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Defective Interfering Particles of Parvovirus H-1

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Defective interfering particles of the parvovirus H-1 were produced by serial propagation at high multiplicities of infection. Such particles interfere with the synthesis of capsid proteins and infectious virus of standard H-1. The interference is sensitive to UV irradiation, dependent on the multiplicity of the challenge virus, and is active in heterotypic infections against parvovirus H-3 or LuIII. Defective interfering particle genomes have alterations characterized by integral numbers (1 to 10 or more) of a 60-base-pair addition in the neighborhood of the origin of replicative-form DNA replication and deletions that are located primarily within two regions, 32 to 44 or 80 to 90 on the genome map. Some of the implications of these findings are discussed.

Defective interfering (DI) particles have not been reported previously for parvoviruses, yet they have been described for most viruses where efforts have been made to detect them. Such particles are nonviable mutants that require a helper virus to replicate (review in ref. 7). Defective viruses are interfering if they reduce the production of nondefective wild-type virus during coinfection, by a nontrivial mechanism that operates after the initial adsorption. Many DI particles are deletion mutants of the standard virus (7, 8).

I have prepared DI particles of the parvovirus H-1 by serial propagation of standard H-1 at high multiplicities of infection (MOI) in the presence of additional H-1 helper virus at each passage. The DNA genome of H-1 DI particles are heterogeneous as detected by alterations in their physical maps, and these changes depend in part on the particular helper virus used for their propagation. Interference generated by H-1 DI particles is multiplicity dependent and acts before or during the transcription and/or translation for viral capsid proteins. A similar cross-interference for viral protein synthesis has been described for heterotypic infections with homologous parvoviruses (17). Most of the H-1 DI particles are defective in replicative-form (RF) DNA replication (RF *rep*$^-$) in the absence of helper virus, a property which has been used to demonstrate the existence of a *trans*-acting RF *rep* gene product required for RF DNA replication (S. L. Rhode III, *in* D. Ward and P. Tattersall, ed., *Replication of Mammalian Parvoviruses*, in press).

MATERIALS AND METHODS

Preparation of DI particles. A stock of wt H-1 produced in secondary hamster embryo cultures was used to initiate a series of virus propagations at high MOI in NB cells (simian virus 40-transformed human newborn kidney cells). The first infection was made at an MOI of 150. The virus was harvested at 48 h postinfection (p.i.) and concentrated 10-fold to a final volume of 1.8 ml by adjusting the medium to pH 6.7 with 0.1 N HCl and collecting virus and cell debris by low-speed centrifugation. Virus particles were then liberated from the cells by adjusting the pH to 8.0 with 0.5 N NaOH and subsequently freezing and thawing the sample three times (4). Low-speed centrifugation of this material yielded a supernatant containing concentrated virus. Subsequent propagations were made by infecting 100-mm dishes of 2×10^6 NB cells per dish with 0.2 ml of the concentrate of serially passed virus with additional helper virus at an MOI of 10. Helper virus was added at each propagation to ensure complementation of defective genomes, which otherwise might eliminate their helper virus and themselves by interference of helper virus replication. After 18 serial passages, two infections were made with 0.5 ml of the culture lysate without concentration to increase the volume of the preparation. This preparation was called DI-wt. Two other preparations, called DI-wt-*ts*14 and DI-wt-DI-1, were made by substituting the H-1 mutants *ts*14 and DI-1, respectively, for wt H-1 as the helper virus after passage 14. Preparations of DI particles at passage levels greater than 18 were made without the concentration step. The nomenclature used here for DI preparations records after the DI prefix the names of the helper viruses used in the order of their appearance.

UV irradiation. The DI preparation was placed in a 100-mm petri dish in a volume of 4 ml and irradiated for 15-s intervals, with the lid removed, at 12 inches (ca. 30.5 cm) from a UV source (GE 15-W germicidal lamp).

Interferon. Human interferon was prepared in MRC-5 fibroblasts as described (5). Interferon was assayed by inhibition of H-1 hemagglutinin (HA) production in NB cells. The titers were taken as the reciprocal of the dilution producing one-fourth the HA of the control. Samples that were contaminated with

H-1 were neutralized with H-1-specific antiserum or by irradiation with UV light for 60 s as described above. Treatment of a standard human interferon prepared in MRC-5 fibroblasts with this dosage of UV light did not reduce its titer.

Preparation of radiolabeled DI RF DNA. DI RF DNA was labeled with [^{32}P]orthophosphate, extracted, and prepared for digestion with restriction endonucleases as previously described (16). Generally, 0.5 ml of a DI virus stock was used to infect each 100-mm petri dish of NB cells, and helper virus was added at an MOI of 10.

Physical mapping studies. Digestion with restriction endonucleases and gel electrophoresis of the digests were carried out as noted in a prior publication (16). In this study I will initiate use of a standard terminology for parvovirus physical maps that was proposed at the Cold Spring Harbor Symposium on Parvoviruses, 13 to 15 May 1977 (described in D. Ward and P. Tattersall, ed., *Replication of Mammalian Parvoviruses*, in press, appendix). Briefly, the physical map is calibrated from 0 to 100 with 100 at the 5′ terminus for the virion (minus) strand (molecular right end; 16). Cleavage fragments are denoted by the coordinates of the cleavage sites at their ends (left/right). For example, H-1 *Eco*RI B will be called *Eco*RI 0/21. For easy reference to the previously described physical map of H-1, the terminology by letter is also included here (16).

