JOURNAL OF VIROLOGY, Jan. 1988, p. 47–53
0022-538X/88/010047-07$02.00/0

Vol. 62, No. 1

Effects of 5′-Terminal Modifications on the Biological Activity of Defective Interfering RNAs of Sindbis Virus

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Received 30 June 1987/Accepted 14 September 1987

We have been studying defective interfering (DI) genomes of the RNA enveloped virus Sindbis virus. Deletion mapping of a DI cDNA demonstrated that only sequences at the 3′ and 5′ termini of the genome are required for the DI RNA to be biologically active. We constructed a series of cDNAs that transcribe DI RNAs differing only in 5′-terminal sequences. Two of the 5′ termini identical to ones found in naturally occurring DI RNAs are the 5′ terminus of the virion RNA (DI-549) and the first 142 nucleotides from the 5′ terminus of the subgenomic 26S mRNA attached to the 5′ terminus of the virion RNA (DI-15). The latter has a 42-nucleotide deletion from nucleotides 25 to 66 in the 26S RNA sequence. These DI RNA transcripts were biologically active, but one (DI-526) which did not have the 42-nucleotide deletion of DI-15 was not replicated. The DI RNA isolated after the presumed amplification of the DI-526 transcript had deleted the first 54 nucleotides of the 26S RNA sequences. The 5′ terminus of Sindbis virion RNA contains a stem and loop region that is conserved among alphaviruses. An 11-nucleotide deletion in DI-549 that disrupted this stem and loop rendered this DI RNA inactive. In contrast, this same deletion in DI-15 and one that removed an additional 100 nucleotides of the virion 5′ terminus did not prevent its amplification. We did not detect by computer analysis any common secondary structures among the biologically active DI RNAs that distinguished them from those RNAs that were not amplified. Our results support the conclusion that tertiary structure or the ability of the RNA to adapt its structure upon interaction with protein is important in the recognition process.

Defective interfering (DI) particles are deletion mutants of viruses primarily noted for their ability to interfere with the replication of homologous or closely related viruses (2, 3, 14). Their genomes provide a valuable tool for analyzing sequences that are required for replication and encapsidation. The deleted genomes are less complex than those of the parental virus, and although they need not retain coding information, they must contain sequences that serve as recognition signals for replication and encapsidation, and they can be used to help identify these sequences.

We have been analyzing DI genomes derived from the RNA enveloped virus Sindbis virus. In a previous study, we demonstrated by deletion mapping of a DI cDNA that most of the sequences in the genome are not required for the expression of the biological activity of the DI RNA (7). Only deletions at the 3′ terminus in the 19 nucleotides that are highly conserved among alphaviruses (12) and in the 162 nucleotides at the 5′ terminus are able to prevent the DI RNA from being replicated and packaged.

Most of the DI RNAs that we isolated by high-multiplicity passaging of virus have nucleotides 10 to 75 of the rat $tRNA^{Asp}$ at their 5′ terminus (9, 10). The CCA at the 3′ end of the tRNA is covalently attached to either nucleotide 31 or nucleotide 23 of the 5′ terminus of the virion RNA. But these are not the only sequences found at the 5′ terminus of naturally occurring DI RNAs (17). One DI RNA population has a 5′ terminus identical to that of the virion genome. Another DI population, referred to as DI-1, has a more unusual 5′ terminus consisting of 100 nucleotides from the 5′ terminus of the subgenomic 26S mRNA attached through a C residue to the 5′ terminus of the virion RNA. It also has a 42-nucleotide deletion encompassing nucleotides 25 to 66 of the 26S RNA.

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The subgenomic 26S RNA serves as the mRNA for the structural proteins of the virus. It is identical in sequence to the 3′ one-third of the virion RNA (12). The template for transcription of 26S RNA is the full-length minus strand (16). The replicase-transcriptase complex must bind to a particular region of the minus strand at or near the start of 26S RNA to initiate transcription. The 21 nucleotides just upstream from the 26S RNA sequences are a highly conserved region among alphaviruses, and they have been proposed to be the recognition signal for binding the transcriptase (11). Our previous identification of 26S sequences at the 5′ terminus of a naturally occurring DI RNA can be explained by models similar to those proposed for the generation of DI RNAs of vesicular stomatitis virus (5, 14). After initiating transcription of 26S RNA, the enzyme complex would fall off the original template but would then be able to reinitiate synthesis at the 3′ terminus of a minus strand.

In this paper, we present a more detailed analysis of those DI RNAs that have either the 5′ terminus of the 26S RNA or the same 5′ terminus as the virion RNA. We were able to focus exclusively on this region of the DI genome because the DI RNAs prepared from cloned cDNAs differ only in 5′-terminal sequences. In addition to making deletions in these 5′ termini, we constructed a DI cDNA which has the 5′ terminus of the 26S RNA intact. A comparison of this DI RNA with the one containing the 42-nucleotide deletion enabled us to inquire whether the deletion was an accidental event or was essential for the DI RNA to be biologically active.

