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Studies of Defective Interfering RNAs of Sindbis Virus With and Without tRNA^{Asp} Sequences at Their 5′ Termini

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Three of six independently derived defective interfering (DI) particles of Sindbis virus generated by high-multiplicity passaging in cultured cells have tRNA^{Asp} sequences at the 5′ terminus of their RNAs (Monroe and Schlesinger, J. Virol. 49:865–872, 1984). In the present work, we found that the 5′-terminal sequences of the three tRNA^{Asp}-negative DI RNAs were all derived from viral genomic RNA. One DI RNA sample had the same 5′-terminal sequence as the standard genome. The DI RNAs from another DI particle preparation were heterogeneous at the 5′ terminus, with the sequence being either that of the standard 5′ end or rearrangements of regions near the 5′ end. The sequence of the 5′ terminus of the third DI RNA sample consisted of the 5′ terminus of the subgenomic 26S mRNA with a deletion from nucleotides 24 to 67 of the 26S RNA sequence. These data showed that the 5′-terminal nucleotides can undergo extensive variations and that the RNA is still replicated by virus-specific enzymes. DI RNAs of Sindbis virus evolve from larger to smaller species. In the two cases in which we followed the evolution of DI RNAs, the appearance of tRNA^{Asp}-positive molecules occurred at the same time as did the emergence of the smaller species of DI RNAs. In pairwise competition experiments, one of the tRNA^{Asp}-positive DI RNAs proved to be the most effective DI RNA, but under identical conditions, a second tRNA^{Asp}-positive DI RNA was unable to compete with the tRNA^{Asp}-negative DIs. Therefore, the tRNA^{Asp} sequence at the 5′ terminus of a Sindbis DI RNA is not the primary factor in determining which DI RNA becomes the predominant species in a population of DI RNA molecules.

Defective interfering (DI) particles are deletion mutants that accumulate during high-multiplicity passaging of standard virus as well as during persistent infections of cultured cells (16). These defective genomes are replicated efficiently by enzymes encoded by standard viral genomes, and therefore should retain sequences or structures that can be recognized by viral enzymes. Sequence analysis of DI RNAs from different viruses, particularly from vesicular stomatitis virus (6, 13), influenza virus (2), and the alphaviruses Sindbis virus (12) and Semliki Forest virus (7, 8) show that various deletions, rearrangements, and base substitutions are present in DI molecules which function effectively in competition with standard viral genomes. A determination of viral sequences retained by DI genomes is providing an understanding of how such molecules can be generated and should lead to the identification of those regions of the genome that are required for replication and packaging.

We have been studying DI particles of Sindbis virus. The virion genome is a positive-strand RNA, the entire sequence of which has been determined (17, 25). The 5′ two-thirds of the molecule codes for the proteins involved in the replication and transcription of the RNA (19, 26). The 3′ one-third of the genome contains the genes which code for the structural proteins of the virion, but these proteins are translated in infected cells from a subgenomic mRNA (26S RNA) which is identical to the 3′ one-third of the virion RNA (26). The complete sequence of a DI genome of Sindbis virus shows that this molecule contains repeats and rearrangements of the standard genome as well as deletions (12). Similar features also have been found in the DI RNAs of the related alphavirus Semliki Forest virus (7, 8). Much of the sequence of these DI genomes is derived from the 5′ regions of the standard genome, although the first 50 to 100 nucleotides from the 3′ end are also conserved. A remarkable feature of some of the DI genomes of Sindbis virus is the covalent attachment of nucleotides 10 to 75 of tRNA^{Asp} at the 5′ end (11). Three of four DI genomes generated in chicken embryo fibroblasts have tRNA^{Asp} sequences, but two DI RNAs generated in baby hamster kidney (BHK) cells do not (12).

In the present study, we determined the sequences of the 5′ termini of three tRNA^{Asp}-negative DI RNAs. These results showed that although the sequences varied, they were always derived from the viral genome. We also began to examine the role of tRNA^{Asn} sequences in the amplification and interfering properties of DI RNAs. These studies suggest that the tRNA^{Asp} sequences at the 5′ terminus do not determine the efficacy of a DI RNA.

MATERIALS AND METHODS

Generation of DI particles. DI-1 and DI-4 particles were generated by serial undiluted passages of Sindbis virus on BHK cells (12, 20). DI-2 particles were generated by undiluted passaging on chicken embryo fibroblasts (10). The standard virus (from an early passage) was plaque purified three times, and two clones from the last plaque purification were picked to generate DI-3 and DI-5 by passaging at a constant standard multiplicity of 50 PFU per cell on chicken embryo fibroblasts. A third clone was picked to generate DI-6 by undiluted passages. DI RNAs of alphaviruses often show size heterogeneity. Although we refer to a particular DI RNA isolate, each independent passaging gave rise to a population of DI RNAs. The predominant species of RNA often varied with continued passaging.

