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Isolation of Vesicular Stomatitis Virus Defective Interfering Genomes with Different Amounts of 5'-Terminal Complementarity

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I isolated at least 30 different vesicular stomatitis virus defective interfering (DI) genomes, distinguished by chain length, by five independent undiluted passages of a repeatedly cloned virus plaque. Labeling of the 3' hydroxyl ends of these DI genomes and RNase digestion studies demonstrated that the ends of these DI genomes were terminally complementary to different extents (approximately 46 to 200 nucleotides). Mapping studies showed that the complementary ends of all of the DI genomes were derived from the 5' ends of the nondefective minus-strand genome. Regardless of the extent of terminal complementarity, all of the DI genomes synthesized the same 46-nucleotide minus-strand leader RNA.

Vesicular stomatitis virus (VSV) is a negative-strand virus and contains all of its genetic information in a single RNA chain, which is approximately 12,000 nucleotides long and is complementary to the viral monocistronic mRNA's. This RNA chain, which sediments at 42S, is never found in infected cells as free RNA, but occurs as a helical nucleocapsid in which the RNA is complexed tightly with the viral N protein and is associated less tightly with the viral NS and L proteins. The nucleocapsid structure is the functional viral genome, and it contains the enzymatic activities that are necessary for the production of active viral mRNA's, including polyadenylation, capping, and methylation, in addition to the RNA polymerase activity (1, 24).

The molecular events associated with the replicative cycle of this virus are as follows. Briefly, upon entry into a host cell the viral nucleocapsid acts as a template for the synthesis of the viral mRNA's. This reaction, called primary transcription, can take place in the absence of the protein synthesis and is thought to be similar to the synthesis of viral mRNA by purified virions in vitro. After translation of the primary viral transcripts, the infecting nucleocapsid can act as a template for the replication of the viral genome; this replication takes place through the synthesis of a full-length complementary RNA chain, the plus-strand antigenome, which is found only in the form of nucleocapsids and never as free RNA. The synthesis of the antigenome nucleocapsids leads to a large amplification of the genome nucleocapsids, which can serve both as templates for further mRNA synthesis (secondary transcription) and as viral genomes for mature progeny virions.

Defective interfering (DI) particles, which are deletion mutants that can replicate only in the presence of nondefective (ND) helper virus and concomitantly interfere with the replication of the ND helper virus, are frequently present in VSV stocks. It is thought that these DI particles are generated as rare aberrations during the replication of the ND genome. Although the multiplicity of infection (MOI) probably has little influence on the frequency of DI genome generation events, DI genomes accumulate to sizeable fractions of the virus progeny only after successive high-MOI passages, because the replication of these genomes depends on coinfection of the host cells with ND virus. When coinfection (mixed virus infection) takes place, DI particles outgrow ND virions, presumably because they have a competitive advantage over ND genomes during genome replication.

The majority of DI particles described to date have been derived from the 5' ends of ND minus-strand genomes (5' DI genomes) and, interestingly, do not contain simple internal deletions, as might have been expected. At their 3' ends these 5' DI genomes contain the complements of the 5' ends; i.e., they contain inverted terminal repeats and can self-anneal to form circles that contain the complementary ends as double-stranded stems (9, 19, 20).

It is thought that such 5' DI genomes have been generated during minus-strand genome synthesis by the crossing over of the viral replicase from the plus-strand antigenome template to a point near the 5' end of the nascent minus-

strand chain, which is used as a template for the completion of the nascent genome chain (6, 13). This mechanism of generation of DI genomes is referred to as the copy-back mechanism, and the DI genomes are referred to as 5′ copy-back DI genomes.

Recently, Keene et al. (8) described a DI genome (designated DI-LT2) that was present in their DI-LT stock; most of this genome was derived from the 3′ end of the ND genome, but it still contained complementary ends that were derived from the 5′ end of the ND genome. These authors suggested that the 5′ complementary ends of DI-LT2 may have been generated not by the replicase copying-back its own nascent strand as a template (nascent strand jump), but by the replicase jumping to and continuing RNA synthesis on a separate template (separate strand jump). These two generation mechanisms can account equally well for the 5′ complementary ends of DI-LT2. Regardless of the exact manner in which DI-LT2 or other 5′ complementary end DI genomes have been generated, a consequence of this arrangement of sequences on 5′ complementary end DI genomes is that both plus- and minus-strand DI genomes contain at their 3′ ends the 3′ end of the ND antigenome, which is thought to account for the replicative advantage of these DI genomes.

The extents of terminal complementarity of only a few different DI genomes have been determined to date (20, 22), and these fell within a rather narrow range (45 to 55 nucleotides), suggesting that the replicase may be crossing over to a specific sequence during DI genome generation (22). In an effort to understand the generation and replication of DI genomes better, I extended these studies to the examination of a much larger number of DI genomes, all of which were derived from the same parental ND virus.