MATERIALS AND METHODS

Plasmids. KDI-25 and KDI-549 were described previously (7). These plasmids have the same DI cDNA sequences except that at the 5′ terminus KDI-25 has nucleotides 10 to 75 of $tRNA^{Asp}$ and KDI-549 has the 5′-terminal sequence of Sindbis virion 49S RNA. The 5′ termini of the DI RNA

TABLE 1. Some of the plasmids used in this study and a description of the 5′ termini of their DI RNA transcripts

Plasmid[a]	5′ terminus of the RNA transcripts[b]
KDI-25	$tRNA^{Asp}$ (7)
KDI-549	Identical to the 5′ terminus of the 49S virion RNA (7)
KDI-549/011	Identical to DI-549, except that nucleotides 16 to 26 of the 49S virion RNA are deleted
KDI-526	The 5′-terminal 142 nucleotides of 26S mRNA are attached to the 5′ terminus of the 49S virion RNA
KDI-15	Identical to DI-526, except that nucleotides 25 to 66 in the 26S RNA sequence are deleted. This 5′ terminus is identical to that of a naturally occurring DI RNA (17)
KDI-15/011	Identical to DI-15, except that nucleotides 116 to 126 are deleted. This deletion is comparable to the one in DI-549/011
KDI-15/111	Identical to DI-15, except that nucleotides 116 to 226 are deleted

[a] All plasmids are designated with the prefix K.
[b] Diagrams of the DI RNAs are shown in Fig. 7. The deletions and junctions were verified by sequencing.

transcribed from these and other plasmids are described in Table 1 (also, see Fig. 7). PiK2, obtained from H. Huang (Washington University), contains the 5′-terminal 130 nucleotides of the Sindbis 49S RNA sequence positioned directly downstream from the promoter for the SP6 DNA-dependent RNA polymerase. CTS253 is a derivative of KDI-25 in which 1,689 internal nucleotides of the DI genome were replaced with a 1,492-nucleotide fragment from pSV2-cat including the gene for the bacterial protein chloramphenicol acetyltransferase (CAT) (6).

Construction of KDI-15. KDI-15 construction is described in Fig. 1. DI-1 RNA was prepared from particles obtained by infection of cells with passage 17 of a DI-1 particle stock (17). The RNA was purified on an oligo(dT) column before use. Primer extension to prepare the correct single-stranded cDNA is illustrated in Fig. 1A. The PiK2 DNA was linearized at the single *Hph*I site located directly upstream from the 5′ terminus of the virion 49S cDNA sequence (Fig. 1B). The DNA was treated with T4 DNA polymerase to generate single-stranded 5′-protruding ends at least 10 nucleotides long. An oligonucleotide of 21 nucleotides, referred to as the bridging primer, was synthesized by the Washington University Oligonucleotide Synthesis Laboratory. It has the following sequence:

5′-TGACACTATAGATAGTCAGCA-3′

The 5′ half of this primer contains sequences from the SP6 promoter and is complementary to one of the protruding 5′ ends of the PiK2 fragment. The 3′ half of the primer is composed of sequences from the 5′ terminus of 26S RNA and is complementary to the 3′ terminus of the cDNA extension product (Fig. 1C). The bridging primer was phosphorylated by treatment with polynucleotide kinase. A mixture containing 10 pmol of this phosphorylated bridging primer, 0.086 pmol of PiK2 fragment, and 10 pmol of the cDNA extension product in a volume of 10 μl containing 150 mM KCl and 10 mM Tris hydrochloride (pH 7.5) was heated to 65°C for 10 min and allowed to cool to room temperature. The mixture was then adjusted to 100 mM KCl, 30 mM Tris hydrochloride (pH 7.5), 15 mM $MgCl_2$, 2 mM dithiothreitol, and 250 μM ATP in a final volume of 20 μl and incubated with the Klenow fragment of DNA polymerase I and T4 DNA ligase for 45 min at room temperature and then for 4 h at 14°C. After phenol-chloroform extraction, the sample was digested with *Bst*XI, heat inactivated at 70°C for 10 min, ethanol precipitated, and

