

Continuing Coevolution of Virus and Defective Interfering Particles and of Viral Genome Sequences during Undiluted Passages: Virus Mutants Exhibiting Nearly Complete Resistance to Formerly Dominant Defective Interfering Particles

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We quantitatively analyzed the interference interactions between defective interfering (DI) particles and mutants of cloned vesicular stomatitis virus passaged undiluted hundreds of times in BHK-21 cells. DI particles which predominated at different times in these serial passages always interfered most strongly (and very efficiently) with virus isolated a number of passages before the isolation of the DI particles. Virus isolated at the same passage level as the predominant DI particles usually exhibited severalfold resistance to these DI particles. Virus mutants (Sdi⁻ mutants) isolated during subsequent passages always showed increasing resistance to these DI particles, followed by decreasing resistance as new DI particles arose to predominate and exert their own selective pressures on the virus mutant population. It appears that such coevolution of virus and DI particle populations proceeds indefinitely through multiple cycles of selection of virus mutants resistant to a certain DI particle (or DI particle class), followed by mutants resistant to a newly predominant DI particle, etc. At the peak of resistance, virus mutants were isolated which were essentially completely resistant to a particular DI particle; i.e., they were several hundred thousand-fold resistant, and they formed plaques of normal size and numbers in the presence of extremely high multiplicities of the DI particle. However, they were sensitive to interference by other DI particles. Recurring population interactions of this kind can promote rapid virus evolution. Complete sequencing of the N (nucleocapsid) and NS (polymerase associated) genes of numerous Sdi⁻ mutants collected at passage intervals showed very few changes in the NS protein, but the N gene gradually accumulated a series of stable nucleotide and amino acid substitutions, some of which correlated with extensive changes in the Sdi⁻ phenotype. Likewise, the 5' termini (and their complementary plus-strand 3' termini) continued to accumulate extensive base substitutions which were strikingly confined to the first 47 nucleotides. We also observed addition and deletion mutations in noncoding regions of the viral genome at a level suggesting that they probably occur at a high frequency throughout the genome, but usually with lethal or debilitating consequences when they occur in coding regions.

The earliest studies of defective interfering (DI) particles of RNA viruses by Henle and Henle (16), von Magnus (55), and Mims (32) established clearly that serial undiluted passage of virus in embryonated eggs or in mice led to strong homologous interference by DI particles. Undiluted passage of virus provides the high input multiplicities of infectious helper virus needed to amplify and maintain the replication of newly generated DI particles. Palma and Huang (37) first demonstrated in cell culture that continuous passage of virus leads to regular cyclical waves of high and low virus yields due to a coupled, out-of-phase cyclic production of DI particles. This cycling is due to an increase in DI particles and strong interference with virus yields, followed regularly by greatly reduced DI particle yields as helper virus levels become limiting due to the DI particle interference. Although the mechanism could not be understood at the time, Mims (32) observed what is now recognized as clear virus-DI particle cycling interactions during serial undiluted passages of virus in mice. Cave et al. (9, 10) recently presented strong evidence that virus-DI particle cycling interactions may occur during the course of an acute virus infection in a single animal. Because of the stochastic nature of such cycling, this

finding has great importance for viral immunity, viral diagnostics, and the varying outcomes of virus infections. There is increasing evidence that DI particles can influence acute virus infections in vivo (3, 9, 10, 12, 45) and that they probably can do so in nature (4, 45). Continuous or intermittent interactions between viruses and their DI particles also appear to be a major factor influencing many persistent infections (for reviews, see references 18, 23, 30, 42, and 47).

In 1977, Kawai and Matsumoto (28) reported the appearance of rabies virus mutants resistant to DI particles which had been present earlier in the course of persistent infection of BHK-21 cells in vitro. Since then, such DI particle-resistant mutants (Sdi⁻ mutants) have been reported to arise during persistent infections by vesicular stomatitis virus (VSV) (19), lymphocytic choriomeningitis virus (27), Sindbis virus (56), and West Nile virus (8). Even bacteriophage ϕ 1 produces Sdi⁻ types of mutants (14). A sensitive assay system for quantitating VSV DI particle interference has shown that Sdi⁻ mutants do not arise by a single mutation of infectious virus; rather, the virus acquires increasing resistance to a particular DI particle in a stepwise manner during either persistent infection or serial undiluted passages in vitro (19-21). At least two virus complementation groups can contribute to DI particle resistance (21). The Sdi⁻ phenotype is rather specific for a particular DI particle, or DI particle type, because DI particles newly generated by an Sdi⁻

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mutant virus can interfere strongly with the parental virus (21). Thus, there can occur a coevolution of different Sdi⁻ mutant viruses and their associated DI particles during serial undiluted passages and during persistent infections (21). In the present study, we characterized the quantitative, stepwise responses of evolving Sdi⁻ mutants to three different DI particles which were the predominant DI particles at different times during prolonged serial undiluted passages of VSV. It was shown that the stepwise coevolution of virus and DI particles recurs repeatedly and probably indefinitely and that some of the Sdi⁻ mutants can become essentially completely resistant (over 250,000-fold resistant) to a formerly dominant DI particle.

With DNA phage ϕ 1, Enea and Zinder (14) used heteroduplex mapping and sequencing to identify the two mutated sites in their IR (Sdi⁻ type) mutants of this male-specific filamentous phage. Both mutations were in gene II, which encodes a replicase-associated, site-specific nicking enzyme required for ϕ 1 replication (along with host cell enzymes). Since DI particle interference with VSV occurs mainly at the level of replication-encapsidation (24, 38, 41, 44, 52), it would be expected that the large transcriptase-replicase (L protein) (5, 13, 36, 49) gene, the polymerase-associated (NS protein) gene (5, 11, 13, 22, 26), or the nucleocapsid (N protein) gene (1, 2, 6, 17, 39, 41) or a combination of these genes would be involved as sites of Sdi⁻ mutations. We show below that the N protein frequently undergoes mobility shifts during Sdi⁻ evolution, so the N genes of a number of Sdi⁻ mutants were sequenced. The NS protein exists in a number of different phosphorylation states and may be involved in regulation of VSV transcription (22, 53). Isaac and Keene (25) mapped different NS protein binding sites of viral 3' ends and compared them with DI particle 3' ends; they suggested that the NS binding may facilitate initiation of RNA transcription and replication by the L protein. Therefore, we also sequenced the NS gene and both the 5' and 3' ends of the Sdi⁻ mutants. The results show that the NS protein was not generally involved in Sdi⁻ mutations, that the N gene slowly accumulated stable mutations associated with the Sdi⁻ phenotype, and that many mutations in protein genes must map elsewhere, presumably in the 6.4-kilobase L protein gene (49). We also showed that virus 5'-end sequences involved in replication-encapsidation interactions continued to evolve extensive base substitutions during the virus-DI particle coevolution, whereas the 3' ends remained relatively very stable.

