particles (9) was used throughout these experiments. It was prepared in chick embryo fibroblasts (9) and purified (21) as previously described. The preparation was estimated to contain approximately 4×10^8 active particles/ml by assuming that the number of active particles in 1 optical density at 260 nm (OD_{260}) unit of T particles is equal to three times the number of PFU contained in 1 OD_{260} unit of standard virus. This adjustment is based on the fact that T particles contain one-third the amount of RNA of standard virions (11) and that the effective infectivity to particle ratios of the two particles are assumed to be approximately the same. Contamination of this T particle preparation by standard virions was no greater than 6.80×10^4 PFU/ml.

Protocol for infections. In general, infections were carried out as previously described (8) with 1-ml samples containing 4×10^6 CHO cells in 13 by 100-mm test tubes and incubated in a 37 C water bath. Cells were kept in suspension with small magnetic spinning bars. Where indicated, just before infection with standard B particles, cycloheximide and T particles were added. Immediately following these additions, 8×10^7 PFU of B particles (multiplicity of infection [MOI] = 20 unless otherwise indicated) and 10 μ g of actinomycin D were added to each tube. Half an hour after addition of B particles radioactive uridine was added in an equal volume of medium. To assay incorporation of radioactive uridine, acid-precipitable radioactivity in 0.1-ml samples from each tube was assayed in either a Packard Tri-carb or a Beckman LS-233 scintillation counter.

Cytoplasmic extracts and sucrose gradient centrifugation. At the termination of some experiments, cytoplasmic extracts were made from the remaining cells in each of the test tubes by washing three times in Earle's saline solution and finally suspending the cells in reticulocyte standard buffer [0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 7.4; 0.01 M NaCl; 0.0015 MgCl₂] containing 1% Nonidet P-40. Cytoplasm was separated from nuclei by centrifugation at $400 \times g$ for 2.5 min at 4 C. To deproteinize RNA in the cytoplasmic extract 1% sodium dodecyl sulfate was added, and separation of the viral-specific RNA species was obtained on 15 to 30% sucrose gradients containing 0.5% sodium dodecyl sulfate as described previously (8).

Materials. Uridine-5-³H at 28.2 Ci/mmmole, adenosine-2,8-³H at 15.5 Ci/mmmole, and uridine-2-¹⁴C at 55.6 mCi/mmmole were purchased from New England Nuclear Corp. Cycloheximide was obtained from Calbiochem. Actinomycin D was obtained from Merck Sharpe and Dohme in vials containing 0.5 mg of (d) actinomycin and 20 mg of mannose. Nonidet P-40 was a kind gift from David Baltimore.

RESULTS

Intracellular RNA synthesis by VSV in the presence of cycloheximide. To demonstrate early viral RNA synthesis in CHO cells either cycloheximide or puromycin at greater than 50 μ g/ml could be used as the inhibitor of protein synthesis. Cycloheximide was the choice in these experiments because it appeared to be less toxic for infected CHO cells and inhibition was readily reversed by washing the cells. When 50 μ g of cycloheximide per ml was used to treat CHO cells, there was a rapid decrease of labeled amino acid incorporation into acid-insoluble products so that the residual rate of protein synthesis was 5 to 8% of that in untreated cells. Inhibition was maintained for longer than 3 hr after addition of the inhibitor, and higher concentrations of cycloheximide did not appreciably increase the extent of inhibition. Although in the following experiments cycloheximide was added to cells only just before addition of B particles, identical results were obtained if cells were pretreated for 30 min with cycloheximide.

When CHO cells were infected with B particles and treated at the same time with cycloheximide as well as actinomycin D, cumulative incorporation of ¹⁴C-uridine into acid-insoluble material was approximately 10% compared to similarly infected cells not treated with cycloheximide (Fig. 1). Background incorporation by uninfected cells exposed only to actinomycin D was also lowered by the addition of cycloheximide, thereby unmasking more viral RNA synthesis in the infected cells.

