

Replication of viral RNA by a defective interfering vesicular stomatitis virus particle in the absence of helper virus

(3' half of viral genome/primary transcription/replicative intermediate RNA/ribonucleoprotein synthesis/autointerference)

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ABSTRACT The genome of a defective interfering particle (DI_{LT}) derived from the heat-resistant strain of vesicular stomatitis virus is expressed *in vivo* without the assistance of infectious helper virus. The rates of RNA synthesis in the presence of cycloheximide (primary transcription) are the same when infections are with equal numbers of physical particles of DI_{LT} or virus. With this treatment, DI_{LT} synthesizes only 12-17S mRNAs as characterized by size, polarity, and polyadenylation. In the absence of cycloheximide, DI_{LT}-infected cells produce not only these mRNAs but also a 28S RNA species. This RNA, which represents one half of the viral specific RNA, contains newly synthesized full-length (+) and (-) strand DI_{LT} RNA. Both strands are found intracellularly as ribonucleoprotein complexes. Without cycloheximide present, the rate of RNA synthesis by DI_{LT} was less than that by virus. This curtailment is most likely due to the inability of DI_{LT} to synthesize L protein mRNA. An expanded role for defective interfering particles in infection is discussed.

The genomes of defective interfering particles derived from the 5' end of vesicular stomatitis virus (VSV) are not expressed in cultured cells and are replicated only when helper virus is present (1-4). Investigations with several such particles have supported this observation by demonstrating that these particles are not transcribed *in vitro* (5-7). Emerson and Wagner (8) have shown that this deficiency resides in the ribonucleoprotein template and not in the virion transcriptase. It has been proposed that the inability to produce transcripts is related to a deletion of a 3'-terminal transcription initiation sequence (4). Indeed, the RNAs present in these particles have no genetic information equivalent to the 3' end of the VSV genome that is detectable by annealing but do have partial information from the 5' half (9, 10).

Defective interfering particles with genetic information of the 3' end of VSV RNA have been isolated (11, 12). The genome of one of these particles (DI_{LT}) has 3'-terminal sequences complementary to the leader RNA sequence transcribed from the extreme 3' end of VSV (13). Purified DI_{LT} produces transcripts *in vitro* as efficiently as VSV; the transcription products are 12-17S mRNA species (13, 14). Recently, the synthesis of G and M proteins by cells infected with both DI_{LT} and heterologous helper VSV was reported (15).

This paper demonstrates that the infecting genome of pure DI_{LT} is transcribed *in vivo* producing 12-17S mRNA species in the absence of helper virus. When replication is blocked with cycloheximide, VSV and DI_{LT} synthesize equivalent amounts of RNA. In the absence of cycloheximide, DI_{LT} is able to carry out the first steps of replication because new full-length (+) and (-) strand DI particle RNAs and ribonucleoproteins are observed.

MATERIALS AND METHODS

Preparation of Virus Stocks. Monolayers of BHK-21 (C-13) cells (ATCC no. CCL 10) were grown as described (16, 17). VSV_{IND}, the Mudd-Summers strain (18), was prepared by infecting these monolayers at an 0.1 multiplicity of infection in medium with 2% fetal calf serum. After 18 hr, the culture medium was clarified by centrifugation at 10,000 × g for 20 min. Aliquots of the supernatant were frozen at -70°. The DI_{LT} was prepared by infecting monolayers with a third high-multiplicity passage of VSV_{HR} obtained from L. Prevec and purified as described (9).

Analysis of DI_{LT} Virus Preparation. The number of plaque-forming particles in the DI_{LT} inoculum was determined on monolayers of BHK-21 (C-13) cells with a 0.75% methyl cellulose overlay. An internal standard of VSV was added to dilutions of DI_{LT} before plating so that the reduction in plaquing efficiency due to autointerference could be determined. A 50% decrease in the number of VSV plaques was observed. In order to enumerate directly the standard VSV particles contaminating the DI_{LT} preparation, the stock was diluted 1:250 and processed for electron microscopy as described (16). Six negatives were enlarged and fields containing a total of 2722 particles were counted. The minimum number of VSV particles was determined from counts of clearly discernible full-length virus. In addition, other large forms possibly representing distorted VSV or aggregated DI_{LT} were counted for a maximum estimate of VSV. VSV contamination of the DI_{LT} preparation was also analyzed from the relative proportions of the 42S and 28S RNAs—i.e., the full-length vRNA species of VSV and DI_{LT}, respectively (9).