MATERIALS AND METHODS

Cells, virus, and DI particles. BHK-21 cells were used for all virus and DI particle production and all assays. The undiluted passage series was started with the cloned *tsG31* mutant and was a continuation of that described previously by Spindler et al. (51) and Horodyski et al. (21). It was continuously maintained for more than 500 passages, usually with 2 days before harvest and subsequent passage but occasionally with 3- or 1-day harvests if the viral cytopathology was unusually slow or fast. The DI particles studied were the undiluted passage 104 DI particle (p104 DI), p200 DI, and p319 DI. Each was isolated from first-passage amplification of the corresponding undiluted passage supernatant, and each was the predominant DI particle present. All virus strains were isolated by plaque assay directly from specific passage number supernatants. Care was taken to pick plaques of average size and morphology for that passage. They were usually very similar within any particular

passage supernatant but varied, often greatly, between different passage supernatants, sometimes in as few as five passages. One exception was p104, in which plaques of two distinct sizes were clearly present. Two isolates, p104 sp (small plaque) and p104 lp (large plaque), were chosen for study from this population.

DI particle assay. Quantitative resistance levels to specific DI particles were determined by spectrophotometric quantitation of virus and DI particle yields as a function of input DI particle multiplicity, as described by Horodyski et al. (21), but with extra precautions when assaying extremely resistant mutants. For these viruses (resistance of >10,000), assays were always done with DI particles purified at least three times on successive sucrose gradients to eliminate all or nearly all contaminating virus, as described by Doyle and Holland (12). Also, the virus was preabsorbed for 15 min before addition of DI particles to avoid possible receptor binding competition from the huge excess of DI particles (up to 200,000 per cell) used in some assays. Finally, after 0.5 h, unabsorbed DI particles were washed off with three 10-ml rinses with minimal essential medium.

Plaque assays in the presence and absence of DI particles. Plaque assays were done with nearly confluent BHK-21 cells in Corning 25-cm² tissue culture flasks. Standard 10-fold dilutions of the p171 and p287 viruses were absorbed for 0.5 h at 23°C in a total volume of 0.2 ml. p200 DI particles at an effective multiplicity of 300 per cell were simultaneously applied to one-half of the flasks. Incubation was at 33°C with a 0.4% agarose-minimal essential medium overlay, and harvesting was done after 2 or 3 days in different experiments.

Protein gels. Proteins from purified virus were examined by Coomassie brilliant blue staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% Laemmli gels (29) with an acrylamide-BIS ratio of 75:1, as modified by Toneguzo and Ghosh (54).

Viral RNA purification. Virus preparations used for RNA extraction were obtained by infecting BHK-21 cells with the same viral pools used for the phenotypic characterizations described below. The RNA was extracted from purified virions by proteinase K digestion and phenol-chloroform extraction, as described previously (34).

Chemical synthesis of DNA primers. Fourteen complementary primers were used for all sequencing. Initially, primers were synthesized on solid support by using the phosphotriester approach as described by Miyoshi et al. (33) and Ito et al. (26). Later primers were synthesized on an Applied Biosystems DNA synthesizing machine. The primers used for sequencing the 3'- and 5'-terminal regions were ACCAACAAACAGAAGCA and CAGGAATGATTGA ATGG, respectively. All other primers are indicated on sequence maps (see Fig. 6 and 7).

Primer extension using reverse transcriptase. Gel-purified DNA primers (1 to 10 ng) end labeled with ³²P at the 5' position were annealed to 3 to 5 μ g of viral RNA and then extended with avian myeloblastosis virus reverse transcriptase (obtained from Life Sciences, Inc.), as described by O'Hara et al. (34).

Sequencing of cDNA reverse transcripts. All sequencing was done by the method of Maxam and Gilbert (31), as described by O'Hara et al. (34). Each base was sequenced an average of three or four times.

RESULTS

Selection of DI particles for characterization. Earlier work by Horodyski et al. (21) had shown a stepwise accumulation

of resistance to the original DI particle (ST-2) in both persistent infection and an undiluted lytic passage series (but not in a dilute lytic passage series in which DI particle levels are low). In the undiluted passage series, resistance reached a maximum of about 800-fold (ST-2 DI) at p150, and the resistance phenotype had almost disappeared (resistance = 2.5; ST-2 DI) at p171. We hypothesized that there might be continual cycles of such escape from newly evolving DI particles, and previously reported results with p34 DI and p80 DI lent credence to this hypothesis (21). To test whether such cycles of resistance continue in similar fashion indefinitely with continued undiluted passages, we characterized three DI particles predominant at about 100-passage intervals: p104, p200, and p319. In each case, they were tested for resistance or sensitivity to standard virus isolates taken immediately before, at the same time as, and at several times (passages) after the time of isolation of each of these DI particles.

Resistance to p104 DI particles. We initially analyzed sensitivity to p104 DI; the results are shown graphically in Fig. 1. The p80 virus is extremely sensitive to this DI particle despite being about 600-fold resistant to the original ST-2 DI (21). As mentioned in Materials and Methods, the p104 supernatant produced a mixture of large and small plaques, and therefore two isolates were picked and multiply recloned for study. The large-plaque isolate (p104 lp) regularly grew to titers about five times that of p104 sp but was considerably less resistant to the predominant DI particle. This suggests