Dependence of cycloheximide-resistant RNA synthesis on multiplicity. Because nucleic acid synthesis measured in the presence of cycloheximide and actinomycin D is thought to be due only to the polymerase associated with input virus, increasing the concentration of virions should increase the synthesis of RNA. Therefore, at 3 hr after infection with different multiplicities, RNA synthesis, measured in the presence of cycloheximide, was compared to total RNA synthesis in the absence of cycloheximide. At a multiplicity of 20, RNA synthesis in the presence of cycloheximide accounted for about 10% of total RNA synthesis (Fig. 20 cf. Fig. 1). As long as all the cells were infected, the amount of cycloheximide-resistant RNA synthesis increased proportionately to the amount of added virions up to a multiplicity of 40. However, increasing the virion concentration beyond a multiplicity of 40 did not yield the expected increase in activity. What causes this saturation effect is not known. In contrast, total viral-specific RNA synthesis in the absence of cycloheximide ap-

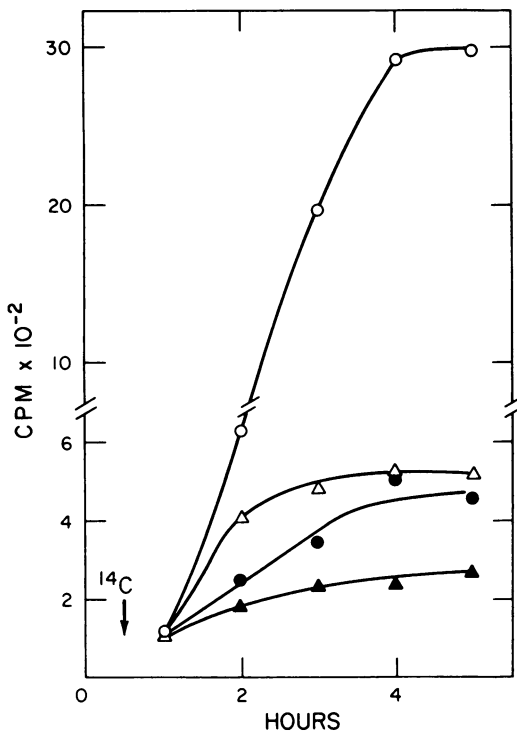


FIG. 1. Cumulative viral RNA synthesis in the presence and absence of cycloheximide. Samples of infected (Δ) and uninfected (\blacktriangle) Chinese hamster ovary cells were exposed to $50 \mu\text{g}$ of cycloheximide per ml. Control cells similarly infected (\circ) and uninfected (\bullet) were not treated with cycloheximide. Each sample received $0.3 \mu\text{Ci}$ of ^{14}C -uridine per ml.

peared to be virtually independent of input multiplicity as long as most of the cells were initially infected (Fig. 2).

Intracellular transcription. To make sure that the RNA synthesis detected in the presence of cycloheximide was a measure of only viral transcription, it would be necessary to demonstrate that the infected cells synthesized only messenger RNA by the following criteria. The RNA (i) sediments in sucrose at both 28S and 13 to 15S; (ii) is single stranded; and (iii) is completely complementary in nucleotide sequence to virion RNA (8, 14).

Figure 3 shows a sucrose-gradient separation of the 28S and 13 to 15S RNA species synthesized in the presence of cycloheximide and labeled with ^3H -uridine from 0.5 hr to 3 hr after infection. No RNA species sedimenting faster than 28S was detected either in the gradient or in the pellet fraction of the centrifuge tube. When each of the fractions was treated with ribonuclease A and T1 all of the RNA species

