Complementation for Replicative Form DNA Replication of a Deletion Mutant of H-1 by Various Parvoviruses

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Received 7 December 1981/Accepted 8 February 1982

A defective interfering genome of the parvovirus H-1, called *dl1*, has been isolated and characterized. The only alteration in *dl1* that has been detected is a 300-base-pair deletion at map position 38. Replicative form DNA replication of *dl1* requires the RF *rep* function of a helper virus and the parvoviruses H-1, H-3, MVM, or MVM(i), but not LuIII complement *dl1* for replicative form DNA replication.

Defective interfering (DI) genomes of the parvovirus H-1 were found to be heterogeneous genomes modestly deleted by up to 10 to 12% and to contain 58-base-pair (bp) tandem repeats originating in the map region 91 to 95 (5, 8). DI particles required a helper virus to replicate replicative form (RF) DNA (6). In this study I have explored this problem further by using a cloned deletion mutant of H-1, *dl1*, that has the advantage of being a deleted DI genome that appears to be homogeneous in size and lacks any additions at map positions 91 to 95. The deletion, which is approximately 300 bp, allows the quantitation of *dl1* RF DNA replication in mixed infections with various helper viruses because *dl1* RF DNA can be separated from standard length genomes by agarose gel electrophoresis. *dl1* was originally detected as a deleted genome in a stock of virus generated from a single plaque of an H-1 *ts14* revertant at the restrictive temperature 39.5°C. *ts14* is an H-1 mutant defective in RF DNA replication (7). A number of spontaneous revertants of *ts14* with normal rates of RF DNA replication were isolated by plaque assay at the restrictive temperature after three serial passages at 39.5°C. Examination of the RF DNA replication of these revertants by agarose gel electrophoresis revealed that one of them contained *dl1* as well as a standard length genome. Continued serial passage of this virus stock was carried out by inoculating 100-mm petri dishes of NB cells that were tipped up at about 30° with 0.2 ml of undiluted virus suspension and adsorbed for 0.5 h. The dishes were returned to the horizontal position, 10 ml of medium was added, and the dishes were incubated at 37°C until the cytopathic effect was complete. At the second passage the cell lysate contained only 4×10^5 PFU/ml. The RF DNA produced by this stock was almost entirely *dl1*, and the yield of *dl1* RF DNA was increased by the addition of wild-type (wt) H-1 helper virus (data not shown).

To test whether *dl1* was defective, the *dl1* stock was serially passaged two times, with a multiplicity of infection (MOI) of 0.01 PFU per cell at each passage. The presence of *dl1* in the second passage stock was then assayed by generating RF DNA and analyzing it by agarose gel electrophoresis. Surprisingly, this preparation was almost entirely *dl1*. Because *dl1* might have been in large excess in the initial stock this experiment was repeated, except that the serial passage was repeated three times at an MOI of 0.001 at each passage. Examination of the RF DNA produced by each passage showed only wt RF DNA in the second and third passages. This indicated that *dl1* was probably defective and required a helper virus. As an additional test for defectiveness, plaques were generated from a *dl1* stock, and 20 well-isolated plaques were picked, propagated, and then their RF DNAs were characterized by gel electrophoresis. In 15 of 20 clones, *dl1* RF DNA was present in various proportions with helper virus DNA, and the remaining 5 were standard length RF DNA. Because H-1 *dl1* was in excess, yet no plaque isolates were *dl1* only, *dl1* probably does not form plaques and thus is defective. The virus stock produced by one of these plaques, which generated mostly deleted RF DNA, was used for the remaining experiments in this study.

The DI particles of H-1 generated by serial high multiplicity passage expressed their interference by inhibition of wt virion protein synthesis, which was easily monitored by a hemagglutination assay. *dl1* was similarly tested, and when NB cells were infected with wt H-1 at an MOI of 3 in the presence or absence of the *dl1* virus stock (MOI of 0.6 PFU per cell), the hemagglutination assays at 48 h postinfection were 2^{17} and 2^{24} U/ml, respectively. Thus, coinfection with *dl1* reduced the hemagglutination assay yield of wt virus by 2^{-7} U/ml. In conjunction with this, polyacrylamide gel electrophoresis of the pro-

teins synthesized during infection with *dl1* revealed only a decrease in yield of viral proteins VP1 and VP2, with no evidence for truncated viral proteins.

The location of the deletion in *dl1* was examined by restriction enzyme digestion of *dl1* RF DNA. Restriction of *dl1* RF DNA with *Hind*III (cleaves wt H-1 at map position 51) and *Eco*RI (cleaves at map position 21) indicated that the deletion mapped between map units 21 and 51. Map units are on a scale of 0 to 100, with 0 at the 3' terminus of the virion strand. RF DNAs of wt H-1, *dl1*, and DI-wt passage 30, labeled with [³²P]orthophosphate, were prepared and digested with *Hha*I (5). The restriction map of wt H-1 to the restriction endonuclease *Hha*I is described elsewhere (8). The digests were analyzed by electrophoresis in a 1.8% agarose gel

FIG. 1. Electropherogram of *Hha*I digests of the RF DNA of wt, DI-wt (passage 30) and *dl1*. Electrophoresis was carried out in a slab gel of 1.8% agarose at 400 V/h. Portions of this experiment were previously reported (5). The *Hha*I digests for DI-wt and *dl1* are not complete, accounting for the high-molecular-weight fragments. The numbers in the left margin refer to the map positions of the fragments; 0 is at the 3'-terminus of the virion strand, and 100 is at the 5'-terminus.