Gel electrophoresis of nuclear proteins. NB cultures labeled with L-[^{35}S]methionine in 60-mm petri dishes were washed once with Tris-buffered saline and lysed with 2 ml of 25 mM Tris (pH 7.5) with 0.14 M NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.5% Nonidet P-40 at 4°C. The nuclei were collected by centrifugation, lysed, and fractionated in a 10% acrylamide gel with a 5% acrylamide stacking gel as described (12).

RESULTS

Preparation of DI particles. A preparation of H-1 that was produced in a secondary passage of hamster embryo cells and was known to contain at least one variant (16) was used for serial propagation at a high MOI in NB cells as described in Materials and Methods. On passage 20, the DI-wt virus stock titered 4×10^7 PFU/ml, and a control of wt H-1 helper virus without DI virus produced 5×10^8 PFU/ml (Table 1). Two other preparations of DI particles, DI-wt-*ts*14 and DI-wt-DI-1, were made by six serial passages with *ts*14 or DI-1 helper viruses beginning with DI-wt at passage 14. The HA and infectivity titrations of these DI preparations are summarized in Table 1. *ts*14 H-1 was chosen as a helper virus because it is defective in its RF DNA replication, and it was expected that it might enhance the DI particle-to-helper virus ratio of the stock produced (17). DI-1 is a viable defective virus that produced very small plaques, which was isolated from the DI-wt preparation (see below).

The DI particles both inhibit HA synthesis and reduce infectious virus production of their helper viruses 1 to 2 logs compared to control preparations. DI-wt-*ts*14 was the most potent interfering preparation of the three described in this report.

Properties of the interference. Interference by competition among particles for a limited number of virus receptors at the cell surface is considered trivial and should be ruled out before characterizing particles as DI particles. One method of doing this is to show that interference occurs even when the standard virus is adsorbed before the DI virus. The results of an experiment in which cultures were inoculated with DI-wt-*ts*14 at various times before or after infection with wt H-1 are shown in Table 2. DI-wt-*ts*14 interfered with both HA synthesis and PFU production even when applied 4 h after wt H-1. Therefore, competition at the cell surface for virus receptors is not the mechanism of interference. The magnitude of the interference with HA synthesis by DI-wt-*ts*14 was reduced to

TABLE 1. *Infectivity and HA of DI virus preparations and their helper virus without DI virus*[a]

Prepn	DI		Helper virus	
	PFU/ml	Log_2 HA	PFU/ml	Log_2 HA
DI-wt	4×10^7	16	5×10^8	19
DI-wt-*ts*14	1×10^6	12	9×10^7	20
DI-wt-DI-1	9×10^6	19	6×10^7	22

[a] Infectivity and HA titer of various DI preparations and their helper viruses propagated without the DI were determined. DI-wt-*ts*14 and *ts*14 plaque assays were performed at 33°C, but the serial passages were done at 37°C.

TABLE 2. *Interference by DI-wt-ts14 as a function of time of infection*[a]

Relative time of infection with DI-wt-*ts*14 (h)	Log_2 HA (% inhibition)	PFU/ml (% inhibition)
No wt H-1	13 (—)	$10^{1.8}$ (—)
−4	15 (94)	$10^{6.9}$ (93)
−2	14 (97)	$10^{6.8}$ (95)
0	14 (97)	$10^{6.6}$ (96)
+2	15 (94)	$10^{7.0}$ (92)
+4	15 (94)	$10^{7.1}$ (90)
No DI-wt-*ts*14	19 (0)	$10^{8.1}$ (0)

[a] Parasynchronous cultures of NB cells were infected at the completion of the 16-h methotrexate treatment ($t = 0$) with wt H-1 at an MOI of 3. DI-wt-*ts*14 (0.2 ml) was added at various times from 4 h before to 4 h after wt H-1; DI-wt-*ts*14 at −4 or −2 h contained methotrexate. The cultures were collected at 54 h p.i. for titration of HA, and infectivity was determined by plaque assay at 37°C.

50% by increasing the multiplicity of the wt H-1 to 50 per cell. This implies that the interference resulted from some type of competitive interaction of DI particles and wt H-1.

Another standard test for true interference is to show that the interference is sensitive to inactivation of the DI particle nucleic acid by UV irradiation (Table 3). It is apparent that DI-wt-*ts*14 interference was very sensitive to UV irradiation, and a dose that reduced DI-wt-*ts*14 infectivity (PFU per milliliter) to a surviving fraction of about 10^{-3} eliminated any detectable interference phenomena. Parvoviruses have been shown to be sensitive to interferon, although they are not efficient inducers of interferon production (10). It is possible that DI particles induce interferon and thereby inhibit the protein synthesis of the standard virus. This would imply that the interferon in DI preparations is sensitive to UV irradiation at the dose levels used here. This was tested by preparing human interferon in MRC-5 fibroblasts by induction with poly(I)·poly(C) (5) and exposing it to UV irradiation. The standard human interferon had a titer of 5×10^3 U/ml against H-1, and, after 60 s of UV, four times the dose that eliminated DI-wt-*ts*14 interference, there was no reduction in titer. Similarly, medium of NB cultures infected with DI-wt-*ts*14 at 6 and 12 h p.i. had no detectable interferon when assayed with H-1. If interferon were induced by DI particles in the same infectious cycle as the interference they cause, it would be present at 6 or 12 h p.i., which is just prior to or during H-1 HA synthesis (Rhode, *in* D. Ward and P. Tattersall, ed., *Replication of Mammalian Parvoviruses,* in press). Thus the interference produced by H-1 DI preparations is not due to interferon.