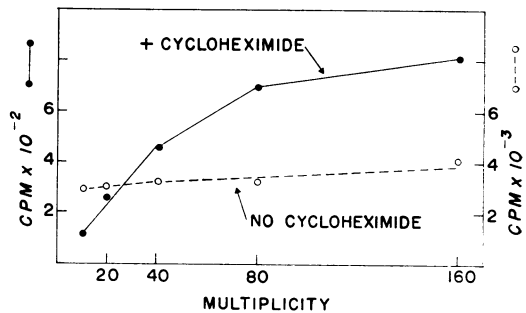


FIG. 2. Total viral RNA synthesis at different multiplicities of infection in the presence and absence of cycloheximide. Samples of Chinese hamster ovary cells were either treated with $100 \mu\text{g}$ of cycloheximide (\bullet) per ml or not treated (\circ) and infected at the indicated multiplicities. Each sample received $0.6 \mu\text{Ci}$ of ^{14}C -uridine per ml. Only the 3-hr sample of 0.1 ml of each of the infected samples is shown here.

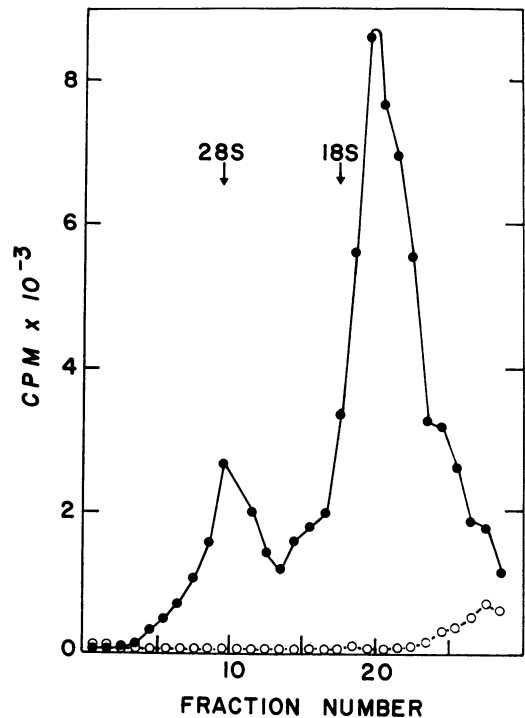


FIG. 3. Sucrose-gradient separation of RNA species made in the presence of cycloheximide. A sample of Chinese hamster ovary cells infected with B particles treated with $100 \mu\text{g}$ of cycloheximide per ml and labeled with $100 \mu\text{Ci}$ of ^3H -uridine per ml. At 3 hr after infection the cells were harvested and the cytoplasmic RNA separated on sucrose gradients containing 0.01% sodium dodecyl sulfate. After centrifugation half of each fraction was digested with ribonuclease and the RNA acid precipitated as previously described (10). (\bullet) ^3H -uridine-labeled RNA; (\circ) ribonuclease-digested labeled RNA.

became acid soluble (Fig. 3), indicating that 28S and 13 to 15S RNA species were single stranded.

Annealing of this labeled RNA made in the presence of cycloheximide to excess unlabeled virion RNA showed that the intracellular RNA was almost completely (97%) complementary in base sequence to virion RNA (Table 1). Also, when ^3H -adenosine-labeled virion RNA was annealed to excess cytoplasmic RNA from in-

fecting cells treated with cycloheximide all of the virion RNA became ribonuclease-resistant, whereas virion RNA alone labeled with ^3H -adenosine was completely ribonuclease sensitive (Table 1). These results indicate that in the presence of cycloheximide only RNA complementary to virion RNA was transcribed intracellularly and that the virion RNA was transcribed completely.

Absence of newly synthesized nucleocapsids in the presence of cycloheximide. To demonstrate that no nucleocapsids containing 28S RNA were synthesized in the presence of cycloheximide the uridine-labeled ribonucleoproteins made by VSV in the presence or absence of cycloheximide were separated in sucrose gradients containing ethylenediaminetetraacetate. In the absence of cycloheximide there were three major RNA-containing species which have been previously identified as either nucleocapsids at 120S or messenger ribonucleoproteins at 65S and 30S (8). In the presence of cycloheximide only, the two messenger ribonucleoprotein species were detected (Fig. 4). The absence of any newly synthesized nucleocapsids in cycloheximide-treated cells further indicated that the RNA synthetic activity was indeed viral transcription and most likely due to the virion-associated transcriptase (1).

