

# Synthesis of a small RNA in cells coinfecting by standard and defective interfering particles of vesicular stomatitis virus\*

(leader RNA/viral interference/viral RNA species)

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**ABSTRACT** A small RNA, containing approximately 50 nucleotides, is synthesized by cells coinfecting with standard vesicular stomatitis virus and its defective interfering (DI) particles. Infection of cells by standard virus or DI particles alone does not lead to synthesis of significant amounts of small RNA. The RNA is initiated at its 5' end with (p)ppXp and is not polyadenylated at the 3' end despite a content of 51% adenosine. It has sequences complementary to the genome of a DI particle. The synthesis of the small RNA correlates with the replication of the genome of DI particles with molar ratio small RNA/genome RNA of DI particles >50. When replication of DI genomes is prevented by the addition of cycloheximide or prior UV irradiation of DI particles, small RNA is not synthesized in coinfecting cells. These results indicate that the small RNA is not the result of transcriptional initiation and that it may relate to interference mediated by DI particles.

Among the vesicular stomatitis virus (VSV)-specific RNA species synthesized during infection, a small RNA, sedimenting at approximately 6 S in sucrose gradients, has been identified and related to the synthesis of defective interfering (DI) particles (2). More recently, small RNAs that are synthesized *in vitro* by purified VSV DI particles have been characterized and sequenced (3-8). These small RNAs resemble the "leader" sequence described by Colonna and Banerjee (9-11) in that they contain 46-48 nucleotides with a (p)ppAp at the 5' end and they lack poly(A) at the 3' end.

The function of these VSV-specific small RNAs is unknown. To study their function, conditions were sought for reproducibly detecting their synthesis in infected cells. Neither standard infectious VSV nor DI particles of VSV alone led to the synthesis of any small virus-specific RNA. Only when cells were coinfecting with standard and DI particles was a small RNA detected. The synthesis of this small RNA correlated directly with the replication of the genome of DI particles. Both RNA species were absent from coinfecting cells when cycloheximide was present or when DI particles were irradiated with UV light prior to infection. These data can be interpreted in terms of interference with the growth of standard VSV by DI particles.

## MATERIALS AND METHODS

**Cells and Viruses.** Suspension cultures of baby hamster kidney (BHK) cells, originally obtained from Amiya Banerjee, were grown in Joklik's modified minimal essential medium supplemented with 5% fetal calf serum. Cloned and sucrose gradient-purified standard infectious particles of VSV of the Indiana serotype (San Juan strain) and two different DI particles derived from the same San Juan standard VSV were used (12, 13). The DI-T particle has been characterized in detail (14-18). It is one-third the size of standard VSV and contains sequences

from the 5' end of the genome. DI0.52 particles were selected from cloned VSV by multiple undiluted passages in mouse L cells and then subsequent growth in BHK cells. DI0.52 particles are twice the size of DI-T particles and also map at the 5' end of the VSV genome (unpublished observations). Both DI particles were sucrose gradient-purified and their multiplicities were determined as described (19).

**Labeling of Infected Cells.** BHK cells were infected at a multiplicity of 20 with either standard VSV or DI particles alone or were coinfecting with equal amounts of standard VSV or DI particles at a total multiplicity of 40. Detailed procedures for infection and labeling with <sup>32</sup>P in the presence of actinomycin D have been published (1).

**Preparation and Analysis of RNA.** RNAs from VSV particles or from infected cells were extracted as described (1). To analyze total cytoplasmic RNA, ethanol-precipitated RNA was resuspended in 5  $\mu$ l of deionized H<sub>2</sub>O and mixed with an equal volume of double-strength electrophoresis buffer [6 M urea, the dyes xylene cyanol (XC) and bromphenol blue, and 0.01% sucrose]. To denature the large RNA species fully, they were first treated with 90% dimethyl sulfoxide and heated at 50°C for 30 min. This treatment was not necessary for small RNA.