TABLE 3. *Inactivation of DI-wt-ts14 interference by UV irradiation*[a]

Virus	PFU/ml (% inhibition)	Log_2 HA (% inhibition)
wt H-1	1×10^8 (0)	18 (0)
wt H-1 + DI-wt-*ts*14 (0 UV)	3×10^7 (73)	15 (88)
wt H-1 + DI-wt-*ts*14 (15-s UV)	1×10^8 (0)	18 (0)
wt H-1 + DI-wt-*ts*14 (30-s UV)	1×10^8 (0)	18 (0)

[a] Parasynchronous NB cultures were infected with 0.2 ml/petri dish of DI-wt-*ts*14 with the following titers: 1×10^6 PFU/ml (0 UV); 1.6×10^3 PFU/ml (15-s UV); <25 PFU/ml (30-s UV); or mock infected. After 2 h of incubation, the cultures were infected with wt H-1 at an MOI of 3 and incubated at 37°C for 48 h. The yields of infectious virus were titrated, and hemagglutination was determined by the usual methods.

A multiplicity-dependent cross-interference between H-1 and parvovirus H-3 or LuIII for viral protein synthesis has been described (17). Therefore, I tested DI-wt-*ts*14 for heterotypic interference for HA synthesis in cultures infected with H-3 or LuIII (Table 4). It was not necessary in this experiment to test the HA of each virus produced in the mixed infection by neutralization assays, because DI-wt-*ts*14 reduced the gross yield of HA in comparison to the controls and therefore it must have reduced the H-3 or LuIII HA yields. This finding indicates that H-1 DI particles interfere with HA synthesis of H-3 or LuIII, as previously found for wt H-1 or *ts*14 H-1 (17).

H-1 capsids consist of two types of polypeptides, VP1 and VP2′ (or VP2) (11). One or both of these are the HA, the synthesis of which is reduced by coinfection with DI particles. An experiment was done to determine whether both VP1 and VP2′ synthesis are reduced during infection with DI particles. NB cultures infected with wt H-1, *ts*14, or DI-wt, or just mock infected, were labeled with L-[^{32}S]methionine, and the nuclear proteins were analyzed by gel electrophoresis (Fig. 1). A small amount of VP2′ was produced by DI-wt, but the DI-wt preparation was clearly defective in the synthesis of polypeptides the size of VP1 and VP2′. No accumulation of cytoplasmic antigens was detected by immunofluorescent staining, as described below. It has not been determined whether the DI particles are defective in transcription of viral mRNA or in its translation.

Monolayers of NB cells infected with wt H-1 plus DI-wt or wt H-1 alone at the same MOI were compared by immunofluorescent staining. The percentage of antigen-positive nuclei was reduced from 94% for wt to 51% for wt plus DI-wt. Many of the cells judged positive in the DI-wt-infected culture stained less intensely than the control. The HA titer produced by DI-wt

TABLE 4. *Nonhomologous interference by DI-wt-ts14*[a]

Virus	Log_2 HA (48 h p.i.)	% Inhibition
H-3	19	
H-3 + DI-wt-*ts*14	15	94
LuIII	19	
LuIII + DI-wt-*ts*14	15	94

[a] Asynchronous NB cultures were infected with H-3 or LuIII at an MOI of 3 with and without 0.2 ml of DI-wt-*ts*14 (10^6 PFU/ml). Cultures were harvested for HA titration at 1 or 49 h p.i. Values shown are log_2 HA and percentage of inhibition of HA synthesis. The HA titers at 1 h p.i. were 5 or less.

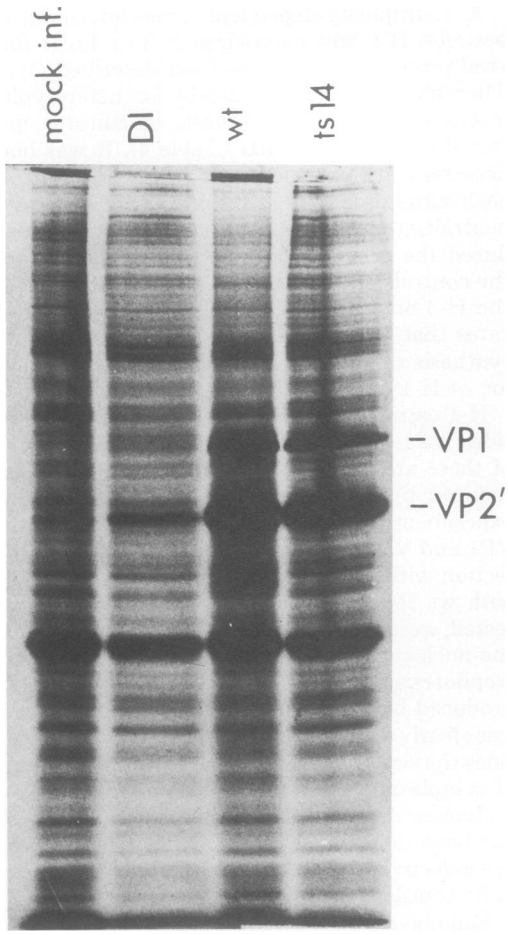

FIG. 1. *Polyacrylamide gel electrophoresis of nuclear proteins of cells mock infected or infected with wt, DI-wt, or ts14 H-1. Parasynchronous NB cultures were mock infected or infected with wt, DI-wt, or ts14 H-1 at an MOI of 5 to 10. The cultures were incubated in medium containing L-[^{35}S]methionine (20 μCi/ml; specific activity, 400 Ci/mmol) and minimal essential medium plus amino acids without methionine from 12 to 16 h p.i. Nuclei were prepared, and total nuclear lysates were subjected to electrophoresis in a slab gel of 10% acrylamide. The figure represents an autoradiogram of the dried gel. The positions of the H-1 polypeptides VP1 and VP2' were determined by including a lysed sample of purified H-1 in an adjacent lane.*

was only 1% (2^{-7}) of that produced by the wt virus. Thus the number of cells producing antigen and the amount of antigen per cell were reduced by DI particles. The fate of cells infected with DI particles in which wt H-1 is not present or its functions are not expressed is not known with certainty. It is clear that the interference delays or prevents the development of cytopathological changes in infected cultures. When different amounts of DI-wt-*ts*14 were added to cultures receiving a constant challenge dose of *ts*14, the cultures infected with the higher concentrations of DI-wt-*ts*14 showed a reduction in cell death as defined by uptake of the vital stain, neutral red. The long-term fate of cultures infected with DI particles and subsequently protected from infection with the helper virus is under study.