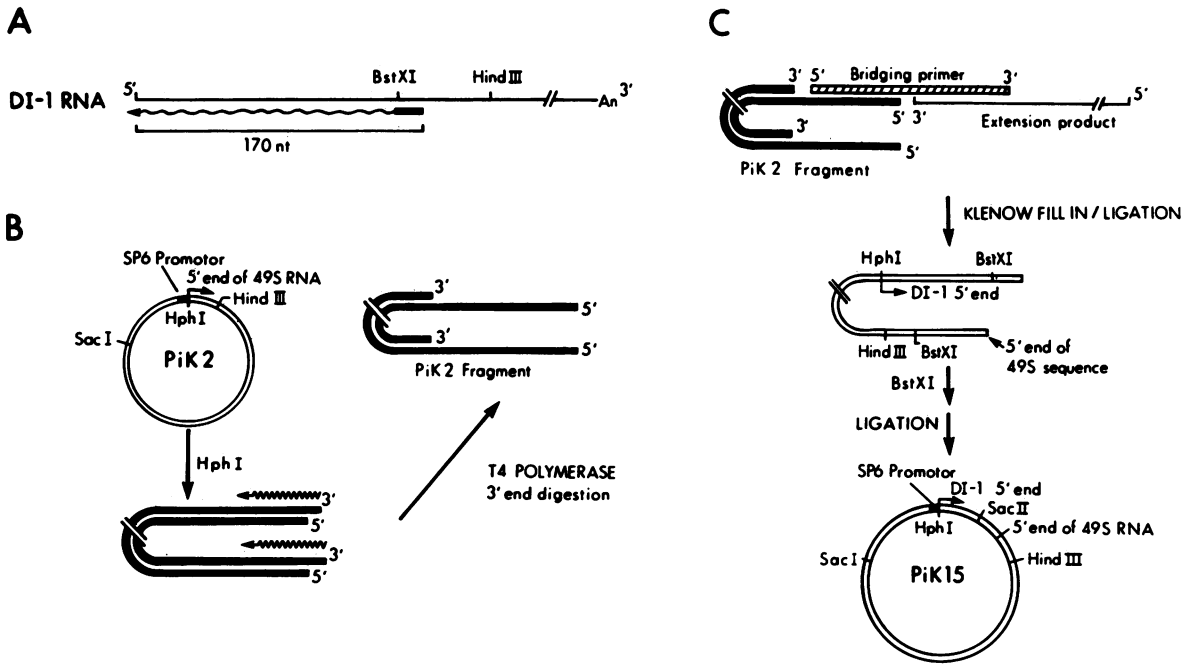


FIG. 1. Construction of PiK15. The details of each of these steps are described in Materials and Methods. PiK15 is the intermediate plasmid; the complete DI cDNA clone is KDI-15. (A) Primer extension on DI-1 RNA. (B) Preparation of the PiK2 fragment. (C) Assembly of PiK15 with the 170-nucleotide extension product, the PiK2 fragment, and the synthetic bridging primer. nt, Nucleotides.

incubated with T4 DNA ligase (Fig. 1C). Bacteria transformed with the correct plasmid were detected by colony hybridization with ^{32}P-labeled bridging primer as a probe. The intermediate plasmid referred to as PiK15 was shown to have the predicted sequence by sequence determination by the dideoxynucleotide chain termination method (15). The final plasmid, designated KDI-15, was obtained by ligation of the *Sac*I-*Hin*dIII fragment of PiK15, indicated in Fig. 1C, with a *Hin*dIII-*Sac*I fragment from KDI-25 (7).

Construction of KDI-526. The construction of plasmid KDI-526 was identical to that described for KDI-15 with the following exceptions. (i) The primer extension of Fig. 1A was done with 26S RNA as the template, and the primer used was complementary to nucleotides 147 to 157 of the 26S RNA. (ii) The intermediate plasmid, analogous to the one shown in Fig. 1C, was obtained by blunt-end ligation without any prior restriction enzyme digestion. (iii) The final plasmid KDI-526 was obtained by ligation of the *Sac*I-*Sac*II fragment of the intermediate plasmid with a *Sac*II-*Sac*I fragment from KDI-15. Both KDI-15 and KDI-526 constructs were screened by colony hybridization with the bridging primer as a probe. The base-composition-independent hybridization method described by Wood et al. (20) was followed.

Construction of deletion clones. The 11-base deletions in KDI-549 and KDI-15 begin at an *Rsa*I site at base 15 in the 49S cDNA sequence and extend downstream to a *Hin*fI site at base 25. KDI-549/011 was constructed by ligation of the following three fragments: (i) a 273-base-pair (bp) fragment from PiK2 extending from the *Sac*I site (Fig. 1B) to the appropriate *Rsa*I site; (ii) a 100-bp fragment from PiK2 extending from the *Hin*fI site to the *Hin*dIII site (Fig. 1B); and (iii) a 4,080-bp fragment from KDI-25 extending from the *Hin*dIII site to the *Sac*I site. The construction of KDI-15/011 was identical except that fragment (i) was obtained from PiK15. This fragment and the 4,080-bp *Hin*dIII-*Sac*I fragment were ligated together to obtain the KDI-15/111 plasmid.

Construction of clones containing the chloramphenicol acetyltransferase (*cat*) gene. CTS253 has been described previously (6). Both KDI-15CT and KDI-526CT were derived from CTS253 by replacing the 508-bp *Sac*I-*Hin*dIII segment in CTS253 with the corresponding *Sac*I-*Bal*I segments from KDI-15 and KDI-526, respectively. The *Hin*dIII site in CTS253 is downstream from the *Bal*I site (base 245) and the 51-base conserved sequence (7). The *Bal*I-*Hin*dIII junction regions of KDI-15CT and KDI-526CT were sequenced by the dideoxynucleotide chain termination method (15) to establish that they were correct.

Transcription and transfection assays. Transcription and transfection assays, including the isolation of cellular RNAs and the analysis by agarose gel electrophoresis, were identical to those we have described previously (7).

Isolation of RNA for chemical sequencing. Cellular DI RNAs were prepared from five 100-mm dishes of infected chicken embryo fibroblasts. The DI RNAs were extracted from the cells after 10 h of infection as described previously (18) and purified on oligo(dT) columns. The method of Maxam and Gilbert (8) was used to sequence the cDNA extension products of DI-15/011, DI-15/111, and DI-526 RNAs.

