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Vesicular stomatitis virus defective interfering particle containing a muted internal leader RNA gene

(initiation sites/termination sites/generation of defective particles/autointerference)

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ABSTRACT **The RNA of a unique long defective interfering particle (DI-LT_2) derived from the heat-resistant strain of vesicular stomatitis virus (VSV) contains 70 nucleotides at its 3′ end that are complementary to the 5′ end of the VSV RNA. Following this region of terminal complementarity, there is a precise copy of the 3′ end of the nondefective VSV RNA. The sequence homology between the DI-LT_2 RNA and the 3′ end of VSV RNA extends for at least 60 bases and probably for most of the length of the DI-LT_2 RNA. The DI-LT_2 particle is capable of transcription *in vitro* but produces only a short RNA [defective interfering (DI) particle product], which is encoded by the extreme 3′ terminus of the DI RNA. Neither leader RNA nor capped VSV mRNAs are synthesized by DI-LT_2, although competent templates for these are present. These data suggest that the 3′-terminal initiation is a prerequisite of the production of competent transcripts and that the sequence coding for leader RNA is not, by itself, sufficient for initiation. We propose a model for the origin of this DI particle, involving specific termination and resumption of replication, which is similar to that described previously for another class of DI particle RNAs.**

Vesicular stomatitis virus (VSV) gives rise to defective interfering (DI) particles, which contain RNAs from which portions of the viral genome have been deleted. There are at least three, possibly four, classes of DI particle RNAs, which are structurally dissimilar with respect to the portions of the genomic RNA retained and the amount of self-complementary sequences contained in the RNA (1–5).

The first and major class of defective particle RNAs has retained the 5′ portion of the VSV genome and contains RNA molecules of various sizes (0.1–0.5 of the VSV RNA). These DI particle RNAs have complementary terminal base sequences, a property not shared by the VSV RNA. Both hybridization studies and direct sequence analysis have shown that the DI RNAs contain a 46- to 48-nucleotide sequence at their 3′ termini that is not found on the parental viral genome (6–10). Recently, we have analyzed several DI RNAs of this class and hypothesized that a specific internal polymerase recognition site located 43–48 nucleotides from the 5′ end of VSV RNA was involved in the origin of the terminal complementarity (11). This hypothesis is consistent with the model of Leppert *et al.* (9), which predicts that the complementary ends of the DI particle RNAs result from an aberration of viral replication—the polymerase and nascent daughter strand detach from the nucleocapsid template and the polymerase resumes synthesis using its own daughter strand as a template, thereby generating the complementary terminus.

A second class of DI particle contains an RNA that is homologous to the 5′ portion of the VSV genome and is covalently linked to the complement of this RNA. Under nondenaturing conditions this DI RNA exists as a hairpin duplex structure in which virtually all bases are paired (3, 12). The molecular origin of this type of DI RNA has not been established yet, but the sequence near the "turnaround region" (13) suggests that the DI RNA was generated by copying both strands of a replication fork.

The third class of DI particles appears to be a true deletion mutant in which the DI has retained most of the 3′ half of the VSV genome and a small portion of the 5′ terminus (14, 15). This DI particle, having a genome that closely resembles the 3′ half of the VSV genome, is fully capable of *in vitro* and *in vivo* transcriptive RNA synthesis (16, 17). Recently, we (14) and others (15) have noted that the original seed stocks of this type of DI (DI-LT) contain a small amount of a second DI, DI-LT_2, which is preferentially amplified during repeated high multiplicity passage of the seed stock. This DI, like the original DI-LT, has a genome derived from the 3′ half of the VSV genome and has retained a small portion of the 5′ terminus of the VSV genome. However, unlike the former DI, the extreme 3′ terminus of the genome is a 70-nucleotide sequence that is the complement of the 5′ terminus. Because the terminal complementarity of the DI-LT_2 RNA extends for 70 rather than 45–48 base pairs, it is difficult to understand how the same internal RNA polymerase recognition site could have been involved in the origin of this and the first class of DI particles. Indeed, the base sequence that resembles the RNA initiation site found in position 43–48 from the 5′ end of the VSV RNA is not present at 60–70 nucleotides (18).