Physical mapping studies of DI RF DNA. The RF DNAs of DI particles were surveyed for large changes in their genomes by examining the fragments produced by digestion with restriction endonucleases. DI-wt, DI-wt-*ts*14, and DI-DI-1 RF DNA labeled in vivo with [^{32}P]orthophosphate was produced in NB cells and digested with *Eco*RI, *Hin*dIII, *Hae* II, or *Hpa* II (not shown). The cleavage fragments were the same as those from wt H-1 DNA (16).

The electropherogram of the digestion products of DI-wt and wt RF DNA to *Hin*dII and *Hae* III is shown in Fig. 2. It is evident that the DI-wt DNA is heterogeneous. The *Hin*dII right-end fragments, *Hin*dII 91.3/95.7 (D), 95.7/99.9 (E), and 95.7/99.9* (F), appeared to be the same for DI-wt and wt. There was considerably less *Hin*dII 83.5/91.3 (C) in the DI-wt preparation, and, in addition, there were many discrete fragments present in DI-wt that were not found in standard virus DNA. The digest with *Hae* III revealed that the DI-wt preparation contained very little wt DNA (helper virus), since the *Hae* III 83.6/100 (B), 36.6/46.3 (D), and 77.2/83.6 (F) fragments were markedly reduced or absent (Fig. 2). The *Hae* III 0/31.9 (A) fragment was present but heterogeneous, whereas the *Hae* III 46.3/59.5 (C), 63.9/74.5 (E), 32.6/36.6 (G), and 59.5/63.2 (H) fragments were relatively well conserved. The map location of the DI-wt *Hae* III fragment migrating between *Hae* III 46.3/59.5 (C) and 63.9/74.5 (E) is unknown.

The genomes of DI particles apparently contain many alterations in sequences characterized by conservation of standard virus sequences in some regions, such as in the area of the left end 0 to 20% (*Hin*dII, *Eco*RI, or *Hae* II) and at the extreme right end from 95 to 100% (*Hin*dII), with variations in other regions. The nature of some of these changes will be considered in the following sections.

Additions near the replication origin. The pattern of *Hin*dII fragments in DI-wt digests suggested that the *Hin*dII cleavage site at 95.7 on the map between D and E is conserved, while the site at 91.3 between C and D varies in position. A clearly established genome change that occurs in the DI population is a 60-base-pair (bp) addition in *Hin*dII 91.3/95.7 (D), which produces the *Hin*dII D' fragment of the viable

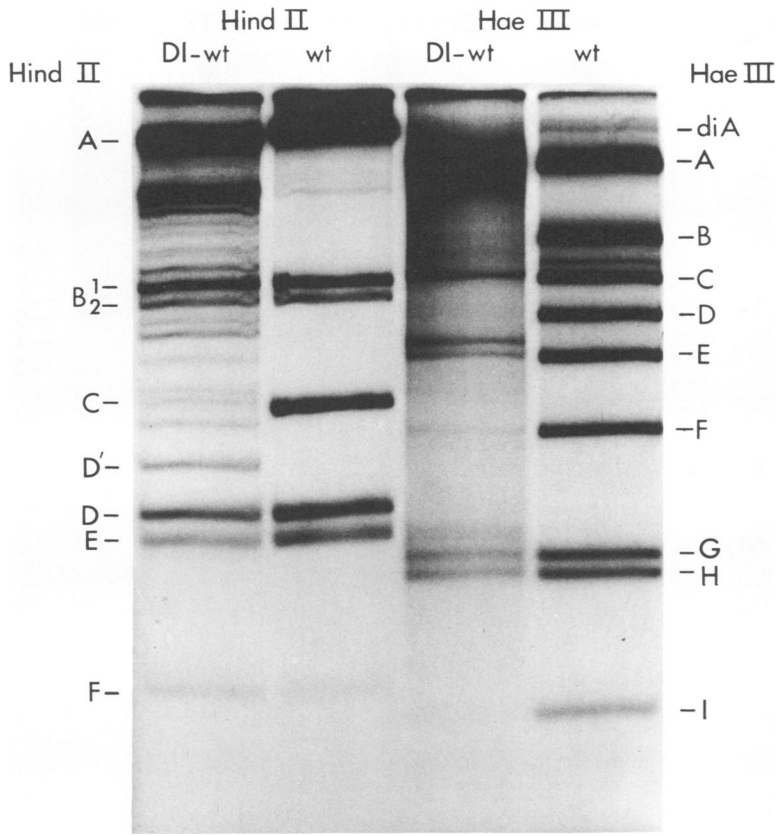

FIG. 2. *Electropherogram of HindII or Hae III digests of wt or DI-wt RF DNA. The ^{32}P-labeled RF DNA of wt H-1 or DI-wt at passage 20 was digested to completion with HindII or Hae III. The digests were fractionated in a 3% acrylamide-0.5% agarose gel for 560 V·h and dried, and an autoradiogram was made. Hae III fragments J, K, and L were lost from this gel.*