Primer extension and sequence analysis of cDNAs. The 5′ end sequences of the DI RNAs were determined from the primer-extended cDNAs by the chemical method of Maxam and Gilbert (9), using the modifications of Smith and Calvo (22). Three different primers were used. The first two were restriction fragments obtained from the SS01 clone (12). One

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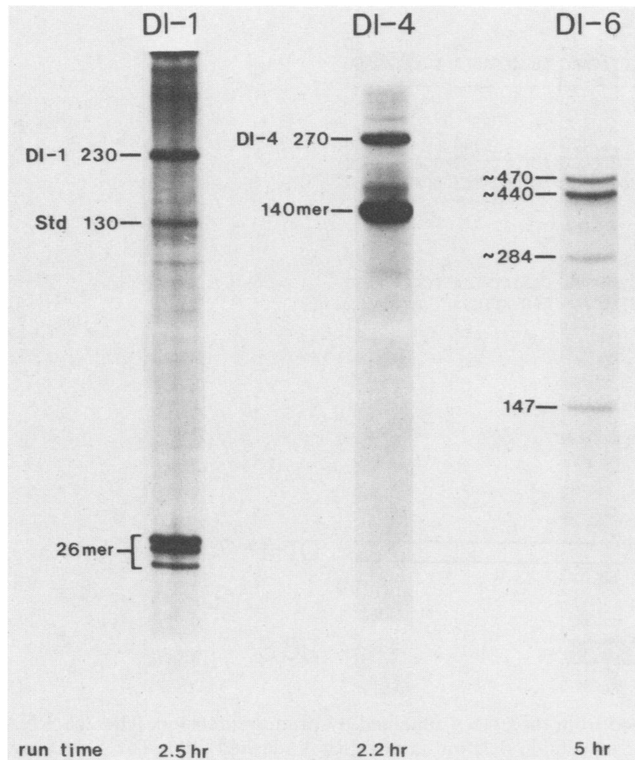

FIG. 1. Polyacrylamide gel of cDNA extension products from DI-1, DI-4, and DI-6 RNA. In each case the primer was labeled at the 5′ terminus with [^{32}P]ATP, using polynucleotide kinase. The primers are described in the text. The size of the cDNA extension products as determined by sequence analysis is indicated. For DI-6, we did not determine the entire sequence of the extension products; therefore the sizes are approximate. Std, Standard.

was a 26-base-pair *Hin*fI/*Hin*dIII fragment described previously (11), and the second was a 140-base-pair *Hin*dIII/*Hpa*II fragment. We obtained extension products by using either of these primers with DI-1 and DI-4 RNAs. We were only able to obtain significant extension with DI-6 RNA by purifying the RNA on oligodeoxythymidylate-cellulose to remove contaminating rRNA. We were then able to use a single-stranded synthetic oligonucleotide of 17 nucleotides (17-mer) (provided by S. Adams, Monsanto Co.) as a primer. Primer extension with the 26-base-pair fragment was described previously (11). Hybridization with the 140-base-pair fragment was carried out at 50°C overnight, but other conditions were the same. For hybridization with the 17-mer, 12 μg of DI RNA (12 pmol) and 0.008 μg of the 17-mer (25 pmol) labeled at the 5′ terminus with ^{32}P (10^9 dpm/μg) were mixed in a total volume of 8.75 μl of 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA. This solution was heated to 95°C for 1 min before adding 1.25 μl of 2 M KCl. The sample was then heated again for 2 min at 95°C, placed in a small beaker of water at 95°C, and allowed to cool to room temperature over a period of 45 min. cDNA synthesis was started by adding the nucleotide buffer mixture to a final concentration of 100 mM Tris-hydrochloride (pH 8.3)–10 mM dithiothreitol–5 mM MgCl–50 mM KCl–10 mM phosphate–1 mM each dNTP–25 U of reverse transcriptase in a volume of 50 μl.

Determination of DI concentration in a given DI stock. All the DI stocks used in the competition experiments were prepared on chicken embryo fibroblasts to eliminate any phenotypic host differences. The viral RNAs were labeled at 37°C in the presence of 1 μg of actinomycin D and 10 μCi of [^{3}H]uridine per ml and isolated from extracellular particles by extraction with phenol and chloroform. The ratio of DI to standard RNA in a given DI particle stock was determined by cutting out and counting the DI RNA and standard RNA bands resolved by agarose gel electrophoresis after fluorography of the gel. Assuming that DI and standard particles contain the same amount of RNA (5), the DI particle concentration was calculated as follows: DI concentration = standard virus titer × DI RNA counts per minute/standard RNA counts per minute.