In vitro translation. The DI RNAs used for in vitro translation were transcribed in the presence of 1 mM m7G(5′)ppp(5′)G to cap the DI RNA transcripts (4). Before translation, the DI RNA transcripts were treated with 30 μg of DNase I per ml at 37°C for 15 min. In vitro translation was done with the rabbit reticulocyte in vitro translation kit from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). DI RNAs (500 ng) were translated in the presence of [^{35}S] methionine, using the specifications of the manufacturer.

Transfection and assay for CAT activity. Chicken embryo fibroblasts in 35-mm dishes were simultaneously transfected with 1 μg of DI transcript and infected with Sindbis virus at a multiplicity of infection of 30. Incubation was at 30°C for 18 h. The preparation of extracts and the assay for CAT activity were those described by Gorman et al. (1) except that 0.1 μCi of [^{14}C]chloramphenicol (20 μM final concentration) was used in a final volume of 100 μl. The reaction time was extended to 2 h to increase the signal of CAT activity at first passage.

RESULTS AND DISCUSSION

Deletion analysis of DI RNAs that have the 5′ terminus of virion 49S RNA. We demonstrated previously that DI RNAs transcribed in vitro are amplified after transfection and passaging (7). We cloned the cDNA of a DI RNA directly downstream from the promoter for the SP6 bacteriophage DNA-dependent RNA polymerase. This cDNA, referred to as KDI-25, was transcribed into RNA and then transfected into chicken embryo fibroblasts simultaneously infected with helper Sindbis virus. Although only a small fraction of the cells received DI RNA, essentially all the cells were infected with virus so that the DI RNA could be amplified by the proteins produced by the helper virus. After one to two passages, DI-25 RNA became the major viral RNA species in infected cells. A DI RNA (DI-549) that had the 5′ terminus of the 49S virion RNA but the rest of the sequences identical

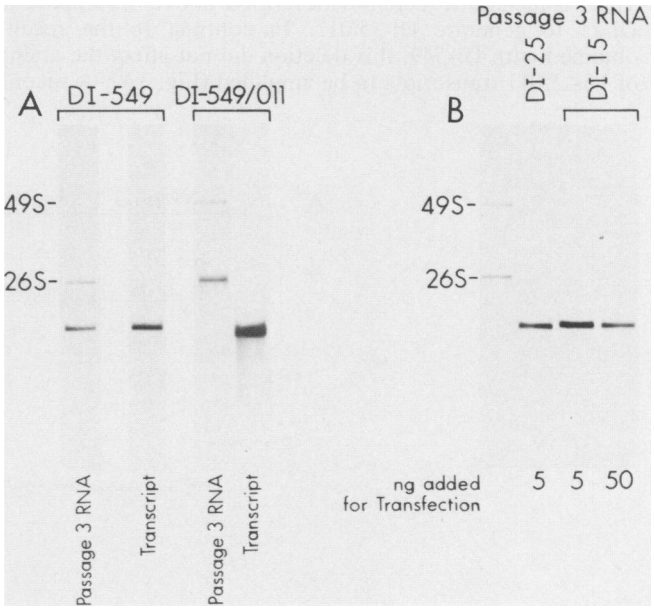


FIG. 2. Analysis of DI-549, DI-549/011, and DI-15 transcripts after transfection and passaging. RNA transcripts were transfected into chicken embryo fibroblasts in the presence of helper Sindbis virus, and the viral RNAs synthesized during the formation of passage 3 were analyzed by agarose gel electrophoresis. (A) Cells were transfected with 1 μg of transcript, using DI-549 transcript or DI-549/011 transcript. (B) Cells were transfected with the amount of transcript indicated. Lane 1 shows the pattern of RNA for cells infected with Sindbis virus alone.

to the sequence of DI-25 was also amplified after transfection and passaging (7) (Fig. 2A).

The 5′ terminus of Sindbis virion RNA is a region of the virion genome that is only weakly conserved among alphaviruses, but the first 44 nucleotides form a stem and loop structure that is conserved at the 5′ termini of different alphaviruses (13). We constructed KDI-549/011 by making a deletion in KDI-549 from nucleotides 15 to 25 to determine whether disrupting this stem and loop structure in a DI RNA would affect the ability of the RNA to be amplified. DI-549 and DI-549/011 transcripts were transfected into chicken cells in the presence of helper virus. The cells were incubated for 16 h (passage 1), and a fraction of the supernatant fluid containing virus was used to infect a new monolayer of cells. The medium from this infection was harvested 16 h later (passage 2), and viral RNAs synthesized during the formation of passage 3 were analyzed. DI-549 RNA was clearly detected during the formation of passage 3, whereas DI-549/011 was not (Fig. 2A).

The 11 nucleotides that are essential for the amplification of DI-549 are not present in DI-25, the DI RNA with $tRNA^{Asp}$ sequences at the 5′ terminus. We constructed another cDNA clone, KDI-15, identical to KDI-25 except that the 5′ terminus was that of the naturally occurring DI-1 RNA that has 100 nucleotides of the deleted subgenomic 26S RNA attached to the 5′ terminus of virion RNA. The same 11-nucleotide deletion was made in DI-15 to determine whether a different 5′ terminus could also make these particular nucleotides superfluous.