Temperature optimum for intracellular VSV transcription. Because the *in vitro* VSV polymerase reaction has a temperature optimum of 31 C (7), the temperature optimum was determined for the intracellular transcriptase activity. Figure 5 shows that the intracellular activity occurred best at 34 to 37 C and not at 31 C. Similarly, total viral RNA synthesis in the absence of cycloheximide occurred optimally at 34 to 37 C and not at 31 C (E. K. Manders, unpublished observations).

Effect of T particles on transcription. To determine if defective (T) particles could directly inhibit the intracellular transcriptase activity associated with input standard B particles, an approximately equivalent concentration of T and B particles was added to CHO cells immediately after the addition of cycloheximide. For comparison, the same concentration of T and B particles was added to CHO cells which were not treated with cycloheximide. Although in this experiment T inhibited the growth of plaque-forming B particles by more than 99% and total ^{14}C -uridine incorporation by about 90% (Fig. 6b), none of the incorporation in the presence of cycloheximide was affected by the presence of T particles (Fig. 6a). This apparent lack of inhibition of early transcription by standard virus was not due to transcriptase activity contributed by T particles themselves, because there was no

TABLE 1. Complementary between intracellular RNA made in the presence of cycloheximide and virion RNA^a

Labeled RNA		Unlabeled RNA		Ribonuclease resistance (%)
Source	Counts/min	Source	μg	
Infected cytoplasm ^b	2,000	B particle ^c	4	97.3
Infected cytoplasm ^b	1,000	B particle ^c	1	90.5
Infected cytoplasm ^b	1,000			0.0
Uninfected cytoplasm ^d	24,000	B particle ^c	1	3.2
Uninfected cytoplasm ^d	24,000			2.4
B particle ^e	10,000	Infected cytoplasm ^f	250	106.0
B particle ^e	10,000	B particle ^c	1	0.6
B particle ^e	10,000			0.6

^a RNA from the various sources was purified either by phenol-extraction or centrifugation through sucrose gradients containing sodium dodecyl sulfate and then precipitated with ethanol (10). Mixtures containing labeled and unlabeled RNA species in a total volume of 0.2 ml of $2 \times \text{SSC}$ (standard saline citrate: 0.15 M NaCl, 0.015 sodium citrate) containing 0.01% sodium dodecyl sulfate were boiled for 3 min and then annealed for 1 hr at 70 C. Digestion of half of each sample with ribonucleases A and T1 was as previously described (10).

^b Total cytoplasmic RNA from 2×10^7 CHO cells infected with B particles in the presence of 100 μg of cycloheximide per ml and labeled for 4 hr with 10 μCi of ^3H -uridine per ml.

^c RNA from purified B particles.

^d Total cytoplasmic RNA from 2×10^7 uninfected CHO cells not exposed to any inhibitors and labeled for 4 hr with 10 μCi of ^3H -uridine per ml.

^e RNA from purified B particles grown on 2.4×10^8 CHO cells labeled with 100 μCi of ^3H -adenosine per ml.

^f Total cytoplasmic RNA from 1.2×10^8 unlabeled CHO cells infected with B particles and incubated in the presence of 100 μg of cycloheximide per ml for 4 hr.