MATERIALS AND METHODS

Generation of DI genomes. The titers of a stock of the Mudd-Summers Indiana strain of VSV were determined on BHK cells. A single plaque was isolated, diluted 10^5 times, and then replated. This process was repeated six times. A single plaque was then removed, diluted with 6 ml of minimal essential medium, and divided into six 1-ml portions, which were designated stocks A to F. The original virus stock before the multiple plaque purification procedure was designated stock T. The various virus stocks were then passed undiluted or at 1:10 dilutions on BHK cells in duplicate cultures. One culture from each duplicate set was labeled with 20 μCi of [^{3}H]uridine per ml in the presence of 0.5 μg of actinomycin D per ml, the other set was not labeled and was used for subsequent passages. ^{3}H-labeled virus particles were pelleted from the medium, and the presence of DI genomes was monitored by sodium dodecyl sulfate (SDS)-sucrose gradient sedimentation of the virus particle RNA.

Isolation and end-labeling of virus particle RNA. Pelleted virus particles were disrupted by adding 1 M NaCl and 1% Nonidet P-40, and the viral nucleocapsids were isolated by equilibrium centrifugation on CsCl density gradients, as previously described (14). The nucleocapsid bands were diluted 3- to 10-fold and then recovered by pelleting in an ultracentrifuge (90 min, 59,000 rpm, SW60 rotor). The resulting nucleocapsid pellets were dissolved in 100 μl of TNE (25 mM Tris-chloride, pH 7.4, 50 mM NaCl, 1 mM EDTA) containing 1% SDS and 500 μg of proteinase K per ml and phenol-chloroform extracted twice, and the RNA was recovered by ethanol precipitation.

Total nucleocapsid RNA was labeled at the 3′ hydroxyl end with ^{32}pCp and RNA ligase, as previously described (3). The reaction mixture was diluted fivefold with TNE containing 10 mM EDTA, 0.2% SDS, and 500 μg of proteinase K per ml, phenol-chloroform extracted twice, and chromatographed on Sephadex G-50. The RNA in the excluded volume was recovered by ethanol precipitation.

To isolate preparative amounts of 3′-end-labeled DI stem RNA, the RNA ligase reaction mixture (20 μl) was diluted with 100 μl of 2.5× buffer A (1× buffer A contained 150 mM NaCl, 10 mM Tris-chloride, pH 7.4, 1 mM EDTA) supplemented with 0.1% SDS and 10 μg of RNase A per ml and then incubated for 10 min at 25°C. A 1-μl amount of 10% SDS and 7 μl proteinase K (10 mg/ml) were added and the incubation was continued for 15 min. Then the reaction mixture was processed as described above.

VSV virion 42S RNA was 5′-end-labeled by capping with [α-^{32}P]GTP and vaccinia virus guanylyl transferase (kindly provided by Bernard Moss) in a reaction mixture containing 25 mM Tris-chloride (pH 7.8), 2 mM $MgCl_2$, 1 mM dithiothreitol, and 12.5 mM GTP for 30 min at 37°C (17). The RNA was recovered from the reaction mixture as described above and characterized as described below.

RESULTS

Generation of multiple DI genome. Holland et al. (5) have shown that when standard VSV which has been purified of contaminating DI particles by multiple successive plaque isolations is passaged at a high MOI, DI particles of different sizes are generated in an apparently random fashion. On the other hand, Kang et al. (7) have presented evidence that a given clonal isolate of ND virus always produces the same pattern of DI particles in a given host cell type. To reexamine this question and possibly generate a large number of different DI particles for subsequent study, I repeated the above-described experiment, using the protocol of Holland et al. (5).

A stock of standard VSV was plaque purified six times in succession, a single virus plaque was isolated and homogenized in minimal essential medium, and six equal portions of this purified clonal isolate were passaged independently on BHK cells at a high MOI (stocks A to F). For purposes of comparison, the original ND virus stock was also passaged at a high MOI without

prior purification by successive plaque isolations (stock T). The appearance of DI particles during high-MOI passage was monitored by velocity sedimentation of [^{3}H]uridine-labeled virus particle RNA on SDS-sucrose gradients. In all of the independent high-MOI passages, DI genomes became visible by passage 4, and these genomes represented a sizeable fraction (25 to 75%) of the total virus particle RNA (by weight) at passage 5. To examine the size distribution of the virus particle RNAs, [^{3}H]uridine-labeled RNAs from five of the clonal isolates (stocks A, B, D, E, and F) and from uncloned isolate (stock T) independent passages were electrophoresed on denaturing acid-urea agarose gels (15). Figure 1 shows the distribution of the nucleocapsid RNAs that were isolated from virus particles at passage 5 by equilibrium CsCl density gradient centrifugation (see above). Each independent