We have determined the 3′-terminal base sequence of the DI-LT_2 RNA. The 3′ end of this RNA is the precise complement of the 5′ end of the VSV RNA for exactly 70 nucleotides. After 71 bases, the 3′ end of the *nondefective* genome is present. The nondefective sequence includes the entire leader gene, the intervening sequence, and the beginning of the *N* gene. Our sequencing data do not extend beyond this point, but earlier annealing studies (14, 15) showed that the DI-LT_2 RNA contains most, if not all, of the genetic information from the 3′ portion of the VSV genome. Thus, the DI-LT_2 RNA contains both the defective and nondefective 3′ RNA terminal sequences covalently linked to one another. We have investigated whether the DI-LT_2 is capable of leader RNA and mRNA synthesis as is the original DI-LT particle (16, 17). Our results show that the internal nondefective sequence does not produce transcription products *in vitro* and suggest the possibility that the polymerase requires a terminal sequence in order to initiate transcription.

We suggest a model for the origin of the DI-LT_2 RNA species that utilizes specific sites for termination and resumption of rep-

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Abbreviations: VSV, vesicular stomatitis virus; DI particle, defective interfering particle; AdoMet, S-adenosylmethionine; PEI, polyethyleneimine.

lication by the VSV polymerase. This model has features similar to those described previously for the origin of the most abundant class of DI particles (9) and provides a more unified concept for the origin of DI particles with complementary termini.

MATERIALS AND METHODS

Virus and Cell Growth. VSV and DI particles were grown in BHK (baby hamster kidney) cells and purified as described (3, 14).

Isolation of RNA and Determination of Its Sequence. Viral RNAs were extracted and labeled and their sequences were determined as described (19, 20). DI-LT_2 "panhandle" was isolated by chromatography over Sephadex G-150 in 0.4 M NaCl after annealing and complete digestion with RNase T1 at 0.1 unit/ml and calf intestinal alkaline phosphatase at 0.1 unit/ml for 30 min at 37°C. The duplex RNA was extracted with phenol and labeled at its 3′ ends with pCp and RNA ligase and analyzed by the "wandering spot" method as described (7).

***In Vitro* Transcription.** γ-Thio-ATP terminating transcripts were prepared with approximately equal numbers of VSV (48 μg), DI-LT_2 (23 μg), and DI-T (11.4 μg) particles. The transcription conditions were the same as previously reported (21) except that thio-ATP (1 mM) replaced ATP; GTP and UTP were present at 0.1 mM, and [^{32}P]CTP (20 mCi/μmol; 1 Ci = 3.7 × 10^{10} becquerels) was present at 0.05 mM. All transcription reactions had the same protein concentration (200 μg/ml). Transcription was allowed to proceed for 3 hr and was terminated by the addition of sodium dodecyl sulfate to 1% and 50 μg of tRNA. The mixture was extracted with phenol and chloroform and the RNA was separated from unincorporated triphosphates with Sephadex G-50. Transcripts that bore triphosphate termini were purified by affinity chromatography using a 6-ml column of Affi-Gel 501 (Bio-Rad) according to the method of Smith *et al.* (22). RNAs bearing thiotriphosphate termini were precipitated with salt and ethanol, rinsed with 80% (vol/vol) ethanol, dried under reduced pressure, dissolved in 25 μl of 0.02 M sodium citrate buffer, pH 5.0, containing 1.5 units of RNase T1, and incubated for 20 min at 50°C. The digest was applied to a 20% polyacrylamide gel containing 8 M urea as described (19). The larger T1 oligonucleotides (28-mer, 21-mer, and 13-mer) are identified in Fig. 3. The minor T1 oligonucleotides apparent in the digest of the VSV transcripts are derived from the thiotriphosphate-bearing mRNA transcripts that copurify with leader RNA in this system. Transcripts to be analyzed for the presence of caps were prepared as above but *S*-adenosyl[^{3}H]methionine (Ado[^{3}H]Met) (2 μM, 5–15 mCi/mmol) was included where indicated. The specific activity of the CTP was 0.5 mCi/μmol and ATP replaced thio-ATP in the reactions where indicated. The transcription reactions were terminated by the addition of sodium dodecyl sulfate to 0.5% and the transcripts were separated from unincorporated substrates by chromatography with Sephadex G-50. The RNA in the excluded fractions was purified further by extraction with phenol and chloroform followed by precipitation with ethanol. Unfractionated transcripts were digested in 50 μl of 0.01 M NH_4 OAc, pH 6.0, containing 30 μg of nuclease P1 for 2 hr at 37°C. Four microliters of 0.2 M Tris·HCl, pH 8.0, and 4 μl of calf intestinal alkaline phosphatase at 100 units/ml were added and the incubation was then continued for an additional hour at 37°C to remove all terminal phosphates. Digests were applied to 20 × 40 cm polyethyleneimine (PEI)-cellulose thin-layer sheets. Ionophoresis was carried out in phosphate buffer, pH 7.5, containing 7 M urea, according to Keller and Crouch (23). The PEI-cellulose sheets were dried and cut into 0.5-cm strips, and radioactivities were measured. The ^{3}H incorporation shown in Fig. 4 tracked with the standards, m^7GpppA and m^7GpppAm, which were not resolved from one another by this system.