FIG. 2. Fluorogram of agarose gel electrophoresis of *dl1* RF DNA at various MOIs of helper virus. Replicate NB cell cultures were infected with *dl1* (endogenous H-1 helper virus at an MOI of 1) and with various amounts of exogenous H-1 as in Table 1. The MOIs of helper virus shown are: (1) 1, (2) 10, (3) 30, (4) 300; and, in lanes 5 to 7, cultures received helper virus only at MOIs of: (5) 10, (6) 30, and (7) 100. Electrophoresis was carried out in a 1.8% agarose gel at 100 V for 16 h. Fluorography was as described in (1). The *dl1* RF DNA has a relative mobility of 1.05 compared with that of H-1 RF DNA.

(Fig. 1). *dl1* RF DNA was missing the *Hha*I 20.5/38.0 (930 bp) and 38.0/52.9 (790 bp) fragments as previously reported for DI-wt (5). *dl1* has a new fragment, with a mobility of 1,400 bp, which indicates that the deletion of 300 bp encompasses the *Hha*I site at 38.0. Examination of the remaining *Hha*I fragments of *dl1* in acrylamide gels showed them to be the same size as those of wt H-1 (data not shown).

The effect of helper viruses at various MOIs on the RF DNA replication of *dl1* was tested. NB cells were infected with a constant amount of *dl1* and coinfectd with wt H-1 at MOIs of 1, 10, 30, 100, and 300 PFU per cell. Viral DNAs were labeled with [³H]thymidine, extracted, and subjected to electrophoresis in a 1.8% agarose gel. A representative fluorogram of one of these experiments is shown in Fig. 2. The yields of *dl1* and the H-1 helper virus were quantitated by excising the regions containing the appropriate DNA and measuring the amount of ³H by liquid scintillation spectrometry (Table 1).

TABLE 1. Yield of RF DNA (counts per minute)^a

wt MOI	<i>dl1</i>	wt (+ <i>dl1</i>)	wt
1	500	200	
10	2,100	600	3,400
30	3,400	1,500	7,000
100	3,800	3,400	15,000
300	3,500	3,800	

^a Replicate NB cell cultures (3×10^6 cells per 60-mm petri dish) were partially synchronized by treatment with methotrexate for 12 h before infection. The cultures were infected with a constant amount of *dl1*, with its endogenous helper virus (MOI of 1), and with various amounts of wt H-1 to provide MOIs of 1, 10, 30, 100, and 300. Three dishes received only the helper virus at an MOI of 10, 30, or 100. The viral DNAs were labeled with [³H]thymidine at 5 μ Ci/ml and 10^{-6} M in the presence of 5-fluorodeoxyuridine at 0.5 μ g/ml from 12 to 17 h postinfection. The *dl1* RF DNA was separated from wt RF DNA by gel electrophoresis (Fig. 2). After fluorography, the regions of gel containing each type of RF DNA were excised, and the ³H was quantitated by liquid scintillation spectrometry.

The yields of ³H-labeled *dl1* RF DNAs were increased in a dose-dependent fashion by modest levels of helper virus (MOIs, 1 to 100), but they began to decrease at the highest level of helper virus (MOI 300). The yields of wt RF DNA or even the sum of wt + *dl1* RF DNAs were depressed in the mixed infection compared with that of wt alone.

In a similar experiment, the heterologous parvovirus MVM was tested and found to be an efficient helper virus for H-1 *dl1* RF replication, whereas LuIII was not (Fig. 3). H-3 and MVM(i) (3, obtained from P. Tattersall, Yale University, New Haven, Conn.) were also found to complement *dl1* for RF replication (data not shown).

To detect complementation for later steps in virus replication such as progeny DNA synthesis and encapsidation by a heterologous helper virus, *dl1* was propagated in THK22A cells (Simian virus 40-transformed hamster kidney obtained from Joan Kaplan, Harvard University, Boston, Mass.), with MVM as a helper virus for two serial passages. If *dl1* is complemented by MVM for all of its defective functions, phenotypic mixing will occur, and *dl1* will be encapsidated by the capsid proteins of the MVM helper virus. In that event, the yields of *dl1* RF DNA will be sensitive to neutralization of the virus inoculum with anti-MVM antisera. For this assay, H-3 was added to the cultures at the time of infection to ensure the presence of sufficient helper virus (Fig. 4A and 4B). The results show that the MVM-*dl1* passage 1 virus contained large amounts of *dl1* neutralized by MVM antiserum, but not by H-1 antiserum or normal hamster serum. For MVM-*dl1* passage 2, a small

quantity of deleted RF DNA was generated by the MVM + *dl1* virus, and it was decreased by the MVM antiserum. As a control, H-1 + *dl1* virus showed elimination of *dl1* RF DNA by H-1 antiserum, but not with anti-MVM or normal serum (Fig. 4A). Thus, MVM not only supports RF DNA replication of H-1 *dl1*, but it also complements for progeny DNA synthesis and encapsidation. The reason that the *dl1* in the MVM-*dl1* passage 2 produced a reduced and broadened RF DNA band is unknown, unless MVM complements at reduced efficiency in

