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Structure and Genetic Complexity of the Genomes of Herpesvirus Defective-Interfering Particles Associated with Oncogenic Transformation and Persistent Infection

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The complexity and structural organization of defective-interfering (DI) particle DNA of equine herpesvirus type 1 (EHV-1) have been elucidated by using restriction enzyme and Southern blot hybridization analyses. DI particles were generated by serial high-multiplicity passage of EHV-1 in L-M cells, and total viral DNA was extracted from virus purified from supernatants of these serial passages. EHV-1 DI particle DNA was quantitatively separated from standard (STD) DNA by several cycles of CsCl isopycnic banding in a vertical rotor. Restriction endonuclease digestion profiles of pure DI DNA were completely different from the mapped patterns observed for EHV-1 STD DNA. Digestion of pure defective DNA with restriction enzymes (*Bgl*II, *Eco*RI, and *Xba*I), for which there are few or no cleavage sites within the S (short) region of the EHV-1 STD genome, yielded high-molecular-weight supermolar DNA bands, suggesting that a large subgenomic repeat unit was present in defective DNA. DNA blot hybridization analysis with the *Bgl*II supermolar fragment of defective DNA, intact DI particle genomic DNA, and EHV-1 STD DNA restriction enzyme fragments as ^{32}P-labeled probes indicated that the EHV-1 DI particle genome originates predominately from the STD DNA S region (0.77 to 1.00 map units) and to a lesser extent from the left terminus of the unique long (U_L) region (0.00 to 0.05 map units). None of the EHV-1 DNA sequences associated to date with EHV-1 oncogenesis (0.32 to 0.38 map units; O'Callaghan et al. *in* B. Roizman [ed.], *Herpesviruses*, in press; Robinson et al., Cell **32**:204–219, 1983, and Proc. Natl. Acad. Sci., U.S.A., **78**:6684–6688, 1981) were detected in the DI particle DNA. The importance of these data with regard to DNA replication of DI particles and the role of DI particles in one model system of EHV-1 oncogenic transformation are discussed.

Defective-interfering (DI) particles have been characterized for a variety of viral systems (3, 10, 13, 14, 16, 17, 41). Typically, DI particles appear after serial undiluted high-multiplicity passage of standard (STD) virus. By definition, DI particles interfere with STD virus replication, although paradoxically they require STD virus as a helper for their own replication. The ability of DI particles to interfere with STD virus replication has led to the suggestion that they may participate in certain disease states involving virus persistence (17). In support of this, DI particles have been shown to mediate persistent infection in a variety of experimental systems such as rhabdoviruses (1, 25), influenzavirus (7), togaviruses (39), and recently herpesviruses (6, 23; D. J. O'Callaghan, G. A. Gentry, and C. C. Randall *in* B. Roizman, ed., *Herpesviruses*, in press). Furthermore, recent studies from this laboratory have indicated that herpesviral DI particles are involved in the coestablishment of both oncogenic transformation and persistent infection in permissive hamster embryo cells (6, 32; O'Callaghan et al., in press). In these studies, we were able to demonstrate that permissive hamster embryo cells which had been oncogenically transformed by equine herpesvirus type 1 (EHV-1) viral preparations enriched for DI particles were virus producers (i.e., persistently infected). Subsequently, it was shown that these cultures of oncogenically transformed hamster cells released both EHV-1 infectious STD virus and DI particles. Repeated efforts to demonstrate in these persistently infected cells the presence of temperature-sensitive viral mutants or the production of interferon in amounts above those present in control cells were unsuccessful, thus indirectly suggesting that EHV-1 DI particles are responsible for the maintenance of the persistent infection (O'Callaghan et al., in press).

The relatively recent association of DI particles with altered host responses to virus infection, such as persistent infection and neoplastic transformation, has made it particularly important to characterize the molecular features of DI particle genetic material. Several herpesviruses have been shown to generate DI particles upon high-multiplicity passage, and defective genomes have been characterized for some of these herpesviruses, including HSV-1 (19, 34, 37), HSV-2 (11, 12), pseudorabies virus (27), human cytomegalovirus (38), herpesvirus saimiri (9), and herpesvirus ateles (8). In general, defective herpesviral DNA has an aberrant buoyant density compared with the corresponding STD viral DNA, and often these defective DNA molecules are deletion mutants that are composed of multiply reiterated sequences of low complexity.

EHV-1 DI particles have been generated in our laboratory both in vivo in LSH hamsters (4) and in vitro in L-M cell suspension cultures (6, 13, 14, 21, 23; O'Callaghan et al., in press). Initial characterization of EHV-1 defective DNA by using both systems yielded identical results. The defective genome was shown to possess a molecular weight similar to that of STD EHV-1 DNA, to have a greater buoyant density (1.724 versus 1.716 g/cm^3), and to consist of highly reiterated sequences. Subsequent analysis of Henry et al. (13) demonstrated that defective DNA decreased in sequence complexity at higher passage numbers, a phenomenon that correlated with an increase in relative interference capacity.

In this paper, we describe the results of restriction enzyme

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analyses and Southern blot hybridization studies which indicate that the genome of the EHV-1 DI particle being investigated in our laboratory is comprised primarily of S-region DNA sequences which are reiterated several times to form an entire defective molecule; unique long (U_L) parental EHV-1 DNA sequences are also present in the defective DNA but are those that originate only from the far left terminus of the EHV-1 STD genome (map units 0.0 to 0.05). Furthermore, since other studies from our laboratory suggest that viral DNA sequences associated with transformations are located within the U_L region of the STD EHV-1 genome (0.32 to 0.38 map units; 29–31; O'Callaghan et al., in press), these results enable us to delineate more precisely the role of EHV-1 DI particles in oncogenic transformation resulting from infection of permissive hamster embryo cells with live virus preparations enriched for DI particles.

MATERIALS AND METHODS

Cells and viruses. EHV-1 (Kentucky A strain) was propagated in L-M suspension cell cultures as described elsewhere (22, 24). Passage of STD EHV-1 was performed at low multiplicity of infection (0.005 PFU per cell). The purity of viral DNA preparations was monitored by restriction enzyme analysis and isopycnic banding in the analytical ultracentrifuge (13; O'Callaghan et al., in press).

