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In vitro synthesis of messenger RNA by a defective interfering particle of vesicular stomatitis virus

(transcriptase/autointerference)

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ABSTRACT **A defective interfering particle derived from the heat-resistant strain of vesicular stomatitis virus was analyzed for the presence of virion-associated RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) activity. The RNA synthesizing capacity of the defective particles *in vitro* was similar to that of the wild-type virus. Characterization of the RNA produced *in vitro* indicated that the defective particles were able to synthesize vesicular stomatitis virus leader RNA and four virus mRNA species that sediment at 12–18 S. These RNA products were identical to the mRNAs synthesized *in vitro* by the wild-type virus in regard to size, polyadenylation, capping, and methylation. In contrast to the wild-type virus, the purified defective particles did not synthesize the large mRNA species sedimenting at 31 S *in vitro*. Possible mechanisms of homotypic and heterotypic interferences shown by this defective particle are discussed.**

Repeated high-multiplicity passage of many animal viruses yields defective interfering particles (DI) (1). These DI particles contain a part of the viral genome and normal viral structural proteins, reproduce only in the presence of helper viruses, and interfere specifically with the intracellular growth of homologous viruses (1). In the vesicular stomatitis virus (VSV) system, various types of DI particles are generated when different wild-type (*wt*) stocks of the same serotype are used to infect different host cells (2–6). The size of the RNA genomes of VSV DI particles range from 10 to 50% of the *wt* genome RNA which has a molecular weight of 4×10^6 (2–6). The RNAs of all the DI particles studied so far contain base sequences complementary to the 31S VSV mRNA species, except for a DI particle obtained from the heat-resistant mutant of VSV Indiana serotype (7, 8) and the DI particles generated from VSV-infected baby hamster kidney cells (5).

The observation that the DI particles contain the same proteins (9, 10), including the transcriptase (11), as the *wt* viruses yet are incapable of self-replication suggests that the genome RNA of the DI particles may lack the nucleotide sequences essential for initiation by the virion-associated transcriptase. However, there are reports that purified DI particles can synthesize RNA *in vitro*, although the RNA products synthesized were not characterized (12, 13). The genome RNA of DI particles appears to be directly involved in the process of interference because UV-irradiated DI particles lose this capacity (14). Thus, it seems that the DI particles are replicated at the expense of the *wt* virus by the continuous use of the replicase function supplied by the *wt* virus. The precise intracellular role of the DI particles in interference is still unclear.

A DI particle of the heat-resistant strain of VSV, commonly known as "long truncated particle" (abbreviated here as DI-LT), contains a homogeneous single-stranded RNA genome of approximately half the length of the *wt* VSV genome (15) and contains base sequences complementary to the VSV 12–18S mRNA species (7, 8). Recent experiments have indicated that VSV 12–18S mRNA species (which contain four mRNA species coding for the viral structural proteins G, M, NS, and N) (16) map contiguously on the VSV genome RNA in the order 3′-N-NS-M-G (17, 18). This indicates that the genome of the DI-LT has been generated by deletion of approximately half of the VSV genome RNA from the 5′-terminal portion. This DI particle is also unique since it exhibits heterotypic inhibition with the New Jersey serotype of VSV (7, 19). Recent work by Ball and White (17) and Abraham and Banerjee (18) have indicated that with *wt* VSV, transcription proceeds from a single site at the 3′ terminus of the genome RNA and that the mRNAs are formed sequentially, possibly by a processing mechanism. Moreover, Colonno and Banerjee (20) have demonstrated that a small RNA molecule (designated "leader RNA") is synthesized first during transcription and is released prior to synthesis of the mRNAs. Mapping studies have indicated that the leader RNA is coded for at the 3′ terminus of the VSV genome RNA (21) and that the base sequences complementary to the leader RNA presumably represent the recognition site for the virion-associated transcriptase in the *wt* VSV.

Because the DI-LT described above appears to be generated from the 3′-terminal half of the *wt* genome RNA, it was of interest to investigate DI-LT genome RNA for the presence of nucleotide sequences complementary to the leader RNA. If the sequences are indeed present, then the transcriptase in the DI-LT particles should be capable of initiating transcription similar to the *wt* virus. In this communication, we demonstrate that purified DI-LT particles behave similarly to the *wt* virus in their capacity to synthesize RNA *in vitro* with the exception of that portion of the gene that is deleted from its genome.