Virus Infections. BHK-21 cells adapted for growth in suspension cultures were cultivated in Eagle's minimal essential medium modified for suspension culture and supplemented with 5% fetal calf serum, penicillin and streptomycin (5 units/ml each), 2 mM glutamine, and twice the normal amount of vitamins. When the culture reached a density of 5 to 10 × 10⁵ cells/per ml, the cells were concentrated by centrifugation at 2000 × g for 3 min and resuspended at 6 × 10⁶ cells per ml in suspension medium plus 0.014 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer at 37°. The concentrated cultures were incubated at 37° in a shaker water bath. Actinomycin D (10 μg/ml) was added 30 min prior to the virus inoculum. Cycloheximide treatment was begun 10 min before infection. Virus was added at the specified number of physical particles per cell, and the shaker speed was reduced

Abbreviations: VSV, vesicular stomatitis virus; DI_{LT}, defective interfering particle from heat-resistant strain of VSV; DI_T, defective interfering particle from 5' end of VSV genome.

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Table 1. Purity of DI_{LT} inoculum

| Assay | Titer, × 10 ⁻¹⁰ /ml |
|---|-----------------------------------|
| DI _{LT} physical particles: ^a | |
| Electron micrographs | 95 |
| VSV physical particles: ^a | |
| Electron micrographs | 0.07–0.28 |
| From plaque-forming units | 0.04–0.09 |
| Plaque-forming units ^a | 0.008 |

^a Physical particles and plaque-forming units were estimated as described in *text*.

during a 20-min adsorption period. Labeling was initiated with the addition of [³H]uridine (specific activity, 23 mCi/μmol; Schwarz/Mann) to a final concentration of 20 μCi/10⁶ cells. At the same time the cells were diluted to 3 × 10⁶ cells per ml and the shaker speed was increased to ensure suspension of the cells.

Preparation of Cytoplasmic Extracts, Ribonucleoproteins, and RNA. At 4 hr after infection, cells were harvested by centrifugation at 2000 × *g*, for 3 min. Cytoplasmic extracts were prepared as described (10) with 0.01 M Tris-HCl, pH 7.5/0.01 M NaCl/0.02 M EDTA as the swelling buffer. For analysis of ribonucleoproteins, the cytoplasmic extract was layered directly on 5–20% sucrose gradients in the same buffer containing 1 M NH₄Cl. Centrifugation was at 40,000 rpm for 2 hr at 4° in a Beckman SW 41 rotor. For preparation of RNA, the cyto-