Generation of EHV-1 DI particles. The methods for the generation of EHV-1 DI particles have been described previously (6, 13, 14, 23; O'Callaghan et al., in press). Undiluted cell-free supernatants from serial high-multiplicity passages of DI stock pools were used to infect increasing amounts of L-M cells in a stepwise fashion. All passages were made with 200 ml of undiluted virus per 10^9 L-M cells in Yelp medium supplemented with 5% fetal bovine serum. At 48 to 72 h postinfection, virus was purified by polyethylene glycol 6000 precipitation of cell-free supernatant virus followed by several cycles of rate-velocity centrifugation in Dextran −10 gradients (24).

Isolation of pure defective DNA. Purified viral particles pelleted at 31,000 × *g* for 1 h were resuspended in TE buffer (0.01 M Tris-hydrochloride [pH 7.4], 0.001 M EDTA) and treated with sodium dodecyl sulfate (final concentration, 1% [wt/vol]) and Pronase (1 mg/ml) for 4 h at 37°C (13). After incubation, the DNA was extracted twice with 80% phenol in TE buffer and finally with equal volumes of phenol and chloroform-isoamyl alcohol (24:1, vol/vol). The DNA was dialyzed overnight against 4 liters of 0.1× TE buffer with several changes of buffer. DNA was mixed with CsCl to achieve a final density of 1.700 g/cm^3 (DNA concentration, 10 to 15 μg/ml). A small amount of ^{3}H-labeled EHV-1 STD viral DNA was added as density marker. The DNA was centrifuged at 35,000 rpm (108,000 × *g*) for 20 h at 20°C in a Beckman V Ti-65 vertical rotor. The gradients were collected into scintillation vials in 10-drop fractions, and each fraction was counted after the addition of 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.).

CsCl analytical ultracentrifugation. The purity of DI DNA preparations was demonstrated by isopycnic banding in a model E analytical ultracentrifuge at 140,000 × *g* for 24 h at 22°C (4, 13). The gradients were photographed, and the buoyant densities of virus DNAs were determined by previously reported procedures (22).

Restriction enzyme analysis and Southern blot hybridization of DI DNA. Samples of purified DNA were digested to completion with a threefold excess of the desired restriction enzyme in the appropriate buffer (as recommended by Bethesda Research Laboratories, Gaithersburg, Md.) for 2 h at 37°C, and the resultant fragments were electrophoresed in 0.7% agarose. After electrophoresis, gels were stained with ethidium bromide (0.5 μg/ml) for 1 h and photographed in shortwave UV light (35). Gels containing viral DNA fragments were alkali denatured, neutralized, transferred to hybridization filters (GeneScreen, New England Nuclear Corp.) in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate), and immobilized by baking in vacuo at 80°C for 2 h by the method of Southern (36). Filters were hybridized to 0.5 × 10^6 to 1.0 × 10^6 cpm of ^{32}P-labeled (by nick translation; 26) EHV-1 restriction enzyme fragments (specific activity, 0.5 × 10^8 to 1.0 × 10^8 cpm/μg) at 62°C in an incubation mixture consisting of 4× SSC, 1× Denhardt mixture (0.02% each bovine serum albumin, polyvinylpyrolidone, and Ficoll), 0.1% sodium dodecyl sulfate, 50 μg of yeast RNA per ml, and 50 μg of denatured salmon sperm DNA per ml. Washing was performed three times with 0.3× SSC for 1 h at 58°C. Filters were air dried and were allowed to expose Kodak X-Omat AR film at −70°C for various times with Dupont Cronex Lightening Plus intensifying screens.

In vivo radiolabeling of viral DNA with $H_3{}^{32}PO_4$. L-M cells infected with DI particle stock pools in serial high-multiplicity fashion as described above were collected by centrifugation after a 2-h viral attachment period and were suspended at a concentration of 2 × 10^7 cells per ml in phosphate-free Eagle minimal essential medium supplemented with 3% fetal bovine serum. At 7 h postinfection, cells were again collected, suspended at a concentration of 2 × 10^6 cells per ml in fresh phosphate-free medium containing $H_3{}^{32}PO_4$ at a final concentration of 50 μCi/ml, and incubated at 37°C. Cell cultures were harvested at 72 h postinfection, and viral DNA was isolated as described above. The specific activities of viral DNA ranged from 1 × 10^5 to 2 × 10^5 cpm/μg.

Restriction enzyme analysis of radiolabeled DNA. Four micrograms of pure $^{32}PO_4$-labeled DI DNA was digested with either *Eco*RI, *Xba*I, *Bgl*II, or *Bam*HI and then was electrophoresed through 0.65% agarose cylindrical gels (Sea Plaque; FMC Corp., Marine Colloids Div., Rockland, Maine). Gels were cut into 1-mm cross-sections, and each slice was counted by the Cerenkov method and then in Aquasol after solubilization (15).

RESULTS

Generation of DI particles and detection of defective EHV-1 DNA. EHV-1 DI particles were generated by serial undiluted passage in L-M suspension cells as described above. Viral DNA was isolated from the resulting mixed particle preparations containing both EHV-1 STD virions and DI particles. The presence of defective DNA was confirmed by CsCl buoyant density analysis in the model E analytical ultracentrifuge. As shown in Fig. 1B, these viral DNA preparations contained both EHV-1 STD DNA (1.716 g/cm^3) and the more dense EHV-1 DI DNA species (1.724 g/cm^3). As a reference *Micrococcus luteus* DNA was also added (density, 1.731 g/cm^3). Restriction enzyme analysis of mixed STD and DI particle DNA preparations demonstrated the presence of novel high-molecular-weight bands (Fig. 2, arrows, lanes 4, 6, 8, and 10) which were not present in pure STD DNA digests (Fig. 2, lanes 3, 5, 7, and 9). The relative amount of DI particle DNA present in mixed EHV-1 DNA preparations varied significantly in the various DI particle passages, and for this study DI passages that contained at least 40% defective DNA, as judged by CsCl analytical centrifugation analysis, were chosen.