For the separation of virion RNA and mRNA (large RNAs), 1.5% agarose/6 M urea, pH 3.8 was used (1, 20). Electrophoresis was at 100 V at 4°C until the XC was 5 cm from the bottom of the gel. Then, the 1.5% agarose gel was dried and exposed to x-ray film as described (1).

For the separation of small RNAs, 10% polyacrylamide gels containing 7 M urea in Tris/borate/EDTA buffer at pH 8.3 (21) were constructed with a thickness of 1.5 mm between two 20  $\times$  40 cm glass plates. Electrophoresis was at 700 V at room temperature until the XC marker reached 11-14 cm from the origin. Then, the wet polyacrylamide gel was covered with SaranWrap and exposed to x-ray film at -70°C with intensifying screens as described (1).

**Materials.** All materials were obtained as described (1, 19).

## RESULTS

**Separation and Identification of Virus-Specific RNAs on Gels.** Because VSV-specific RNA species are known to range in size from approximately 46 bases to 12 kilobases (2, 16, 22), two different gel systems were used: (i) 1.5% agarose gels to separate genome-sized and mRNA, and (ii) 10% polyacrylamide gels to separate RNAs smaller than 1 kilobase. These systems separated all of the VSV RNA species.

In the presence of actinomycin D, uninfected cells as well as DI-T particle-infected cells do not incorporate <sup>32</sup>P into RNA

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Abbreviations: VSV, vesicular stomatitis virus; DI, defective interfering; BHK, baby hamster kidney; XC, xylene cyanol dye.  
\* This paper is no. 9 in a series. Paper no. 8 is ref. 1.

species larger than tRNA (2). VSV-infected cells synthesized five mRNA species (L, G, N, NS, and M) as well as genome-sized (40S) RNA (Fig. 1, lane a). These mRNAs have been identified by their insolubility in 2 M lithium chloride, binding to oligo(dT)-cellulose columns, and annealing to virion RNA (refs. 1 and 2; unpublished observations). They have also been characterized by their RNase T1-resistant oligonucleotides (1). When cells were coinfecting with standard VSV and either one of two DI particles, mRNA synthesis occurred, although at a decreased rate compared to cells infected with standard VSV alone (Fig. 1, lanes b and c). In addition, in coinfecting cells, instead of the genome (40S RNA) of standard VSV, the RNAs specific to DI particles predominated. These results confirm previous analyses of VSV RNAs on sucrose gradients (2, 17). The intracellular DI-specific RNAs, representing (+) and (-) strands, migrated identically after treatment with glyoxal to the RNA extracted from their respective DI particles (ref. 23; unpublished observations).

The banding pattern of small RNAs from uninfected cells and those infected with standard virus or DI particles alone were identical when the cells were treated with actinomycin D (Fig. 2, lanes a-d). Only when cells were coinfecting with standard VSV and either one of two DI particles was there an extra band evident (Fig. 2, lanes e and f). This band, indicated by an arrow in the figure, migrated faster than 4S RNA, suggesting that it contained RNA with 40-60 nucleotides. Material migrating slower than 5S RNA on these 10% polyacrylamide gels represented the large virus-specific RNAs.

When the band containing small RNA was cut out of the gels and the radioactivity in it was compared to that in the band containing the (-) strand of DI-T particles, the molar yield of small RNA was in excess. In several experiments, small RNA incorporated 20,000-25,000 cpm which calculated to a molar yield  $\geq 50$ -fold that of the RNA of DI particles.

