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The Termini of VSV DI Particle RNAs Are Sufficient to Signal RNA Encapsidation, Replication, and Budding to Generate Infectious Particles

ASIT K. PATTNAIK,*,[1] L. ANDREW BALL,† ALISON LEGRONE,† AND GAIL W. WERTZ†

*Department of Microbiology and Immunology, University of Miami School of Medicine, P.O. Box 016960 (R-138), Miami, Florida 33101; and †Department of Microbiology, University of Alabama at Birmingham Medical School, Birmingham, Alabama 35294

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Infectious defective interfering (DI) particles of the negative-stranded RNA virus vesicular stomatitis virus (VSV) have been recovered from negative-sense transcripts of a plasmid that contains a full-length cDNA derived from the DI-T particle genome. In order to determine the *cis*-acting sequences necessary for RNA replication, encapsidation, and budding and to approximate the minimal size of RNA that can be packaged into infectious particles, we constructed a series of internal deletions in the DI cDNA to generate plasmids that could be transcribed to yield RNAs which ranged in size from 2209 nucleotides down to 102 nucleotides. All the deletion plasmids retained at least 36 nucleotides from the 5′-terminus and 51 nucleotides from the 3′-terminus of the DI genome. In cells expressing the five VSV proteins, the deleted DI RNAs were examined for their ability to be encapsidated, to replicate, and to bud to produce infectious DI particles. An RNA as small as 191 nucleotides, which contained 46 nucleotides from the 5′-end and 145 nucleotides from the 3′-end of the DI genome was encapsidated, replicated, and budded at least as efficiently as the full-length wild-type DI RNA. In contrast, a 102-nucleotide RNA that contained only the 51 nucleotides from the 5′-end of the DI RNA and its perfect 51-nucleotide complement at the 3′-end replicated poorly and failed to bud infectious DI particles. However, an RNA with an insertion of 1499-nucleotide "stuffer" sequences of non-VSV origin between the two 51-nucleotide complementary termini not only replicated but also budded infectious particles. These data show that the signals necessary for RNA encapsidation, replication, and packaging into infectious DI particles are contained within the 5′-terminal 36 nucleotides and the 3′-terminal 51 nucleotides of the DI RNA genome. Furthermore, the results show that a heterologous sequence can be replicated and packaged into infectious particles if it is flanked by the DI RNA termini.

Defective interfering (DI) particles of vesicular stomatitis virus (VSV) are generated during high multiplicity passage of the virus in cells in culture. The genomes of these DI particles have large portions of the genomic RNA deleted and they vary significantly in structure and size (*1–5*). The DI particles interfere specifically with the replication of the standard homologous virus and play a major role in the establishment and maintenance of persistent infection (*1*, *6–10*). One of the best characterized DI particles of VSV is the DI-T particle which was generated from the San Juan strain of the Indiana serotype of VSV. The RNA of the DI particle is 2208 nucleotides in length and contains at its 5′-terminus 2163 nucleotides from the 5′-terminus of the VSV genome and at its 3′-terminus, an inverted complement of the first 45 nucleotides of the 5′-terminus of the VSV genome (*3*). Since the genome of the DI-T particle contains only a portion of the L gene, it lacks the genetic information to code for any of the viral proteins. However, it is an excellent template for RNA replication and has been used to examine the role and requirement for viral proteins in RNA replication and the assembly and budding of infectious particles (*11–17*).

We have demonstrated that by transfecting a specialized transcription plasmid (pDI) containing a cDNA copy of the DI RNA, it is possible to generate the DI RNA from pDI with a 3′-terminus identical to that of the authentic DI RNA. The DI RNA from pDI, if synthesized intracellularly in the presence of the five VSV proteins expressed by cotransfection of individual plasmids, can be encapsidated by the nucleocapsid protein N, replicated by the polymerase proteins P and L and in the presence of the matrix protein (M) and the glycoprotein (G), be assembled to generate infectious DI particles (*18*). To determine the *cis*-acting sequences necessary to signal RNA encapsidation, replication, and budding, we generated a series of internal deletion mutants of pDI and tested the ability of the mutants to be replicated and to bud to yield infectious particles.