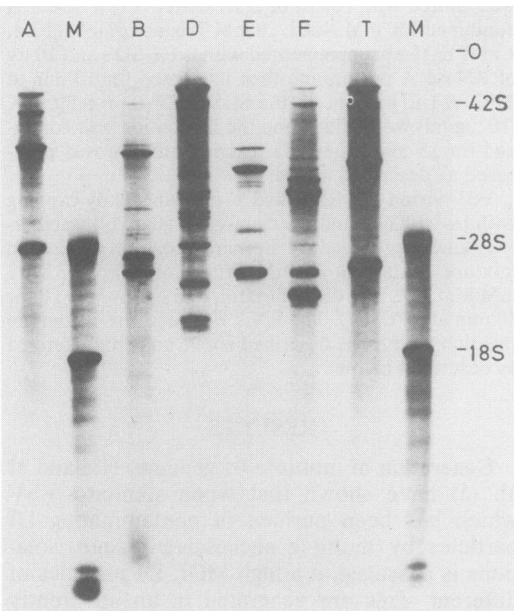

FIG. 1. Comparison of DI genomes from independently passaged virus stocks by denaturing agarose gel electrophoresis. [^{3}H]uridine-labeled nucleocapsid RNAs from passage 5 virus particles of the various independently passaged virus stocks (see text) were isolated as described in the text. Equal amounts (approximately 60,000 cpm) of the RNA samples in 10 μl of ET buffer (1 mM EDTA, 10 mM Tris-chloride pH 7.4) were denatured by adding 20 μl of dimethyl sulfoxide and heating for 1 min at 55°C, and the RNAs were electrophoresed on a 1.5% acid–urea agarose gel (12) for 16 h at 30 mA. The gel was then impregnated with PPO (2,5-diphenyloxazole), dried, and exposed to X-ray film. The positions of VSV 42S RNA and BHK cellular rRNA's are indicated on the right. The letters at the top indicate the various independently passaged virus stocks (see text). Lanes M contained [^{14}C]uridine-labeled total BHK cellular RNA.

passage of each starting clonal isolate (stocks A to F) generated multiple different DI genomes during high-MOI passage; these genomes were also different from the multiple DI genomes generated when the uncloned virus stock (stock T) was passaged. In some independent passages, only two or three different DI genomes were visible (stock A), whereas in others more than 10 different DI genomes were detected by this method (stocks D and F). Curiously, although at least 30 different DI genomes were generated in six different passages, no DI genomes smaller than 2,500 nucleotides were observed. This may have reflected the constraints imposed by BHK cells on the assembly of DI genomes into mature virus particles. In addition, once a particular pattern of DI genomes in a given independent passage became detectable, this pattern was stable during any further high-MOI passage (data not shown), suggesting that the sizes of the DI genomes did not account for the replicative advantage among DI genomes. The band which migrated slightly slower than 42S RNA appeared to be an artifact of aggregation since it varied quantitatively from gel to gel and was also found when only ND virion RNA was electrophoresed (data not shown). These results agreed well with the results of previous work on the generation of VSV (5) and Sendai virus (10) DI genomes and demonstrated that a given virus clone in a given host cell was not predisposed to generate a particular DI genome, as long as the starting virus stock was adequately purified of contaminating DI particles (see below).

Characterization of complementary ends of DI genomes. Many VSV DI genomes are derived from the 5′ end of the ND minus-strand genome and contain complementary ends (21). It is thought that the extent of this complementarity at the end of a DI genome reflects the point at which the viral replicase crosses over from the template to the nascent strand to copy back the nascent strand during the generation of the DI genome with an inverted terminal repeat (6, 13). Schubert et al. (22) and Perrault et al. (20) examined four such 5′ end copy-back DI genomes to determine the extents of the complementary ends and found that they fell within a relatively narrow range (45 to 55 nucleotides), suggesting that the replicase may cross over to a specific signal on the nascent chain during DI genome generation. However, since the four DI genomes used in these studies were isolated from different parent strains of the Indiana serotype, at different times, and in different laboratories, the lengths of the complementary ends of these DI genomes may have reflected the individual conditions of generation and amplification. Therefore, it was of interest to determine the lengths of the complementary ends of a

"matched set" of DI genomes isolated under identical conditions from the same parental virus clone.