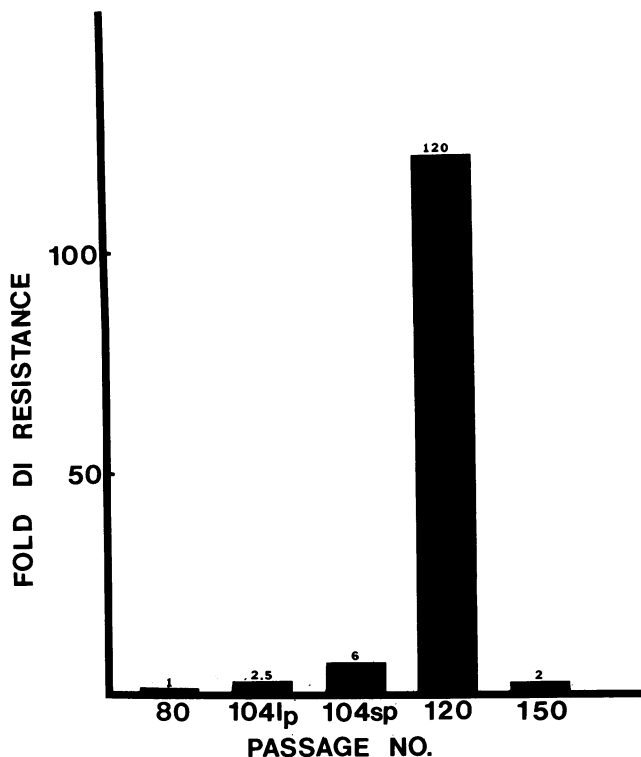


FIG. 1. Quantitation of resistance of evolving Sdi^- mutants of VSV to DI particles isolated from undiluted p104. Fold resistance was determined as described previously (21) and in Materials and Methods. The Sdi^- mutant viruses tested were representative clones isolated at the indicated passage number (abscissa) in the undiluted passage series (see Materials and Methods). The DI particle used was the most abundant DI particle present at p104 of the undiluted passage series.

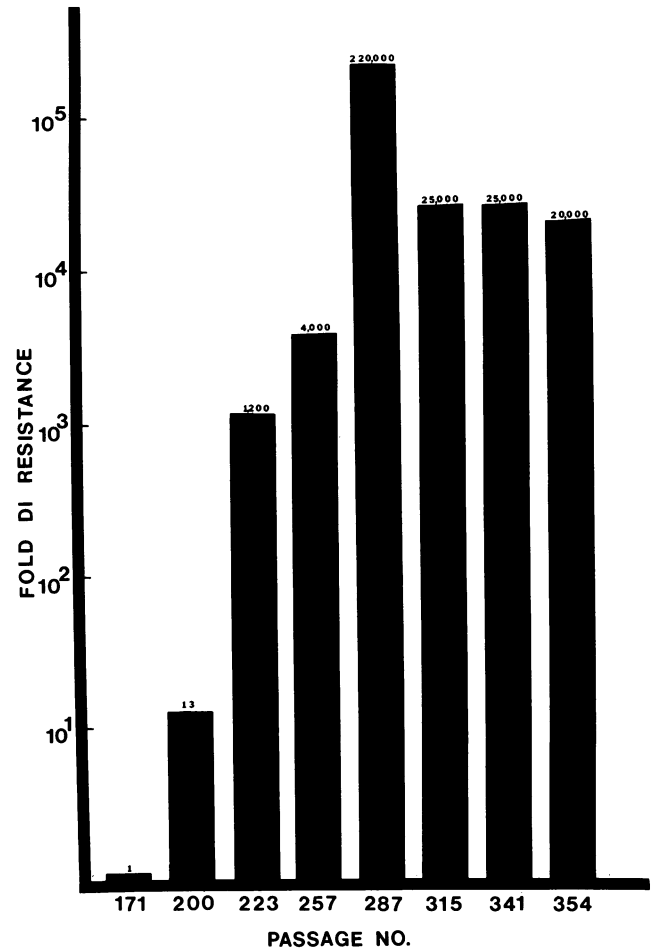


FIG. 2. Quantitation of resistance of evolving Sdi^- mutants of VSV to DI particles isolated from undiluted p200. Conditions were the same as those described in the legend to Fig. 1, except that the DI particle which was predominant at p200 was used as the test particle.

that the increased resistance enabled the slower-growing virus to compete. By p120, only 16 passages later, the resistance had peaked at about 120-fold. At passage 120 and at all other passages in which peak resistance was observed, new clones from the original passage supernatant were picked, recloned, and tested to assure that we were characterizing representative virus clones. In each case, the resistance levels were very similar to that of the original clone, with the largest differences being two- to threefold, which is very small in comparison with the differences in resistance between different passage numbers. Finally, between p120 and p150 the resistance dropped from 120- to 2-fold, a factor of 60, which was even more precipitous than the drop from 80- to 50-fold resistance shown by these same viruses relative to the original DI particles (21).

Resistance to p200 DI particles. A similar pattern of mutation to resistance to p200 DI particles was observed, but the peak resistance was enormously higher than had ever been seen (Fig. 2). The virus from p171 was highly sensitive, and the p200 virus was 13-fold resistant. In only 23 passages after the p200 DI particle isolation, the virus exhibited 1,200-fold resistance, and the resistance kept increasing until at p287 a virus essentially totally resistant (over 220,000-fold) was

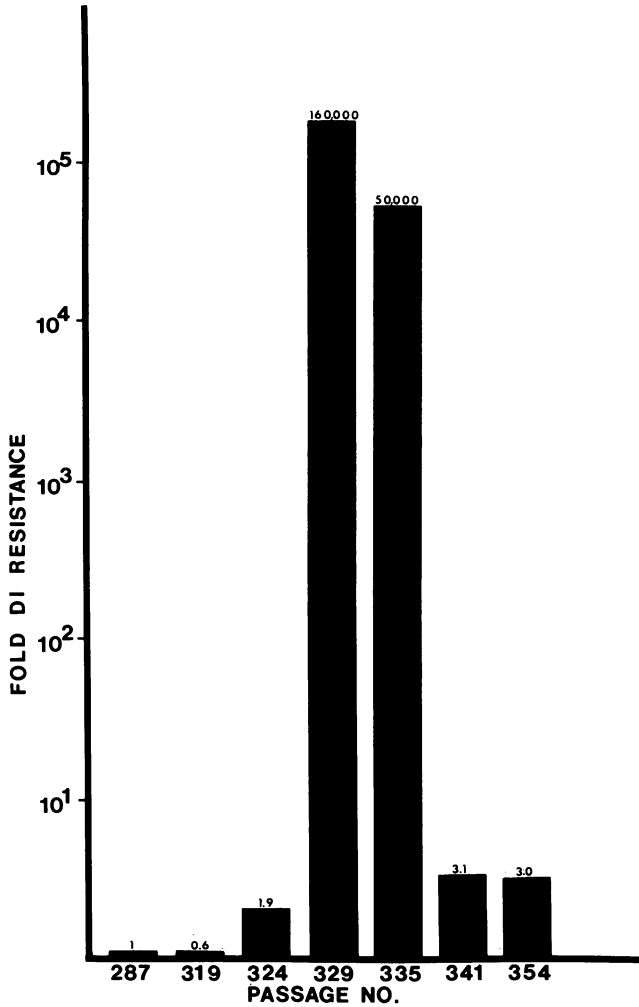


FIG. 3. Quantitation of resistance of evolving Sdi⁻ mutants of VSV to DI particles isolated from undiluted p319. Conditions were the same as those described in the legend to Fig. 1, except that the test DI particle used was isolated at undiluted p319.

isolated. This virus required enormous quantities of p200 DI particles (e.g., over 100,000 per cell) to show small but reproducible levels of interference and DI particle replication. Once again, the resistance levels later declined, but this time only slightly. Isolates from p394 and p470 were also tested (data not shown) and still exhibited strong resistance to this DI particle.