MATERIALS AND METHODS

Growth and Purification of Virus Particles. VSV (Indiana serotype) particles were grown in baby hamster kidney cells (BHK-21, clone 13) adapted to suspension culture, in the presence or absence of [^{3}H]uridine and were purified as described previously (22, 23). VSV DI-LT particles were prepared by infecting monolayers of these cells (4) with a third high-multiplicity passage of a heat-resistant strain of VSV obtained from L. Prevec, McMaster University, Hamilton, ON. One-tenth of the monolayers were labeled with [^{3}H]uridine (specific activity, 23 mCi/μmol) at 5 μCi/ml. After 18 hr, culture medium from virus-infected monolayers was clarified by centrifugation at $8000 \times g$ for 20 min at 4°. The virus was concentrated 25-fold by a Millipore molecular filtration cassette system. The concentrated virus was layered directly on 15-ml potassium tartrate gradients 15–45% (wt/wt) (21) and centrifuged for 2 hr at 24,000 rpm at 4° in a Spinco SW-27 rotor. The

Abbreviations: DI, defective interfering; VSV, vesicular stomatitis virus; DI-LT, long truncated DI particle; BHK, baby hamster kidney cells; $NaDodSO_4$, sodium dodecyl sulfate.

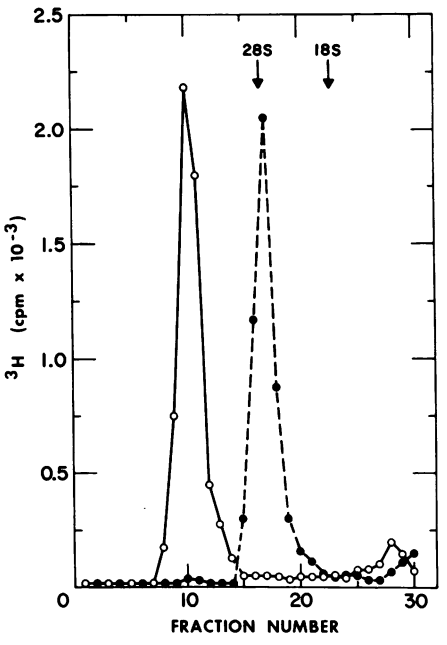

FIG. 1. Velocity sedimentation analysis of *wt* VSV and DI-LT particle RNAs. [^{3}H]Uridine-labeled virions were disrupted with 0.5% (wt/vol) $NaDodSO_4$, and the genome RNA of the *wt* (○) and DI-LT (●) particles was analyzed directly by sedimentation through a 15–30% (wt/vol) sucrose gradient containing 10 mM Tris·HCl (pH 8.0), 0.1 M NaCl, 1 mM EDTA, 0.5% $NaDodSO_4$. Centrifugation was for 17 hr at 21,000 rpm in a Beckman SW40 rotor at 24°. Gradients were fractionated (0.4 ml) directly into counting vials and assayed for radioactivity by the addition of 0.5 ml of H_2O and 8 ml of Aquasol 2. Positions of [^{3}H]uridine-labeled 18S and 28S ribosomal RNA sedimented in a parallel gradient are indicated by arrows.

virus bands were collected and the [^{3}H]uridine-labeled DI-LT particles were separated from the *wt* virus by rate zonal sedimentation in 10–40% (wt/vol) sucrose gradients as previously described (4).

***In Vitro* RNA Synthesis.** *In vitro* RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) reactions (0.2 ml) contained 50 mM Tris·HCl (pH 8.0), 0.1 M NaCl, 5 mM $MgCl_2$, 4 mM dithiothreitol, 0.05% (vol/vol) Triton N-101, 10 mM S-adenosyl-L-methionine, 0.1 mM UTP, 1 mM ATP, CTP, and GTP, and 10 μCi of [^{3}H]UTP (10 Ci/mmol). For [β,γ-^{32}P]ATP labeling, 40 μCi of [β,γ-^{32}P]ATP (25 Ci/mmol) was added and the concentration of unlabeled ATP was lowered to 0.4 mM. Reaction mixtures were incubated with purified *wt* VSV or [^{3}H]uridine-labeled DI-LT particles, 0.05 mg/ml for 5 hr at 30°.