At passage 5 virus particles from my independently passaged stocks were removed from the growth medium by centrifugation, and their nucleocapsids were isolated by equilibrium centrifugation on CsCl gradients (see above). This procedure removed contaminating cellular RNA, such as tRNA, which pelleted through the CsCl gradient under these conditions. The 3′ hydroxyl ends of the total nucleocapsid RNAs were then radiolabeled with ^{32}pCp by using RNA ligase, and the end-labeled RNAs were recovered after Sephadex G-50 chromatography. A nearest-neighbour analysis of these RNAs showed that approximately 90% of the radioactivity appeared as Up, as expected for VSV ND and DI genomes (data not shown). To examine the lengths of the complementary ends of the DI genomes present in the independently passaged virus stocks, the 3′-end-labeled RNAs were digested with RNase A in a high-salt solution, and the remaining RNAs were electrophoresed on a nondenaturing polyacrylamide gel. Figure 2 shows that each stock did indeed contain 3′-end-labeled RNA which was resistant to RNase digestion (stem RNA). The lengths of these stem RNAs varied from 46 to 200 base pairs (bp) (Fig. 3); however, except for stocks D and F, a single size class predominated in each stock. In addition, the lengths of the complementary ends represented distinct size classes rather than a continuum of sizes, and no complementary ends smaller than 46 bp (an apparent lower limit) were detected.

To examine whether these complementary ends of the DI genomes contained perfectly matched complementary strands, as might be expected of copy-back DI genomes, or whether they contained mismatches that led to single-stranded breaks, which could not be detected under nondenaturing conditions, I also examined the 3′-end-labeled strands in the stem RNAs as single strands. The stem RNA bands of a preparative gel such as that shown in Fig. 2 were recovered from the gel, denatured with dimethyl sulfoxide, and electrophoresed on a 7 M urea denaturing gel along with similarly treated DNA restriction fragments as chain length markers (Fig. 3). In the case of stocks D and F, which contained more than one stem RNA, only the largest stem RNA was examined. Except for stock D, all of the 3′-end-labeled strands electrophoresed at the position expected for an intact single strand, indicating that the stem RNAs from which these strands were isolated did not contain any mismatches that were sensitive to RNase A digestion. On the other hand, the 200-bp stem RNA isolated from stock D gave rise to a unique series of end-labeled single-stranded RNAs under denaturing conditions, indicating that these double-stranded RNAs did contain hidden breaks and were not perfectly matched complementary strands. An analogous situation in which the complementary ends of a DI genome were shown to contain point mutations

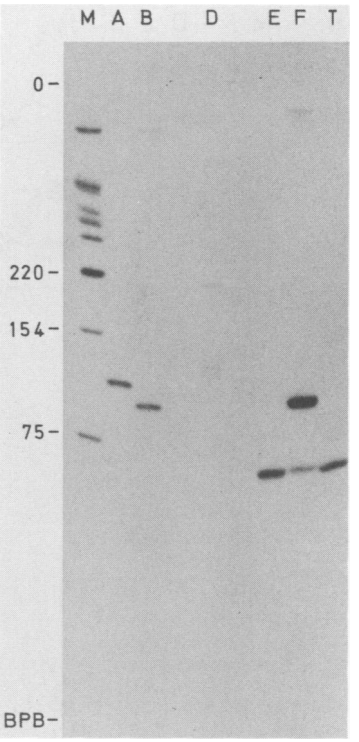

FIG. 2. Characterization of DI genome stem RNAs by polyacrylamide gel electrophoresis. The various 3′-end-labeled nucleocapsid RNAs from the independently passaged stocks (see text) in 10 μl of ET which contained 10,000 to 20,000 cpm of end-labeled RNA and from 1 to 10 μg of carrier tRNA were digested with 100 μl of 2.5× buffer A supplemented with 0.1% SDS and 10 μg of RNase A per ml for 10 min at 25°C. The RNase was digested with 600 μg of proteinase K per ml in 0.2% SDS for 15 min at 25°C, 20 μg of carrier tRNA was added, and the remaining RNA was recovered by ethanol precipitation. The RNAs were dissolved in 10 μl of TNE containing 12% glycerol and 0.04% bromphenol blue and electrophoresed on a 12.5% polyacrylamide sequence gel (15). The gel did not contain urea and was electrophoresed slowly (16 h at 300 V) to avoid excessive heating, which could have denatured short double strands. The letters at the top indicate the various independently passaged stocks. Lane M contained 5′-end-labeled *Hin*F-digested pBr322 (used as a reference marker). At the left, O and BPB indicate the origin and the position of the bromphenol blue dye marker, respectively, and the numbers indicate the chain lengths of the three smallest DNA restriction fragments.