Competition between two preparations of DI particles. Chicken embryo fibroblasts were initially infected with standard Sindbis virus at a multiplicity of 40 PFU per cell and with tRNA-positive and tRNA-negative DIs each at a defined multiplicity of infection. The particles released from the cells from this initial passage were then passaged undiluted four times with the addition of standard virus at a multiplicity of 20 PFU per cell. To label the intracellular viral RNAs, plates (60-mm diameter) of chicken embryo fibroblasts were infected with 0.5 ml of each of the competition passages in the presence of 10 μCi of [^{3}H]uridine and 1 μg of actinomycin D per ml.

Blot hybridization analysis of viral RNA. The RNA samples were resolved on 1% agarose gels, electrophoretically transferred to activated paper, and hybridized to the nick-translated $tRNA^{Asp}$ probe as described previously (12).

RESULTS

5′-terminal sequences of three DI RNAs of Sindbis virus. The three DI RNAs which do not contain $tRNA^{Asp}$ sequences at the 5′ terminus are from defective particle isolates designated DI-1, DI-4, and DI-6 (12). DI-1 was generated in BHK cells (20) and had been used in our studies to establish persistent infections (28). DI-4 was also generated in BHK cells. DI-6 was obtained by high-multiplicity passaging of Sindbis virus on chicken embryo fibroblasts. We determined the 5′-terminal sequences of these DI RNAs by primer extension and chemical sequencing of their cDNAs. Several different primers were used. For DI-1, the primer was the 26-base-pair restriction fragment described previously (11). It contains sequences complementary to nucleotides 104 to 129 of the standard virion RNA. Primer extension on DI-1 RNA gave two major products (Fig. 1). The band of 130 nucleotides most likely represents the product obtained from extension on the contaminating standard virion RNA present in the preparation since it was the same size as and had the sequence of the standard virion cDNA extension product. It might, however, represent an extension product from a DI RNA with a standard 5′ terminus. The sequence of the cDNA product of 230 nucleotides is shown in Fig. 2A. This sequence contained 100 bases from the 5′ terminus of the subgenomic 26S mRNA, with a deletion from nucleotide 24 to 67 (Fig. 2B). The 26S RNA sequences were covalently attached to the exact 5′ terminus of the standard virion RNA. There was, however, an insertion of a cytosine residue between the 26S RNA sequence and the 5′-terminal A of the standard RNA. This insertion was observed in two independent sequencing experiments.

Both DI-4 and DI-6 RNAs were purified by rate-zonal centrifugation in a sucrose gradient to remove contaminating standard virion RNA before primer extension. DI-6 RNA was also purified from contaminating rRNA by chromatography on oligodeoxythymidylate-cellulose. The latter purification appeared to increase the yield of the extension

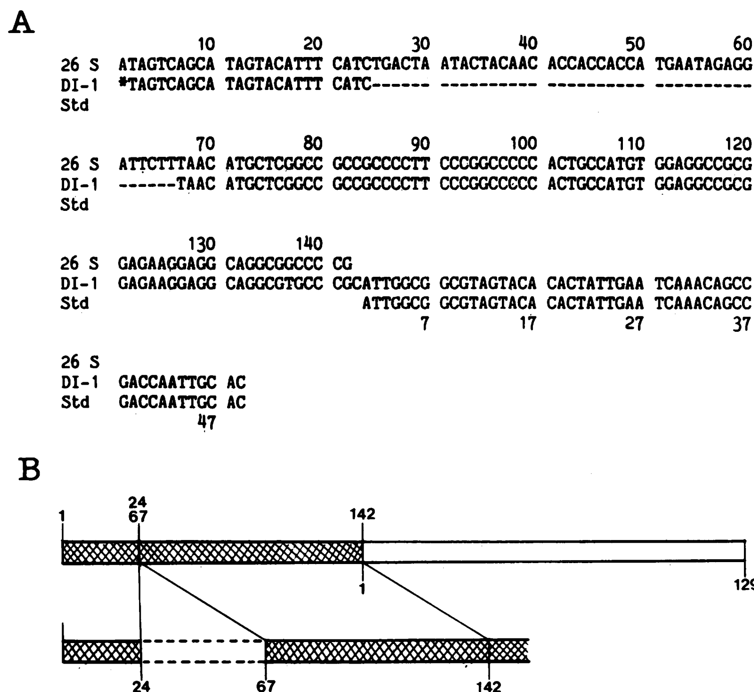

FIG. 2. (A) Nucleotide sequence of the DI-1 RNA 5′ terminus deduced from the cDNA obtained by primer extension. The 26S RNA sequence is aligned with that of the 5′ end of DI-1 RNA to show the 42 nucleotide deletion in DI-1 RNA (dashed line). The 5′-terminal sequence of standard RNA (Std) is also aligned with that of DI-1 RNA to show that the 26S RNA sequence is attached to the standard 5′ end with the intervention of a C residue in the DI-1 RNA. An asterisk represents nucleotides not determined. (B) Diagram comparing the 5′-terminal sequences of DI-1 and 26S RNA.

products. Primer extension on DI-4 gave one major product, whether the primer was a 140-base-pair restriction fragment complementary to nucleotides 130 to 269 (Fig. 1) or the 26-base-pair fragment which had been used with DI-1. The sequence of the 130 3′ nucleotides of the cDNA when converted to the complementary strand was identical to that of the standard RNA 5′ terminus.