DI RNAs with 26S RNA sequences at the 5′ terminus. The construction of KDI-15 is described in Fig. 1 and in Materials and Methods. DI-15 differs from DI-25 only in the 5′-terminal sequences and was amplified after transfection and passaging to the same extent as DI-25 RNA (Fig. 2B). The 11-nucleotide deletion described above was made in DI-15 to generate DI-15/011. In contrast to the results obtained with DI-549, this deletion did not affect the ability of DI-15/011 transcripts to be amplified (Fig. 3A). A second deletion beginning at the same upstream site as the 11-nucleotide deletion but extending downstream for 111 nucleotides was also made in DI-15 to generate DI-15/111. This deleted RNA was also amplified to the same extent as DI-25 and DI-15 (Fig. 3A).

To determine whether the amplified RNAs still retained the original deletions, we sequenced the RNA isolated from cells during the formation of passage 3. After purification of the cellular RNAs, cDNAs were prepared by primer extension. The extension products obtained from both the transcripts and the cellular RNAs were essentially the same size (Fig. 3B). Primer extension on RNA transcripts gives rise to products that are one nucleotide longer than the products obtained by extension on cellular RNAs. The transcripts have one extra G residue at their 5′ termini which may be removed during replication. The sequence of the extension products from the cellular RNAs showed that they contained the junction regions expected for the 11-nucleotide and the 111-nucleotide deletions (data not shown).

DI RNA transcripts with the 5′ terminus identical to that of 26S RNA were prepared from a cDNA construct referred to as KDI-526. In contrast to the results obtained with DI-15 RNAs, DI-526 RNA was amplified very poorly (Fig. 4A). DI RNA was detected at passage 3 only when the original transfection was done with 1 μg of transcript. The extent of amplification was variable, and in some experiments DI RNAs were not detected. Further analysis showed that the amplified DI RNA differed from the input transcript. The cDNA product obtained by primer extension of the cellular RNA was a different size from the product obtained by primer extension of the transcript (Fig. 4B). Chemical sequencing of the RNA showed that the first 54 nucleotides of the 26S RNA were deleted (data not shown). Thus, amplification of the RNA transcript was most likely preceded by a deletion event.

Assay for DI RNA replication in transfected cells. We considered the possibility that DI-526 was not amplified because it could not be encapsidated. The encapsidation

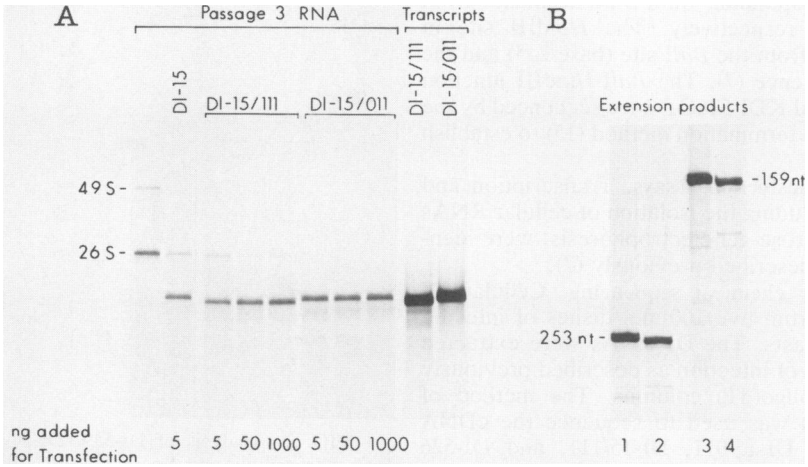


FIG. 3. Analysis of the transcripts of two DI-15 mutants containing deletions in the 49S RNA 5′-terminal region. (A) Agarose gel electrophoresis of DI transcripts and passage 3 RNAs. Cells were transfected with the amount of transcript indicated in the figure. Lane 1 is the pattern obtained from cells infected with Sindbis virus alone. (B) cDNA extension products prepared from transcripts and passage 3 RNAs. A 15mer complementary to nucleotides 248 to 262 of 49S RNA was used to prepare the cDNA extension products from DI-15/111 transcript (lane 1) and passage 3 RNA (lane 2). A 21mer complementary to nucleotides 49 to 69 of 49S RNA was used to prepare the cDNA extension products from DI-15/011 transcript (lane 3) and passage 3 RNA (lane 4). The cDNAs were labeled with [^{35}S]dATP during synthesis. Samples were analyzed by electrophoresis in a 6% polyacrylamide gel for 8 h (lanes 1 and 2) or 4 h (lanes 3 and 4). The sizes of the extension products were determined from coelectrophoresed sequencing ladders. nt, Nucleotides.