defective virus DI-1 (17). Many of the *Hin*dII fragments of DI-wt or DI-wt-*ts*14 appeared to differ in size by about 60 bp. It is possible that the 60-bp addition between 91.3 and 95.7 may be a reiteration that tends to be tandemly repeated, with the overall genome size reduced by compensating deletions. In preliminary experiments it was determined that the restriction endonuclease *Hpa* I, which cleaves at GTTAAC, one of the four sequences cleaved by *Hin*dII (3, 9), cleaves H-1 at a single *Hin*dII cleavage site at 91.3. When DI-wt at passage 30 was digested with *Hpa* I, the standard *Hpa* I 91.3/100 fragment was produced, along with a series of fragments representing additions by integral multiples (n) of 60 bp (Fig. 3, lanes 1 and 2). Some additional new fragments were found at the position for fragments of about 1,200 to 1,400 bp. RF DNA of H-1 DI-wt particles was prepared from virus at passage levels 5 and 10 and also digested with *Hpa* I (data not shown). The same type of 60-bp addition as described above was present as early as passage 5, except that the smaller ones with $n = 1, 2, 3$ were predominant. It occurred to me that such a specific mechanism of generating DI DNA would be likely to produce similar changes in the DI DNA for the parvovirus H-3, which has a high degree of homology to H-1 (17). Therefore a preparation of H-3 DI virus was prepared by 10 serial propagations as for H-1. The *Hpa* I digest of H-3 DI produced the standard *Hpa* I 92.4/100 (B) fragment and a series of fragments representing additions by integral multiples (n) of 60 bp as for H-1 (Fig. 4). The first addition in this series would produce a *Hin*dII cleavage pattern much like H-1, since the H-3 *Hin*dII 92.4/95.7 (E) fragment is increased by 60 bp to equal in size the H-1 *Hin*dII 91.4/95.7 (D) fragment. Thus both H-1 and H-3 appear to generate very similar additions at the right end of the DI genome.

Deletions in the left half of the genome. The RF DNA of DI genomes are the same size as wt DNA or smaller by as much as 10%, based

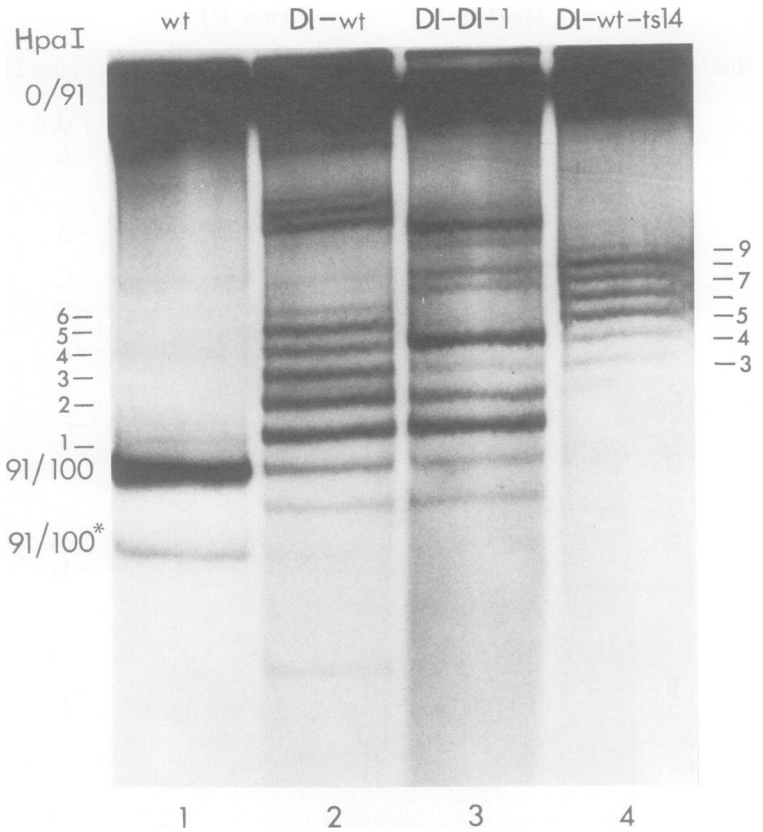

FIG. 3. *Electropherogram of Hpa I digests of wt H-1, DI-wt, DI-DI-1, and DI-wt-ts14 RF DNA. The ^{32}P-labeled RF DNA of wt H-1, DI-wt (passage 30), DI-DI-1 (passage 10), and DI-wt-ts14 (passage 30) were prepared as previously described (13). The Hpa I digests were subjected to electrophoresis in a slab gel of 3% acrylamide-0.5% agarose for 720 V·h and dried, and an autoradiogram was made. The Hpa I 91.3/100 fragment is the right-end fragment, and Hpa I 91.3/100* is the same fragment with the inverted self-complementary sequence in the foldback configuration (16). The foldback fragments of the Hpa I DI fragments with additions are coincident in mobility with the fragments in the series that are smaller by two additions.*