The construction of pDI has been reported (*18*). The DI cDNA contains 2209 nucleotides rather than 2208 nucleotides of the wt DI RNA, as it contains an additional A residue at position 22 from the 5′-end. A series of deletions was generated by the use of restriction enzyme recognition sites within the DI cDNA in pDI (Fig. 1). The sequences at the deletion junctions were determined by nucleotide sequencing of the deleted plasmid DNAs. These deletions were such that the negative-sense RNA transcripts made by T7 RNA polymerase would range in

[1] To whom correspondence and reprint requests should be addressed. Fax: (305) 548-4623.

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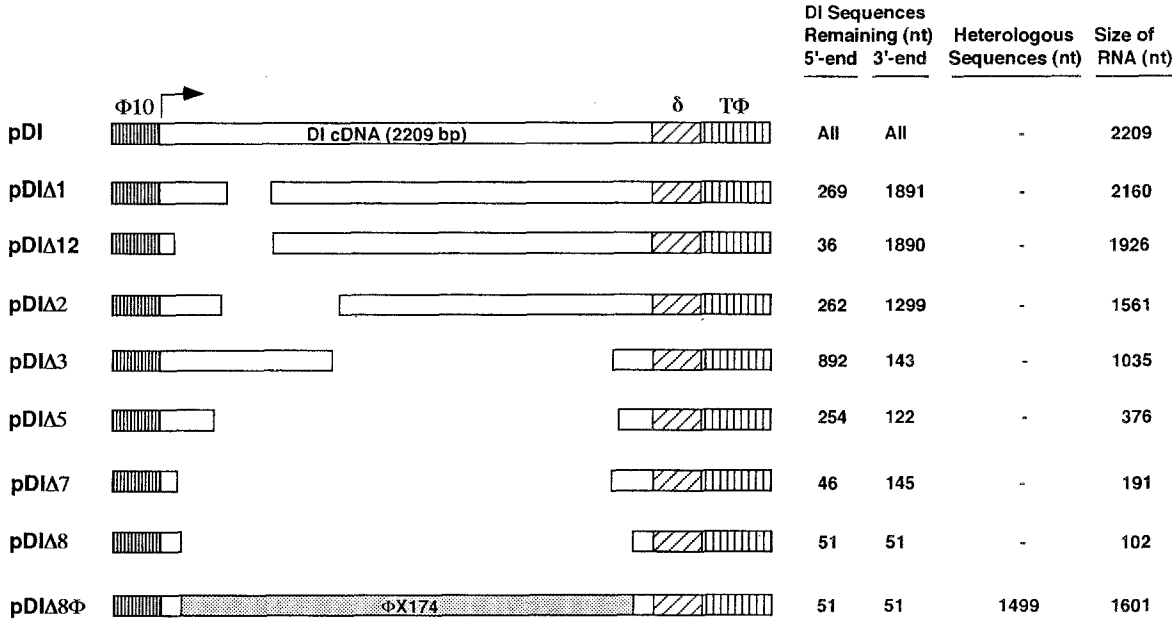

FIG. 1. pDI deletion mutants and their derivatives. ϕ10 and Tϕ correspond to T7 RNA polymerase transcription initiation and termination signals. δ corresponds to self-cleaving ribozyme sequence from hepatitis delta virus. Arrow shows the position and direction of transcription by T7 RNA polymerase.