Purification of defective DNA. Analysis of defective viral genomes has been hindered by the inability to separate

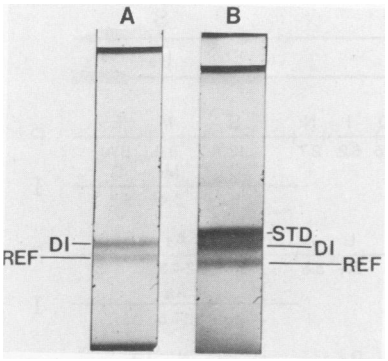

FIG. 1. Isopycnic banding of pure DI particle DNA and total DNA preparations (STD DNA and DI particle DNA). Total viral DNA was obtained from purified particles of supernatants of serial high-multiplicity passages of EHV-1. DI particle DNA was purified by CsCl isopycnic banding in a vertical rotor as described in the text. (A) UV absorbance profile of DI particle DNA after its purification by CsCl isopycnic banding in a vertical rotor. DI particle DNA, p = 1.724 g/cm^3; REF refers to reference DNA, which was *M. luteus* DNA, p = 1.731 g/cm^3. (B) UV absorbance profile of total viral DNA (STD DNA, p = 1.716 g/cm^3 and DI particle DNA, p = 1.724 g/cm^3). REF refers to reference DNA, which was *M. luteus* DNA, p = 1.731 g/cm^3.

defective particles from standard virions. Previous work in this laboratory demonstrated that EHV-1 defective particles could not be separated from standard virions by ultracentrifugation techniques relying on differences in buoyant density or sedimentation coefficient. In addition, it was observed that defective DNA could not be separated from standard viral DNA by rate velocity centrifugation since the majority of defective molecules were similar in size to STD virus DNA as judged by sedimentation analysis in sucrose density gradients (13) and measurement of DNA molecules by electron microscopy (manuscript in preparation). The observation that defective DNA has a significantly greater buoyant density than STD DNA suggested that DI DNA could be qualitatively separated from STD DNA by CsCl isopycnic centrifugation in a vertical rotor as described above. Graphical analysis of fractions obtained by vertical rotor buoyant density centrifugation of radiolabeled viral DNA preparations indicated that separation was achieved; two easily discernible viral DNA peaks were present corresponding to the 1.724-g/cm^3 DI DNA species and the less dense 1.716-g/cm^3 STD DNA species (data not shown).

The absence of STD viral DNA in the purified defective DNA preparations was demonstrated by three methods. First, CsCl buoyant density analysis in the model E analytical ultracentrifuge revealed that pooled fractions of defective

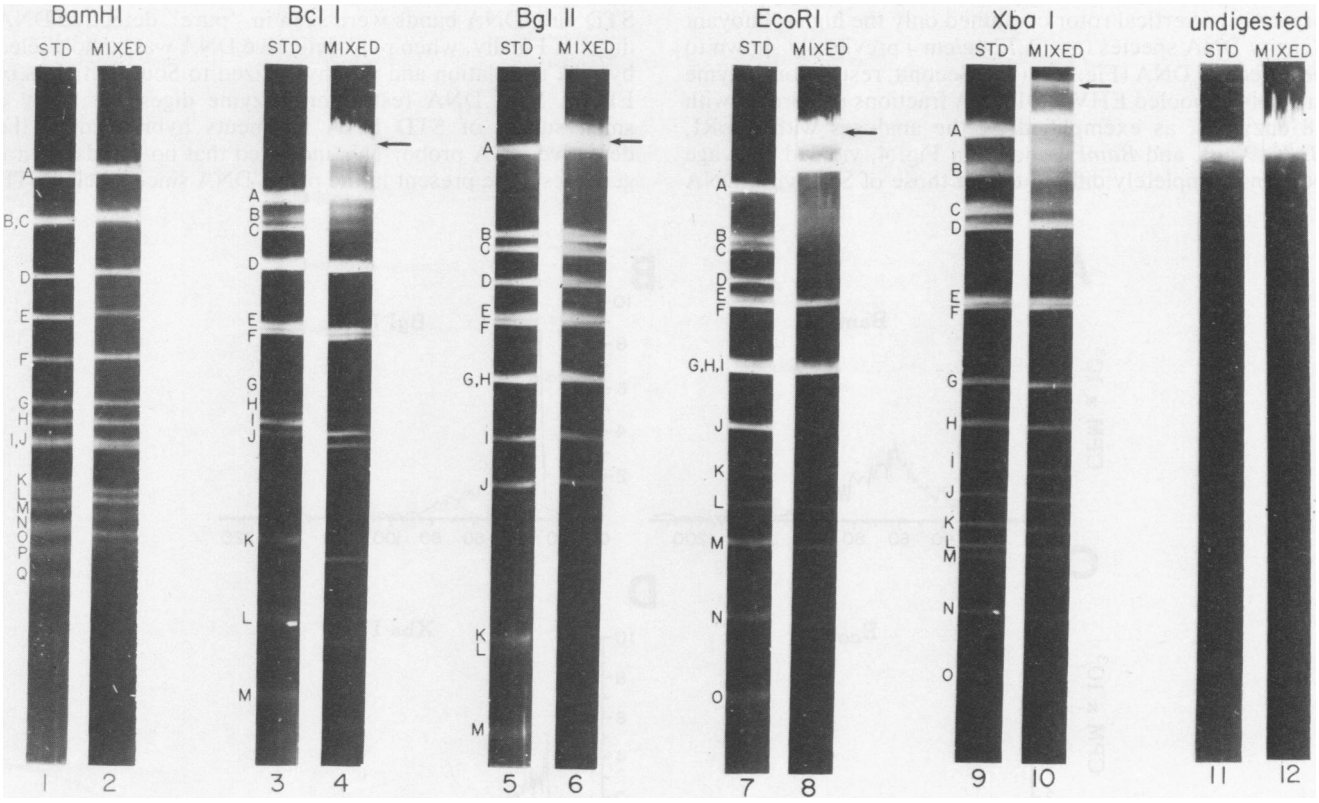

FIG. 2. Restriction endonuclease digestion of mixed viral DNAs. Viral DNA was isolated from particle preparations derived from serially passaged virus stocks enriched for DI particles and was digested with the desired restriction enzyme. The resultant fragments were electrophoresed through cylindrical agarose gels as described in the text. Lanes 1, 3, 5, 7, and 9 show pure STD EHV-1 DNA digested with a single restriction enzyme as labeled at the top of the figure (see Fig. 3 for cleavage map). Lanes 2, 4, 6, 8, and 10 show mixed EHV-1 DNA (i.e., both STD and DI DNAs) digested with a single restriction enzyme as labeled at the top of the figure. Lanes 11 and 12 show undigested STD and mixed DNAs, respectively. Arrows indicate high-molecular-weight supermolar DNA bands characteristic of DI particle DNA and not present in STD DNA digests.