on their electrophoretic mobility in agarose gels. Because the additions at the right end of the molecule are accompanied by deletions in both the left and right halves of the molecule, the net result of these alterations is little change in the total RF size. The deletions in the left half are found between 21 and 51 on the genome map, as illustrated in an electropherogram of a sequential *Hin*dIII (cleaves at 51) and *Eco*RI (cleaves at 21) digest (Fig. 5). The DI-wt-*ts*14 genomes, which have larger additions than DI-wt, also have larger left-side deletions. The DI-DI-1 DNA was not cleaved well by *Hin*dIII. Perhaps this occurred because the deletions in DI-DI-1 might include the *Hin*dIII cleavage site, though this was not confirmed. The location of these deletions was determined more precisely by digesting DI DNA with *Hha* I (Fig. 6). The map positions of the *Hha* I fragments on the H-1 genome have been determined by analyzing double enzyme digestions and incomplete digestions, as previously described for *Hae* III (unpublished data). The *Hha* I 20.5/38.0 (930 bp) and 38.0/52.9 (790 bp) fragments are markedly decreased from the DI DNA digests, with new fragments appearing at about 1,400 bp. Thus the majority of these deletions include a deletion of the *Hha* I cleavage site at position 38, which divides these two *Hha* I fragments. The deletions are heterogeneous, since the bands of the new fragments are not all sharply defined (Fig. 5 and 6), but their location is highly restricted to the region 32 to 44 on the genome map.

The deletions in the right half of the molecule occur just to the left of the *Hpa* I cleavage site (11) and manifest themselves (i) by the fact that the right side (*Hin*dIII 51/100) does not increase in size by the amount expected on the basis of the additions (Fig. 5) and (ii) by the loss of fragments such as *Hin*dII 83.0/91.3 (C) or *Hae*

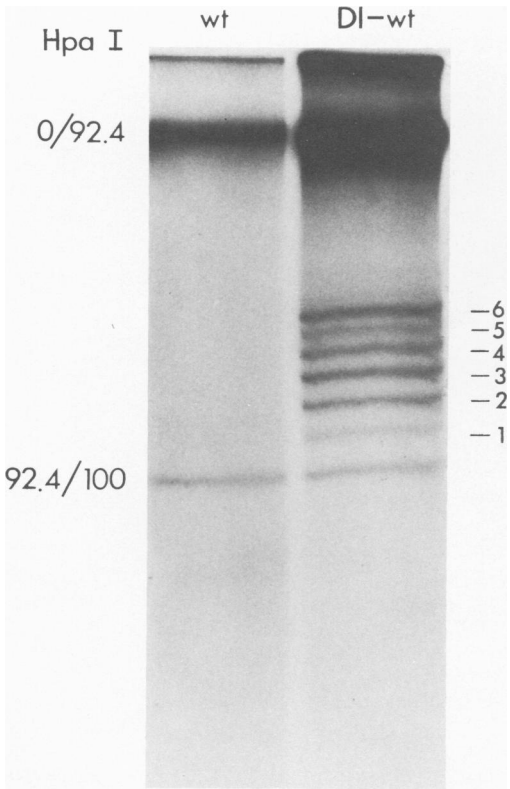

FIG. 4. *Electropherogram of an Hpa I digest of wt H-3 and DI-wt H-3 RF DNA. RF DNA labeled with [^{32}P]orthophosphate was prepared from NB cells infected with H-3 or DI-wt H-3 at passage level 10. The RF DNA of each was digested to completion and analyzed by electrophoresis in 3% acrylamide-0.5% agarose for 540 V·h. The series of Hpa I fragments from the DI-wt H-3 DNA differ in size by about 60 bp, based on the mobility of H-3 HindII marker fragments subjected to electrophoresis in this same gel (not shown).*

III 77.2/83.6 (F) (Fig. 2). Until a number of the DI particles are cloned and mapped, it will not be known what combinations of additions and deletions occur in a given molecule.

Sequence alterations determined by the helper virus. A comparison of the *Hpa* I fragments of DI-wt and DI-wt-*ts*14 at passage 20 (not shown) and at passage 30 revealed a significant difference (Fig. 3, lanes 2 and 4). DI-wt-*ts*14 RF DNA yielded little *Hpa* I 91.3/100 even though its helper virus *ts*14 contains a normal *Hpa* I 91.3/100 fragment (17). This change was associated with an increase in the recovery of fragments representing $n = 5, \cdots, 8$ of the 60-bp additions. Because *ts*14 was a competent helper virus for the RF DNA replication of RF DNA with 0 or 1 addition, the factors that selected against D particles with this type of DNA did not appear to be acting during RF DNA replication (Rhode, *in* D. Ward and P. Tattersall, ed., *Replication of Mammalian Parvoviruses*, in press). The additional constraints imposed on DI particle production are that progeny DNA synthesis and encapsidation must be directed by helper virus capsid protein. Therefore, it is possible that a mutant *ts*14 capsid protein selected against viral DNA that had $n = 0, \cdots, 4$ 60-bp additions. This was tested by comparing the *Hin*dII cleavage patterns of DI-wt after a single passage with *ts*14 with those of DI-wt propagated with wt helper virus. The distribution of fragments from DI-wt was not affected appreciably by a single passage with *ts*14 (data not shown). Therefore the stage of replication in which *ts*14 selected a different population of DI particles was not determined by this analysis of a single cycle of propagation.

The genome changes in DI particles characterized by additions were found to be in transition, because DI-wt-*ts*14 at passage 30 produced RF DNA with $n = 5, \cdots, 8$ 60-bp additions as the most prominent forms, whereas at passage 20, $n = 3, 4, 5$ were predominant (Fig. 3 and data not shown). Similarly, DI-wt had additions of $n = 1, \cdots, 5$ at passage 30 and at passage 50, $n = 3, \cdots, 6$ were predominant. It appears that with continued serial propagation the number of additions gradually increases, and the use of *ts*14 as the helper virus tends to produce a population of DI genomes with a higher average number of additions than that obtained with wt H-1 as a helper virus.