We used a synthetic primer of 17 nucleotides complementary to nucleotides 131 to 147 of the standard virion RNA to analyze the 5′ end of DI-6 RNA. Four cDNA products were detected (Fig. 1). The smallest product (147 nucleotides) had a sequence complementary to the standard 5′ terminus (Fig. 3). The sequence of the 284 and 440 nucleotide fragments showed rearrangements of the standard 5′ end as dia-

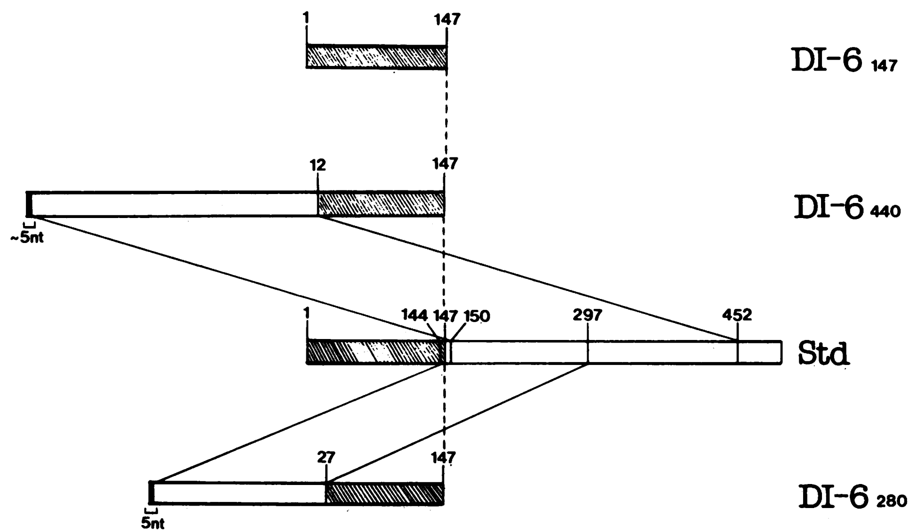

FIG. 3. The 5′-terminal sequence organization of DI-6 RNAs. The 5′ end sequences of DI-6 RNA species were determined from the cDNAs extended from a 17-nucleotide synthetic primer hybridized to the DI-6 RNAs (Fig. 1). The 5′-terminal nucleotide of the synthetic primer corresponds to nucleotide 147 in the standard RNA (Std). Symbols: ▨, standard 5′ end up to and including the priming site; ▭ standard sequence normally downstream from the priming site. The first two 5′ nucleotides of $DI\text{-}6_{147}$ and approximately the first five 5′ nucleotides of $DI\text{-}6_{284}$ and $DI\text{-}6_{440}$ were not determined.