RESULTS

Unusual Extent of Self-Complementarity in DI-LT_2 RNA. The original DI-LT seed stock contains at least two species of DI particles whose RNAs can be identified by electron microscopy, agarose gel electrophoresis, or analysis of the terminal sequences (14, 15). The RNA species differ in size by only 8–10%. The smaller species (DI-LT RNA) is a linear molecule when visualized in the electron microscope and has retained the original 3′ and 5′ ends of the VSV RNA. The larger species has complementary termini and can circularize under conditions used for electron microscopy. Subsequent passages of the original DI-LT seed stock contained different amounts of the two DI particles, depending upon the conditions of the passage. We examined stocks of purified DI particles for the two classes of RNA by electrophoresis on methylmercury agarose gels and obtained one stock that contains the larger DI-LT_2 RNA with complementary termini and only traces of the smaller DI-LT RNA.

Terminally labeled DI-LT_2 RNA was prepared and its sequence was determined by using the chemical methods described previously (19, 20). The sequence from the 3′ terminus to position 132 was determined. Fig. 1 shows the sequence starting at position 43 from the 3′ end. It is evident that only one sequence was derived and that the trace amounts of the linear RNA in no way compromised the sequencing. The sequence was found to be the precise complement of the 5′ end of the Mudd–Summers strain of VSV RNA for 70 nucleotides

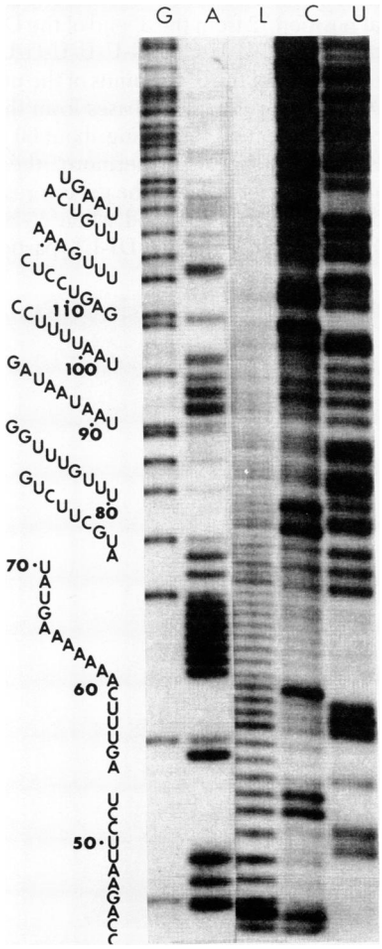

FIG. 1. Chemical RNA sequence analysis of DI-LT_2 RNA, starting at position 43 from the 3′ end. The L indicates a "ladder" generated by formamide cleavage at every ribonucleoside.