Resistance to p319 DI particles. The initial tests were done at time points (passage numbers) spaced about 15 to 30 passages apart and were unusual in that they showed only slight resistance changes. Further examination at more time points revealed an extremely abrupt cycle of mutation to resistance (Fig. 3). Once again, the virus isolated before p319 DI particle isolation was extremely sensitive. Notice, however, that the sensitive virus was p287, the same virus which was over 220,000-fold resistant to p200 DI. This underscores the fact that DI particle resistance (or Sdi⁻ phenotype) has meaning only in relation to a particular DI particle (or class of DI particles). The coincident virus, p319, isolated at the same time as this DI particle showed no resistance, unlike all other cases examined. The p324 virus had much larger plaques but little change in resistance, whereas the p329

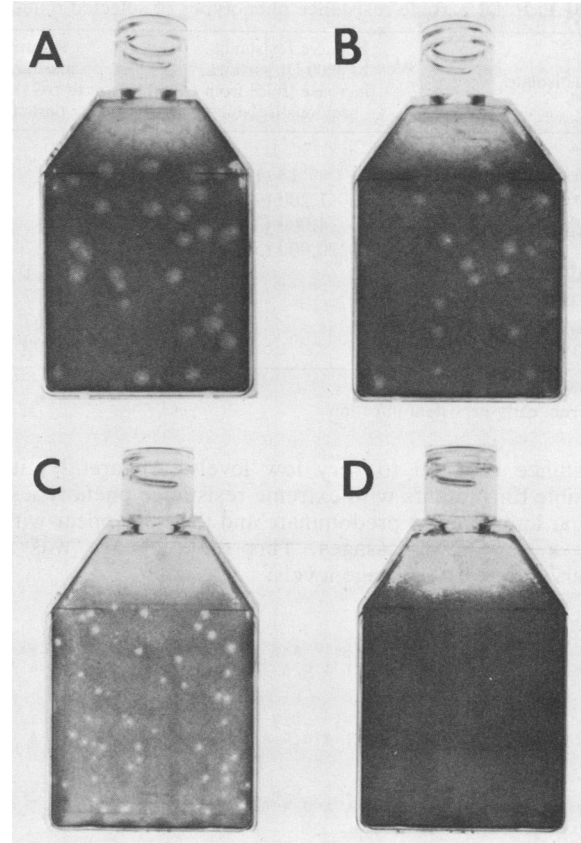


FIG. 4. Plaque assays of p171 and p287 viruses in the presence and absence of p200 DI particles at a multiplicity of 300 per cell. (A) p287 virus alone; (B) same amount of p287 as in panel A, plus p200 DI particles; (C) p171 virus alone; (D) 20,000-fold more p171 virions than in panel C plus p200 DI particles.

supernatant again gave very small plaques which showed enormous resistance (about 160,000-fold). Five passages later, the p335 virus had much larger plaques and 50,000-fold resistance, and after only five more passages, to p341,

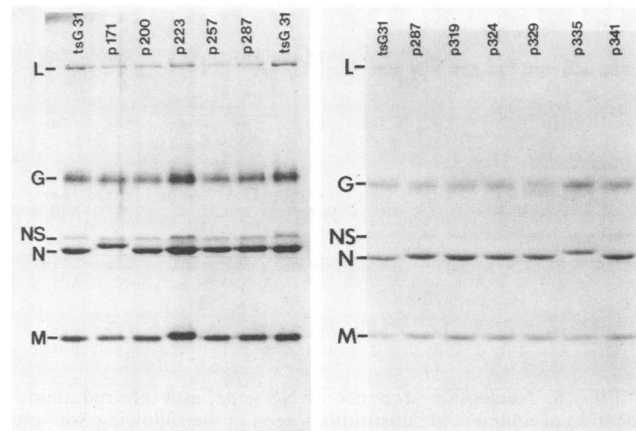


FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel analysis of proteins from purified tsG31 and Sdi⁻ undiluted passage series viruses between p171 and p341. G, Glycoprotein.

TABLE 1. DI particle resistance phenotypes of selected mutants

Virus isolate	Relative resistance to p200 DI particle (increase [fold] from previous isolate)	Relative resistance to tsG31 DI particle
p171 ^a	1	
p200 ^a	13 (13)	
p223 ^a	1,200 (92)	
p257 ^a	4,000 (3.3)	
p287 ^a	220,000 (55)	
tsG31 ^b		1
p12, clone 3 ^b		77
34-day sp ^b		67
75-day sp ^b		90

^a From undiluted passage series.

^b From early persistent infection.

resistance dropped to very low levels. Apparently, it is possible for mutants with extreme resistance phenotypes to appear and come to predominate and then disappear within only a very few passages. This rapid change was not observed at early passage levels.

Plaque assays in the presence of DI particles. The extreme resistance of the p287 virus to p200 DI suggested that it might be possible to show directly by plaque assay the resistance or sensitivity of different virus isolates. The p171 virus was very sensitive to p200 DI, and the p287 virus was almost totally resistant; therefore, we examined the efficiency of plaque formation by each in the presence of p200 DI particles added at a multiplicity of 300 per cell. Representative examples for both viruses with and without DI particles are shown in Fig. 4. The p287 virus produced approximately normal-sized plaques in unchanged numbers with this DI particle, whereas p171 plaque production was completely blocked even at extreme input concentrations of the virus (i.e., >10⁷ PFU per flask). The experiment was repeated three times, and in each case, p171 plaque production was completely blocked, whereas p287 plaque counts and size remained unchanged in the presence of p200 DI. Plaques from the p171 virus plus DI particles were never observed, even after 5 days.