L cells extracts, prepared as described by Friedman *et al.* (24), contained 7 mg of protein per ml and had an absorbancy at 260 nm of 28 units/ml. VSV leader RNA was synthesized *in vitro* in the presence of [α-^{32}P]GTP and purified as previously described (20). The procedures used for phenol extraction, Sephadex G-50 chromatography, oligo(dT)-cellulose chromatography, polyacrylamide gel electrophoresis, and isolation of released product RNA from virus cores have been detailed previously (18, 20). [^{3}H]UTP was purchased from Amersham/Searle, Urbana, IL, [β,γ-^{32}P]ATP from International Chemical and Nuclear, Irvine, CA, and Aquasol 2 and [^{3}H]-uridine from New England Nuclear, Boston, MA.

RESULTS

The DI-LT particles have been shown to contain the 3′-terminal region of the *wt* VSV genome RNA by previous mapping studies (7, 8). It was of interest to determine whether the DI-LT

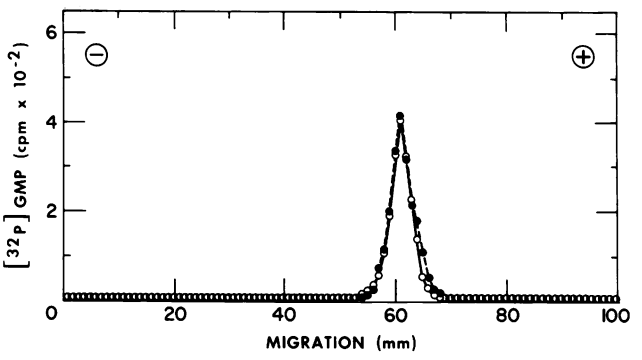

FIG. 2. Protection of [^{32}P]GMP-labeled leader RNA by genome RNA of *wt* VSV and DI-LT particles. [^{32}P]GMP-labeled leader RNA (1 ng) was used in hybridizations containing 1.5 μg of gradient-purified *wt* (○) or DI-LT (●) genome RNA. Hybridizations were performed in 0.12 ml of 0.3 M sodium citrate/0.3 M NaCl (pH 7.0) at 75° for 2 hr. The RNA duplexes were ethanol-precipitated, resuspended in 50 μl of 20 mM sodium acetate (pH 4.5)/0.3 M NaCl, and digested with RNase T2, 30 units/ml for 30 min at 37°. The digestion was stopped by the addition of $NaDodSO_4$ to 0.5%, and the RNA sample was diluted to 0.2 ml with 60% (vol/vol) glycerol/40 mM Tris·HCl, pH 7.8/20 mM sodium acetate/2mM EDTA and analyzed by electrophoresis on 20% polyacrylamide gels for 14.5 hr at 10 mA as previously described (21). Gels were sliced into 1-mm fractions in a Gilson automatic gel crusher; the fractions were incubated at 50° in 0.6 ml of 15% H_2O_2 for 16 hr and assayed for radioactivity in 7.5 ml of Aquasol 2.

particle also contained RNA sequences that code for the leader RNA which has also been mapped at the 3′ end of the *wt* VSV genome RNA (21). DI-LT particles were grown in BHK-21 cell monolayers in the presence of [^{3}H]uridine and purified as described in *Materials and Methods*. In order to demonstrate that the DI-LT preparation was free of *wt* VSV particles, aliquots of both the [^{3}H]uridine-labeled DI-LT preparation and the [^{3}H]uridine-labeled *wt* VSV preparation were disrupted with sodium dodecyl sulfate ($NaDodSO_4$) and the genome RNA was analyzed by velocity sedimentation on sucrose gradients. The DI-LT preparation yielded a single peak of RNA which sedimented coincident with the 28S ribosomal RNA marker (Fig. 1). Less than 1% of the DI-LT genome RNA sedimented at the position of 42S genome RNA extracted from the *wt* VSV particles, demonstrating the purity of the DI-LT particle preparation. Moreover, electron microscopy and infectivity tests also indicated that the DI-LT preparation contained less than 0.5% *wt* virus (data not shown).

VSV leader RNA was synthesized *in vitro* in the presence of [α-^{32}P]GTP, purified as previously described (20), and used in hybridizations with 28S DI-LT genome RNA and 42S *wt* VSV genome RNA purified as in Fig. 1. The hybrids were treated with RNase T2 to degrade any single-stranded RNA regions and analyzed on 20% polyacrylamide gels. Both the 28S and 42S genome RNAs were able to form RNase-resistant hybrids with [^{32}P]GMP-labeled VSV leader RNA which migrated in gels at identical positions (Fig. 2). Therefore, both the *wt* VSV and DI-LT particle genome RNAs contain the same RNA sequence that codes for the leader RNA.