(18). To determine the precise point at which the complementarity between the 5′ and 3′ sequence ended, the DI-LT_2 RNA was annealed and the terminal duplex was isolated after digestion with RNase T1 and calf intestinal phosphatase. The isolated duplex with short single-stranded RNA appendages extending to the next G residue was terminally labeled with pCp and RNA ligase. Under these conditions, both strands of the duplex were labeled at their 3′ termini, but to different extents, because one terminus is single stranded and the other is double stranded. Fig. 2 shows a sequence analysis of this RNA by the wandering spot method. In addition to the original 3′ end of the RNA (HO-U-G-C-U-U-C-U-G-G-U... 5′), the sequence 3′ ...G-A-A-A-U-U-C-A-U-A-C-U-U-U-U... 5′ was found. The latter sequence is identical to the sequence previously reported for positions 63–77 from the 5′ end of the VSV genome and includes the L mRNA polyadenylylation site (18). An abbreviation of this sequence is shown below. The precise length of the panhandle region is obtained by comparing this sequence with that of the 3′ end of the DI-LT_2 RNA derived from Fig. 1:

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                    70
5′ pppA-C-G...U-U-U-U-C-A-U-A-C-U-U-A-A-A-G
     . . ....  . . . . . .
3′ HO-U-G-C...A-A-A-A-G-U-A-U-A-U-G-C-U-U-C-U-G-U-U-U-G
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It is readily apparent from the sequences that the DI-LT_2 RNA has a 70-nucleotide-long terminal complementarity (panhandle).

Internal Leader RNA Gene. The base at position 71 that follows the complementary region is an A residue. Following this and beginning at position 72 from the 3′ end of the DI-LT_2 RNA is the sequence ...U-G-C-U-U-C-U-G-U-U-U-G-U-U-U-G-G ... which is the sequence of the 3′ terminus of the nondefective VSV RNA (7, 20, 24). More than 130 bases from the 3′ end of DI-LT_2 RNA were determined, including about 60 nucleotides of the nondefective sequence. Furthermore, the pattern of bands in each of the lanes for as far as the gel can resolve (about 350 bases) is identical to that obtained when 3′-labeled VSV RNA is used (20). We conclude that the DI-LT_2 particle contains a large RNA with termini that are complementary for 70 nucleotides. The 3′ portion of this complementary region is immediately followed by the polymerase initiation site, leader gene, intervening sequence, and *N* gene of the VSV genome.

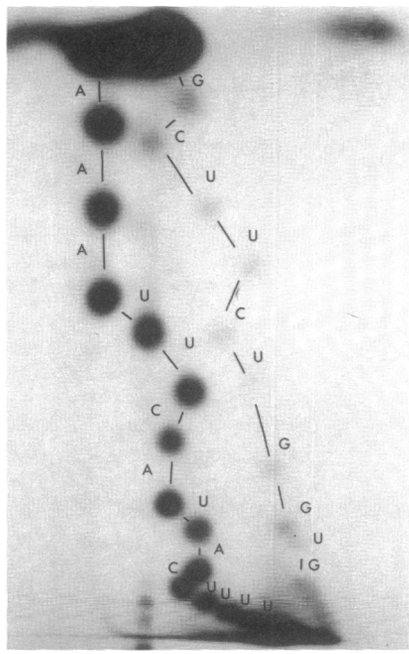

FIG. 2. Wandering spot analysis of the panhandle duplex from DI-LT_2. The isolated duplex was 3′ terminally labeled, subjected to partial alkali digestion, and analyzed by high-voltage electrophoresis in the first dimension and homochromatography in the second dimension.

Transcription Products from DI-LT_2. The presence of both the defective terminal sequence and the internal leader RNA gene raised the possibility that DI-LT_2 particles might be capable of synthesizing both the DI particle product (a 46-base-long RNA encoded by the defective terminal region) and leader RNA. Furthermore, the sequence determination and hybridization studies indicate that the 3′ portion of the *N* cistron is also intact and that this DI contains most of the genetic information of the 3′ half of the VSV genome. Consequently, mRNA transcription from this DI, similar to that observed with DI-LT, might occur.