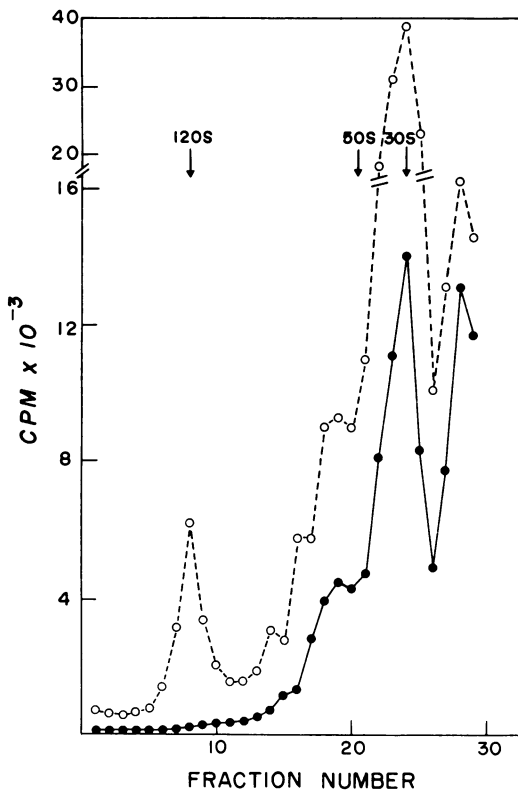


FIG. 4. Viral ribonucleoproteins made in the presence and absence of cycloheximide. Each of two samples made up of twice the usual number of CHO cells was either treated with 100 μg of cycloheximide per ml (●) or not treated (○) and infected with B particles at a multiplicity of 40 in the presence of actinomycin D. At 30 min after infection at 35 C, 2 ml of medium containing 40 μCi of ^3H -uridine were added to each sample. Cells were harvested at 3.5 hr after infection, and the cytoplasmic extracts in 0.02 M ethylenediaminetetraacetate (EDTA) were layered over gradients of 15 to 30% sucrose in NEB (0.01 M Tris, pH 7.4; 0.01 M NaCl; 0.02 M EDTA). The gradients were centrifuged for 6 hr at 27,000 rev/min at 4 C in a Beckman SW27 rotor. Fractions were collected and assayed as previously described (10).

detectable RNA synthesis by T alone at this concentration, whether the cells were treated with cycloheximide or not.

To further rule out the contribution of RNA synthesis directed by T particles in cycloheximide-treated cells, the labeled RNA from an experiment similar to the one shown in Fig. 6 was deproteinized and examined on sucrose gradients containing sodium dodecyl sulfate. Viral RNA in the cycloheximide-treated cells had almost identical patterns whether the cells were infected with B alone or with both B and T particles

(Fig. 7a). The control patterns from infected cells not treated with the inhibitor show the usual pattern of total RNA synthesis by B alone and the dramatic shift to T particle-directed RNA synthesis when T was added to cells together with B particles (Fig. 7b; cf. 20).

Effect of prior incubation of T particles on transcription. To show that the lack of inhibition by T on transcription was not simply due to an inhibition of some hypothetical function of T particles by cycloheximide, cells were preinfected with T particles for 1 hr before the addition of cycloheximide, B particles, and actinomycin D. Such an experiment was possible because of the previous demonstration that infection of cells with defective interfering particles before B particles not only inhibits viral growth but increases the interference over that observed when