Analysis of proteins of Sdi⁻ mutants. Because of the extreme resistance of the Sdi⁻ mutants, rising and falling between p171 and p341, we examined them for protein

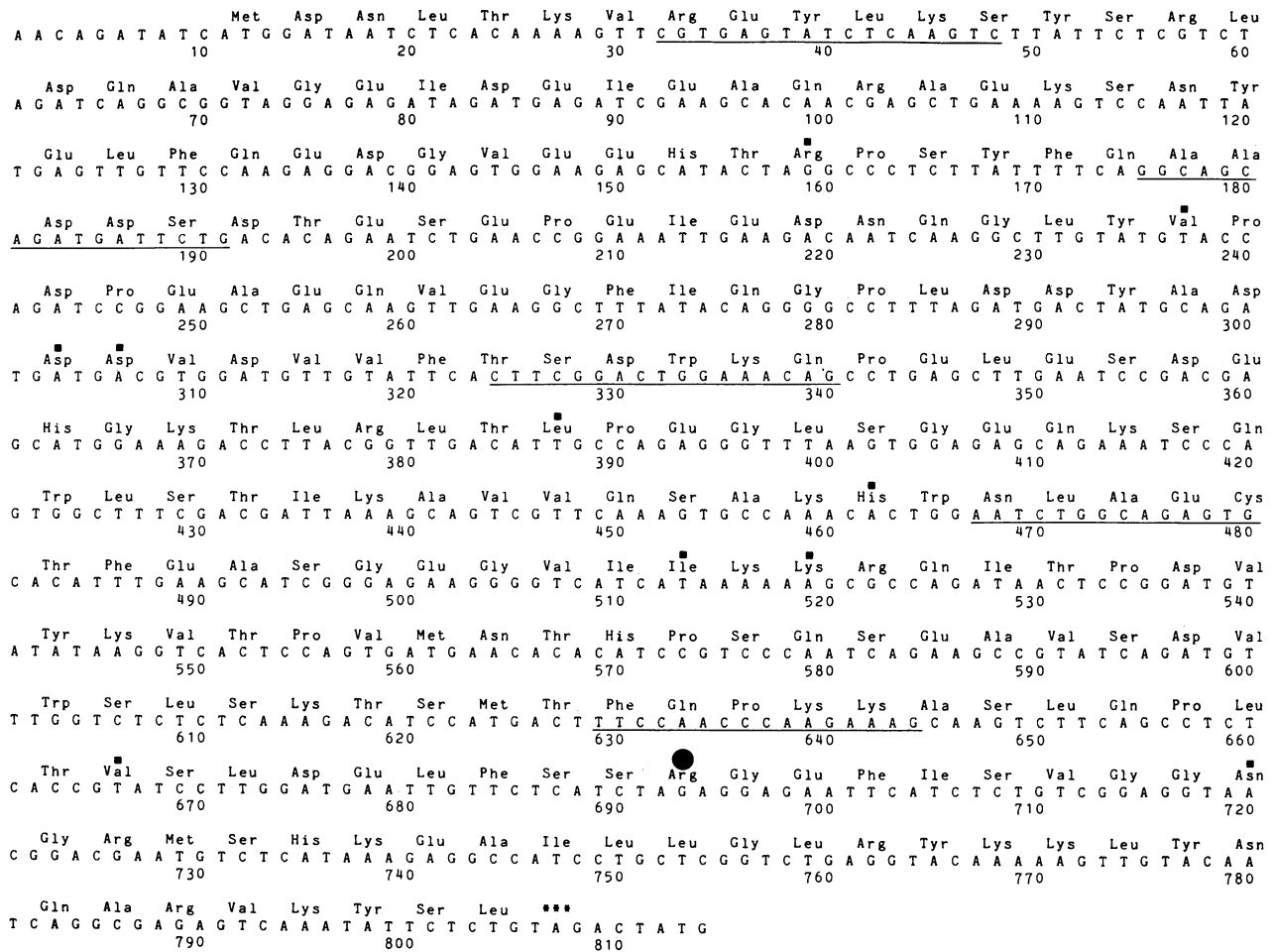


FIG. 6. Nucleotide sequence of NS gene, and inferred amino acid sequence, of tsG31 mutant of Glasgow strain of VSV-Indiana with location of amino acid substitutions seen in the following Sdi⁻ mutants: p171, p200, p223, p257, and p287 (from the diluted passage series); and 34-day sp and 75-day sp (from early persistent infections). The only amino acid change occurring in any of these mutants was at amino acid 228 (Arg to Glu) (●). Amino acid differences from the sequence of the San Juan strain of VSV-Indiana, as reported by Gallione et al. (15), are indicated (■). The underlined bases indicate the deoxyoligonucleotide primers used for reverse transcription (see Materials and Methods). All mutations in these Sdi⁻ mutants are listed in Table 2.

TABLE 2. NS gene mutations

Virus isolate	Nucleotide position(s) ^a and change	Amino acid position and change
34-day sp	22; CTC → CTA	4; no change
p171	692, 693; AGA → GAA ^b	228; Arg → Glu
p200	692, 693; AGA → GAA	228; Arg → Glu
p223	692, 693; AGA → GAA	228; Arg → Glu
p257	692, 693; AGA → GAA	228; Arg → Glu
p287	692, 693; AGA → GAA 241; CCA → CCG	228; Arg → Glu 77; no change

^a The positions refer to those in Fig. 6.

^b These mutations first appeared at p171 and were stably maintained through p287.

mobility changes by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Viral proteins from purified *tsG31* virions were compared first with proteins of isolates from p171 to p287 and then with those of isolates from p287 to p341 (Fig. 5). Several clear differences were observed in the N protein, with definite shifts between *tsG31* and p171, p171 and p200, p329 and p335, and p335 and p341. A few smaller, less certain mobility shifts may also have been present. These changes suggested the occurrence of a number of mutations or modifications in the N protein. The presence of these mobility shifts in the N protein bands could be significant in light of recent evidence linking the N protein to a crucial role in control of VSV replication (1, 2, 6, 17, 39, 40, 43). We therefore sequenced the N (and NS) protein genes of these *Sdi*⁻ mutants; the results are presented below.

Selection of mutants for sequencing. Five selected *Sdi*⁻ mutants from the studies discussed above are listed in Table 1 along with their relative resistance to p200 DI. Note the magnitude of the change in resistance at each step of this series, which ranged from 3.3- to 92-fold. Because of the extraordinary resistance at p287 and the stepwise increase in resistance between p171 and p287, we sequenced regions of the five corresponding mutants plus several others. The p12, clone 3 (undiluted passage series), 75-day sp (CAR 4 carrier cells), and 34-day sp (CAR 21 carrier cells) mutants were described previously by Horodyski et al. (21). Each showed significant resistance to the original *tsG31* DI particle (Table 1), and each was isolated very early in persistent infections (from independent sources), thus minimizing nonrelevant mutational drift. The 34-day sp and 75-day sp mutants are prototypes of the two complementation groups of *Sdi*⁻ mutants seen by Horodyski et al (21). We also sequenced the parental virus for all of these mutants, *tsG31*, which is an M gene temperature-sensitive mutant of VSV-Indiana, Glasgow strain (46).