To distinguish between the synthesis of leader and DI particle product RNAs, we have made use of the size of the larger RNase T1 oligonucleotides that are characteristic for each of the RNA transcripts. Leader RNA yields a single large RNase T1 oligonucleotide, a 28-mer, while DI particle product yields two unique oligonucleotides, a 21-mer and 13-mer (8, 25, 26). To minimize the contribution of large RNase T1 oligonucleotides derived from other RNAs, the transcripts to be analyzed were prepared with γ-thio-ATP, an analog of ATP that contains a sulfhydryl group on the γ phosphate. This analog readily substitutes for ATP in the VSV transcription reaction (ref. 27; unpublished experiments), and the synthesized thiotriphosphate-terminated RNA transcripts can be purified by affinity chromatography with mercury-agarose. Because relatively few of the mRNA transcripts bear triphosphate termini, this procedure effectively purifies the leader and DI particle product RNA away from extraneous RNAs.

Fig. 3 shows the results of such an experiment. Labeled transcripts were prepared by using approximately equal numbers of VSV, DI-LT_2, and DI-T particles. The VSV and the DI-T served as a source of leader RNA and DI particle product RNA, respectively, for comparison. Thiotriphosphate-terminated transcripts were collected by affinity chromatography and digested with RNase T1, and the total products of the experiment were applied to a 20% polyacrylamide gel. From the autoradiogram shown in Fig. 3, it is clear that leader and DI particle product RNAs are readily distinguishable by this method, that DI-LT_2 synthesizes approximately as much DI particle product as the DI-T particles do, and finally, that DI-LT_2 does not synthesize significant amounts of leader RNA. Although not visible in the figure, there is a very small amount of the 28-mer present in the DI-LT_2 lane. However, the amount is so small that it is easily accounted for by the contaminating VSV (0.8%) or DI-LT (3–4%) present in the preparation of DI-LT_2. Both of these contaminants can synthesize leader RNA.

To examine whether mRNAs are synthesized by the DI-LT_2 particle, we have analyzed the transcripts for the presence of methylated cap structures. Transcripts from equal numbers of VSV or DI-LT_2 particles that were doubly labeled with Ado[^{3}H]Met and [α-^{32}P]CTP were digested with nuclease P1 and calf intestinal phosphatase. The resultant digestion products were separated by ionophoresis on thin-layer sheets of PEI-cellulose (23) under conditions that separate m^7GpppA and m^7GpppAm from inorganic phosphate and nucleoside monophosphates. Fig. 4 summarizes the results of this experiment. The VSV transcripts contained 1940 cpm of ^{3}H that tracked with authentic cap m^7GpppA and m^7GpppAm structures. The upper panel of Fig. 4 shows that transcripts from an equal number of DI-LT_2 particles contained only 65 cpm of ^{3}H label in the cap structures (note change in scale). This comparison shows that the DI-LT_2 preparation synthesized only 3.5% as many methylated caps as did an equal number of VSV particles. Because

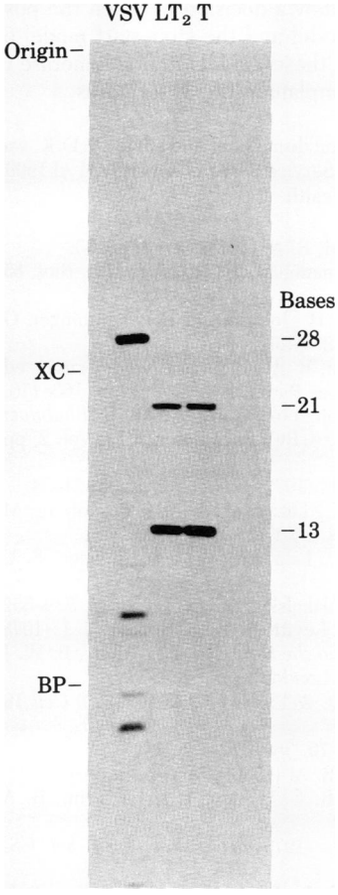

FIG. 3. Acrylamide gel electrophoresis of *in vitro* transcription products from DI-LT_2 made in the presence of [α-^{32}P]CTP and γ-thio-ATP. Transcripts from DI-T, DI-LT_2, and VSV were prepared, digested with RNase T1, and fractionated on a polyacrylamide gel. XC, xylene cyanol; BP, bromphenol blue.

the DI-LT_2 preparation has trace contaminants of both VSV (0.8%) and the linear DI-LT (3–4%), a small amount of cap synthesis by the mixture is expected. We conclude from these experiments that the DI-LT_2 particle does not synthesize leader RNA or capped mRNAs *in vitro*, although its genome contains genes for both types of transcripts.