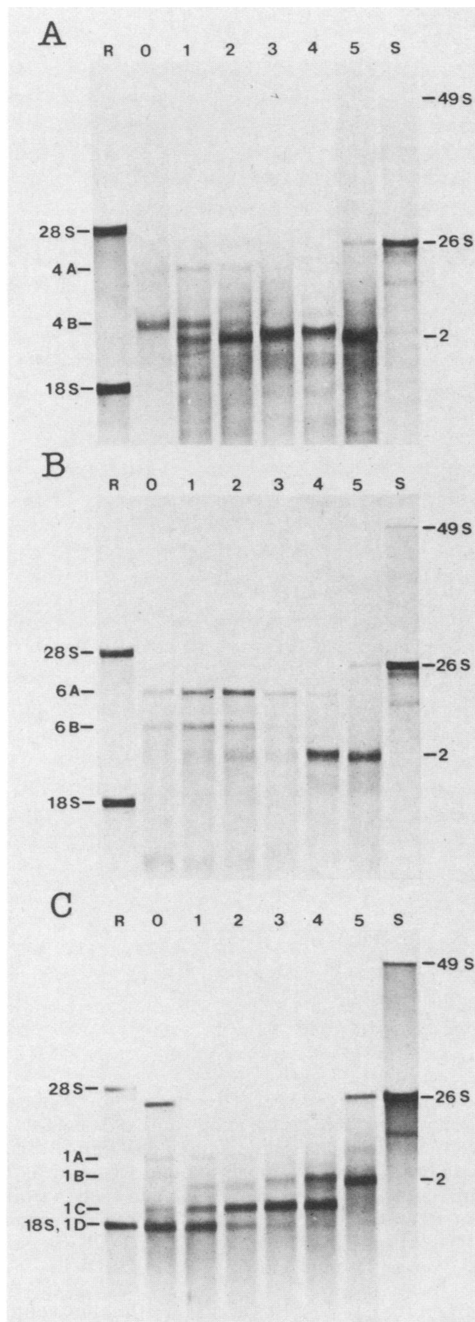

FIG. 4. Competition between DI-2 and tRNA^{Asp}-negative DIs. The infection was initiated with a multiplicity of standard virus of 40 PFU per cell, with DI-2 at a multiplicity of 1 DI per cell and with the tRNA^{Asp}-negative DI at a multiplicity of 20 DI per cell. The [^{3}H]uridine-labeled intracellular RNAs isolated from the infected cells were denatured with glyoxal and electrophoresed on a 1% agarose gel for 5 h. A, DI-2 versus DI-4; B, DI-2 versus DI-6; C, DI-2 versus DI-1. Lanes: 1 to 4, competition passages 1 to 4, respectively; R, ribosomal markers; lane O, RNA isolated from DI-4 particles (A) and RNA isolated from cells infected with the tRNA^{Asp}-negative DI (B and C); 5, RNA isolated from cells infected with DI-2; S, RNA isolated from cells infected with standard Sindbis RNA. The different DI RNA bands from the same DI source are indicated by letters after the DI number, with the letter A corresponding to the largest DI RNA.

grammed in Fig. 3. We were unable to read a sequence for the 470-nucleotide fragment, suggesting that it was heterogeneous. The finding that there were three, and probably four, distinct 5′ terminal sequences in the DI-6 RNA population indicates that these DI RNAs are more heterogeneous at the 5′ terminus than the other DI RNA sequences that we determined. The significance of this is not clear and may only indicate that there is no selection for one of these 5′ sequences to become predominant over the others.

We do not know whether all of the RNAs in each DI population contained the 5′-terminal sequences determined. Any DI RNA that did not contain sequences complementary to the primer would not be detected in our experiments. Two points, however, should be noted: (i) the tRNA^{Asp}-negative DI RNAs were first shown to be negative by blot hybridizations; thus, they did not contain tRNA^{Asp}-positive species; (ii) two different primers were used in determining the sequence of the 5′ terminus of D1-4. The same single sequence was obtained with both primers.

Competition between Sindbis DI RNAs with and without tRNA^{Asp} sequences at their 5′ termini. Our finding that one-half of the DI RNAs generated and three of four of those generated in chicken embryo fibroblasts had tRNA^{Asp} sequences at their 5′ termini suggests that these sequences may contribute to the selective advantage that DI RNAs have in competition with standard virion RNA. As one step in our efforts to understand whether tRNA^{Asp} has a role in Sindbis viral RNA replication, we asked whether the presence of these sequences at the 5′ terminus of a DI RNA provides a selective advantage among DI RNA molecules. We carried out a series of experiments in chicken embryo fibroblasts and in BHK cells to determine whether DI RNAs with tRNA^{Asp} sequences would have a selective advantage over those with other sequences at the 5′ terminus.

The procedure used to calculate the concentration of DI particles in the different preparations is described above. Since the values are an estimate rather than an accurate measure of DI particle concentration, we set up the competition experiments at an initial ratio of tRNA^{Asp}-positive DI particles to tRNA^{Asp}-negative DI particles of 1 to 20 to ensure that in the first infection by the two DI particle populations the DI particles with tRNA^{Asp} sequences were present at a significantly lower concentration than that of the other DI particles. The results of competition between the tRNA^{Asp}-positive DI-2 and DI-1, DI-4, or DI-6 are shown in Fig. 4. In each case, DI-2 could be detected in the first passage and soon became a predominant species in all of the competitions. In these experiments, as well as when DI-2 was passaged alone, the predominant DI RNA band changed in size, and in some cases more than a single DI RNA species accumulated (Fig. 5). It is known that DI RNAs of alphaviruses are heterogeneous in size and may change with continued passaging (3, 12). Thus, to establish that the presumed DI-2 RNA band or bands were actually RNAs containing tRNA^{Asp} sequences, we analyzed the passages from each competition by blot hybridizations and showed that these RNAs did contain tRNA^{Asp} sequences. The data from passage 4 are shown in Fig. 5. DI-2 RNA had almost completely replaced the DI-4 and DI-6 RNA species by this passage. DI-2 RNA amplified extensively in competition with DI-1 RNA, but one of the DI-1 RNA bands (DI-1C) also became a predominant species. The passaging of DI-1 (Fig. 4 and 6) also shows that smaller DI RNAs can be replaced by larger DI RNAs, a result noted previously with DI RNAs of both Sindbis virus (1, 27) and Semliki Forest virus (3). The tRNA^{Asp}-positive DI-2 RNA also proved to be an effective

DI-RNA in competition experiments carried out in BHK cells (data not shown).