A DI preparation (DI-DI-1) was also made with DI-1 as the only helper virus by 10 serial passages of DI-1. The DI-DI-1 DNA has *Hpa* I cleavage fragments that are similar to DI-wt except that the relative proportions of each type differ (Fig. 3, lane 3). Whether the relative amount of the various types of altered genomes is subject to chance variations or is strictly determined by the helper virus is not known.

DISCUSSION

DI particles of the parvovirus H-1 were produced during serial high-multiplicity propagation of wt H-1 or certain H-1 mutants. Many of these DI particles produce replicative form DNA, which requires the RF *rep* gene product of a helper virus for its replication (Rhode, *in* D. Ward and P. Tattersall, ed., *Replication of Mammalian Parvoviruses*, in press). They also appear to be defective in synthesis of the capsid polypeptides VP1 and VP2′, and therefore require complementation with a helper virus for progeny DNA synthesis and encapsidation as well. The DI particles reduce the yield of HA

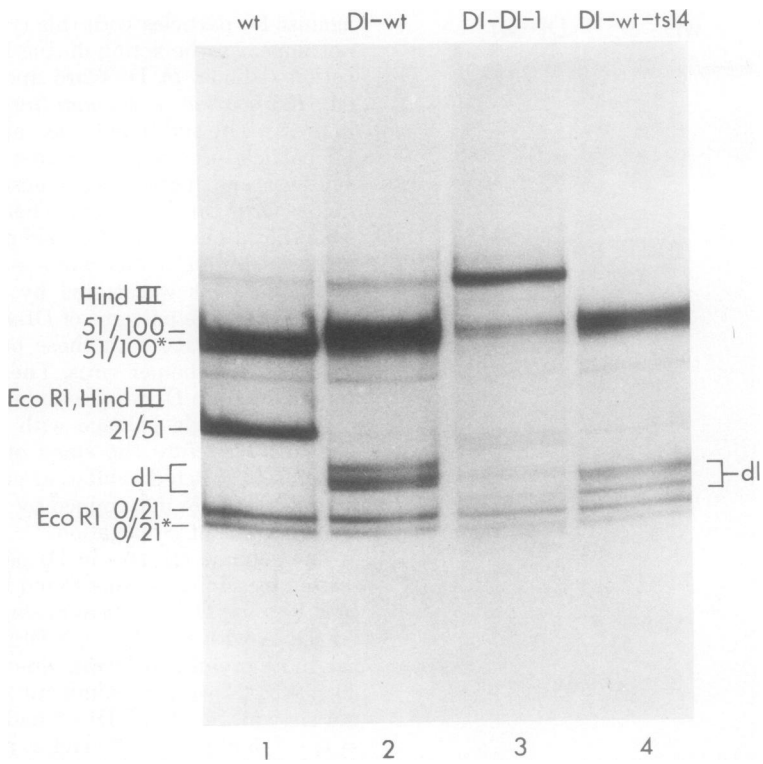

FIG. 5. *Electropherogram of a HindIII and EcoRI sequential digestion of the RF DNA of wt, DI-wt, DI-DI-1, and DI-wt-ts14. The ^{32}P-labeled RF DNA preparations described in Fig. 4 were digested with HindIII followed by EcoRI and analyzed by electrophoresis in a 1.8% agarose gel until the bromophenol blue dye reached the bottom. The autoradiogram of the dried gel is shown. The deletion fragments of the DI particle RF DNA are labeled dl. Some of the fragments with deletions appear to be homogeneous, since the bands are sharply defined. The variations in size due to the variable number of additions and deletions in the right half of the molecule caused the HindIII 51/100 fragments of the DI-wt and DI-wt-ts14 preparations to produce fuzzy, ill-defined bands with a slightly slower mobility than wt HindII 51/100.*

and infective virus production of standard wt H-1 in mixed infections. This interference manifests itself after adsorption to the plasma membrane receptors and before translation of the mRNA for HA or VP1 and VP2′ polypeptides. The interference is expressed against two other parvoviruses, H-3 and LuIII, in heterotypic infections and is reduced by increasing the multiplicity of the standard virus. Thus the interference by DI particles is similar to the interference between wt H-1 and its mutant, *ts*14, and H-3 or LuIII in heterotypic infections (17). It is likely that the nucleic acid of the DI particle is the active agent, since the interference is very sensitive to inactivation by UV irradiation. There was no evidence for an induction of interferon by the DI particles. Our results suggest a mechanism for this interference by which the DI particle genomes, defective for viral protein synthesis, act as competitors with standard virus for some rate-limiting requirement for transcription (or possibly translation) of viral mRNA. This interference may be analogous to superinfection exclusion described for some bacteriophages (6).

The RF DNAs of DI particles were used for characterization of their genomes by physical mapping techniques. It was found that the DI RF DNAs were slightly heterogeneous in electrophoretic mobility in agarose gels. If the mobility differences reflect only changes in length, then the faster-migrating species were about 10% smaller than wt H-1, and any that migrated more slowly (e.g., DI-1) differed in size by only very small amounts (1 to 2%). Analysis of the fragments produced by infection with DI preparations and helper virus at an MOI of 5 to 10 was predominantly DI-particle DNA. For example, digestion of a DI-wt preparation with *Hae* III produced little or no *Hae* III 83.6/100 (B) or 36.6/46.3 (D) fragments.