DISCUSSION

Structure and Possible Origin of DI-LT_2. Previous work (14, 15) showed that the original seed stocks of DI-LT contain at least two different species of DI particles. The most abundant DI particle was large (about 50% of the size of VSV, had an RNA that contained the genetic information of the 3′ half of the VSV genome, was capable of *in vitro* and *in vivo* transcription (16, 17), and was able to interfere heterotypically with some, but not all New Jersey serotype VSV strains (28, 29). Analysis of the RNA derived from this particle showed that it retained the 3′- and 5′-terminal sequences of the parental genome but had undergone a deletion that began 320 nucleotides from the 5′ end and was wholly contained within the *L* gene. We refer to this DI particle as DI-LT.

Upon repeated passage of the original seed stock, another DI particle becomes dominant among the progeny. Like DI-LT, this particle has a genome that is derived principally from the 3′ end of the VSV genome and has undergone a deletion in the *L* gene, but one that may be different from that of DI-LT (see below). The RNA of this particle is about 500 bases longer than that of DI-LT and unlike DI-LT RNA has termini that are complementary. We refer to this particle as DI-LT_2.

In the present communication we show that the complementarity of the termini is unusual in that it extends for 70 nucleotides rather than 45–48 nucleotides as found in other DI particle RNAs (11). Furthermore, the complementary region

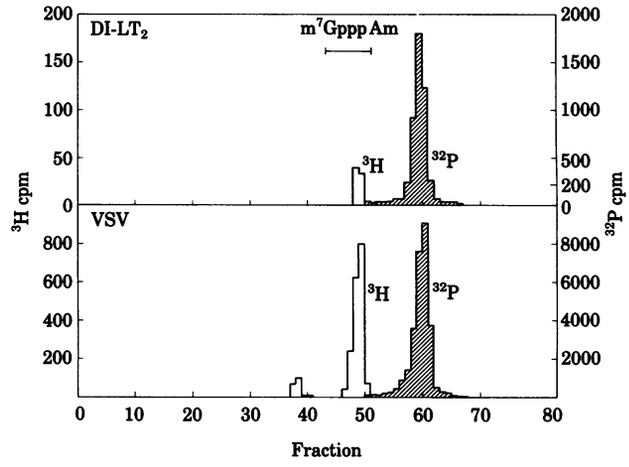

FIG. 4. Analysis of VSV and DI-LT_2 transcripts for the presence of methylated cap structures. Ado[^{3}H]Met- and [α-^{32}P]CTP-labeled transcripts were digested with RNase P1 and alkaline phosphatase, and the resulting digests were fractionated by PEI ionophoresis.

is followed on the 3′ side by a complete leader RNA gene and the beginning of the *N* gene. Hybridization experiments suggest that the homology to the VSV genome is uninterrupted and may extend for the next 5 kilobases and include the four smaller genes in their entirety. However, DI-LT_2 RNA is not the result of the simple addition of a 70-base complementary sequence onto the 3′ end of DI-LT RNA because they differ in size by 500 nucleotides. It is possible that DI-LT_2 arose by either multiple additions to the DI-LT RNA or, alternatively, by a single addition to a deletion DI that is larger than DI-LT. In either case a multistep process must be invoked to account for both the deletion and the 70-nucleotide addition at the 3′ terminus. At present it is not known which of the two steps occurred first.

A model for the addition of a sequence at the 3′ terminus of DI particle RNAs has been proposed by Leppert *et al.* (9). However, a study of the relevant sequences of the DI-LT_2 RNA suggests that a different mechanism may have operated in the origin of the 3′ terminus of the DI-LT_2 RNA. In this model (Fig. 5), a nascent minus strand and polymerase detach from the plus strand template at the sequence 3′ ... U↓G-A-A ... 5′, 70 bases from the 3′ end of the template. The termination at a GAA is consistent with other examples of termination sites in VSV and DI particle RNAs. As discussed previously (20), this sequence alone is not sufficient to specify termination or processing, because the same sequence occurs at many other locations in VSV RNA. Other elements of the termination or processing signals are not known as yet. The polymerase–nascent strand complex resumes synthesis at the 3′ end of another minus strand and thereby adds a normal (+) sense sequence onto the end of the 70-nucleotide (−) sense fragment. The complement of this product, which is shown in the next step in the figure, has a 70-nucleotide sequence at its 3′ end followed by the normal 3′-terminal sequence. The origin of the A residue in position 71 is not explained by the above model. However, the VSV polymerase has been shown to insert A residues in transcripts that are not specified by the template (18, 25, 30–33), and perhaps a similar phenomenon occurred here.