In contrast to the results obtained with DI-2 particles, we found that a second $tRNA^{Asp}$-positive DI particle preparation, DI-3, did not compete well with the other DI-particles (Fig. 6). Since DI-2 RNAs survived and usually predominated when the initial ratio of DI-2 to $tRNA^{Asp}$-negative DIs was 1 to 20, this same ratio was used in the competition experiments with DI-3. The disappearance of DI-3 RNA was followed both by examining the [^{3}H]uridine-labeled RNA bands and the $tRNA^{Asp}$-containing RNA bands. DI-3 RNA bands were only detected in the first passage in competition with DI-1 (Fig. 6C). They persisted, but in decreasing concentration, in competition with DI-4 and DI-6. The effectiveness of the three $tRNA^{Asp}$-negative DI-RNAs was similar to that seen in competition with DI-2; i.e., DI-1 was superior to DI-6, which was better than DI-4 in competition with DI-3. In contrast, when DI-3 was passaged at a multiplicity of infection of one in competition with standard virus, the defective RNA soon amplified to a high level (data not shown).

Evolution of $tRNA^{Asp}$-positive DI RNAs of DI-3 and DI-5. In a previous study of DI-3, we showed that the first DI-RNA to appear at passage 7 when Sindbis virus was passaged at a multiplicity of infection of 50 was about one-half the size of standard RNA and did not contain $tRNA^{Asp}$ sequences (12).

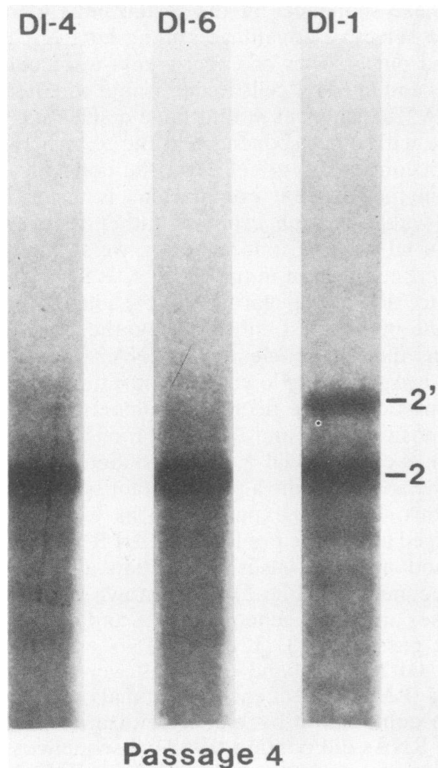

FIG. 5. Blot hybridization of passage 4 from competition experiments between DI-2 and the three $tRNA^{Asp}$-negative DIs. The probe was a restriction fragment from a plasmid carrying the rat $tRNA^{Asp}$ gene (12, 21). Passage 4 for DI-2 versus DI-4 and DI-6 corresponds to the identical passage in Fig. 4. Passage 4 for DI-2 versus DI-1 comes from a different experiment and shows that two species of $tRNA^{Asp}$-positive RNA were generated in that passaging; two species were not detected in the passaging analyzed in Fig. 4. Lanes: 1, DI-2 versus DI-4; 2, DI-2 versus DI-6; 3, DI-2 versus DI-1.