The majority of the changes in the DI genomes were caused by additions in the region

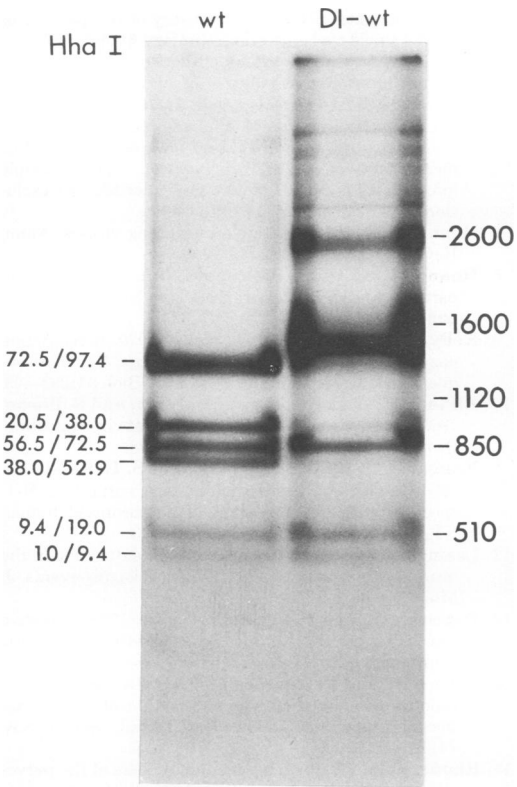

FIG. 6. *Electropherogram of Hha I digests of the RF DNA of wt and DI-wt (passage 30). Electrophoresis was carried out in a slab gel of 1.8% agarose for 400 V·h. The determination of the physical map of H-1 for Hha I will be described elsewhere. Illustrated here are the loss of fragments Hha I 20.5/38.0 and 38.0/52.9 in the DI-wt preparation and diffuse new bands at mobilities equivalent to 1,400 and 2,500 bp. The Hha I fragments and HindIII plus EcoRI fragments (not shown) were used for size markers, and values noted are in base pairs.*

91–95.7 (*Hin*dII D) with deletions in the area 80–91 and/or in 32–44. The DNA of the extreme right end with its inverted self-complementary sequences (95.7–100) and the left end (0–21) appeared to be highly conserved. It is likely that some form of nonreciprocal recombination generates the altered genomes that emerge in the DI particle preparations. For example, the deletions could be generated by intramolecular recombination at two homologous regions between 32 and 44 (14). It is not known whether the conservation of overall length is the result of specificity in the mechanism producing the aberrant genomes or of constraints imposed on DNA size by the encapsidation process.

The additions occurring at the right end in the neighborhood of the replication origin (18, 19) appear to be generated by a very specific mechanism. DI preparations digested with *Hpa* I, which cleaves standard DNA once at 91.3, exhibit a series of fragments with incremental increases in size of about 60 bp. This series was observed to go as high as 10 additions if each step is in fact an increment of 60 bp. The analyses of DI genomes for other viruses have shown that they may have tandem repeats of the origin of replication (1, 2). Some *cis*-acting sequences in the DI particle DNA seem to confer a selective advantage on these DNAs, since they increase at the expense of standard viral DNA under these conditions of propagation. Tandem repeats of the origin of replication could account for such an advantage. Thus the additions observed here with H-1 DI DNA are compatible with this explanation, but it will require further study to prove this. It is interesting that the parvovirus H-3, which is about 96% homologous to H-1 (17), has a *Hin*dII fragment in this region of the genome that is 60 bp smaller than that of H-1. This suggests that wt H-1 may have one more tandem repeat of this 60-bp sequence than wt H-3.

This addition event apparently is not rare, since it was found in two independently cloned viable H-1 viruses. One is DI-1, which is a small plaque-forming mutant isolated from the DI-wt virus stock. The other isolate is a revertant of the mutant *ts*14, which is RF *rep*$^+$ and only slightly temperature sensitive for plaque formation (17; unpublished data). Evidence for this addition was also observed in *Hin*dII digests of RF DNA produced by standard virus (16), even after three serial plaque purifications. Accompanying these additions are deletions just to the left of the *Hpa* I or *Hin*dII cleavage site at 91.3. These cause a disappearance of the *Hin*dII 83.5/91.3 (C) fragment and prevent the right side of the molecule (*Hin*dII 51/100) from increasing in size in proportion to the size of the additions.

Deletions (up to 450 bp) were found in the left half of the molecule between 32 and 44 on the map. Because there appeared to be no variation in the left-end fragments produced by *Hin*dII, *Hae* II, or *Eco* RI, the region from 0 to 21 was conserved.

A more detailed analysis of the genomic changes in DI particles will require cloning of various DI particles so that the ambiguities of mixed populations can be avoided. I have partially purified one deletion mutant with a 320-bp deletion at 38 that does not have an addition between 91.3 and 95.7. Thus deletions in the left half are not necessarily coupled to the additions in the right half.

Variations in the type of helper virus used to propagate the DI particles resulted in some dif-

ferences in the DI genomes produced. Although nearly all of the DI particles had the additions at the right end and deletions between 32 and 44 and immediately to the left of 91.3, they varied in the relative amounts of each type, depending on the helper virus. With *ts*14 as helper virus the DI particles had $n = 5, \cdots, 8$ as the most abundant number of 60-bp additions, and with wt as helper the most common types were $n = 1, \cdots, 5$. Also DI-wt-*ts*14 had larger deletions (by about 100 bp) in the left half of the genome than were found in DI-wt at the same passage level. Whether these two differences are coupled, and which is the primary change, are unknown. It is tempting to speculate that the *trans*-acting inhibition of RF replication shown by *ts*14 (17) selects for high numbers of tandem repeats at the origin in the DI population.

DI particles play a role in persistent infections with some viruses (13), and their possible significance in the persistent infection of hamsters surviving neonatal infection with H-1 is under study (20). Further analysis of the DI particles of H-1 should help to identify the *cis*-acting sequences required for H-1 DNA replication and aid in the mapping of the structural genes for H-1 proteins.

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