FIG. 3. Fluorogram of agarose gel electrophoresis of *dl1* DNA with LuIII, MVM, or H-1 as helper virus. THK22A cells were cultured in 100-mm petri dishes at 5×10^6 cells per dish. The cultures were partially synchronized by treatment with methotrexate for 10 h before infection. Replicate plates were infected with *dl1* (H-1 helper virus [hv] at an MOI of 1) and, for lane 1, no additional helper virus; 2, LuIII (MOI of 10); 3, MVM (MOI of 6); and 4, H-1 (MOI of 40). The cultures were labeled with [³H]thymidine (5 μ Ci/ml, 10^{-6} M plus 5-fluorodeoxyuridine at 0.5 μ g/ml from 12 to 17 h postinfection). Viral DNAs were extracted as previously described (4), and the electrophoresis was carried out in a 1.4% agarose gel at 50 V for 16 h. The positions of monomer RF DNA of helper virus and *dl1* are indicated by hv and *dl1*, respectively; di indicates dimer RF DNA.

FIG. 4. Fluorograms of agarose gel electrophoresis of *dl1* RF DNA produced after treatment with specific antisera. *dl1* was propagated serially for two passages in THK22A cells with MVM or H-3 added as helper virus (hv) at an MOI of 10 (MVM-*dl1* or H-3-*dl1*). The *dl1* virus stocks were incubated with hamster antisera before infection and labeling of THK22A cultures as in Fig. 3. Virus stock (0.4 ml) was incubated with 50 μ l of antiserum for 1 h at 37°C. The hemagglutination inhibition titer of the anti-MVM was 2^{-15} to MVM, the anti-H-1 titer was 2^{-16} to H-1, the anti-H-3 titer was 2^{-14} to H-3, and the nonimmune serum (N.S.) was 0 to all of the viruses. In (A), MVM was treated with anti-MVM (lane 1) or N.S. (lane 5) as a control. To ensure adequate helper virus for any *dl1* which survived neutralization, H-3 was added at an MOI of 30 to all of the *dl1* infections. The H-1-*dl1* stock after neutralization is in lanes 2 to 4, and the MVM-*dl1* (passage 2) is in lanes 6 to 8. In (B), MVM-*dl1* (passage 1) was similarly treated (lanes 1 to 3) and H-3-*dl1* (passage 2) is shown after treatment with anti-H-3 antiserum or nonimmune serum (lanes 4 and 5). The MVM-*dl1* and H-3-*dl1* were analyzed on separate gels, and only the bottom portion of the gels are shown. The DNA species are identified on the margins: (A) hv RF, monomer helper virus RF DNA; *dl1* RF, monomer *dl1* RF DNA; (B) di RF, dimer RF DNA; * indicates a heat-labile configuration of viral DNA not fully characterized that is prominent in this experiment. Electrophoresis was in a 1.6% agarose gel at 100 V for 16 h.

comparison with H-1. A similar phenotypic mixing was obtained with H-3 and *dl1* (Fig. 4B).

In summary, the DI deletion mutant of H-1, *dl1*, requires the RF *rep* function of a helper virus to replicate its RF DNA. The homologous virus H-1 and the heterologous viruses H-3, MVM, MVM (i), but not LuIII, complement *dl1* for RF replication. H-3 and MVM were also found to be capable of complementing *dl1* for the functions required for progeny DNA synthesis and encapsidation. *dl1* is strongly interfering to its helper virus, yet it does not contain extra copies of the tandem repeats near the replication origin characteristic of H-1 DI genomes (5, 8). Therefore, I conclude that these tandem repeats do not effect the interference. It should be noted that the major promoter for H-1 has been reported to be at map position 40 (2). My preliminary sequencing data show that the canonical promoter sequence TATAAA (sense strand) is only 20 nucleotides proximal to the *HhaI* site at 38 (S. L. Rhode, unpublished data). Thus, it is likely that the deletion in *dl1* has disrupted this promoter and caused a defect in the synthesis of both of the capsid proteins, VP1 and VP2. This lesion also seemed to be present in DI genomes generated by serial high multiplicity passage and may be an important property of DI particles (5).

I thank Jessica Bratton and Jeanne Helft for expert technical assistance.

This work was supported by Public Health Service grants CA25866 and CA26801 from the National Institutes of Health.

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