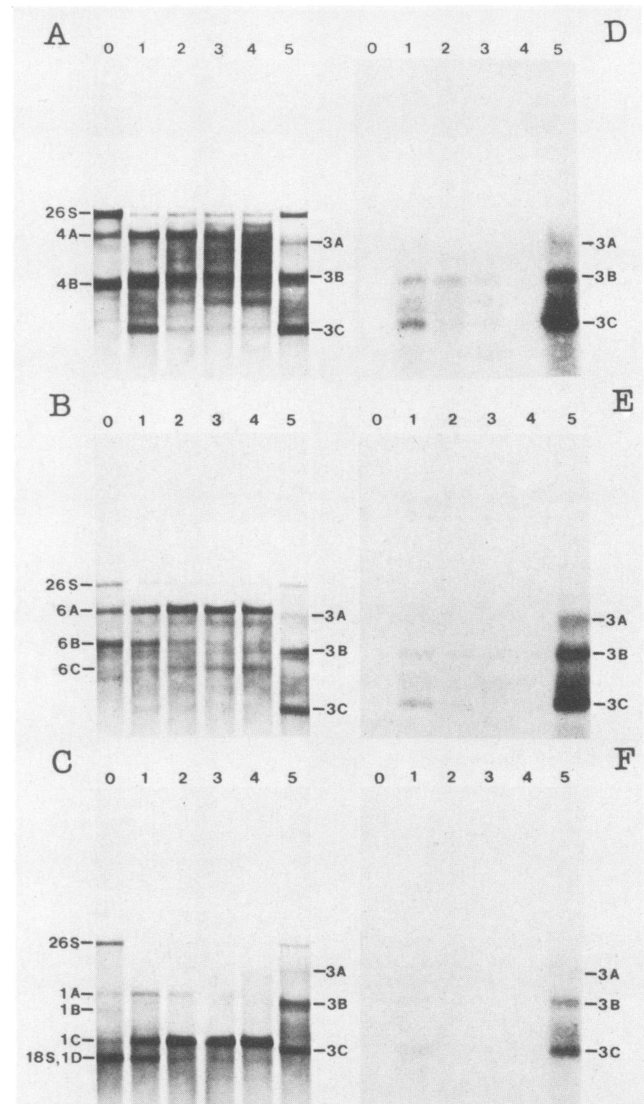

FIG. 6. Competition between DI-3 and $tRNA^{Asp}$-negative DIs. The infection was initiated with a multiplicity of standard virus of 40 PFU per cell, with DI-3 at a multiplicity of 1 DI per cell and with the $tRNA^{Asp}$-negative DI at a multiplicity of 20 DI per cell. The same experimental protocol was as for Fig. 4. A to C shows the fluorography of the RNA samples, and D to F show the corresponding blot hybridizations with a $tRNA^{Asp}$ probe. A and D, DI-3 versus DI-4; B and E, DI-3 versus DI-6; C and F, DI-3 versus DI-1.

We examined the DI-3 RNAs accumulating between passage 6 and passage 12 to determine when the $tRNA^{Asp}$ sequences were first detected and to which DI RNAs they were added. We also analyzed the evolution of the $tRNA^{Asp}$-positive DI RNA of DI-5 generated in chicken embryo fibroblasts. The large DI RNA (5L in Fig. 7) was first seen as a single clear band in passage 5; the DI band labeled 5A was not detected until passage 6. In both cases, the appearance of $tRNA^{Asp}$-positive DI RNAs occurred at the same time as did the emergence of small DI RNAs (Fig. 7). Our data could not distinguish, however, between the selective generation of DI RNAs with $tRNA^{Asp}$ sequences and the generation of many different small DI RNAs at levels too low to detect, followed by selection of those DI RNAs containing $tRNA^{Asp}$ sequences. The continued evolution of the $tRNA^{Asp}$-positive DI RNAs was shown clearly in passaging of DI-5 where, by

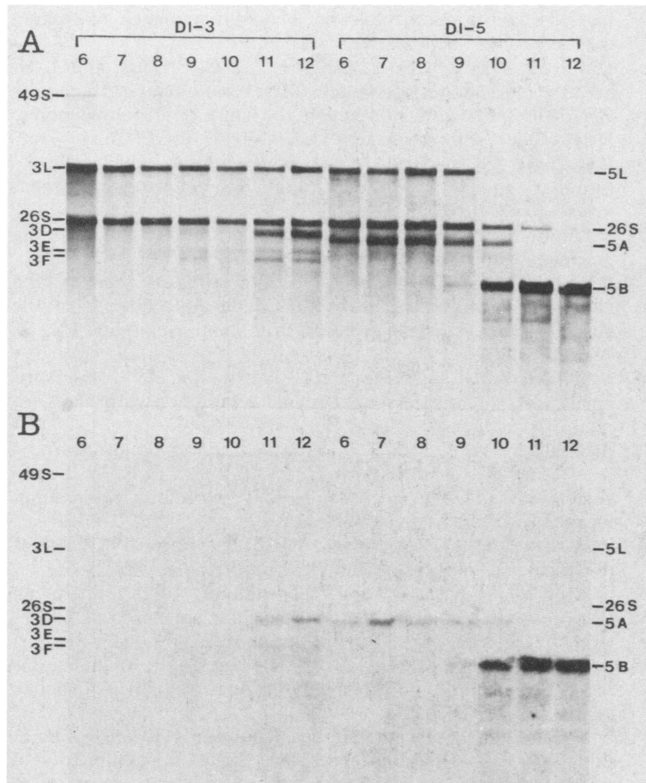

FIG. 7. Evolution of DI-3 and DI-5. Intracellular RNAs were isolated from cells infected with the passage indicated and a multiplicity of standard virus of 50 PFU per cell. Passage numbers are indicated above the lanes. A, Fluorography of RNA samples; B, blot hybridization of the same samples, using a probe for $tRNA^{Asp}$ (12, 21).

passage 11, the first $tRNA^{Asp}$-positive DI RNA to be generated was completely replaced by another smaller species.