size from 2160 nucleotides (pDIΔ1) down to 191 nucleotides (pDIΔ7), which is less than 9% of the length of the DI genomic RNA. In each of the deletion mutants, however, at least 36 nucleotides from the 5′-terminus and 51 nucleotides from the 3′-terminus of the DI RNA were retained. Thus the termini of these RNAs were complementary for 36 to 45 nucleotides. The plasmid pDIΔ8 was constructed by using the *Afl*II site at position 2158 in the DI cDNA sequence and two plasmids containing the DI cDNA in opposite orientations. Exchange of restriction fragments generated by digestion with *Afl*II and another restriction enzyme with a unique site within the vector sequence created pDIΔ8 containing 51 nucleotides at each terminus (Fig. 1). The transcript generated from pDIΔ8 is therefore a 102-nucleotide-long RNA whose termini are perfectly complementary for 51 nucleotides, 6 more than the 45 nucleotide-terminal complementarity present in the wild-type DI RNA. The plasmid pDIΔ8 was further modified by introducing an *Afl*II fragment of 1499 bp of bacteriophage ϕX174 RF DNA into the unique *Afl*II site at the center of the two 51-nucleotide termini to generate the plasmid pDIΔ8ϕ. All plasmids contained the T7 promoter positioned to transcribe the negative-sense DI RNA with two additional nucleotides (GG) at the 5′-terminus. The plasmids also contained the antigenomic ribozyme of hepatitis delta virus positioned to cleave the 3′-terminus of the transcripts immediately after the last VSV-specific nucleotide. All of these deleted plasmids and derivatives were examined for synthesis of RNA by the T7 RNA polymerase both *in vitro* and in transfected cells infected with the recombinant vaccinia virus (vTF7-3) expressing the T7 RNA polymerase (*19*) as described before (*18*). In each case, RNAs of appropriate sizes were generated following cleavage of the primary transcript by the ribozyme (data not shown).

The ability of the RNAs transcribed in cells from each of the various pDI deletion constructs to be encapsidated and replicated by the VSV proteins N, P, and L was analyzed. BHK-21 cells infected with vTF7-3 were transfected with plasmid DNAs containing the genes for the VSV proteins N, P, and L and the indicated pDI wild-type or deletion plasmids. The cells were exposed to [^{3}H]uridine for 6 hr at 5 or 16 hr post-transfection. Results obtained by labeling at either of the two times were qualitatively the same. Cytoplasmic extracts were prepared and subjected to immunoprecipitation by anti-VSV antibodies which selects for encapsidated RNAs (*18*). The RNAs were recovered from immunoprecipitated nucleocapsids and analyzed by electrophoresis in agarose–urea gels as described previously (*18*). RNA is separated in these gels (run at pH 3.0) on the basis of both size and base composition, thereby allowing distinction of positive- and negative-sense RNA and direct visualization of replication of the negative-strand RNA to yield a positive-strand RNA. A fluorogram of such a gel is shown in Fig. 2. The results showed that RNA generated from each of the deletion constructs was encapsidated and replicated. Replication of the negative-sense RNA transcript is evident from the presence of a faster migrating RNA, which has been shown to correspond to the positive-sense product of RNA replication (*18*). A positive-sense RNA in addition to the negative-sense RNA was visible in all