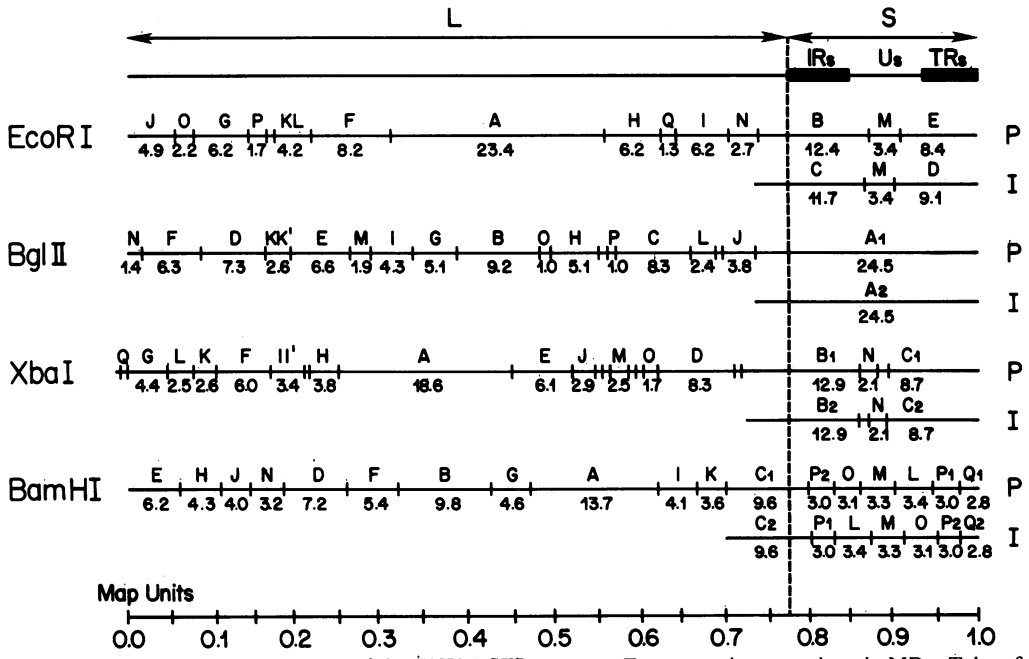

FIG. 3. Restriction endonuclease cleavage maps of the EHV-1 STD genome. Fragment sizes are given in MDa. Taken from Henry et al. (15) with permission.

DNA obtained by CsCl buoyant density centrifugation in a preparative vertical rotor contained only the higher buoyant density DNA species (p = 1.724 g/cm^3) previously shown to be defective DNA (Fig. 1A) (13). Second, restriction enzyme analysis of pooled EHV-1 DI DNA fractions performed with 18 enzymes, as exemplified by the analyses with *Eco*RI, *Bgl*II, *Xba*I, and *Bam*HI shown in Fig. 4, yielded cleavage patterns completely different from those of STD viral DNA (15); these patterns were unique to defective DNA, and no STD viral DNA bands were seen in "pure" defective DNA digests. Finally, when pure defective DNA was radiolabeled by nick translation and was hybridized to Southern blots of EHV-1 STD DNA restriction enzyme digestions, only a small subset of STD DNA fragments hybridized to the defective DNA probe. This indicated that no standard viral genomes were present in the probe DNA since labeled STD

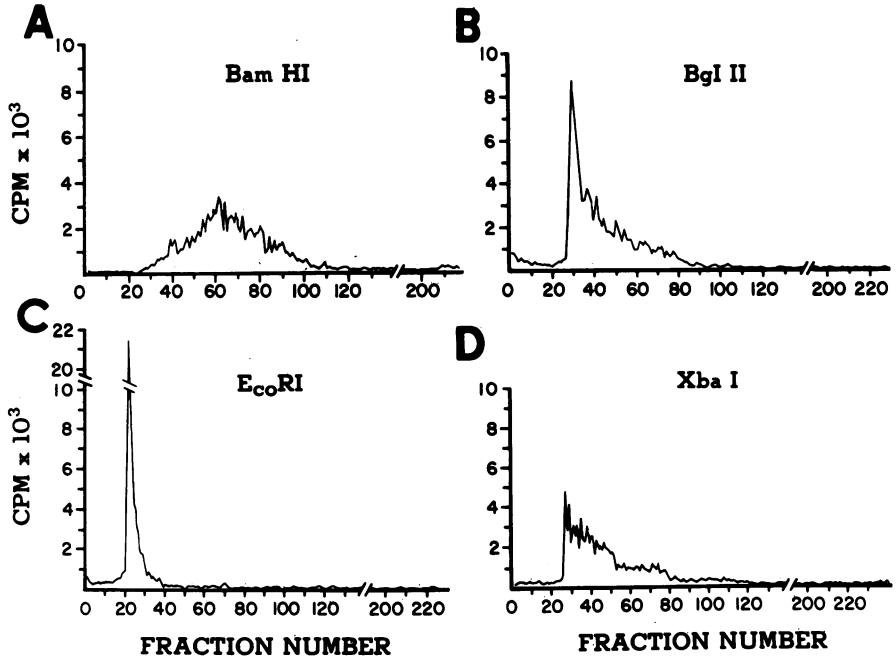

FIG. 4. Restriction enzyme analysis of $^{32}PO_4$-labeled EHV-1 DI DNA. Pure $^{32}PO_4$-labeled EHV-1 DI particle DNA was digested with a single restriction enzyme, and the resultant fragments were electrophoresed through cylindrical agarose gels. Gels were sliced into 1-mm cross-sections, and each slice was counted by the Cerenkov method. Enzymes used were *Bam*HI (A), *Bgl*II (B), *Eco*RI (C), and *Xba*I (D).

viral genomes would be expected to hybridize to all fragments in the blot.