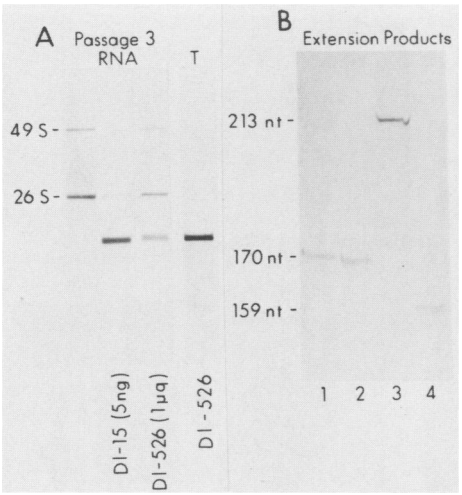
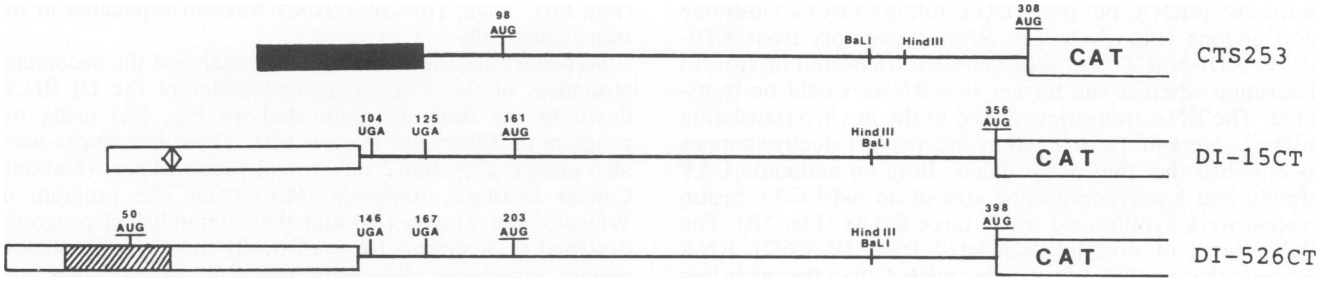


FIG. 4. Analysis of DI-526 transcript. (A) Agarose gel electrophoresis of passage 3 RNA as described in the legend to Fig. 3. (B) cDNA extension products prepared from DI-15 transcript (lane 1), DI-15 passage 5 cellular RNA (lane 2), DI-526 transcript (lane 3), and DI-526 passage 5 cellular RNA (lane 4). The 21mer described in the legend to Fig. 3 was used to prepare these extension products. nt, Nucleotides.

process does discriminate against 26S RNA since cells infected with Sindbis virus contain both 49S virion RNA and 26S RNA, yet only 49S RNA is encapsidated. 26S RNA is identical to the 3′ one-third of 49S RNA. Therefore, the basis for this discrimination might be the presence of a 5′-terminal sequence or structure in 49S RNA that facilitates encapsidation or a 5′-terminal sequence or structure in 26S RNA that inhibits the packaging of 26S molecules. The transfection and passaging assay requires that a DI RNA be both replicated and packaged for it to accumulate by passage 3. We have recently developed a sensitive assay that can detect the replication of DI RNAs after transfection by their ability to be translated. This assay depends on inserting the bacterial *cat* gene into the DI cDNA so that the transcribed RNA is translated in the proper reading frame. We originally replaced nucleotides 242 to 1928 of the KDI-25 plasmid with 1,492 nucletoides from the pSV2-cat plasmid to create the plasmid CTS253 (6). When RNA transcribed from this plasmid was transfected into cells in the presence of helper virus, the newly replicated RNA translated enough CAT protein for the enzymatic activity to be detected in cell extracts. The DI RNA with these foreign sequences was amplified, and both RNA and CAT activity were detected in cells in later passages.

We replaced the internal portion of both KDI-15 and KDI-526 with a fragment containing the *cat* gene to generate KDI-15CT and KDI-526CT, respectively. There are two AUGs which serve as initiator codons for the synthesis of CAT protein in these RNAs (Fig. 5A). The upstream AUG is the one at which the synthesis of the nonstructural proteins of Sindbis virus begins (13). Initiation at this codon should give rise to a fusion protein between the first amino acids of the nonstructural protein nsP1 and the CAT protein. This fusion protein translated from CTS253 RNA was detected and identified as a fusion protein by immunoprecipitation not