NS gene sequencing. Maxam-Gilbert sequence analysis of cDNA reverse transcripts from the relevant genomic area was used to determine the sequence of the *tsG31* NS mRNA (Fig. 6) along with the predicted amino acid sequence. The length of 265 amino acids and the reading frame used are consistent with the VSV-Indiana (San Juan) sequence as reported by Gallione et al. (15). Compared with the San Juan strain NS gene sequence, the *tsG31* sequence has 23 nucleotide differences resulting in 10 amino acid differences (5 conservative) (Fig. 6). The complete NS gene sequence was also determined for seven *Sdi*⁻ mutants; the mutations are

listed in Table 2. No changes were seen in the early *Sdi*⁻ mutants, 34-day sp or 75-day sp, and the only amino acid substitution in the undiluted passage series mutants (amino acid 228, Arg to Glu) was present in p171 and maintained through p287.

Sequences of the N gene. The determined RNA sequences (and inferred amino acids) for the N gene of the original *tsG31* virus and derived mutants are shown in Fig. 7. The *tsG31* Glasgow strain has only nine nucleotide differences from the N gene sequence of the San Juan strain of VSV-Indiana as determined by Gallione et al. (15), and only three result in amino acid substitutions. We determined the complete N gene sequence for nine relevant *Sdi*⁻ mutants; the observed mutations are listed in Table 3. Again, no mutations were found in the early *Sdi*⁻ mutants (34-day sp, 75-day sp, and p12, clone 3), but eight N gene mutations accumulated by p287. These resulted in six amino acid substitutions, five of which were nonconservative. Interestingly, two mutations (Lys to Glu at position 46 and Gly to Glu at position 186) appeared during the passage interval (between p200 and p223) in which the most dramatic change in the DI particle resistance phenotype occurred in this passage series (Table 1). Additionally, an adjacent amino acid substitution (Ser to Arg at position 47) was first observed at the same passage (p150) as a sudden drop in resistance to both the p104 DI particle and the original ST-2 DI particle. This suggests that some of the N protein gene mutations might be significant to the evolving *Sdi*⁻ phenotypes.

Sequences of 5' and 3' genomic termini of *Sdi*⁻ mutants. Previous results showed that the earliest *Sdi*⁻ mutants to appear had no mutations in the 5' (or 3') termini but that later they accumulated a series of stepwise, stable base substitutions clustered within the first 47 nucleotides from the 5' end (34). We sequenced the genomic termini of *Sdi*⁻ mutants appearing at intermediate and later times in this undiluted passage series; the results (along with earlier mutations) are presented in Fig. 8. It can be seen that extensive changes in the 5' termini continued to accumulate, and strikingly, they remained clustered within the first 47 bases. It is also striking that seven mutations plus six heterogeneity differences occurred in the 5' termini between p257 and p287, an interval during which a dramatic increase in DI particle resistance also took place. Most mutations in the terminal regions were stably maintained, but several underwent reversion and a number of others were extremely unstable, so that base heterogeneity was repeatedly observed at certain sites. Site heterogeneity may often reflect slow reversion of viruses which were cloned before sequencing (and hence removed from the selective effects of DI particles). In contrast to the 5' termini, the 3'-terminal region showed only one new stable mutation, after p171 (A to T at position 30), for a total of only two mutations from *tsG31* to p287. The p200 virus showed a T to C transition (at position 29 from the 3' end) which reverted by p223.

Noncoding region insertions and deletions. Quite unexpectedly, we found a number of insertion and deletion events in isolates from both persistent infection and the undiluted passage series. The most common were insertions or deletions of a single U at the polyadenylation signal sites, AUAC(U)₇, seen at the 5' end of all VSV genes (48). Either seven or eight U residues were always seen (Table 4), but the number fluctuated frequently. Even more unexpected were the insertion and deletion events near transcriptional start sites (Fig. 9). One NS-M junction insertion produced a direct repeat, and another produced a strange inverted repeat near