Restriction enzyme analysis of radiolabeled pure DI DNA. The cleavage maps for the restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RI, and *Xba*I have been determined for the EHV-1 standard virus genome (15) (Fig. 3). Purified DI DNA was digested with each of these enzymes (Fig. 4), and cleavage patterns of defective DNA were compared with those generated by the digestion of STD EHV-1 DNA with the identical restriction enzymes (Fig. 2). As shown in Fig. 4, restriction enzyme cleavage patterns of the purified DNA of DI particles differed completely from those of STD EHV-1 DNA (Fig. 2, lanes 1, 5, 7, and 9), indicating that defective genomes have a sequence organization distinct from that of STD EHV-1 DNA. Digestion of mixed or pure defective DNA preparations with the enzymes *Bgl*II, *Eco*RI, and *Xba*I generated large supermolar (multiple-copy) bands (Fig. 2, arrows; also see Fig. 4) which were not present in EHV-1 STD DNA digests. In contrast, digestion of EHV-1 DI DNA with the enzyme *Bam*HI yielded a broad band of nonresolvable low-molecular-weight DNA fragments (Fig. 2, lane 2; Fig. 4A). One interesting difference between these enzymes is that *Bam*HI cleaves at multiple sites within the EHV-1 S region, whereas *Bgl*II, *Eco*RI, and *Xba*I cleave at few or no sites within the S region. Digestion of pure DI DNA with more than 18 other enzymes yielded similar polarities in digestion patterns; that is, DI DNA was restricted into either a single high-molecular-weight supermolar band (for example *Bcl*I; Fig. 2, arrow, lane 4) or a large family of small fragments with a cleavage pattern similar to that obtained with *Bam*HI (data not shown). To date, no restriction enzyme has been found which cleaves DI DNA into discrete, relatively small bands amenable to more refined characterization.

Radioactivity profiles of restriction enzyme digests of pure ^{32}P-labeled DI DNA are shown in Fig. 4. Profiles obtained for the enzymes *Bgl*II, *Eco*RI, and *Xba*I (Fig. 4B, C, and D), which generate large supermolar bands upon digestion of pure DI DNA, indicate that at least for the *Bgl*II and *Eco*RI digests a major portion of the total radioactivity in each lane was present as a single radioactive peak corresponding to the high-molecular-weight supermolar DI DNA band.

Complexity of DI particle DNA. The origin and complexity of EHV-1 DI particle genomic DNA were explored with the Southern blot hybridization technique. In initial experiments, the *Bgl*II DI supermolar DNA fragment was isolated from gel slices of low-melting-point agarose as described by Henry et al. (15), ^{32}P-labeled by nick translation, and hybridized to digests of STD EHV-1 DNA. As shown in Fig. 5, only bands originating from the S region of the STD virus genome hybridized with the labeled *Bgl*II DI DNA fragment probe. These fragments were *Eco*RI-B, C, D, E, and M, *Xba*I-B, C, and N, *Bgl*II-A, and *Bam*HI-C, O, P, and Q. (The *Bam*HI-C, *Eco*RI-M, and *Xba*I-N fragments, which are derived almost entirely from unique [nonrepeated] S-region sequences, appeared after longer exposure times.) These results suggest that DI DNA is comprised largely of S-region sequences primarily from the inverted repeats (see Fig. 3). However, since a significant fraction of the total defective DNA sequences were not present in the *Bgl*II DI supermolar band (as judged by radioactivity profiles; see Fig. 4) the presence of U_L DNA sequences in defective genomes could not be ruled out.

To elucidate the complexity of EHV-1 DI genomes further, purified intact molecules of DI particle DNA were ^{32}P-labeled by nick translation and were hybridized against STD EHV-1 DNA digests. As shown in Fig. 6, ^{32}P-labeled genomic DI DNA hybridized predominately to STD restriction enzyme fragments derived from the EHV-1 STD virus S region, a pattern similar to that observed when the ^{32}P-labeled *Bgl*II DI supermolar fragment was used as probe. But in contrast to the results with the DI *Bgl*II fragment probe, the intact DI particle genomic probe also hybridized to fragments derived from the EHV-1 STD virus U_L terminus (Fig. 6, arrows); these fragments included *Bam*HI-E (lanes 1 and 5), *Bgl*II-F and N (lanes 2 and 6), *Eco*RI-J (lanes 3 and 7), and *Xba*I-G and Q (lanes 4 and 8). In all analyses of defective DNA sequences, no L-region DNA sequences other than those which map at the L terminus were detected. In these hybridizations, two separate DI genomic DNA preparations, DI passage X-40H (Fig. 6, lanes 1 through 4) and DI passage X-58H (Fig. 6, lanes 5 through 8) were used as ^{32}P-labeled genomic probes, and identical hybridization patterns were obtained with both of these DNA preparations; however, some band intensities differed, possibly

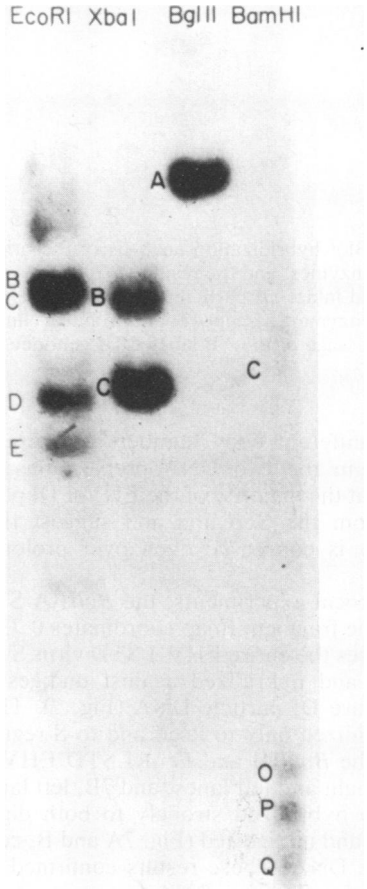

FIG. 5. Analysis of the origin of the *Bgl*II supermolar fragment of DI particle DNA. EHV-1 STD DNA was digested with the appropriate restriction enzymes, and the resultant fragments were electrophoresed through an agarose slab gel and transferred to nitrocellulose. The DI *Bgl*II supermolar fragment which had been purified from cylindrical agarose gel slices after electrophoresis was ^{32}P-labeled by nick translation and hybridized to EHV-1 STD DNA restriction enzyme fragments bound to nitrocellulose under conditions described in the text. EHV-1 STD DNA digests are designated at the top of each lane by the restriction enzyme used in each case.