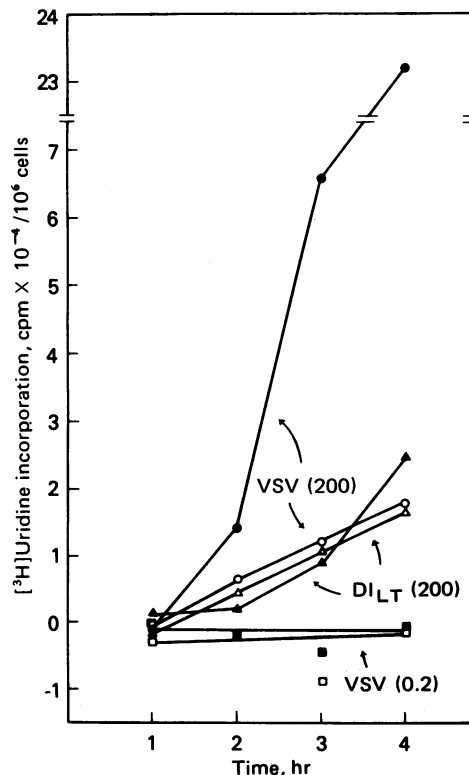


FIG. 1. RNA synthesis by DI_{LT} and VSV in the absence and presence of cycloheximide. BHK-21 suspension cells were prepared for infection as described in *Materials and Methods*. DI_{LT} was used at 200 particles per cell (Δ) and VSV at both 200 (○) and 0.2 (□) particles per cell. Parallel experiments were done with cycloheximide at 100 μg/ml (open symbols). At the indicated times, duplicate aliquots were precipitated with trichloroacetic acid and processed for liquid scintillation counting. Radiolabel incorporated by uninfected control cells was subtracted at each time point and represented about one-third the radioactivity incorporated by DI_{LT}.

plasmic extract was made 1% in sodium dodecyl sulfate and the RNA species were separated on 10–30% (vol/vol) sucrose in 0.1 M NaCl/0.05 M Tris, pH 7.0/1 mM EDTA containing 0.1% sodium dodecyl sulfate (20,000 rpm 16 hr, 20°, Beckman SW 41 rotor). RNA was located by trichloroacetic acid precipitation and was precipitated in 0.4 M NaCl with ethanol. The pellets were resuspended in diethylpyrocarbonate-treated 1 mM Tris, pH 7.2/1 mM EDTA.

Preparation of Viral mRNA for Annealing. Total RNA was extracted from unlabeled VSV-infected cells at 4 hr after infection as described (10). The small amount of (–) strand RNA was sequestered in duplexes with mRNA by self-annealing in 0.4 M NaCl/0.01 M NaP_i, pH 6.8, at 60°. This preparation was annealed to labeled 42S RNA and 12–17S mRNA to verify that only (+) strand RNA was available for duplex formation.

RESULTS

Purity of DI_{LT} Inoculum. In order to ascribe functions such as transcription and replication of RNA to defective interfering particles themselves, the contribution of contaminating standard virions must be negligible. We estimated the amount of VSV in the DI_{LT} stock by several methods. First, because the virus was grown in the presence of [³H]uridine, the relative amounts of ³H-labeled 42S and 28S RNA could be used as a gross estimate. Less than 1% of the RNA sedimented as 42 S, indicating that less than 0.5% of the particles were VSV (9). Second, the size difference between VSV and DI_{LT} is large enough so that they can be distinguished in electron micrographs. With this technique, a maximum of 1/340 and a minimum of 1/1361 physical particles were large enough to be VSV contaminants (Table 1). Third, the number of VSV particles can be estimated from the infectivity (plaque-forming units/ml) and the measured efficiency of plaquing under our conditions, 9–20%. Estimates derived in this way place the level of VSV contamination at between 1/1056 and 1/2375 particles. Comparison of the biological activities (i.e., plaque-forming particles and interfering particles) indicates that there were