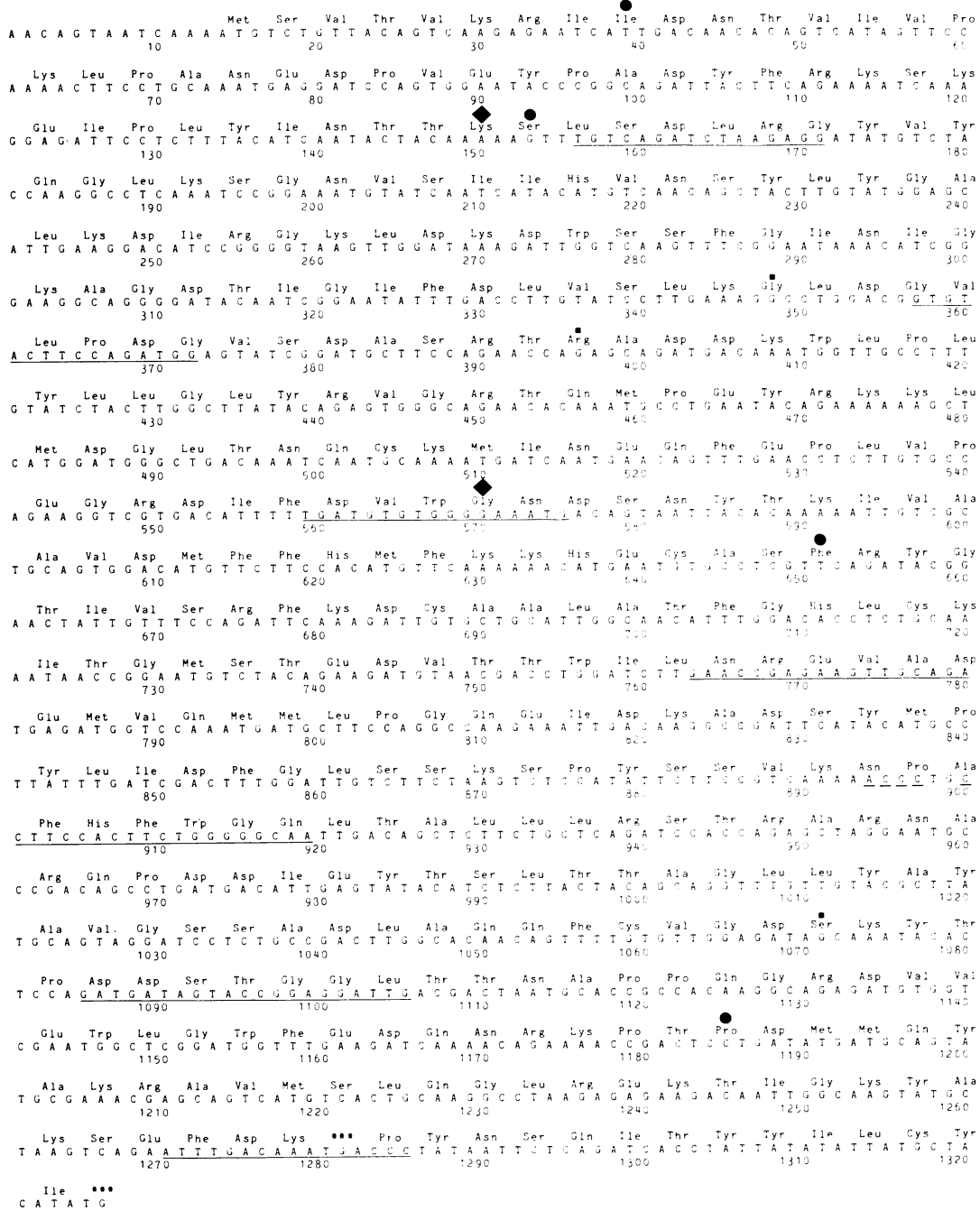


FIG. 7. Nucleotide sequence of N gene, and inferred amino acid sequence, of *tsG31* with the location of amino acid changes seen in the following *Sdi*⁻ mutants: p12 (clone 3), p171, p200, p223, p257, and p287 (from the undiluted passage series); and 34-day sp and 75-day sp (from early persistent infections). Amino acid changes appearing in these mutants are indicated by the following symbols: ●, mutations occurring up to p171; ◆, mutations appearing at p200 or later; ■, changes present in *tsG31* compared with the N gene sequence of VSV-Indiana (San Juan) reported by Gallione et al. (15). The underlined sequences identify N gene primers used for sequencing, and the dashes under bases 894 to 897 and 900 indicate an extremely strong stop during reverse transcription which prevented accurate sequence determination at this site. No N gene mutations were observed in p12 (clone 3), 34-day sp, or 75-day sp (from early independent persistent infections). The bases and amino acids changed in these *Sdi*⁻ mutants are listed in Table 3.

the M mRNA start site (48). This is right before the UU sequence which is repeated within the insertion. It will be interesting to determine whether this insertion affects the 5' terminus or the abundance of the M mRNA or both. In the case of the insertion at the N-NS junction in 4-year CAR 4

virus, a direct repeat (UAA) was again generated, this time in a U+A-rich region upstream from the polyadenylation site. It has been suggested that this site promotes transcriptional initiation (48). Again, quantitation of relative mRNA levels before and after such events might be informative.

TABLE 3. N gene mutations

Nucleotide position and change	Amino acid position and change	Presence in virus isolate:					
		<i>tsG31</i>	p171	p200	p223	p257	p287
38, ATT → CTT	9, Ile → Leu	—	+ ^a	+	+	+	+
149, AAA → GAA	46, Lys → Glu	—	—	—	+	+	+
152, AGT → CGT	47, Ser → Arg	—	+ ^b	+	+	+	+
570, GGA → GAA	186, Gly → Glu	—	—	—	+	+	+
652, TTC → TTA	213, Phe → Leu	—	+	+	+	+	+
754, ACC → ACA	247, no change	—	—	—	—	—	+
934, CTG → CTA	307, no change	—	—	—	—	+	+
1185, CCT → CAT	391, Pro → His	—	+	+	+	+	+

^a Mutation first appeared at p104 (24).

^b Mutation first appeared at p150 (24).

DISCUSSION

The undiluted passages studied averaged 2 days from infection to harvest, so the time covered for all the Sdi⁻ phenotypes analyzed was more than 2 years. It must be emphasized that the DI particles chosen for analysis were not always the only ones observed at the passage studied, but they were the most abundant ones at that time. Likewise, the virus plaques picked for analysis probably represented the more abundant of numerous variants present at the passage level. Furthermore, it should be emphasized that the complex mixtures of virus variants and the DI particles present in the undiluted passage populations may have exerted complementation effects, interference properties, and other behavior not necessarily identical to the observed behavior of virus clones and individual DI particles selected for the one-to-one interactions studied. Nevertheless, we repeatedly observed rather consistent patterns of interaction between any chosen DI particle and cloned viruses isolated before, during, and after the passage level at which the DI particle was observed to predominate in the population.

It was observed in every case but one that virus isolated at the same passage already exhibited slight (severalfold) re-

sistance to the DI particle as compared with virus isolated a number of passages earlier. This is not surprising since the predominant DI particle type had had time to exert selective pressures on most variants present in the same population. Furthermore, we observed with every DI particle tested that virus variants isolated at some subsequent passages exhibited significant resistance to the DI particle, and in some cases the resistance was complete to the point that normal plaques were formed in the presence of very high multiplicities of the DI particle. The specificity of this resistance was remarkable because even complete resistance to the “selecting” DI particle did not necessarily confer resistance to DI particles isolated at other passage levels. This suggests that the DI particles being tested had played a significant role in selecting the variants selectively resistant to them, but in these complex, constantly evolving virus and DI particle populations, it might often have been classes of related DI particles which selected a particular Sdi⁻ mutant virus. For example, the DI particles which selected the completely resistant (p287) Sdi⁻ mutant virus (Fig. 2) must have been closely related (or identical) to the DI particle isolated for analysis at p200. In contrast, the relatively lower level of resistance seen in the virus isolated at p120 (Fig. 1) suggests