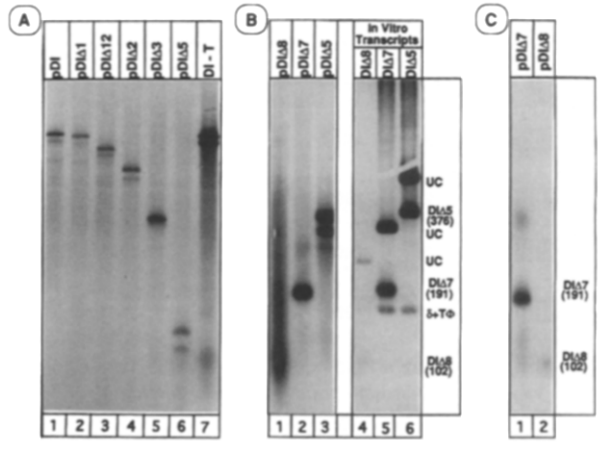

FIG. 2. Intracellular replication of RNA from pDI deletion mutants. BHK-21 cells in 60-mm plates were infected with vTF7-3 (m.o.i. ≃ 10) and subsequently transfected as described previously (*18*) with 3 μg of p-N, 2 μg p-P, 1 μg p-L, and 5 μg of plasmid DNA for each pDI deletion construct as indicated at the top of each lane. Cells were labeled with 20 μCi [^{3}H]uridine per ml for 6 hr at 5 hr post-transfection, RNAs were recovered from nucleocapsids immunoprecipitated from cytoplasmic extracts, and analyzed by electrophoresis in agarose–urea gel. (A) Replication of RNA derived from plasmids pDI, pDIΔ1, pDIΔ12, pDIΔ2, pDIΔ3, and pDIΔ5 (Lanes 1–6, respectively). Lane 7 (DI-T) shows DI RNA replication products from cells transfected with N, P, and L plasmids and superinfected with authentic DI particles as described previously (*13*). (B) Replication of RNA derived from plasmids pDIΔ8, pDIΔ7, and pDIΔ5 (Lanes 1–3, respectively). *In vitro* transcription products made by T7 RNA polymerase in the presence of [^{3}H]UTP from plasmids pDIΔ8, pDIΔ7, and pDIΔ5 are shown in lanes 4–6. The gel in B was electrophoresed for 14 hr to retain smaller RNAs compared to 18 hr for A. (C) Replication of RNA from pDIΔ7 (Lane 1) and pDIΔ8 (Lane 2) in the presence of actinomycin D. Transfected cells were treated with 10 μg of actinomycin D per ml for 30 min at 18.5 hr post-transfection and then labeled for 5 hr with [^{3}H]uridine in the presence of actinomycin D. RNAs were analyzed as described above. "UC" represents uncleaved primary transcript present at the time of assay.

cases, except DIΔ7 (Fig. 2B, lane 2) where the RNAs do not have a significantly different base composition to be resolved from one another, and DIΔ8 (Fig. 2B, lane 1) where the positive- and negative-strands are identical.

Since the negative- and positive-sense DIΔ7 and DIΔ8 RNAs were not resolved from each other, it was not clear whether the RNAs in Fig. 2B, lanes 1 and 2 were the products of replication by the VSV polymerase or simply the encapsidated transcripts generated by the T7 RNA polymerase. To distinguish between these possibilities, we treated the transfected cells with actinomycin D 30 min prior to and during labeling with [^{3}H]uridine. Under these conditions, DNA-dependent RNA synthesis from transfected DNA templates directed by T7 RNA polymerase is completely blocked and the only RNAs synthesized are due to replication of RNA by the VSV RNA polymerase (*18*). Results of such an experiment showed that DIΔ7 and DIΔ8 RNAs were synthesized in the presence of actinomycin D (Fig. 2C, lanes 1 and 2), although DIΔ8 RNA replicated extremely poorly. Furthermore, when the P or L plasmid DNA was omitted from transfection during analysis of replication of these RNAs in the presence of actinomycin D, these RNAs were not synthesized at all (data not shown), confirming that both DIΔ7 and DIΔ8 RNAs were replicated by the VSV RNA polymerase, albeit very less efficient replication of DIΔ8 RNA.

To examine whether the deleted RNAs shown to be encapsidated and replicated above could be assembled to bud infectious particles, cells were infected with vTF7-3 and then transfected separately with each of the pDI deletion constructs along with the plasmids for all five VSV proteins. At 24 hr post-transfection, the culture fluids were collected, clarified and assayed for the presence of infectious DI particles. The assay for the budded DI particles relies on their infectivity and has been described previously (*12, 18*). Briefly, fresh cells were infected with vTF7-3, transfected with the plasmids containing the N, P, and L genes, and then 5 hr later these cells were exposed to supernatant fluids from the previous transfection. The infected cells were incubated in medium containing [^{3}H]uridine for 6 or 16 hr before being harvested and assayed for synthesis of RNA. If infectious particles were released into the supernatant fluids of the first transfection, they would infect the new cells and replicate their genomic RNA using the N, P, and L proteins provided by the transfected plasmids. The results of this assay for infectious particles are shown in Fig. 3. RNAs having the sizes corresponding to the deletion constructs used in the original transfection were synthesized in each of the cultures infected with the appropriate supernatant fluids, with the exception of pDIΔ8. These data indicated that infectious DI particles were released from cells transfected with each of the pDI derivatives, except the smallest derivative, pDIΔ8. No RNA synthesis was detected in cells infected with culture fluids from pDIΔ8-transfected cells (Fig. 3B, lane 2), although the RNA from pDIΔ8 replicated intracellularly (Fig. 2C, lane 2).