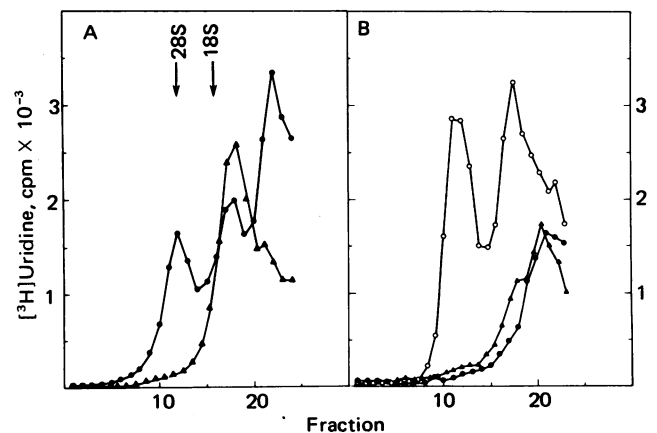


FIG. 2. (A) Sucrose gradient separation of RNA made by DI_{LT} in the absence and presence of cycloheximide. BHK-21 suspension cells without (●) or with (▲) cycloheximide were infected with DI_{LT} at 200 particles per cell. [³H]Uridine (20 μCi/10⁶ cells) was added. At 4 hr, cells were harvested and RNAs were separated on 10–30% (wt/vol) sucrose gradients. Radioactivity in 50-μl aliquots of each fraction was determined after trichloroacetic acid precipitation. Sedimentation of ribosomal RNA markers was determined by measurement of absorbance at 260 nm. (B) Sedimentation of RNAs from uninfected control cells and from cells infected with VSV and DI_{LT}. Cells were infected with DI_{LT} at 200 particles per cell (○) or with VSV at 0.2 particles per cell (▲) in the absence of cycloheximide. The control (●) represents similarly treated uninfected cells. Other procedures were performed as in (A).

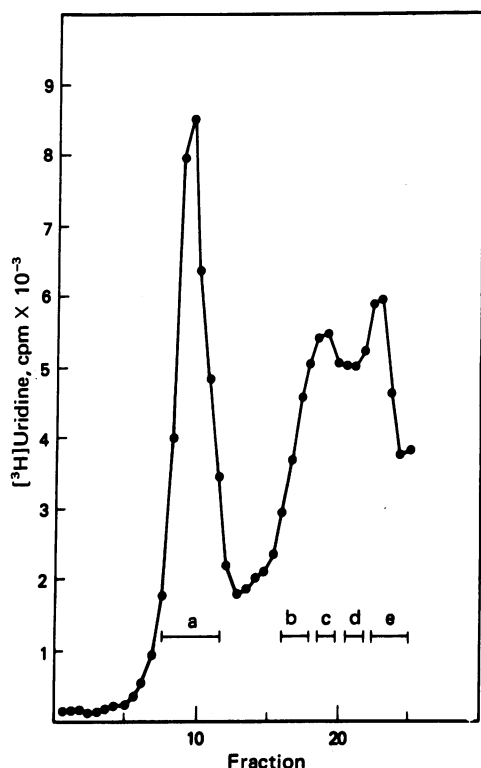


FIG. 3. Sucrose gradient separation of DI_{LT} RNA for characterization experiments. Infection with DI_{LT} in the absence of cycloheximide and RNA preparation were performed as described in Fig. 2. The indicated fractions were combined, ethanol precipitated, resuspended in 1 mM Tris, pH 7.2/1 mM EDTA, and stored at -70° .

about 8000 more biologically active interfering particles than biologically active VSV particles (19). In all work presented here, it was assumed that 1/1000 physical particles in this DI_{LT} preparation was a standard VSV particle.

Total RNA Synthesis by DI_{LT} . Both DI_{LT} and VSV bring into a cell the ribonucleoprotein template and viral proteins required for primary transcription. The infective process of these particles can be limited to primary transcription by inhibiting protein synthesis with cycloheximide (20). This blockade permits the transcriptive abilities of the two types of particles to be compared by measuring the incorporation of RNA precursors by equivalent numbers of particles. As can be seen in Fig. 1, throughout the course of a 4-hr infection in the presence of cycloheximide, DI_{LT} synthesized as much RNA as VSV did. The incorporation by DI_{LT} was not due to VSV contamination because infection with standard VSV at the contaminating level gave no incorporation above that of the uninfected control. For comparison, DI_{LT} incorporation in the absence of cycloheximide was 14% of VSV at 4 hr. In most experiments, DI_{LT} incorporated more label in the absence of cycloheximide than in the presence of cycloheximide at all time points.