Previous studies have demonstrated that the VSV leader RNA is synthesized exactly from the 3′ end of 42S genome RNA and is the first RNA product to appear *in vitro* (21). Because the DI-LT particle contains the same viral proteins as do *wt* VSV particles (9) as well as that region of the genome RNA which codes for the leader RNA, we next determined if the DI-LT particle could also synthesize VSV leader RNA *in vitro*. To detect leader RNA synthesis, we took advantage of the fact that [β,γ-^{32}P]ATP is incorporated solely into the VSV leader RNA

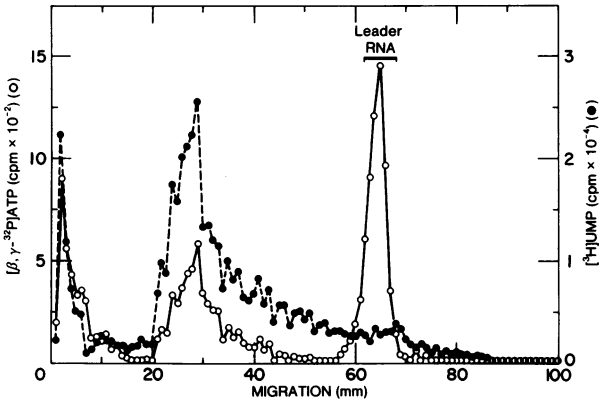

FIG. 3. Synthesis of leader RNA by purified DI-LT particles. DI-LT particles were used to synthesize RNA in a 1-ml reaction mixture containing 200 μCi [β,γ-^{32}P]ATP (25 Ci/mmol) and 25 μCi of [^{3}H]UTP (10 Ci/mmol) for 5 hr at 30°. Product RNA was extracted with NaDodSO$_4$/phenol, purified by Sephadex G-50 chromatography, and analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea as previously described (20). Electrophoresis was for 24 hr at 130 V and 24°. Gels were sliced and assayed for radioactivity in [^{3}H]UMP (●) and [β,γ-^{32}P]ATP (○) as described in Fig. 2. The position of purified [^{32}P]GMP-labeled leader RNA analyzed in a parallel gel is indicated by the bracket.

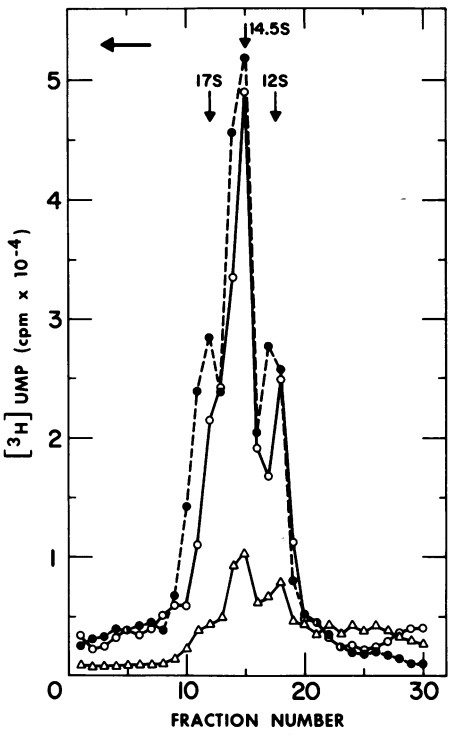

FIG. 4. Synthesis of 12–18S mRNA species by purified DI-LT particles. Standard 0.2-ml polymerase assays containing 10 μCi [^{3}H]UTP were incubated with 10 μg of *wt* VSV particles (○), 10 μg of DI-LT particles (△), or 10 μg of DI-LT particles plus preincubated L cell extract (1.4 A_{260} units/ml) (●) for 5 hr at 30°. Reactions were terminated by the addition of NaDodSO$_4$ to 0.5%, and the entire reaction mixture was layered over a 15–30% (wt/vol) sucrose gradient as described in Fig. 1. Centrifugation was for 17 hr at 33,000 rpm, at which time the gradients were fractionated (0.4 ml) and each fraction was assayed for trichloroacetic acid-precipitable radioactivity.