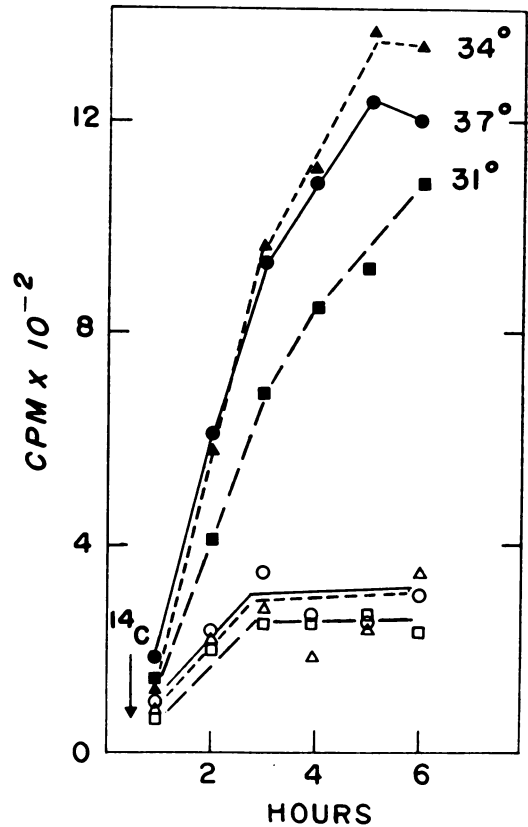


FIG. 5. Effect of temperature of incubation on intracellular transcription. Infected and uninfected Chinese hamster ovary cells were all treated with 100 μg of cycloheximide per ml and incubated at the indicated temperatures. Each sample received 0.6 μCi of ^{14}C -uridine per ml. (●) Infected at 37 C; (○) uninfected at 37 C; (▲) infected at 34 C; (△) uninfected at 34 C; (■) infected at 31 C; (□) uninfected at 31 C.

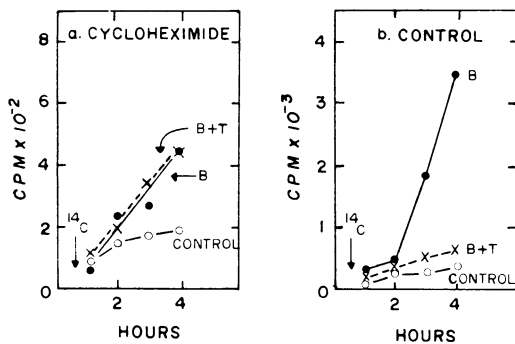


FIG. 6. Effect of T particles on viral RNA synthesis in the presence and absence of cycloheximide. (a) Three samples of Chinese hamster ovary cells, each treated with 100 μ g of cycloheximide per ml were either infected with B particles alone (●), with B and T particles at the same time (X), or not infected (○). (b) Three other samples not treated with cycloheximide were similarly infected. Each tube received 0.6 μ Ci of ¹⁴C-uridine per ml.

cells are infected at the same time with standard virus and defective particles (2). Figure 8 shows that even preincubation of cells with T in the absence of cycloheximide caused no inhibition of this initial transcription. Infected cells similarly pretreated with T particles, but not exposed to cycloheximide, showed that the growth of plaque-forming B particles was inhibited by greater than 99.9%. When the viral RNA products synthesized in the presence of cycloheximide were analyzed in sucrose gradients, only 28S and 13 to 15S labeled RNA were found and in the same amounts, irrespective of whether the cells were infected with B alone, infected with T and B at the same time, or infected with T particles prior to the addition of B particles (Fig. 9). Such unabated synthesis of messenger RNA by input virions in the presence of interfering quantities of defective particles permits the conclusion that viral transcription is not the primary step inhibited by defective interfering particles.

DISCUSSION

If early viral transcription by input VSV is not affected in any way by the presence of T particles, how then is interference accomplished by defective particles? To satisfactorily explain this, it is necessary to account for the alteration in the pattern of newly synthesized viral RNA species along with the inhibition of viral RNA synthesis when there is interference (20). T particles by themselves do not appear to have *in vitro* polymerase activity (4) nor do they synthesize any RNA in CHO cells whether there is cycloheximide present or not (20). When viral antigens

were sought in cells infected only with T particles, none were found (R. R. Wagner, *unpublished observations*). All of these results suggest that T particle RNA is not transcribed into messenger RNA and that it may function only as a replicative entity.