**Partial Characterization of the Small RNA.** To characterize this small RNA made under conditions of coinfection by stan-

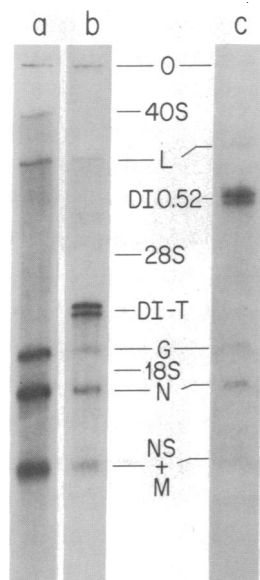


FIG. 1. Separation of large VSV-specific RNA on 1.5% agarose gels. Infected BHK cells were labeled with  $^{32}\text{P}$  at 0.5-5 hr after infection. The total cytoplasmic RNA was extracted and separated on agarose gels. rRNA markers from uninfected BHK cells were co-electrophoresed. Exposures of the gels were chosen to maximize the clarity of the RNA-containing bands; therefore, comparison of the relative amounts of RNA cannot be made here. Lanes: a, infected with standard VSV alone; b, coinfecting with standard VSV and DI-T particles; c, coinfecting with standard VSV and DI0.52 particles.

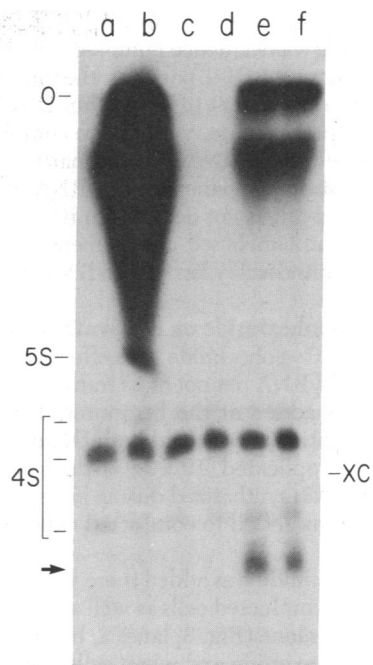


FIG. 2. Separation of a small VSV-specific RNA on 10% polyacrylamide gel. Infected and uninfected BHK cells, all treated with actinomycin D, were labeled with  $^{32}\text{P}$  between 0.5 and 5 hr after infection. RNA was extracted and separated on a 10% polyacrylamide gel. Marker 4S and 5S RNAs from BHK cells were co-electrophoresed. Arrow indicates the position of the small VSV-specific RNA. Lanes: a, uninfected cells treated with actinomycin D; b, infected with standard VSV; c, infected with DI-T particles; d, infected with DI0.52 particles; e, coinfecting with standard VSV and DI-T particles; f, coinfecting with standard VSV and DI0.52 particles.

dard VSV and DI particles, it was eluted from the 10% polyacrylamide gel (1) and its base ratios were determined by electrophoresis at pH 3.5 after complete digestion with RNases T1, T2, and A (24). The small RNA contained 51% adenosine, 21% cytosine, 10% guanosine, and 17% uridine. Electrophoresis of the products on DEAE-paper at pH 3.5 after digestion with RNases T1, A, and T2 indicated the presence of a nucleoside triphosphate and a nucleoside tetraphosphate at a ratio of 1:9, suggesting that the 5' end was initiated during synthesis and not generated by cleavage from a larger RNA molecule.

The small RNA was annealed to RNA from standard VSV, from DI-T particles, and from DI0.52 particles. RNA from DI-T particles annealed to 70-74% of the  $^{32}\text{P}$ -labeled small RNA made by coinfection with standard VSV and DI-T or DI0.52 particles (Table 1). RNA from standard VSV annealed to less than 12% of the small RNA. Mixtures of small RNA and RNA from DI0.52 particles failed to result in greater than 9% hybridization, even after the unlabeled RNA from DI0.52

Table 1. Hybridization of the small RNA to genomic RNAs from standard VSV, DI-T particles, and DI0.52 particles

Tube	Unlabeled RNA	% RNase	
		DI-T product	DI0.52 product
1	None	2.7	2.0
2	Standard VSV	11.5	5.7
3	DI-T particles	70.0	73.7
4	DI0.52 particles	9.4	7.7