and not into VSV mRNA molecules (21). An *in vitro* reaction mixture, containing [β,γ-^{32}P]ATP to measure leader RNA synthesis and [^{3}H]UTP to measure total RNA synthesis, was incubated with purified DI-LT particles. The resulting product RNA was extracted with NaDodSO$_4$/phenol, purified by Sephadex G-50 chromatography, and analyzed on 20% polyacrylamide gels containing 8 M urea (Fig. 3). The [β,γ-^{32}P]-ATP-labeled RNA migrated predominantly as a sharp peak that comigrated with purified [α-^{32}P]GMP-labeled VSV leader RNA analyzed in a parallel gel, indicating that the leader RNA was synthesized. In addition, ^{32}P-labeled RNA was found at the top of the gel, presumably representing transcriptive intermediates, and in a heterogeneous peak that migrated one-quarter of the way into the gel. Because the [^{3}H]UMP-labeled RNA also comigrated with this heterogeneous peak, it may represent mRNA species containing covalently linked leader RNA—i.e., unprocessed RNA.

We next determined the size, by velocity sedimentation on sucrose gradients, of the RNA products synthesized by DI-LT particles and compared them to the size of products synthesized by *wt* VSV particles grown in BHK spinner cells. Because cellular extracts have been shown to aid in the synthesis of VSV mRNAs (17) *in vitro*, [^{3}H]UMP incorporation was followed in the presence and in the absence of L cell extract. DI-LT particles were able to incorporate [^{3}H]UMP radioactivity at a linear rate for 5 hr in the presence or in the absence of cellular extract, with an approximately 3-fold stimulation in RNA synthesis observed in the presence of cell extract (data not shown). Fig. 4 shows that the RNA product synthesized by *wt* VSV particles in the absence of cell extract contained the three size-classes of VSV mRNA sedimenting at 12 S, 14.5 S, and 17 S (23). The RNA product synthesized by DI-LT particles in the presence of cellular extract was identical to that made by the *wt* VSV particle. RNA synthesized by the DI-LT particles in the absence of cellular extract also sedimented in the 12–18S region but, in addition, contained a high proportion of degraded or incomplete RNAs that sedimented slower than 12 S. The specific activity of the virion-associated transcriptase of the DI-LT particles was found to be identical to the corresponding activity of the *wt* VSV purified from coinfected BHK-21 monolayer cells (28 nmol of UMP incorporated per mg of protein per hr). However, as shown in Fig. 4, this value was 34% of the *wt* VSV purified from BHK-21 spinner cells and only after addition of cell extract was it equal to the *wt* activity (86 nmol of UMP incorporated per mg of protein per hr). These results indicate that different *wt* VSV preparations possess dissimilar transcriptase activities, depending on the types of cells used for infection. More importantly, the data show that the presence of a small amount of actively transcribing *wt* VSV (<1%) in the DI-LT preparations cannot account for the amount of RNA synthesis observed.

The RNA synthesized *in vitro* by purified DI-LT particles was further characterized for the presence of poly(A). The [^{3}H]UMP-labeled product RNAs were purified and separated into poly(A)$^+$ and poly(A)$^-$ RNA species by oligo(dT)-cellulose chromatography, and both fractions then were analyzed by velocity sedimentation. All three size-classes of product RNAs contained poly(A), whereas the poly(A)$^-$ RNAs were smaller and sedimented heterogeneously between 6 and 16 S (Fig. 5).

In a separate series of experiments, it was shown that the methyl group of *S*-adenosyl-L-methionine was incorporated into all three size-classes of product RNAs, indicating that they were also methylated (ref. 26; other data not shown). Analysis of the methyl-labeled RNA products by paper electrophoresis after enzymatic digestion indicated that the methylation occurred in the 5′-terminal-blocked structure of the RNAs as ^{7}mGpppA^m_p . . . (ref. 26; other data not shown). Finally, to confirm that the 12S RNA product contained two distinct RNA