The RNA of DI_{LT} is 28 S and anneals to 12–17S mRNAs (9, 19, 21). Therefore, primary transcription is expected to produce 12–17S mRNAs. Any unprocessed (+) strand or full-length RNA synthesized would be expected to have a sedimentation rate of 28 S. Synthesis of such specific RNAs by DI_{LT} was examined by labeling the products with [3H]uridine in the absence and presence of cycloheximide and analyzing them on sucrose gradients (Fig. 2A). A peak of radioactive material appeared at the top of the gradient (fractions 20–24). Most of this material, which also is synthesized by uninfected control cells (Fig. 2B), did not appear to be RNA because it was resistant to RNase

Table 2. Characterization of RNAs

| Sample ^a | % RNase resistance | | | |
|---------------------|----------------------|-------------------|----------------------|------------------------|
| | Initial ^c | Annealed to | | |
| | | Self ^d | VSV RNA ^e | % poly(A) ^b |
| b | 3 | 10 | 78 | 56 |
| c | 4 | 9 | 72 | 9 |
| d | 11 | 19 | 46 | 1 |
| e | 40 | 40 | 49 | 1 |

^a Fractions were pooled into samples as indicated in Fig. 3, ethanol precipitated, and resuspended in 1 mM Tris, pH 7.2/1 mM EDTA.

^b An aliquot of each RNA fraction was adjusted to 0.1% sodium dodecyl sulfate, 0.5 M NaCl, and 0.01 M Tris, pH 7.6, and chromatographed on an oligo(dT)-cellulose column equilibrated with the same buffer but without sodium dodecyl sulfate. Elution was performed as described (17). The percentage of recovered radioactivity that eluted in 0.01 M Tris, pH 7.6, is reported.

^c The sample was heated at 100° for 1 min and then cooled; resistance to RNases A and T_1 (50 μ g/ml and 2 units/ml, respectively) in 0.4 M NaCl/0.01 M NaPi, pH 6.8, was determined at time 0.

^d Annealing was carried out at 60° in 0.4 M NaCl/0.01 M NaPi, pH 6.8. Aliquots were removed at various times and RNase resistance was determined as in e. The percentages represent saturation values (8–22 hr).

^e The same concentrations of samples b–e were used as in d above and VSV RNA was added at a final concentration of 42 μ g/ml. The percentages are values at saturation (2–22 hr).

in dilute salt, even after heating to 100° . A peak of labeled RNA (fractions 15–19) migrated just slower than 18S rRNA and therefore was of a size consistent with its being 12–17S mRNA. Like the synthesis of mRNA primary transcripts, it was not sensitive to cycloheximide. At fractions 10–13 and coincident with 28S rRNA was a third peak that could be newly synthesized DI_{LT} RNA because it was eliminated by cycloheximide treatment. Both the middle and bottom peaks were completely sensitive to RNase, even in the presence of 0.4 M NaCl. The relative amounts of the 28S and 12–17S RNA peaks varied between experiments. The observed extremes are shown in Figs. 2A and 3.

mRNA Synthesis by DI_{LT} . VSV mRNAs have several properties by which they can be identified: size, polyadenylation at their 3' end, a capped and methylated sequence at the 5' end, and polarity (+ sense). The RNAs synthesized by the DI_{LT} particle were analyzed for some of these characteristics. The two peaks sedimenting slower than 18 S were divided into four samples as illustrated in Fig. 3. On the basis of their RNase sensitivity, both initially and after self-annealing, samples b and c contained little (–) strand RNA (Table 2). Annealing to excess 42S VSV RNA revealed that 78% of the RNA in sample b and 72% of the RNA in sample c was messenger sense RNA. The proportion of RNA in samples b and c having poly(A) ends was 56% and 9%, respectively. This decrease in polyadenylation as the S value decreases has been reported (13). Approximately 35% of the material in sample d was (+) RNA. Sample e did not contain any VSV RNA but did contain material similar to that found in uninfected control cells. The presence of methylated and capped structures was not examined.