Unlabeled RNA (4  $\mu\text{g}$ ) was mixed with 10,000 cpm of  $^{32}\text{P}$ -labeled small RNA made under conditions of coinfection with standard VSV and DI-T particles or DI0.52 particles. Annealing was done in a volume of 50  $\mu\text{l}$  at 70°C for 6 hr in 0.30 M NaCl/0.03 M sodium citrate, pH 7 (19). RNase-resistance was determined as described (19).

particles was partially nicked by alkali (4). Although these data indicate that there was sequence homology between the small RNA and the genome of DI-T particles, the failure of the small RNA to anneal to any extent to RNA from DI0.52 particles is difficult to interpret. This may be due to complementary sequences contained in the RNA from DI particles which compete in the annealing with labeled small RNA (25). Such competition most likely does not occur with RNA from standard VSV, because the amount of self-complementarity in RNA from standard VSV is considerably less than in RNA from DI particles (26, 27).

**Effects of Cycloheximide on the Synthesis of the DI-Specific Small RNA.** Cycloheximide is an effective inhibitor of the replication of VSV RNA but not of its transcription (28). When cycloheximide is added at the beginning of infection, only primary transcription occurs, and both DI and standard virus genomes are not replicated (19, 29). To determine whether or not the small RNA is synthesized during primary transcription, cycloheximide was added to coinfecting cells at the initiation of infection.

When cycloheximide was added there was an absence of the small RNA from coinfecting cells as well as from cells infected by standard VSV alone (Fig. 3, lanes a, b, and c). Only in the absence of cycloheximide and when cells were coinfecting by standard VSV and DI-T particles was the small RNA detected (Fig. 3, lane d). These results indicate that the small RNA was not synthesized during primary transcription by VSV, even though DI particles were coinfecting the cells.

**Effects of UV Irradiation on the Synthesis of the Small RNA.** Intracellular interference with the growth of standard VSV by DI particles does not occur if DI particles are irradiated with UV light prior to coinfection of cells (15). To determine

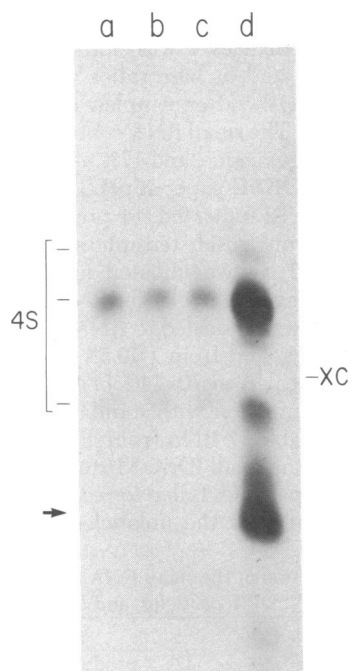


FIG. 3. Effect of cycloheximide on the synthesis of the small VSV-specific RNA. BHK cells that had been treated with cycloheximide (100  $\mu\text{g}/\text{ml}$ ) for 1 hr at 37°C or not treated were infected and labeled with  $^{32}\text{P}$  between 0.5 and 5 hr after infection. The RNA was extracted and separated on a 10% polyacrylamide gel. Lanes: a, infected with standard VSV, treated with cycloheximide; b, coinfecting with standard VSV and DI-T particles, treated with cycloheximide; c, coinfecting with standard VSV and DI0.52 particles, treated with cycloheximide; d, coinfecting with standard VSV and DI-T particles, not treated with cycloheximide.

whether or not the small RNA is synthesized when interference by DI particles is abrogated, DI-T particles were irradiated (2200 ergs/ $\text{mm}^2$ ) and the synthesis of the small RNA was examined.