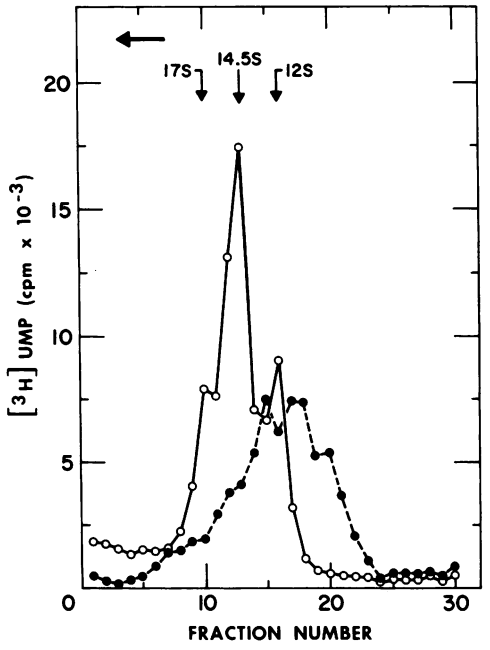

FIG. 5. Velocity sedimentation analysis of poly(A) containing DI-LT product RNA. [^{3}H]UMP-labeled RNA synthesized by DI-LT particles in the presence of L cell extract was purified by Sephadex G-50 chromatography and oligo(dT)-cellulose chromatography as described in *Materials and Methods*. Both the oligo(dT)-bound (○) and unbound (●) product RNAs were analyzed by velocity sedimentation on 15–30% (wt/vol) sucrose gradients as described in Fig. 4.

species, product RNA sedimenting at 12 S was isolated and converted into double-stranded RNA duplexes by annealing to 42S genome RNA. The RNA duplexes were then treated with RNase T2 and analyzed on 5% polyacrylamide gels. This process yielded two distinct RNA species similar to those previously shown (25) for the *wt* VSV 12S mRNA species (data not shown). These results clearly indicate that the product RNAs synthesized *in vitro* by the DI-LT particles contain similar RNA species with the same 5′- and 3′-terminal modifications as their *wt* counterparts and presumably function as mRNAs.

Because the DI-LT genome RNA is identical to the 3′-terminal half of 42S genome RNA, it presumably does not contain the region coding for the *wt* VSV 31S mRNA species. To contrast the *wt* VSV and DI-LT particles in this respect, [^{3}H]-UMP-labeled product RNA was synthesized by *wt* VSV and DI-LT particles in the presence of cellular extract. After incubation at 30°, the nucleocapsid cores and transcriptive intermediates were pelleted in glycerol gradients and the released poly(A)-containing RNAs were then isolated by oligo(dT)-cellulose chromatography and analyzed directly by velocity sedimentation in sucrose gradients. Although the *wt* VSV particle was able to synthesize a 31S species of RNA, no corresponding peak of RNA was found upon analysis of the DI-LT product RNAs (Fig. 6). Thus, the DI-LT particle genome comprises the entire 3′ end of 42S genome RNA through the 17S mRNA region (G protein cistron) and must terminate shortly thereafter in the L protein cistron.

DISCUSSION

The data presented in this paper clearly demonstrate that purified DI-LT particles behave similarlly to the *wt* VSV in respect to their ability to synthesize RNA *in vitro*. Like *wt* VSV, the DI-LT particles can synthesize the leader RNA and four mRNA species *in vitro* in the same relative proportions and

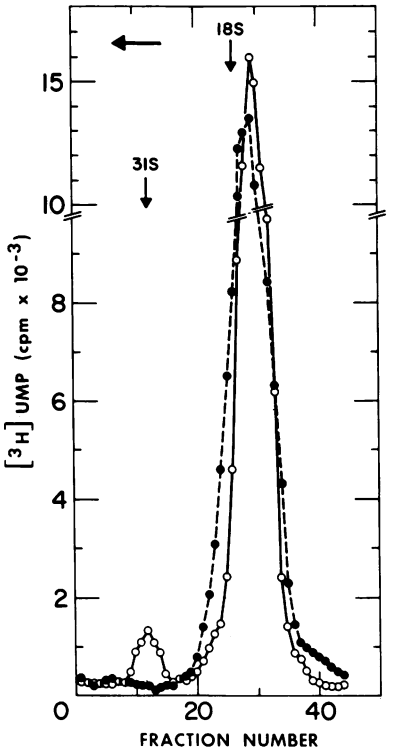

FIG. 6. Absence of 31S mRNA synthesis *in vitro* by DI-LT particles. RNA was synthesized, as described in Fig. 4, by *wt* VSV particles (○) and DI-LT particles with L cell extract (●) for 4 hr at 30°. The reaction mixtures (0.2 ml) were layered onto 0.375 ml of 20% (vol/vol) glycerol in a 0.7-ml adapter tube for the Beckman SW50.1 rotor. After centrifugation for 1 hr at 45,000 rpm to pellet the nucleocapsids and transcriptive intermediates, the top 0.5 ml was removed and diluted to 1 ml with 1% $NaDodSO_4$/1 M NaCl and the poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography as described previously (18). [^{3}H]UMP-labeled RNA that was bound to the column was eluted with H_2O and analyzed directly on 15–30% (wt/vol) sucrose gradients as described in Fig. 1. Centrifugation was for 26,000 rpm for 15 hr.