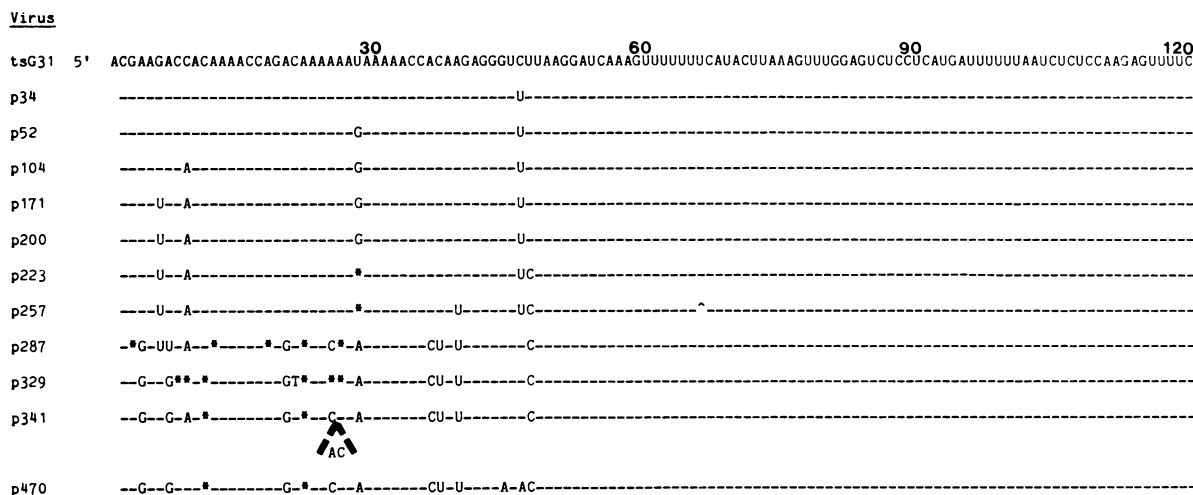


FIG. 8. Comparative sequences of 5' termini of genomic RNA of VSV Sdi⁻ mutants which arose during serial undiluted passages (see Table 1 and Results). The sequences of the 170 terminal bases at the 5' ends of these Sdi⁻ mutants were determined, but only the first 120 bases are shown. The earlier sequences, up to p171, were reported previously (24). Confirmed sequence identities are indicated by dashes. Asterisks indicate sites exhibiting base heterogeneity. The circumsflex at position 67 indicates a U insertion in that area (as shown in Table 4). The terminal base was not obtainable by our sequencing methods. Notice that the p287 virus shows at least seven new mutations plus six heterogeneity changes since p257. Notice also that the p341 virus has an insertion (AC). Because of the nearby mutations, the exact positions of the inserted bases could not be determined, but note that they disappeared by p470 and additional mutations appeared.

TABLE 4. Insertions or deletions at polyadenylation sites

Virus isolate	No. (<i>n</i>) of U's in AUAC(U) _{<i>n</i>} in gene ^a :		
	N	NS	L
VSV-Indiana (San Juan) ^b	7	7	7
<i>tsG31</i>	7	8	7
p171	7	7 (D)	7
p200	7	7	7
p223	7	7	7
p257	7	7	8 (I)
p287	8 (I)	7	7 (D)
p341			7
p470			8 (I)

^a D and I, First appearances of deletions and insertions, respectively.

^b VSV-Indiana (San Juan) data is that determined by Rose (48).

that the tested DI particle (isolated at p104) and closely related DI particles may have played a lesser role in selecting the p120 Sdi⁻ mutant.

Although our sampling of virus and DI particles necessarily allowed examination of only a small fraction of the DI particles and Sdi⁻ variant viruses evolving during these passages, it seems clear that this type of intracellular coevolution can proceed for very long periods of time and probably indefinitely. The unusual rapidity and magnitude of the changes in the resistance phenotype shown in Fig. 3 suggest that the cycles of virus and DI particle coevolution can become greatly accelerated, rather than tending toward equilibrium, after more than 2 years of undiluted serial passages. In view of the occurrence of Sdi⁻ mutants among a wide variety of different virus groups (8, 14, 19, 27, 28, 56), this type of virus-DI particle coevolution may often play a role during acute infections and perhaps even more regularly during certain types of persistent infections.

The sequencing results discussed above show clearly that the polymerase-associated NS protein (5, 11, 13, 22, 25, 36) is not involved in the mutations producing Sdi⁻ phenotypes

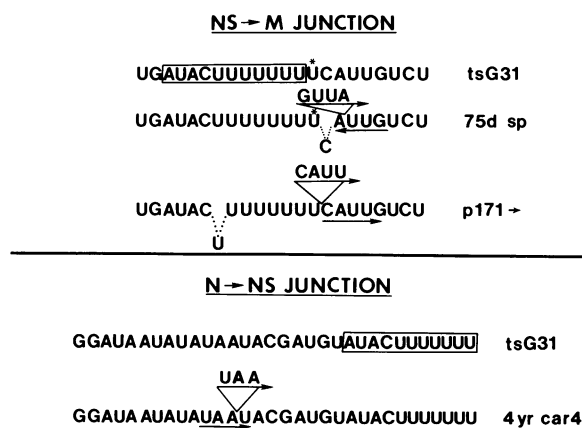


FIG. 9. Small insertions and deletions which appeared in noncoding junction regions of evolving Sdi⁻ viruses during serial undiluted passages and during persistent infection in BHK-21 cells. The boxed sequences at the NS-to-M and N-to-NS junctions delineate the polyadenylation signal proposed by Rose (48). Asterisks indicate the presence of an eight-uridine stretch rather than the expected seven-uridine stretch (48) at the site. Insertions are indicated above the sequence for each mutant, and deletions are indicated below the sequence. Note that two of the three insertions appear to be short direct repeats and the other appears to be a strange pseudo-inverted repeat.

or it is very infrequently involved. The RNA-encapsidating N protein exhibited an accumulation of stable base substitutions, and some of these appeared at passage levels during which profound changes in Sdi⁻ phenotypes also appeared. The VSV 5' termini, and to a lesser extent the 3' termini, have been shown to undergo mutations during persistent infections and undiluted passage series (24, 35, 50, 57). It was shown in this study that base substitutions in the 5' termini continued to accumulate. Strikingly, this extensive sequence alteration was confined to the terminal 47 nucleotides; the region beyond was extremely stable in comparison (Fig. 8). Some of these mutations may facilitate better interactions between the termini and the rapidly mutating replication-encapsidation proteins L and N (1, 7, 17, 39-41). Because viral termini and DI particle termini compete for viral replication-encapsidation proteins, all mutations in viral termini which make a virus more competitive (with the DI particle being tested) should cause a quantitative increase in the Sdi⁻ phenotype. Therefore, it is very likely that many (perhaps all) of the observed mutations in the viral termini contributed to the Sdi⁻ phenotypes observed, and it will be interesting in later sequencing to correlate these extensive mutations with coevolving changes in the L polymerase protein.

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