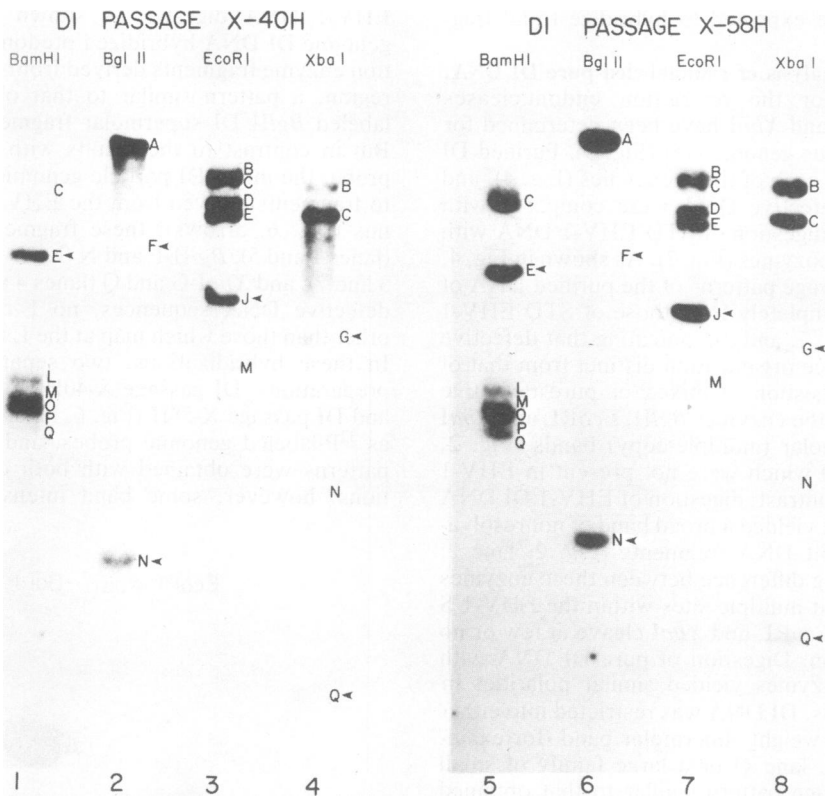

FIG. 6. Blot hybridization analysis of the origin of EHV-1 DI particle genome. EHV-1 STD DNA was digested with the appropriate restriction enzymes, and the resultant fragments were electrophoresed through an agarose slab gel and transferred to nitrocellulose. Vertical rotor-purified intact DI particle DNA (passage X-40H or X-58H) was ^{32}P-labeled by nick translation and hybridized to EHV-1 STD DNA restriction enzyme fragments bound to nitrocellulose. The enzyme used for digestion of EHV-1 STD DNA is designated at the top of each lane. The passage of the ^{32}P-labeled DI genomic probe used is given at the top of each autoradiogram.

reflecting different copy numbers of these identical DNA sequences in the two DNA preparations. These findings confirm that the majority of the EHV-1 DI particle genome is derived from the S region and suggest that DI genomic complexity is conserved even over prolonged passage in culture.

In reciprocal experiments, the *Bgl*II-A STD viral restriction enzyme fragment (map coordinates 0.73 to 1.00), which encompasses the entire EHV-1 STD virus S region, was nick translated and hybridized against undigested and *Eco*RI-digested pure DI particle DNA (Fig. 7). The *Bgl*II-A fragment hybridized only to itself and to S-region-derived fragments in the *Bam*HI and *Eco*RI STD EHV-1 DNA digests (Fig. 7A, right and left lanes, and 7B, left lane). The *Bgl*II-A probe also hybridized strongly to both digested (Fig. 7B, right lane) and undigested (Fig. 7A and B, center lanes) pure DI particle DNA. These results confirmed that DI particle DNA contains S-region DNA sequences.

Recently, EHV-1 DNA sequences associated with oncogenic transformation have been mapped to map coordinates 0.32 to 0.38 within the U_L region of the STD EHV-1 genome (29, 30; O'Callaghan et al., in press). Since EHV-1 DI particles were found to be a necessary component in preparations of live virus used to coestablish both oncogenic transformation and persistent infection of permissive hamster embryo cells (6, 23, 28, 32; O'Callaghan et al., in press), it was of particular importance to determine whether DI particle genomes contained any of these L-region STD viral DNA sequences associated with oncogenesis. Therefore, the *Bgl*II-G and H fragments (map units 0.33 to 0.39 and 0.50 to 0.55, respectively) were nick translated and hybridized against undigested DI DNA (Fig. 7C). As shown in Fig. 7C (right lane), undigested pure DI DNA did not hybridize to the ^{32}P-labeled *Bgl*II-G and H probe, which is derived in part from the EHV-1 transformation-associated sequences. As expected, the *Bgl*II-G and H probe hybridized only to the STD *Eco*RI-A fragment (Fig. 7C, left lane; see Fig. 3), thus demonstrating its specificity. These results demonstrate conclusively that DI DNA does not contain DNA sequences associated with EHV-1 oncogenesis and hence suggest that DI particles may mediate oncogenic transformation in an indirect manner by preventing cytocidal effects of STD virus in these permissive cells and thereby allowing DNA sequences associated with transformation to be expressed.