The above scheme for the origin of the 3′-end DI-LT_2 RNA has important differences from and similarities to the model proposed by Leppert *et al.* (9). The models are different in that one depends upon a resumption of synthesis at an internal position on a template strand, whereas the other specifies that a prematurely terminated polymerase resumes synthesis at the normal 3′-terminal initiation site on the minus strand template. The unifying feature of these two models is that specific re-

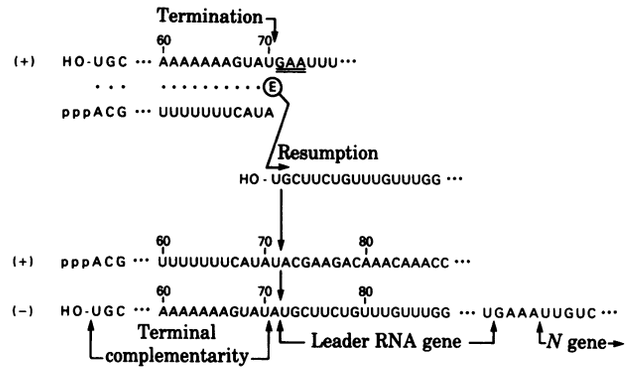

FIG. 5. Model for the origin of the 70-base-long terminal complementarity in the DI-LT_2 RNA. A 70-nucleotide-long nascent minus strand results from a premature termination of transcription. After resumption of transcription at the 3′ end of another (−) strand, the complement of the DI-LT_2 RNA (plus strand) is synthesized. E, polymerase enzyme.

sumption sites for the VSV polymerase allow a transcriptional leap to take place. The end product of the illicit replication events described by both models is a defective viral RNA that has a 3′ terminus different from that of VSV RNA.

Dysfunction of Internal VSV RNA Promoter. Although the DI-LT_2 particles contain the genetic information of the 3′ half of the VSV genome, only the extreme 3′ end of the template is actively transcribed *in vitro*. The only recognizable product is the same small transcript made by most other DI particles. Thus, as with the other DI particles, terminal initiation takes place on the DI-LT_2 template and transcription is terminated at a strong stop signal 46 nucleotides from the 3′ terminus. Surprisingly, the internal leader RNA gene, which starts at position 72, is silent, and no evidence of leader RNA synthesis is detected during *in vitro* transcription. This result suggests that base sequence, by itself, does not specify efficient binding and initiation of the polymerase—some structural or topographical component is also required. Conceivably some unique alteration of the nucleocapsid structure in the vicinity of the internal leader gene prevents efficient initiation at that site. Because the DI-LT_2 nucleocapsid serves as an efficient template for replicative RNA synthesis, the hypothetical alteration would have to affect the initiation but not the elongation of RNA chains. Alternatively, the VSV polymerase may be able to efficiently enter and initiate only at the extreme 3′ terminus of the template. Although there are no clear indications yet as to why the polymerase might initiate only at the terminus, it is possible that some structural feature of the terminus (i.e., a specific protein or a short stretch of RNA protruding beyond the first *N* protein) may be indispensable. Alternatively, the requirement for terminal binding and initiation may result from steric considerations. For example, if transcription requires a perturbation of the nucleocapsid structure such as a partial displacement of the N proteins, that process may be initiated only at the terminus. Irrespective of the causes, effective initiation only at the terminus of the RNA strongly supports the conclusions drawn from the UV inactivation studies that the 3′ terminus plays an important role in the initiation of transcription (34, 35). It should be noted that, as with the results of the UV inactivation studies, the present study does *not* allow a decision between the possibilities of the processing model and the stop–start model of VSV transcription. In both of these models, the polymerase is constrained to "enter" the template at the 3′ terminus.

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