Two possible reasons to account for the finding that the 102 nucleotide DIΔ8 RNA replicated poorly and did not bud particles were that (i) the sequences necessary for efficient encapsidation and replication and/or assembly of DI RNA had been deleted from this molecule or (ii) there is a minimal size of RNA required to assemble and bud infectious particles. Alternatively, the failure to bud particles could be explained by the fact that the 51 nucleotides at each terminus of this RNA were perfectly complementary to each other and therefore, such a molecule could fold back on itself or self-anneal to generate a double-stranded RNA which might be inefficiently encapsidated, replicated, or assembled. However, it should be noted that larger DI RNAs with perfectly self-complementary sequences, such as the genome of DI 011, occur naturally (*20*). We tested whether incorporation of "stuffer" sequences between the two termini of DIΔ8 RNA might restore the ability of the RNA to bud into infectious parti-

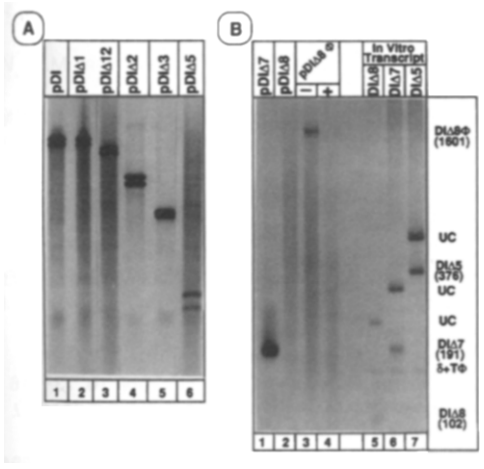

FIG. 3. Assembly and budding of infectious DI particles. BHK-21 cells in 60-mm plates were infected with vTF7-3 and transfected with 6 μg of p-N, 4 μg of p-P, 2 μg of p-L, 5 μg of p-M, 5 μg of p-G, and with 5 μg of various pDI deletion constructs as indicated on top of each lane. Clarified culture fluids harvested from cells at 24 hr post-transfection were used to superinfect cells that had already been infected with vTF7-3 and transfected with N, P, and L plasmids. Replicating RNAs were labeled with [^{3}H]uridine, isolated, and analyzed by electrophoresis as described in the legend to Fig. 2. (A) RNA replication by particles from pDI-, pDIΔ1-, pDIΔ12-, pDIΔ2-, pDIΔ3-, and pDIΔ5-transfected cells. (B) RNA replication by particles from pDIΔ7-, pDIΔ8-, and pDIΔ8ϕ-transfected cells. *In vitro*-synthesized RNAs are shown in lanes 5–7. (–) and (+) indicate particles left untreated or treated, respectively, with anti-VSV antibody (1:100 dilution) for 2 hr at 4° before infection. "UC" indicates primary transcript that was not cleaved by the ribozyme at the time of assay.

cles. We inserted 1499 bp of heterologous DNA from ϕX174 into the unique *Afl*II site in the center of pDIΔ8 to create pDIΔ8ϕ. When transcribed, pDIΔ8ϕ generated a 1601 nucleotide RNA that had the 51-nucleotide termini at either end as its only DI-specific sequences. In contrast to the failure to detect infectious particles from culture fluids of cells transfected with pDIΔ8 plasmid, the supernatant medium harvested from cultures transfected with pDIΔ8ϕ in the presence of all five proteins did contain infectious particles as shown by the replication of a 1601 nucleotide RNA in the infectious particle assay (Fig. 3B, lane 3). The infectivity of pDIΔ8ϕ particles was neutralized when the culture fluid containing these particles was incubated in the presence of anti-VSV antibody (Fig. 3B, lane 4), indicating that these particles were fully assembled and enveloped rather than subviral particles or naked nucleocapsids. Thus, incorporation of 1499 nucleotides of heterologous sequence between the two termini of DIΔ8 RNA resulted in a replicable RNA (DIΔ8ϕ) which could assemble and bud particles. These data demonstrate that the 51 nucleotides from each terminus of the VSV DI RNA are competent to signal RNA replication, encapsidation, and assembly to bud infectious particles and ruled out the possibility that essential *cis*-acting sequences had been deleted from DIΔ8 RNA. In addition, these data show that it is possible to include a heterologous sequence between these termini to produce an RNA that can be encapsidated and replicated by the VSV RNA polymerase.