DISCUSSION

The finding that defective RNAs of Sindbis virus have sequences of a specific tRNA at their 5′ termini indicated that recombinational events involved in generating DI RNAs of alphaviruses were not restricted to viral RNAs. Not all of the DI RNAs have $tRNA^{Asp}$ sequences, however, and we determined the sequence of the 5′ termini of three $tRNA^{Asp}$-negative DI RNAs to determine whether these termini were derived from viral or host nucleic acids. We found several different 5′ termini, all of which were derived from viral genomic RNA sequences. One of the DI RNA samples (DI-4) had the same 5′-terminal sequences as the standard RNA. The DI RNAs from another of the DI particle preparations (DI-6) were heterogeneous at the 5′ end, the sequence being either that of the standard 5′ terminus or rearrangements of regions near the 5′ terminus. This type of rearrangement resembles those previously described for DI genomes of Sindbis virus (12) and Semliki Forest virus (7, 8).

The sequence at the 5′ end of the third DI RNA sample (DI-1) consisted of the 5′ terminus of a subgenomic mRNA, 26S RNA, with a deletion from nucleotide 24 to 67 of the 26S RNA sequence. The presence of the 26S RNA sequences in the DI RNA was a surprise because it had been thought that this region of the genomic RNA was one of the first to be deleted in the generation of alphavirus DI RNAs (24). DI-1 was generated by undiluted passaging, and no intermediate DI RNAs were detected, so we do not know whether the attachment of 26S RNA sequences to the 5′ terminus occurred during the initial deletions or was the result of subsequent recombination with 26S RNA.

A DI RNA with a 5′ terminus of 26S RNA might be transcribed and replicated by the replicase complex that produces 26S RNA. This RNA is transcribed from the full-length minus strand, the site of initiation being two-thirds of the distance from the 3′ end of the molecule. Ou et al. found that the 19 nucleotides preceding the start of 26S RNA and the first two nucleotides of this region are highly conserved among alphaviruses and proposed that this sequence in the minus strand is the transcription recognition site (14). This recognition site is not present at the 5′ end of DI-1 RNA, and therefore it is more likely that the DI-1 RNA is replicated by the enzyme complex that replicates full-length virion RNA.

One conclusion from our sequence analysis was that the 5′-terminal nucleotides can vary extensively, and the RNA can still be replicated by virus-specific enzymes. How much variation can be tolerated at this site is not yet clear. Ou et al. demonstrated that the exact 5′ terminus of several alphaviruses is not highly conserved, suggesting that this site can undergo more modifications than can other regions of the genome (15). In this regard, it is worth noting that some of the small size variants of Qβ virus which show almost no sequence homology to the original virion RNA can be recognized and replicated by the Qβ replicase (18). Although the extreme 5′ end of the alphaviruses shows variations, Ou et al. found that a 51-nucleotide sequence located 150 nucleotides from the 5′ end of Sindbis virion RNA is strongly conserved among alphaviruses and propose that this sequence serves as an important recognition site (15). This latter sequence is also present in DI RNAs (8, 12), strengthening the contention that it is an essential region of the genome.

One of our major goals was to determine whether the presence of $tRNA^{Asp}$ sequences at the 5′ terminus of DI RNAs provided those DI RNAs with an advantage over other DI RNAs. Our initial competition analysis showed that the $tRNA^{Asp}$-positive DI-2 RNA survived and replaced each of the $tRNA^{Asp}$-negative DI RNAs. Each independent passaging to generate DI RNAs produces a population of molecules which may vary in size upon continued passaging. In the case of the $tRNA^{Asp}$-positive DI RNAs, the number and size of the RNA species detected by intrinsic uridine labeling always corresponded to the bands detected by blot hybridizations with a probe for $tRNA^{Asp}$. The survival of DI-2 means the survival of $tRNA^{Asp}$-positive DI RNA, not the retention of a particular size molecule. DI-2 RNA did show a change in size during passaging. The ability of the $tRNA^{Asp}$-positive species to replace the other DI RNAs was observed not only in chicken embryo fibroblasts, in which these DI RNAs were generated, but also in BHK cells. A second $tRNA^{Asp}$-positive DI particle preparation (DI-3) did not survive when the same conditions of competition were tested. Therefore, $tRNA^{Asp}$ sequences per se cannot be a primary factor in determining the ability of a DI RNA to survive and be amplified during passaging in chicken embryo fibroblasts.

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