quantities (Fig. 4). In addition, the mRNAs synthesized by DI-LT are capped, methylated in the presence of *S*-adenosyl-L-methionine, and contain poly(A). In contrast to the *wt* VSV, the DI-LT did not synthesize the 31S mRNA that presumably codes for the viral structural protein L (Fig. 6). Thus, it seems that the inability of DI-LT to replicate *in vivo* is due to the deletion from its genome of the viral L protein gene (27, 28) that is essential for its replication. The results further indicate that the DI-LT particle contains all the necessary components present in the *wt* virus that will allow RNA synthesis *in vitro* with initiation at the 3′ terminus of the genome RNA followed by sequential transcription of four genes (26). These observations are also compatible with previous mapping studies of DI-LT RNA on the *wt* VSV genome RNA (3, 8). Our results, nonetheless, do not rule out the possibility that a part of the gene coding for the 31S mRNA is present in the DI-LT RNA. Experiments have consistently shown a small but significant amount of hybridization of the DI-LT RNA with isolated 31S mRNA (8). It would be of interest to determine if the poly(A)$^-$ RNA products (Fig. 5) contain any 31S mRNA sequences.

The demonstration of *in vitro* transcription by DI-LT particles suggests that these particles may be actively transcribed *in vivo* and may not be genetically silent during autointerference or cell killing by VSV (33, 34). Indeed, it has been shown recently that virus-specific RNAs are synthesized *in vivo* when BHK-21 monolayer cells are infected with DI-LT particles

without helper virus (L. D. Johnson and R. A. Lazzarini, unpublished data). This potential for the expression of DI-LT genetic information may explain the heterotypic inhibition of New Jersey serotype VSV by this Indiana serotype DI particle (19). In cells infected with *wt* VSV and DI-LT, two types of nucleocapsid protein might be simultaneously synthesized and incorporated into nucleocapsid structures. These resulting mosaic nucleocapsids may be substantially poorer templates for the replicase and transcriptase and lead to a decreased yield of progeny particles. Other DI particles that do not contain the cistron for the nucleocapsid protein or are unable to express that information would be incapable of this kind of autointerference. Prevec and Kang (19) have also considered this mechanism for heterotypic interference.

The data presented here, that some DI particles are capable of efficient transcription, also predict that complementation between two different DI particles could theoretically occur *in vito*. A DI particle containing the 5′-terminal half of the *wt* genome RNA and transcribing the 31S mRNA *in vitro* is an excellent candidate as a source of L protein to complement the DI-LT particle during coinfection. The latter particle will provide the rest of the structural proteins for the replication of both. Similarly, the DI-LT particle should be able to complement temperature-sensitive mutants of complementation groups II–IV (defects in the N, M, NS, or G protein genes) (29) and produce progeny temperature-sensitive VSV at nonpermissive temperatures. The group I mutants' (29) defect in the L protein gene would not be expected to be complemented by the DI-LT particles.

Finally, the present data clearly show the necessity for reexamination of all DI particles with respect to their transcriptase and replicase activities *in vitro*. There are conflicting results in the literature regarding these types of studies. Mori and Howatson (13) reported that all the DI particles they investigated synthesized substantial amounts of high-molecular-weight RNA. Others (30–32), however, have been able to demonstrate only limited transcription with various DI particles. In the latter case, the products were shown to be of low molecular weight (2 S in size). The VSV leader RNA will prove to be a powerful tool for screening the various DI particles for the presence of the 3′-terminal sequence of the *wt* genome RNA in the DI genome RNA. Like the DI-LT particles, the positive ones will be potentially useful in the study of the mechanism of DI particle production and their intracellular role in autointerference as well as the mechanism of replication of the *wt* VSV genome.

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