The demonstration that only few nucleotides from the two termini of VSV DI RNA are sufficient to support RNA replication and assembly is in agreement with recent findings in other negative-strand RNA viruses like influenza (*21*), Sendai (*22*), respiratory syncytial virus (*23*), and human parainfluenza virus Type 3 (*24*). It has been demonstrated for these viruses that only 100 to 150 nucleotides or less from each terminus are sufficient to allow replication and assembly of infectious particles containing a foreign gene. Our data on VSV DI RNA suggest that 36 or fewer nucleotides from the 5′-terminus and 51 or fewer nucleotides from the 3′-terminus are sufficient to signal encapsidation, replication, and assembly of infectious particles. The data agree with previous *in vitro* encapsidation studies which localized an encapsidation site for the leader RNA to the 5′ terminal 14 to 19 nucleotides (*25, 26*) and with studies in VSV-infected cells which suggest that the smallest nucleocapsid complex formed during nucleocapsid assembly contains 5′-terminal 65 nucleotides of viral RNA (*27*). It appears that for the negative-strand RNA viruses examined to date, the *cis*-acting signals controlling transcription, replication, and encapsidation reside entirely within the two termini of their genomes. This observation is in direct contrast to what is known for positive-strand RNA viruses. In addition to the terminal sequences, most positive-strand RNA viruses require internal sequences for replication and packaging of their RNA (*28–33*). Short internal sequences of 78 nucleotides for flock house virus RNA 2 (*28*) or 134 nucleotides for coronavirus defective interfering particle RNAs (*29*) or multiple *cis*-acting sequences located internally in Qβ bacteriophage genome (*30*) have been shown to be required for efficient RNA replication. For Sindbis virus also, internal sequences have been shown to be required for encapsidation and replication of the genomic RNA (*33*). Therefore, the *cis*-acting sequences for RNA replication in negative- and positive-strand RNA viruses appear to be located differently.

Our experiments demonstrated that exceedingly small DI RNAs were replicated and budded to generate particles. Among the variety of naturally occurring DI particles of VSV, DI (0.10) containing approximately 1100 nucleotides appears to be the smallest (*1, 4, 5*). DIΔ7 RNA (191 nucleotides), the smallest RNA budded into infectious particles here, is about six times smaller than DI (0.10) RNA and is less than 2% of the genome of VSV. It is possible that the lack of naturally occurring VSV DI particles containing RNAs of this size is because they are outcompeted by other DI particles in the population. For Sendai virus DI particles, small size of the DI genome per se did not confer selective advantage for replication although there is evidence that selection might occur at

envelopment (*34, 35*). It should be possible now to examine questions of DI RNA competition using the cDNA-based system where no wild-type virus is present. The envelopment and relative stability of different size DI genomes during multiple passages also can be examined directly with or without the selective pressure of competition.

It has been reported for Sendai virus DI RNA replication that only DI RNAs whose lengths are multiples of six nucleotides can be replicated (*36*). This finding was interpreted as a need for the nucleocapsid protein to contact exactly six nucleotides (*36*). Estimates of the mass and molecular composition reported for VSV suggest a stoichiometry of approximately nine nucleotides per N protein molecule (*37*) and since VSV has a helical nucleocapsid, it is reasonable to consider a spacing requirement for the N protein. However, in the work presented here, the sizes of all the DI RNAs that replicate efficiently are not multiples of either six or nine. Similarly, the 11,161 nucleotide genome RNA of VSV is not a multiple of either six or nine, and examination of the sizes of several naturally occurring DI RNAs (*4, 5*) shows that not all of them are multiples of either six or nine. Thus, the results presented here show no evidence for a rigid number of nucleotides per nucleocapsid protein molecule for VSV RNA replication, in contrast to the situation with Sendai virus.

Taken together, the studies with the DI deletion mutants reported here show that 36 nucleotides or less from the 5′-terminus and 51 nucleotides or less from the 3′-terminus of the DI RNA are sufficient to signal encapsidation, replication, assembly, and budding of VSV DI particles. Furthermore, a heterologous sequence placed between these termini was encapsidated, replicated, and assembled into infectious particles, providing the first direct evidence that the termini are sufficient to signal these processes.

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