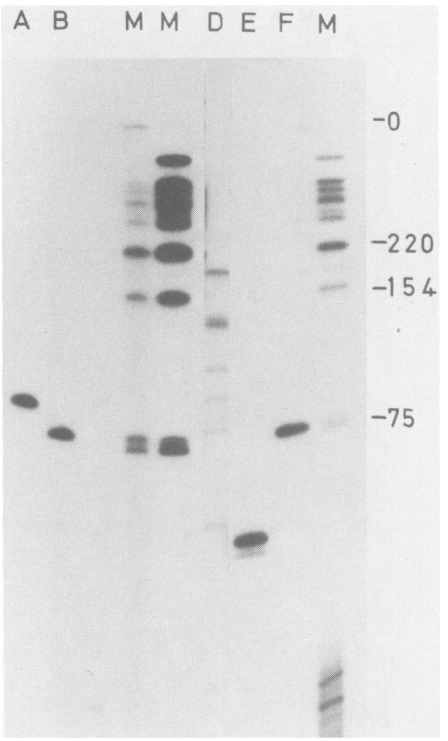

FIG. 3. Characterization of DI genome stem RNA as single strands by polyacrylamide gel electrophoresis. Stem RNA bands from a preparative gel such as that shown in Fig. 2 were excised and recovered from the gel by the method of Maxam and Gilbert (16). In the case of DI genome stem RNAs from stocks D and F, which contained more than one band, only the largest band was removed. The RNAs were recovered by ethanol precipitation and dissolved in 3 μl of ET. Then the RNAs were denatured by adding 6 μl of dimethyl sulfoxide and heating for 1 min at 60°C and electrophoresed on a 12.5% polyacrylamide sequencing gel which contained 7 M urea and was run hot (1,500 V, 2 h) to prevent renaturation of the RNA. The *Hin*F-digested pBr322 markers (lanes M) were denatured similarly with dimethyl sulfoxide before electrophoresis. For an explanation of letters and numbers, see the legend to Fig. 2.

was described recently by Hagen and Huang (4). By comparison with DNA markers, the chain lengths of the end-labeled strands of the stem RNAs were estimated to be as follows: stock A, 86 nucleotides; stock B, 76 nucleotides; stock D, 200 nucleotides; stock E, 46 nucleotides; and stock F, 75 nucleotides.

To demonstrate that the stem RNAs were in fact derived from the 5′ end of the ND minus-strand genome, the following mapping experiment was performed. ND virion 42S RNA was radiolabeled at its precise 5′ end by using [^{32}P]GTP and vaccinia virus guanylyl transferase (17). Characterization of this 5′-end-labeled RNA by nuclease digestion (Fig. 4) demonstrated that all of the radioactivity was contained in an unmethylated cap structure; no radioactivity which migrated at the position of nucleotide monophosphates was detected. Unlabeled stem RNAs were then isolated from the various DI genome groups and mixed with the 5′-end-labeled ND genome probe. The RNAs were denatured, annealed, and digested with RNase in a high-salt solution, and the remaining RNAs were electrophoresed on a polyacrylamide gel. Figure 5 shows that in each case exactly the same bands resulted as in the stem RNAs isolated from 3′-end-labeled DI genomes (Fig. 2). Since the only

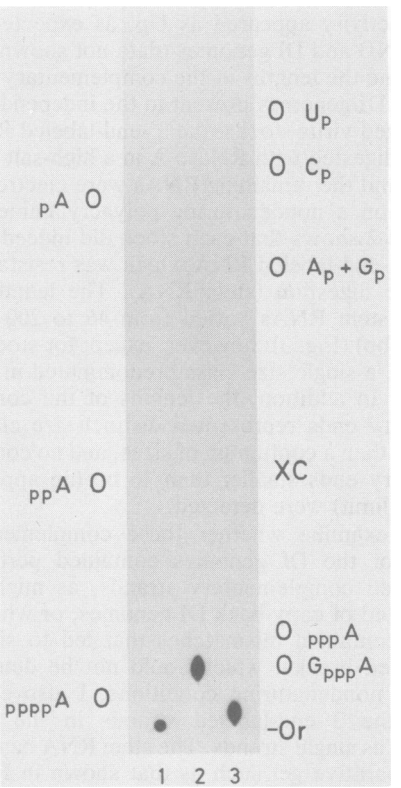

FIG. 4. Characterization of 5′-end-labeled 42S virion RNA. Virion 42S RNA was capped with [α-^{32}P]GTP (see text), and 2-μl samples containing 7,000 cpm were either digested with 10 μg of nuclease P1 in 12 μl of 10 mM sodium acetate (pH 5.3) containing 1 mM ATP (lane 2) or digested with 10 U of RNase T_2 and 4 μg of RNase A in 13 μl of 10 mM sodium acetate (pH 5.3)–1 mM EDTA for 80 min at 37°C (lane 3). The samples were spotted onto Whatman DEAE paper along with nonradioactive nucleotide markers and electrophoresed for 1.75 h at 3,000 V in pyridine acetate buffer (pH 3.5). Lane 1 contained 1 μl of untreated RNA. The nucleotide markers were located under UV light, and the radioactivity was detected by autoradiography.