Fig. 4 shows the separation of both the large and small virus-specific RNA made by cells infected with standard VSV alone, with standard VSV and DI-T particles, and with standard VSV and UV-irradiated DI-T particles, there was loss of the small RNA when DI particles were irradiated. The controls in lanes a and b show the expected absence of small RNA when cells were infected by standard VSV alone and the expected presence of the small RNA when cells were coinfecting. The

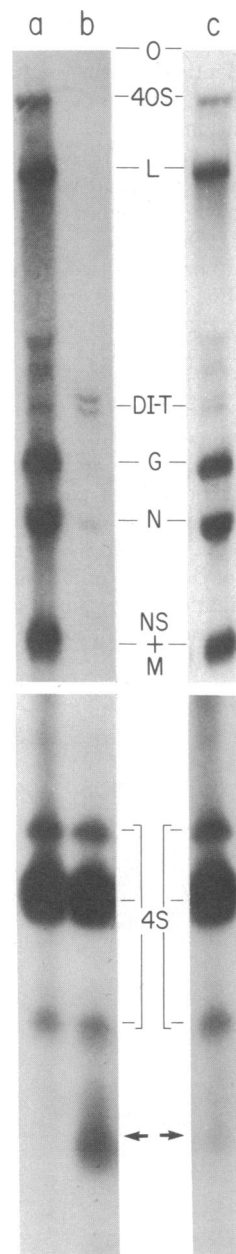


FIG. 4. Effects of prior UV irradiation of DI-T particles on the synthesis of VSV-specific RNA. Infected BHK cells were labeled with  $^{32}\text{P}$  between 0.5 and 5 hr after infection. Cytoplasmic RNA was extracted and divided into equal aliquots. The large RNAs were examined on 1.5% agarose gels (top half of figure) and the small RNAs were examined on 10% polyacrylamide gels (bottom half). Lanes: a, infected with standard VSV; b, coinfecting with standard VSV and DI-T particles; c, coinfecting with standard VSV and DI-T particles, irradiated with UV light at 2200 ergs/ $\text{mm}^2$ .

upper half of Fig. 4 shows the alleviation of the inhibition by DI particles of mRNA synthesis when UV-irradiated DI particles were used in the coinfection (lane c). The amount of mRNA, as well as of 40S RNA, was a measure of the reversal of the interfering effect by prior UV irradiation of DI-T particles.

### DISCUSSION

The results presented here demonstrate an intracellular small RNA that has properties similar to those of products made *in vitro* by VSV DI particles (6, 8). These properties are: (i) a length of 40–60 nucleotides; (ii) a base distribution that is high in adenosine; (iii) the ability to hybridize to the genome RNA of DI-T particles and not of standard VSV; (iv) the presence of a nucleoside tri- and tetraphosphate; and (v) the lack of poly(A). Preliminary sequence results indicate that the small RNA reported here contains the sequence . . . GAGGGUC<sub>OH</sub> which is identical to that published for the *in vitro* products synthesized by DI particles.

Our ability to detect such an intracellular product was enhanced by the use of 10% polyacrylamide gels which permitted its separation from transfer RNA. Also, coinfection of cells, at equal multiplicities of infection, by DI and standard VSV particles led to increased synthesis of the small RNA. DI particles alone did not produce any detectable RNA product. Whether this is due to an insufficient concentration of DI RNA templates intracellularly or to some other reason is not known at present. Standard VSV alone produced less than 1% of the amount made under conditions of coinfection. This residual amount may be due to contamination of standard virus preparations by small, undetectable amounts of DI particles.

The relationship of the small RNA reported here to "leader" RNA is unclear. A small RNA made *in vitro* by preparations of VSV predominantly composed of standard particles has been reported and postulated to be a "leader" sequence resulting from transcriptional initiation and subsequent cleavage (30). Our hybridization results as well as those with cycloheximide and UV irradiation indicate that intracellularly the small RNA appears to be specific to DI particles and not part of the transcript process involving standard VSV.

Its presence correlates directly with the synthesis of the genome RNA of DI particles. Because of this it is tempting to speculate that the small RNA reported here plays some role during interference by DI particles. It may participate as an antiviral agent by binding enzymes or substrates or by blocking specific complementary sequences. On the other hand, small RNA may be an abortive product synthesized as a result of interference with the synthesis of the genome of standard RNA.

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