Support for such a conclusion is found in the genetic studies of Reichmann et al. (17) where T particles do not complement any of the temperature-sensitive mutants of VSV. Also, Sreevalsan's

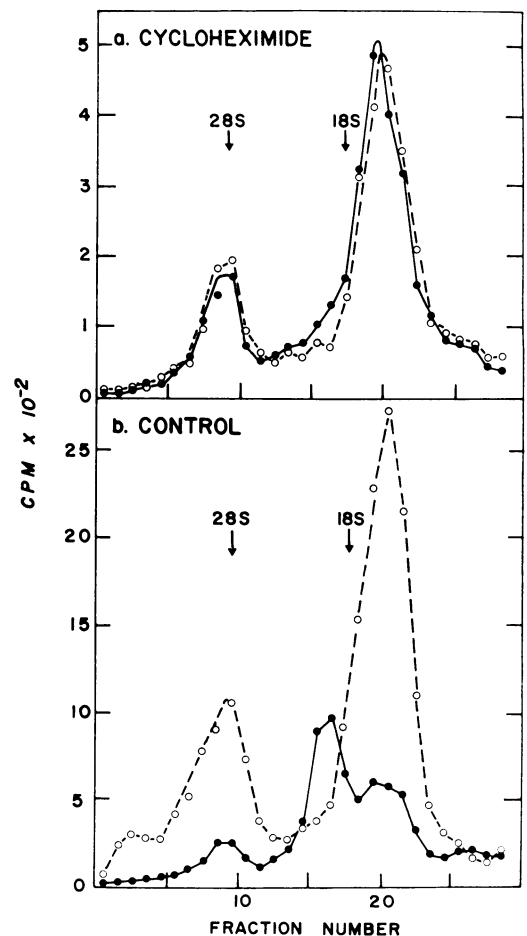


FIG. 7. Sucrose gradient centrifugation of viral RNA made in the presence or absence of cycloheximide and T particles. An experiment identical to the one shown in Fig. 6 but labeled with 1 μ Ci of ¹⁴C-uridine per ml was terminated at 3.5 hr after infection. The cytoplasmic extracts were deproteinized and separated on sucrose gradients containing 0.5% sodium dodecyl-sulfate. (a) Infected with B and T particles in the presence of cycloheximide (●); infected with B particles alone in the presence of cycloheximide (○). (b) Infected with B and T particles (●); infected with B particles alone (○).

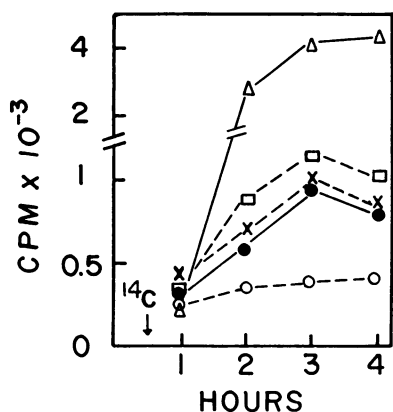


FIG. 8. Lack of inhibition by T particles on transcription by B particles. Two out of five samples of cells were exposed to T particles and all five samples were preincubated for 1 hr at 37 C prior to 0 time of infection with B particles. Each tube received 0.6 μ Ci of 14 C-uridine per ml. (O) Uninfected plus cycloheximide at 0 time; (●) infected with B particles plus cycloheximide at 0 time; (X) pre-exposed to T and infected with B particles plus cycloheximide at 0 time; (□) pre-exposed to T and infected with B particles at 0 time; (Δ) infected with B particles at 0 time.

(19) finding that RNA alone, extracted from T particles, causes interference suggests that viral structural proteins of T particles are unnecessary. Because of these findings it appears likely that T particles interfere at the step of viral RNA replication by competing successfully for replicative enzymes. Such an idea has been proposed previously (20) and the lack of effect of T particles on early transcription by B particles supports that hypothesis. Moreover, studies on another system, Sendai virus, show that defective particles added late in the viral growth cycle preferentially inhibit the synthesis of virion RNA rather than messenger RNA (16). To prove that homologous interference in these two systems is the result of competition for replicative enzymes requires an understanding of the enzyme(s) and template(s) involved during the replication of virion RNA.