Synthesis of (+) and (–) Strand DI_{LT} RNA. The 28S RNA peak from DI_{LT} -infected cells (Fig. 3, sample a) migrated to a position expected of newly synthesized DI_{LT} RNA. However, several other reasonable explanations for this peak were considered. The L protein mRNA coded for by VSV but not DI_{LT} would migrate to this region of the gradient. However, the rate of RNA synthesis by cells infected with VSV at the level that contaminates the DI_{LT} inoculum is the same as the uninfected

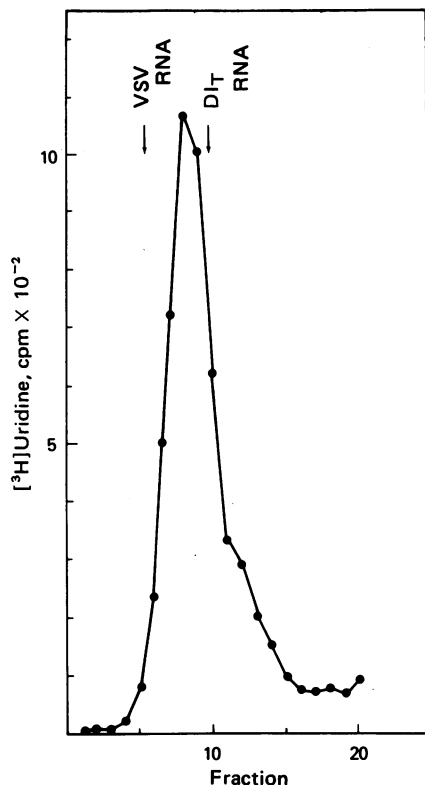


FIG. 4. Sedimentation of 28S RNA in denaturing dimethyl sulfoxide/sucrose gradients. A portion of 28S RNA (Fig. 3, sample a) was again precipitated with ethanol. The pellet was resuspended in 80% dimethyl sulfoxide in 10 mM LiCl/1 mM EDTA/10 mM Tris, pH 7.5. The RNA was layered on 5–20% sucrose gradients in the same buffer and was centrifuged in a Beckman SW 50.1 rotor at 49,000 rpm for 18 hr at 20°. Radioactivity in each fraction was determined by trichloroacetic acid precipitation. The positions of VSV RNA (42 S) and DI_T (19 S) RNA markers were analyzed in identical gradients in the same run.

control (Fig. 1). Analysis of RNA made under these same conditions is shown in Fig. 2B. Infection with VSV at 0.2 particles per cell produces no RNA the size of 28S mRNA or 42S vRNA. If significant amounts of L protein mRNA were present in sample a RNA, these could be detected by annealing with limiting amounts of [^{32}P]RNA derived from a defective interfering particle (DI_T) that contains part of the gene for L protein (9, 10). However, after annealing under our standard conditions for 28 hr, there was no increase in the resistance of the [^{32}P]RNA to RNase. Furthermore, the sensitivity of the 28S RNA to cycloheximide also strongly indicated that it contained no mRNA (Fig. 2A). We conclude from these data that the 28S RNA species is not L protein mRNA.

The 28S RNA peak could also represent transcriptive intermediates—i.e., newly synthesized and incomplete mRNAs that remain attached to the input template by hydrogen bonding after deproteinization. These species usually sediment slightly slower than the template RNA in standard RNA gradients. However, such complexes are dissociated in 80% dimethyl sulfoxide and, because the mRNAs are smaller, they are separated from their template on gradients that also contain 80% dimethyl sulfoxide. The pattern obtained from 28S RNA after such treatment is shown in Fig. 4. Only 7% of the radiolabel in the 28S RNA peak migrated slower than a 19S RNA marker from DI_T particles and could therefore represent nascent or incomplete mRNAs from transcriptive intermediates. Thus, as was indicated earlier by the sensitivity of the 28S peak to the presence of cycloheximide, very little ($\leq 7\%$) of the radiolabel in the large RNA is due to mRNAs.