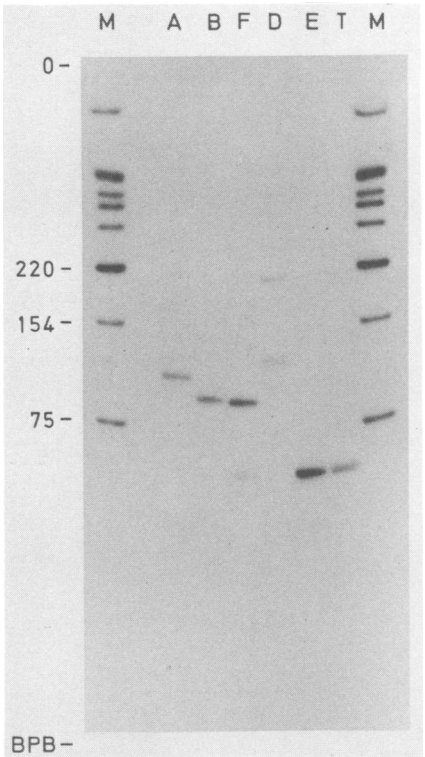

FIG. 5. Annealing of DI genome stem RNA to 5'-end-labeled ND genome RNA. Unlabeled stem RNAs were prepared from the DI genomes present in various independently passaged stocks (see text) and then mixed with equal amounts (4,000 cpm) of 5'-end-labeled 42S ND virion RNA. The mixed RNAs were denatured by adding 2 volumes of dimethyl sulfoxide and heating for 1 min at 60°C, and they were recovered by ethanol precipitation. The RNAs were dissolved in 20 μl of 2× buffer A and annealed for 20 min at 65°C; then they were digested with RNase A and electrophoresed under nondenaturing conditions, as described in the legend to Fig. 2. For an explanation of letters and numbers, see the legend to Fig. 2.

radiolabel was the label in the 5' end of the ND genome probe, this experiment demonstrated that all of the stem RNAs shown in Fig. 2 were in fact derived from DI genomes whose ends contained the 5' end of the ND minus-strand genome and its complement.

Since all of the stem RNAs that I detected were in fact derived from the 5' end of the ND minus-strand genome, presumably by a copy-back mechanism, I examined the nucleotide sequences at the presumptive replicase crossover points to determine whether I could detect similarities of sequences which might constitute a crossover signal. However, an inspection of the nucleotide sequences (23) of these regions at the 5' end of the ND genome did not reveal a particular sequence that was common to the presumptive replicase crossover regions. If my 5' complementary-end DI genomes were in fact generated by a copy-back mechanism (nascent strand jump), it appeared that the viral replicase did not require a unique sequence when crossovers from the template to the nascent strand occurred in the generation of 5' copy-back DI genomes. Analogously, if my 5' complementary-end DI genomes were generated by a separate strand jump mechanism, then it appeared that the replicase did not require a unique sequence from which it could jump to a separate template.

Characterization of minus-strand leader RNAs in various mixed-virus-infected cells. Leader RNAs are short transcripts (approximately 47 nucleotides long) from the exact 3' ends of VSV genome templates. Previous work (14) has shown that standard VSV-infected cells (i.e., cells in which DI genomes cannot be detected by radiolabeling) contain both plus-strand and minus-strand leader RNAs, which are complementary to the 3' ends of ND minus-strand genomes and plus-strand antigenomes, respectively. Compared with standard virus-infected cells, cells infected with a mixture of ND virus and DI particles (mixed-virus-infected cells) contain lower amounts of plus-strand leader RNA, which reflects the lower numbers of ND genome templates present in these cells, and increased amounts of minus-strand leader RNA, which reflects the increased numbers of templates for this leader RNA (i.e., mostly plus- and minus-strand DI genomes) (12). Since my matched sets of DI genomes contained various DI genomes with different size complementary ends, all of which were derived from the 5' end of the ND genome, it was of interest to determine the sizes of the minus-strand leader RNAs synthesized from the various DI genomes.

Leader RNAs can be detected most easily by their ability to anneal to 3'-end-labeled genome probes. In the case of minus-strand leader RNA, stem RNA radiolabeled at the original 3' hydroxyl end of the DI genome is a suitable probe (14). The largest stem RNA obtainable in good yields in the experiments described above was the 86-bp stem RNA obtained from independent passage of stock A. Therefore, DI genomes from stock A were 3'-end-labeled with ^{32}pCp and RNA ligase, and their RNase-resistant stem RNAs were isolated (see above). Minus-strand leader RNAs were assayed by determining their abilities to anneal to the 3'-end-labeled strand of the denatured stem RNA and, after RNase digestion, to convert this strand to a double-stranded RNA whose length was then determined by the length of the minus-strand leader RNA. However, since the starting probe was double-stranded RNA and the complement of

the 3'-end-labeled strand was also present in the annealing reaction mixture, the complementary strand could anneal to the portion of the end-labeled strand that was not covered by the leader RNA. In such a structure, the annealed leader RNA could be displaced by branch migration of the complementary 86-nucleotide strand. To avoid such complications, the annealing of the leader RNA to the denatured stem RNA was carried out for short periods of time in large volumes, so that reassociation of the stem RNA strands was limited and double-stranded RNA formation depended mostly on leader RNA.