DISCUSSION

DI particles of EHV-1 exhibit all the classic properties which distinguish DI particles in general: defectiveness, interference activity, and enrichment (6, 13, 14, 23; O'Callaghan et al., in press). Further, EHV-1 DI particles are necessary components in live STD EHV-1 preparations used for the coestablishment of persistent infection and oncogenic transformation of permissive LSH hamster embryo cells (6, 32; O'Callaghan et al., in press). The presence of EHV-1 DI particles in the transforming virus inoculum and the recent mapping of EHV-1 STD DNA sequences associated with oncogenic transformation to within the 0.3-to-0.4 locus of the L region are developments that have made it particularly

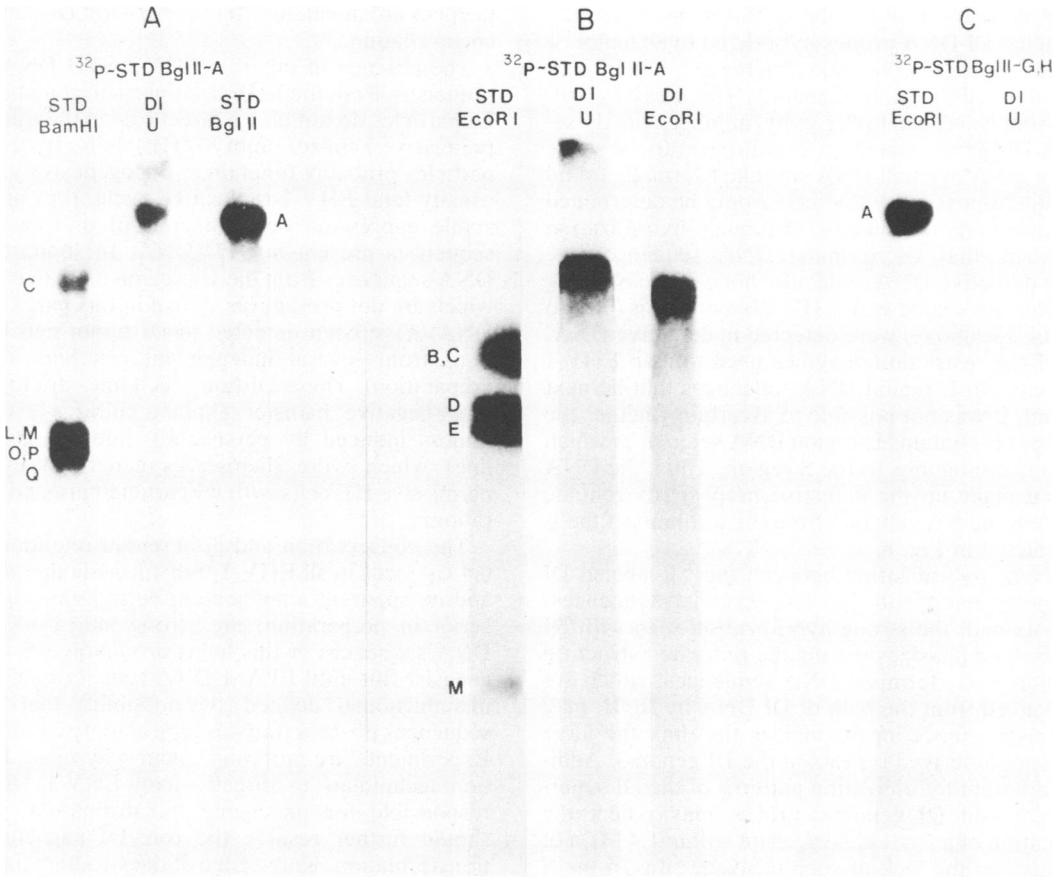

FIG. 7. Blot hybridization analysis of the DI particle DNA with ^{32}P-labeled STD DNA fragments. STD EHV-1 *Bgl*II restriction enzyme fragments (*Bgl*II-A, 0.73 to 1.00 map units, and *Bgl*II-G and H, 0.33 to 0.39 and 0.50 to 0.55 map units) isolated from agarose gel slices were ^{32}P-labeled by nick translation and hybridized against pure DI DNA (digested and undigested) as well as against EHV-1 STD DNA as a control. (A) Left, STD EHV-1 DNA digested with *Bam*HI; center, pure undigested DI DNA; right, EHV-1 STD DNA digested with *Bgl*II. (B) Left, EHV-1 STD DNA digested with *Eco*RI; center, pure undigested DI DNA; right, pure DI DNA digested with *Eco*RI. (C) Left, EHV-1 STD DNA digested with *Eco*RI; right, pure undigested DI DNA.

important to investigate the origin of the DI genome itself. The EHV-1 STD virus genome has been extensively characterized. It has a molecular mass of 92 megadaltons (MDa) and consists of a fixed 72.8 MDa U_L region of unique sequences covalently linked to an invertable 19.2-MDa S region. The S region contains unique DNA sequences (U_s 6.4 MDa) bracketed by identical inverted repeats (6.4 MDa) which enable the entire S region to invert relative to the long region, resulting in two possible isomeric structures (Fig. 3 and 8) (15, 33; O'Callaghan et al., in press).

Restriction enzyme digestion of pure EHV-1 defective DNA yielded banding patterns unique to defective DNA, and no fragments present in STD viral DNA were readily observed. Digestion of DI DNA with enzymes which do not cut or cut only a few times within the EHV-1 S region generated large supermolar high-molecular-weight DNA bands. In contrast, digestion of DI DNA with enzymes that possess multiple cleavage sites within the S region resulted in a nonresolvable smear of low-molecular-weight DNA fragments. These results indicate that the EHV-1 DI genome has a molecular structure distinct from that of STD EHV-1 DNA and further suggest that the DI DNA molecule might consist of large repeated units derived primarily from the EHV-1 STD genome S region. In addition, electron microscopic and thermal denaturation studies (to be published elsewhere) supported restriction enzyme data in suggesting that DI molecules contain a major repeat unit which was reiterated to comprise an entire defective genome.