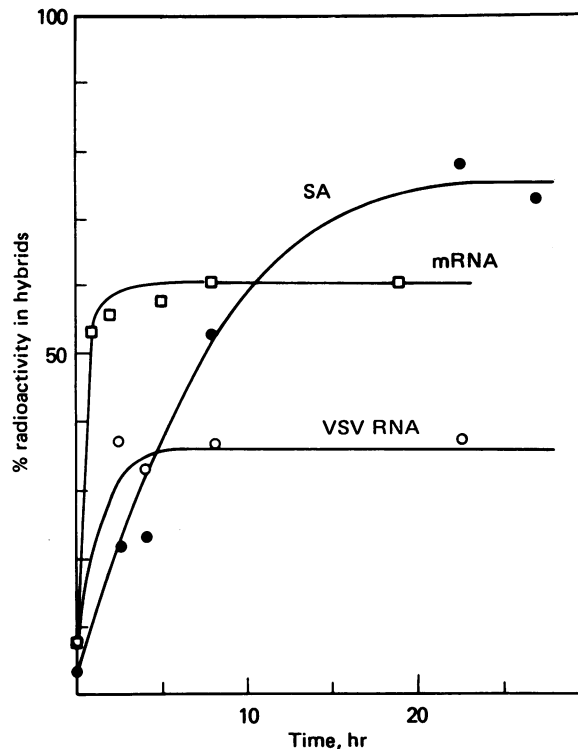


FIG. 5. Characterization of 28S RNA by annealing. Self-annealing at 60° was performed on 28S RNA after heating at 100° for 1 min, cooling, and adjusting to 0.4 M NaCl/0.01 M NaP_i, pH 6.8 and a final volume of 0.25 ml (●). For determination of (+) strand RNA, 28S RNA was incubated with 7.38 μ g of unlabeled DI_T RNA under the above conditions (○). The amount of (–) strand RNA was measured by annealing (without prior heating) the 28S RNA and an excess of viral mRNA prepared as described in *Materials and Methods* (□). At the indicated times, samples were removed for trichloroacetic acid precipitation before and after RNase treatment.

The 28S RNA was also examined for poly(A)-containing RNA. Only 3% of the radioactivity in this peak bound to oligo(dT)-cellulose. This could represent contamination of the 28S RNA with the neighboring mRNAs that are polyadenylated (see Fig. 3). On the other hand, a small amount ($<10\%$) of the RNA could have a poly(A) end (22, 23). These two possibilities have not been resolved. We conclude that the RNA in question is not mRNA but rather a single-stranded full-length RNA species from DI_T .

The presence of (+) and (–) strands and their relative amounts was determined by annealing the labeled 28S RNA to unlabeled (–) and (+) strand RNAs (Fig. 5). In the presence of excess unlabeled DI_T RNA, all (+) strand species will become resistant to RNase by forming duplexes with the added RNA. Under these conditions, 37% of the 28S RNA formed hybrids. Annealing a large excess of mRNA to the 28S RNA indicated that 61% of the RNA was (–) strand. These relative abundances of (+) and (–) strand were confirmed in self-annealing experiments in which, in the absence of added RNA, the strand present in the lesser quantity will govern the amount of self-annealing. Because (+) strand represented 37% of the total radioactivity in the 28S peak, self-annealing should yield 74% of the radioactivity in hybrids, assuming both strands have the same radio specific activity. The value of 77% obtained from such an experiment confirmed this prediction. We conclude from the annealing data that the 28S RNA synthesized in DI_T -infected cells is composed of approximately 37% (+) strand and 61% (–) strand species.

Presence of Ribonucleoprotein Structures. In cells infected

with VSV, the full-length 42S RNA is encapsidated with N protein to form a ribonucleoprotein that is resistant to ribonuclease (22). Similar ribonucleoprotein structures containing defective interfering genomic RNA have been observed in autointerfered infections (16). The presence of full-length (+) and (-) 28S RNA in the cytoplasm of cells infected with only DI_{LT} suggested that these too may be present as ribonucleoproteins. [3H]Uridine-labeled extract of DI_{LT} -infected cells was analyzed on nondeproteinizing sucrose gradients that separate ribonucleoprotein from unencapsidated RNA and on deproteinizing sucrose gradients that separate 28S RNA from viral mRNAs. DI_{LT} ribonucleoprotein was identified by its sedimentation rate and RNase resistance. Slightly more radioactivity was found in the ribonucleoprotein structure than in the 28S RNA (data not shown). Analysis by sedimentation in dimethyl sulfoxide/sucrose gradients of RNA prepared from these ribonucleoprotein fractions indicated that at least 75% was 28S RNA. The remaining RNA made from ribonucleoprotein sedimented slower than 19 S and is presumed to be nascent mRNA. These experiments indicate that 90% of the radioactivity in the 28S RNA is encapsidated in a DI_{LT} ribonucleoprotein.