To obtain the leader RNAs, cytoplasmic extracts of various mixed-virus-infected BHK cells were centrifuged on CsCl gradients; in these gradients the genome nucleocapsids banded in the middle of the gradient, and the RNAs which were not in viral nucleocapsids pelleted through the gradients as free RNA. Similar amounts of the various CsCl pellet RNAs from mixed-virus-infected and uninfected BHK cells were mixed with end-labeled 86-bp stem RNA, and the RNAs were denatured, annealed, and digested with RNase as described above and in the legend to Fig. 2. The remaining RNase-resistant RNAs were then electrophoresed on a nondenaturing polyacrylamide gel. Figure 6 shows that when uninfected BHK cell CsCl pellet RNA or *Escherichia coli* tRNA was used, no radioactive bands were detected, except for a small amount of a doublet band at the position of nondenatured 86-bp stem probe (Fig. 6, lanes 2 and 9). On the other hand, when CsCl pellet RNAs from the six independently passaged mixed-virus infections were added (Fig. 6, lanes 3 to 8), the 46-bp band which previously had been shown to be due to minus-strand leader RNA was present. In particular, I detected no bands which migrated between the 46-bp band and the 86-bp stem probe, even after prolonged exposure of the gel. Since the CsCl pellet leader RNAs were synthesized from various DI genomes, which contained different extents of complementarity at their ends, these results demonstrated that the size of the minus-strand leader RNA was independent of the lengths of the complementary ends in the various DI genomes.

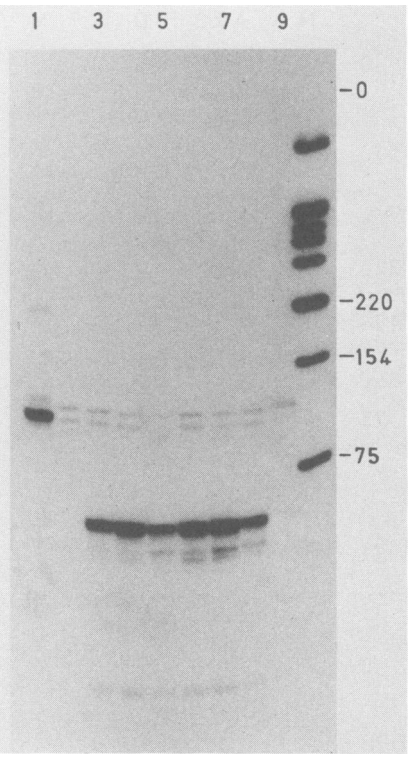

FIG. 6. Examination of intracellular minus-strand leader RNAs from various mixed-virus-infected cells. 3'-End-labeled stem RNA was prepared from the DI genomes present in stock A at passage 5 (see text), and 2-μl amounts of this RNA (8,000 cpm) were mixed with 5-μl volumes containing 40 μg of CsCl pellet RNA from uninfected BHK cells (lane 2), BHK cells infected with stocks A, B, D, E, F, and T at passage 4 (lanes 3 to 8, respectively), and 40 μg of *E. coli* tRNA (lane 9). The RNAs were denatured by adding 15 μl of dimethyl sulfoxide and heating for 1 min at 60°C, and samples were then diluted with 150 μl of 2.5× buffer A and annealed for 2 min at 60°C. A 50-μl amount of 2.5× buffer A containing 50 μg of RNase A per ml was added, and the samples were incubated for 10 min at 25°C. Proteinase K digestion and nondenaturing polyacrylamide gel electrophoresis were performed as described in the legend to Fig. 2. Lane 10 contained *Hin*F-digested pBr322 DNA fragments which were end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. Lane 1 contained 1 μl of the nondenatured 86-bp stem probe.

DISCUSSION

In this paper I show that when a given clonal isolate of VSV is passaged at a high MOI on a given host cell, it has no particular predisposition to generate a given type of DI particle. As Fig. 1 shows, a single isolate of VSV seems to be able to generate an infinite number of different DI genomes in a given host cell type. These results confirm the findings of Holland et al. (5) with VSV and my findings with Sendai virus (10), but they are not in agreement with the findings of Kang et al. (7). However, it should be noted that the experimental protocol used here was the protocol used by Holland et al. (5), which differed from that of Kang et al. (7) in one important aspect. Kang et al. prepared their clonal isolate stock by a single low-MOI passage after successive plaque isolations, whereas Holland et al. and I did not. It seems likely that the different results obtained were due to the difference in protocol and that the identical DI parti-

cles found by Kang et al. were in fact generated during the single low-MOI passage used to prepare the stock and were simply amplified during the subsequent high-MOI passages.