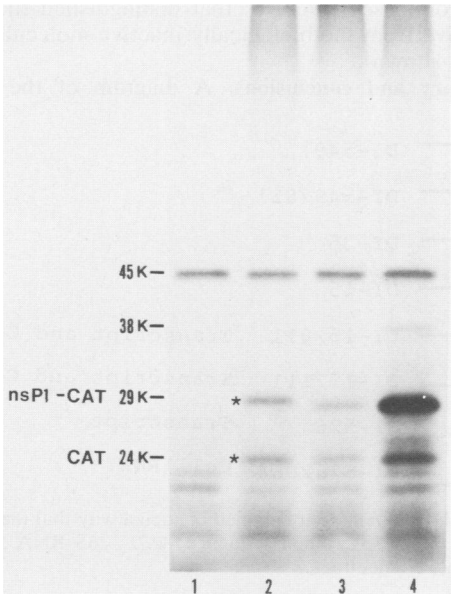


FIG. 5. (A) Diagram of the 5′ termini of the DI RNAs encoding the *cat* gene. The three RNAs are aligned at the *Bal*I site. KDI-15CT and KDI-526CT were constructed by ligation of their respective fragments (see Materials and Methods and Fig. 1) to a *Hin*dIII-*Sac*I fragment from CTS253 that had been filled in at the *Hin*dIII end. This construction led to the deletion of the 12-base *Bal*I-*Hin*dIII segment in CTS253, and this was established by sequence analysis of the junction region. The numbers above the AUG and UGA codons indicate the position of the first nucleotide of the triplet in their respective RNAs. The AUG and UGA codons in each DI RNA are in frame with each other. The diamond near the 5′ end of DI-15CT represents the 42-nucleotide deletion in the 26S RNA sequences. (B) In vitro translation of CTS253, DI-15CT, and DI-526CT RNAs. RNAs were used at a concentration of 500 ng. Proteins were analyzed on a 10% polyacrylamide gel. Lanes: 1, no added RNA; 2, CTS253 RNA; 3, DI-15CT RNA; 4, DI-526CT RNA. The two bands marked with asterisks were identified previously as authentic CAT polypeptide and as the nsP1-CAT fusion polypeptide (6). Numbers on left show molecular weight (K, 10^3).

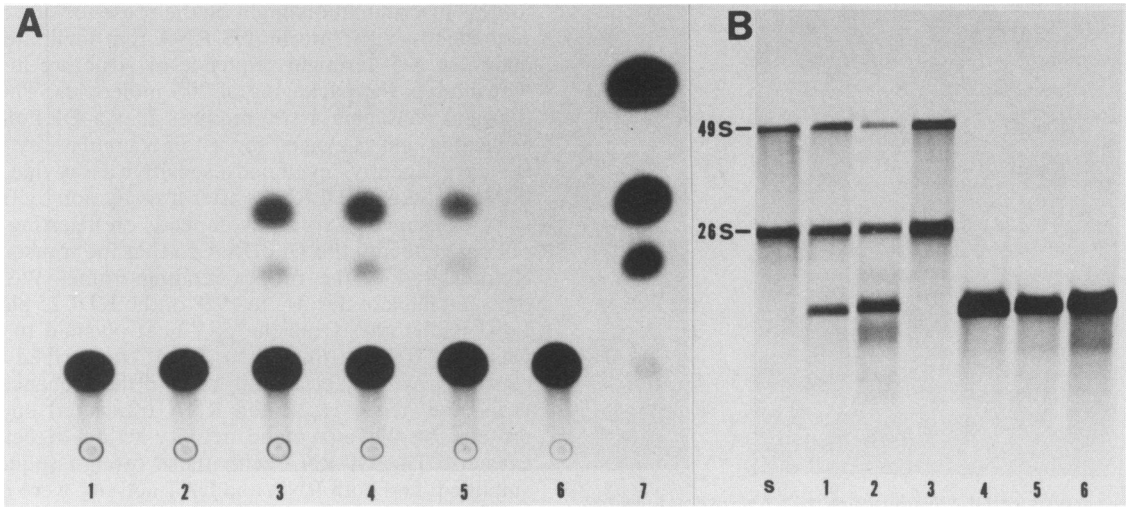


FIG. 6. Analysis of DI-15CT and DI-526CT transcripts. (A) CAT activity in cells transfected with DI RNA. Cells were transfected with 1 μg of DI RNA and except where indicated were also infected with Sindbis virus. Extracts were prepared after 18 h at 30°C and were assayed for CAT activity. Lanes: 1, cells infected with Sindbis virus alone; 2, cells transfected with CTS253 RNA in the absence of added helper virus; 3 and 4, cells transfected with CTS253 RNA; 5, cells transfected with DI-15CT RNA; 6, cells transfected with DI-526CT RNA; 7, bacterial CAT activity. (B) Agarose gel electrophoresis of DI transcripts and passage 3 RNAs. Virus obtained from a transfection experiment as described in panel A was passaged, and RNA was isolated from cells during the formation of passage 3. Lanes: S, Sindbis virus alone; 1, CTS253 passage 3 RNA (positive control); 2, DI-15CT RNA; 3, DI-526CT RNA; 4, 5, and 6, transcripts of CTS253, DI-15CT, and DI-526CT respectively.

only with antibodies directed against CAT, but also with antibodies directed against nsP1 (6). The downstream, in-frame AUG is the initiator codon for the CAT polypeptide. DI-526CT RNA has an additional upstream AUG at nucleotide 50, corresponding to the AUG that initiates translation of the 26S mRNA, but this AUG is followed by two in-frame stop codons (Fig. 5A). The RNA transcripts from KDI-15CT, KDI-526CT, and CTS253 were translated in vitro to determine whether the former two RNAs would be translated. The RNA transcripts added to the in vitro translation mixtures were first examined by agarose gel electrophoresis to establish that they were intact. Both an authentic CAT protein and a polypeptide the size of an nsP1-CAT fusion protein were synthesized by all three RNAs (Fig. 5B). The higher level of proteins translated from DI-526CT RNA suggests that this RNA is a better mRNA than the other two RNAs. Alternatively, DI-526 may be more stable during the translation reaction.