The lack of inhibition of early transcription by defective particles, together with the finding that T particles interfere in the presence of actinomycin D, shows that inhibition by defective particles, at least in the rhabdovirus and paramyxovirus groups, is a distinct interference phenomenon and not in any way mediated through the induction of interferon. This points to the differential site of action between the interference induced by homologous defective particles and the interference induced by inter-

feron. Recently, Marcus et al. (13) showed that chick cells pretreated with interferon do not support transcription by VSV as well as untreated cells. By using human muscle skin fibroblasts, similar results have been obtained (Manders, Tilles, and Huang; *manuscript in preparation*).

In comparing intracellular to in vitro VSV RNA synthesis there are several points to note. In addition to the temperature optimum, intracellular transcription results in 28S and 13 to 15S species of messenger RNA, whereas in vitro RNA synthesis makes only single-stranded RNA smaller than 15S whether the synthesis is accomplished by purified virions (3, 7) or by partially purified nucleocapsid fractions from infected cells (L. Prevec, *personal communication*). Despite complementarity of the in vitro RNA products to virion RNA (1) and the almost complete transcription of virion RNA by the enzyme (3), the in vitro polymerase reaction does not adequately represent early intracellular transcription during the course of VSV replication.

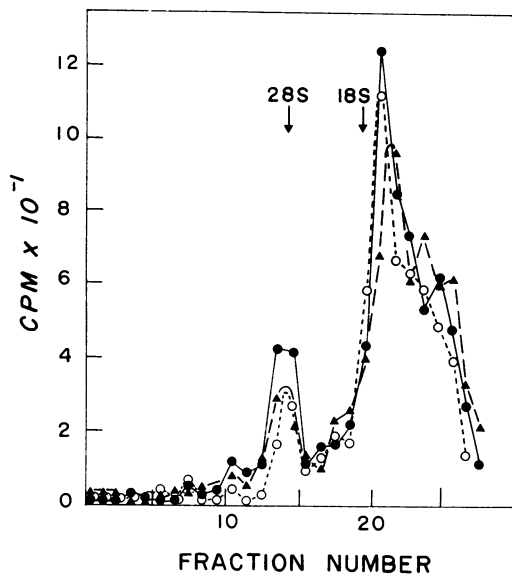


FIG. 9. Identity of intracellular transcription products synthesized by B particles in the presence or absence of T particles. One out of three samples of cells were exposed to T particles, and all the cells in 1 ml of medium were preincubated at 37 C for 1 hr prior to addition of cycloheximide and infection with B particles. One of the tubes which was not previously exposed to T received T particles at the same time as B particles. Cytoplasmic extracts were made after 4 hr of infection and layered on gradients of sucrose-sodium dodecyl sulfate. (●) Infected with B particles alone; (○) infected with B and T particles at 0 time; (▲) pre-exposed to T and then infected with B particles.

Until it is understood why the *in vitro* products differ in size from the intracellular messenger RNA, observations made on the *in vitro* polymerase assay can be extended to viral replication only with caution.

The annealing data presented here lead to several interesting conclusions aside from demonstrating that RNA made in the presence of cycloheximide was complementary to virion RNA. When virion RNA was labeled with ^3H -adenosine and digested with ribonucleases A and T1, essentially no ribonuclease-resistant core was detected. This indicates that virion RNA contains no long sequences of poly A, whereas digestion of ^3H -adenosine-labeled 13 to 15S messenger RNA of VSV showed that these RNA species contained poly A sequences which represented about 25% of the total ^3H -adenosine incorporated into the RNA (Soria and Huang, *unpublished observations*). The presence of poly A in VSV messenger RNA has been suggested by the base composition studies of Mudd and Summers (14). It should be possible to study the function of poly A by using the VSV system where the virion RNA, lacking poly A, serves as a template for the synthesis of messenger RNA which contains poly A.

Another conclusion from these annealing studies is that the genome of VSV appears to be completely transcribed intracellularly by the virion-associated enzyme. It would be safe to generalize for viruses which carry the RNA-dependent transcriptase as part of virions that the first synthetic event after uncoating is copying of the complete viral genome into multiple, complementary messenger RNA species.

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