Studies with both ^{32}P-labeled EHV-1 DI DNA and ^{32}P-labeled EHV-1 STD DNA fragments as probes in Southern blot hybridization analyses demonstrated that EHV-1 DI

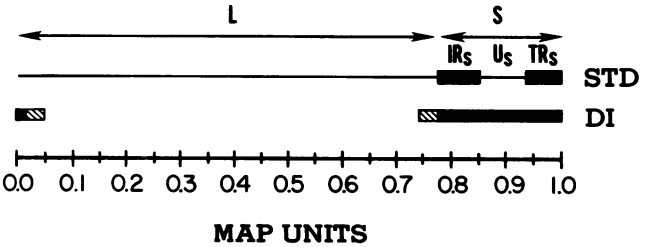

FIG. 8. Origin of the EHV-1 DI particle genome. Shown at top is the structure of the EHV-1 STD virus genome with the long (L), short (S), unique short (U_s), internal repeat (IR_s), and terminal repeat (TR_s) regions labeled. Represented under the EHV-1 STD genome is the origin of the EHV-1 DI particle genome. ■, areas conclusively shown to be present in DI DNA (0.00 to 0.015 and 0.77 to 1.00 map units); ▨, regions which may be present in DI DNA but which require further fine mapping for final determination (0.015 to 0.05 and 0.73 to 0.77 map units).

genomes are composed primarily of S-region DNA sequences. Labeled DI DNA probes hybridized to all major S-region-derived restriction enzyme fragments, albeit only weakly to U_S-derived S-region fragments. This weak hybridization may simply reflect lower copy numbers of U_S sequences in DI DNA; alternately, it could reflect the absence from defective DNA of small DNA stretches normally found in the U_S region, a possibility that could only be determined by DNA sequencing techniques. Although hybridization data did indicate that U_L terminal DNA sequences are present in the defective DNA molecule, none of the STD U_L DNA sequences associated with EHV-1 oncogenesis (0.32 to 0.38 map units, see above) were detected in defective DNA. Since none of the restriction enzymes used to map EHV-1 STD DNA cleave in L-region DNA sequences that lie next to the L-S joint, it was not possible to ascertain whether the DI particle genome contains L-region DNA sequences which are immediately contiguous to the S region. Thus, the DNA sequences that make up the defective herpesvirus genome are derived from the S region and from the terminus of the L region, as depicted in Fig. 8.

The absence of hybridization between the ^{32}P-labeled DI *Bgl*II supermolar band and U_L left terminal sequences, which contrasts with the strong hybridization seen with DI genomic probes, emphasizes the unique reiterated structure of the DI genome. U_L terminal DNA sequences, which are selectively cleaved from the bulk of DI DNA by *Bgl*II, may represent interim connecting sequences that link the large reiterated S-region-derived repeats in the DI genome. Additionally, the different hybridization patterns of the DI supermolar fragment and DI genomic probes may reflect the fortuitous location of a *Bgl*II cleavage site within 1.4 MDa of the L terminus and the lack of such cleavage sites in the S region (see Fig. 3). However, further experiments are necessary to confirm these conclusions.

The inclusion in the EHV-1 defective genome of DNA sequences derived from both ends of the STD virus genome may have important implications for DI particle generation and replication. Considerable evidence has accumulated to suggest that STD herpesvirus genomes are replicated by a rolling circle mechanism (2, 5, 18), probably during a late (second) temporal phase of DNA replication which utilizes head-to-tail concatemeric viral DNA replicative intermediates (2). Recently, cotransfection studies using restricted HSV-1 DI particle DNA have strongly suggested that herpesviral DI genomes are replicated by a rolling circle mechanism (40). In further support of this, these workers have shown that defective DNA rapidly accumulates only late in infection, presumably at a time when the necessary machinery for rolling circle replication has become available. Thus, the presence in the DI genome of left and right terminal viral DNA sequences, which are separated by over 66 MDa in the STD EHV-1 genome, may reflect the initial generation of defective DNA from a circularized STD viral genome template. The unique reiterated structure of herpesvirus DI particle genomes (including the EHV-1 DI particle genome) may render them virtually ideal substrates for rolling circle replication and allow defective molecules to use more efficiently the machinery for rolling circle replication than STD viral genomes, thus in part explaining the interference capacities of defective DNA. Further, as suggested by DNA sequencing and genetic studies on HSV-1 (20), terminal DNA sequences may play essential roles in isomerization, circularization, and packaging of virion DNA. Thus, selective pressure might exist to retain terminal DNA sequences in defective molecules, particularly if these sequences are mandatory for proper viral DNA replication and encapsidation.

The absence of putative EHV-1 STD DNA transforming sequences from the EHV-1 DI particle genome suggests that DI particles do not play a direct role in the transformation of permissive hamster embryo (HE) cells. Instead, EHV-1 DI particles probably function in oncogenesis by tempering the usually fatal EHV-1 replicative cycle, thus allowing for the stable expression (and integration) of transforming DNA sequences present in STD DNA. In support of this, viral DNA sequences from the U_L region (0.30 to 0.55 map units), which are not present (as shown in this paper) in DI particle DNA, have been detected in DI tumor cell DNA preparations from several independent cell lines (manuscript in preparation). These DI tumor cell lines are higly oncogenic, virus-negative hamster explant cultures established from tumors induced by persistently infected transformed cell lines which were themselves generated by infection of permissive HE cells with DI particle-enriched EHV-1 preparations.

The conservation and preferential retention of the 0.3 to 0.4 U_L locus in all EHV-1-transformed (and tumor) cell lines and its apparent amplification during tumorigenesis (manuscript in preparation) are strong indicators that L-region DNA sequences at this locus are involved in EHV-1 oncogenesis. But until EHV-1 DNA transforming sequences are unambiguously defined, the possibility that S-region DNA sequences possess transforming activity cannot be ruled out. Experiments are currently under way using DNA transfection techniques to directly map EHV-1 DNA sequences responsible for oncogenic transformation. These studies should further resolve the role DI particles play in cell transformation. Knowledge of the structure and organization of EHV-1 defective genomes will provide a foundation for any future research aimed at investigating whether DI particle genes or gene products play specific roles in maintaining EHV-1 persistent infection or in enhancing the stable expression of EHV-1 STD DNA sequences during the initial stages of oncogenic transformation.

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