After I obtained a large number of different size DI particles all of which were generated from a single clonal isolate and therefore were a matched set in this respect, I examined the genomes of these DI particles for the presence of complementary ends (end-labeled stem RNAs). Interestingly, although a particular size stem predominated in most of the independently passaged stocks, the lengths of the stems varied over a much wider range than previously reported (19, 21). Stems as long as 200 bp were detected, suggesting that there was no upper limit to the size of the complementary ends for viable DI genomes. In fact, this was not surprising, since previous work had described DI genomes which were composed entirely of complementary ends (the "snap-back" DI genomes) (11, 18). On the other hand, viable DI genomes, appear to have a lower limit to the extent of the complementarity at their ends; no stems smaller than 46 bp were detected. This finding was confirmed in a separate experiment, in which a single clonal isolate of the *ts*G31 mutant of VSV was used to generate a large number of DI genomes (data not shown). Therefore it appears that the extent of terminal complementarity beyond 46 nucleotides is of little importance in determining the competitive advantage among 5′ copy-back DI genomes. The mere presence of complementary ends, which reflects the fact that both plus- and minus-strand DI genomes contain at a minimum the first 46 nucleotides of the 3′ end of the ND antigenome at their 3′ ends, appears to be sufficient to give a replicative advantage to these DI genomes over ND genomes.

The existence of a matched set of 5′ complementary-end DI genomes which contained variable extents of complementarity also allowed me to reexamine the origin of the minus-strand leader RNA. Minus-strand leader RNA is complementary not only to the exact 3′ end of plus- and minus-strand DI genomes, but also to the exact 3′ end of the ND antigenome. Indeed, standard virus-infected cells, in which DI genomes cannot be detected by radiolabeling, also contain minus-strand leader RNA, suggesting that this leader RNA is transcribed from the ND antigenome (12, 14). However, further studies on the quantitation of leader RNA in standard virus-infected cells and mixed-virus-infected cells have demonstrated that the viral polymerase initiates leader RNA synthesis considerably more frequently on DI genome templates than on ND antigenome templates (12). From these experiments, I calculated that the contaminating presence of 1% DI genomes relative to ND genomes in my standard virus-infected cells, which could not be excluded in these experiments, could have accounted for all of the minus-strand leader RNA that I detected. Under these circumstances, I could not demonstrate unambiguously that ND antigenomes were templates for minus-strand leader RNA synthesis. Of course, this raises the possibility that only DI genomes which contain the recombination site created by the replicase during DI genome generation (DI genome minus strands) synthesize minus-strand leader RNA. However, in this latter possibility, where minus-strand leader RNA is the result of a termination signal created by the recombinational event, one would expect that the length of the minus-strand leader RNA would be determined by the site of recombination. The data in Fig. 6 appear to rule out this possibility. Identical size (46′ mer) minus-strand leader RNAs are synthesized from DI genomes in which this recombination site is as little as 46 nucleotides or as much as 200 nucleotides from the 3′ end of the DI minus-strand genome. These data argue that the termination signal which leads to the synthesis of minus-strand leader RNA must in fact be contained within the ND antigenome. Thus, ND antigenomes, as well as DI genomes, appear to be templates for minus-strand leader RNA synthesis.

Recently, I and my co-workers described a model for the control of VSV genome replication in which the viral N protein controls transcription and replication of the minus-strand genome by its ability to bind to the nascent leader RNA and thereby modulate suppression of the leader RNA termination signal (2, 12, 14). In this model, similar termination signals near the 3′ ends of the ND antigenomes and the DI genomes are responsible for the synthesis of minus-strand leader RNA. However, ND antigenomes and DI genomes are templates only for replication (no mRNA's are transcribed from these templates). We suggested that the function of the minus-strand leader RNA termination signal is to prevent RNA synthesis from these templates under conditions where there is not sufficient N protein available to assemble these RNAs into nucleocapsids. The unassembled complementary RNAs in infected cell cytoplasm could anneal to form double-stranded RNA, thereby increasing the antiviral state induced by interferon. The experiments described here were performed not only to gather information on a matched set of DI genomes, but also to test this model indirectly. In the generation of multiple DI genomes whose ends were complementary to different extents, I was particularly interested in whether I could detect DI genomes which contained complementary ends less than 46 nucleo-

tides long. According to the model, since in such cases the minus-strand DI genome has lost its leader RNA termination signal (near position 46 from the 3′ end) by a recombinational event, it would be able to serve as a template for plus-strand DI genome synthesis even in the absence of viral N protein, thus producing free DI genome RNA. Such unassembled DI genomes would anneal intramolecularly to form stem RNA and would activate the interferon system. Therefore, infected cells which contain such DI genomes would produce fewer virus particles, leading to the disappearance of these genomes from the mixed virus population. I believe that the absence of such DI genomes among a large number of independently generated DI genomes offers indirect evidence in support of the model. In any event, the presence of the 46-nucleotide minus-strand leader RNA sequence at the ends of copy-back DI genomes and antigenomes appears to be a minimum requirement for the maintenance of these genomes in mixed virus populations.

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