DISCUSSION

This paper demonstrates that the genome of DI_{LT} is expressed in the absence of helper VSV. Discrete mRNA species, replicative intermediate RNA, and new genomic RNA all are produced. This finding is in direct contrast to results obtained with other defective interfering particles infecting tissue culture cells (1-4). Like these other particles, DI_{LT} has all five of the standard viral proteins, has the ability to interfere with the replication of VSV, and has a genome that is shorter than that of VSV and that anneals to VSV mRNAs (9-11, 21). However, the RNA of DI_{LT} is different in that it is derived from the 3' end of the VSV genome and contains information for the leader sequence and for four of the viral proteins: N, NS, M, and G (9-11, 13). It only lacks a functional gene for L protein.

Although RNA is synthesized, infections with DI_{LT} are still defective. The problem does not reside in primary transcription since that process is equivalent in DI_{LT} - and VSV-infected cells. Although DI_{LT} does not code for L protein and no L mRNA or protein is observed, both replicative intermediate RNA and genomic RNA are made. Hence, it appears that synthesis of new L protein is not necessary for synthesis of full-length genomic RNA—i.e., replication. This does not rule out the possibility that incoming L protein is modified before replication begins. Furthermore, these RNAs are found in ribonucleoprotein structures as is the case for RNA made by the standard virus particle (22, 23). Thus, the presumed template for secondary transcription and amplification [ribonucleoprotein containing (-) strand RNA] is synthesized in DI_{LT} -infected cells. Because these two events do not occur to an appreciable extent, we believe that the transcriptase and replicase functions, although present in the input DI_{LT} particles, are ultimately limiting. This is consistent with the inability of the DI_{LT} genome to code for the L protein, which is part of the transcriptase (9, 10, 24).

The biochemical processes of DI_{LT} have interesting biological potential. For example, VSV is a cell-killing particle. This property depends on transcription of the one-fifth of the viral genome closest to the 3' end and translation into minimally functional N and NS proteins (25, 26). Because DI_{LT} codes for N and NS mRNAs and because mRNAs are produced in DI_{LT} -infected cells, we might expect this virus to be a cell-killing particle. Recently, Marcus *et al.* (19) have shown that DI_{LT} kills cells at about 10% the efficiency of VSV.

Demonstration that the genes of one defective interfering particle are expressed also expands the possible roles for such particles in natural virus infections. Previously, such particles were shown to modulate viral infection in tissue culture. They decreased the yield of infectious particles by interfering with replication of their genome (1, 4). Related studies in animals demonstrated that defective interfering particles from the 5' end of VSV were innocuous when injected intracerebrally in mice (3). Additionally, they were able either to protect totally against the rapid death caused by VSV or to change the nature of disease, depending upon the inoculation schedule. At least part of the protection elicited by these particles appeared to be due to activation of the host immune responses (27). The effect of injection of pure DI_{LT} into animals is not known. The demonstrated cytolytic properties of this particle (19) could produce observable tissue destruction and disease symptoms. The severity, of course, is expected to be less because DI_{LT} is 10% as efficient as VSV in cell killing and because production of new DI_{LT} particles is limited by the inability of this virus to synthesize the L protein. Recently, we have obtained evidence that small amounts of the G, N, and M proteins are synthesized in cells infected with only DI_{LT} (unpublished data). This protein synthesis suggests that infection with DI_{LT} may stimulate the host's protective immune response to a greater degree than found with previously studied defective interfering particles.

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