RNAs transcribed from the three cDNAs containing the *cat* gene were transfected into cells, and extracts were prepared after 18 h at 30°C. Although significant amounts of CAT activity were present in cells transfected with either CTS253 RNA or DI-15CT, no CAT activity was evident in cells transfected with DI-526CT (Fig. 6A). The former two RNAs but not the latter were also amplified on passaging (Fig. 6B). Thus, DI-526CT RNA was not replicated in the transfected cells.

Secondary structure analysis. We analyzed the secondary structures of the 5′-terminal nucleotides of the DI RNAs down to the *Bal*I site (indicated on Fig. 5A) using the program of Zuker and Stiegler (21). These sequences were also analyzed by Shu-Yun Le and Jacob Maizel (National Cancer Institute, Bethesda, Md.) using the program of Williams and Tinoco (19) and their unpublished programs designed to determine the statistically most significant secondary structures. We were not able to recognize any features of these structures that distinguished the biologically active from the biologically inactive molecules (structures not shown).

Summary and conclusions. A diagram of the different

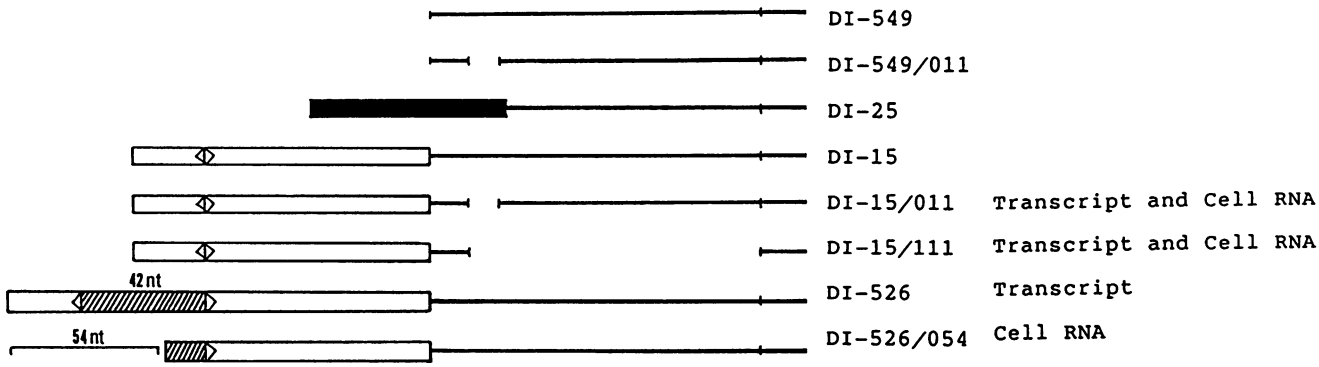


FIG. 7. Diagram of the 5′ termini of DI transcripts and a cellular DI RNA. The DI sequences are aligned in such a way that the same line or box pattern in a given vertical axis represents the same sequence. ——, 49S RNA 5′-terminal sequence; ▨, 26S RNA 5′-terminal sequence; ◇, 26S RNA sequence deleted from DI-1; ▬, $tRNA^{Asp}$ sequence. nt, Nucleotides.

5'-terminal sequences examined in this study is shown in Fig. 7. Only two of these, a small deletion and an insertion, were not amplified in the transfection and passaging assay. An 11-base deletion in DI-549 rendered it biologically inactive, but this same deletion in another context did not destroy the ability of the DI RNA to be amplified. Thus, the DI-15 RNA which has sequences from the 5' terminus of 26S RNA at its 5' end could tolerate this deletion as well as a more extensive deletion of 111 nucleotides with little or no effect on its ability to be amplified. DI-15 contains a 42-nucleotide deletion in the 26S sequences. The restoration of this deleted sequence, that is, the placement of intact 26S sequences at the 5' terminus of the DI RNA, destroyed the ability of the RNA to be replicated. At first it seemed that DI-526 was amplified, albeit poorly. Our sequence analysis, however, showed that the RNA that was amplified was different from the input transcript. On the basis of this result, we conclude that the generation of DI-1, the naturally occurring DI RNA (17), required not only the juxtaposition of 26S RNA sequences with those of the 5' terminus of the virion RNA, but also a deletion of sequences near the 5' terminus of the 26S region.

Our analysis of the 5' termini of DI RNA and the studies of Ou et al. (13) demonstrating that there was not a strong conservation of sequences at the 5' terminus among the different alphaviruses led to the hypothesis that structure rather than sequence is conserved in this region. This remains the most tenable model even though we were unable to detect by computer analysis any common secondary structure among the 5' termini of the biologically active DI RNAs that would distinguish them from those RNAs that were not amplified. The ability to recognize common structural features among RNA molecules often depends on knowing what specific constraints should be put on the secondary structures drawn by the computer (21). Furthermore, both tertiary structure and the ability of the RNA to adapt its structure upon interaction with protein may be important in the recognition process. A more detailed knowledge of the interaction of Sindbis virion RNA and DI RNAs with the replication proteins may be a necessary prelude to identifying the 5' structural features of these RNAs that permit them to be replicated.

ACKNOWLEDGMENTS

We thank Shu-Yun Le and Jacob Maizel (National Cancer Institute) for their help with the computer analysis.

This work was supported by Public Health Service grant AI11377 from the National Institute of Allergy and Infectious Diseases.

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