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Physical Map of the Origin of Defective DNA in Herpes Simplex Virus Type 1 DNA

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The origin of defective DNA (dDNA) of the Patton strain of herpes simplex virus type 1 (HSV-1) was physically mapped with *Bam*HI in the parental DNA. The dDNA obtained from virus passaged at high multiplicities of infection was resistant to cleavage with *Hin*dIII, whereas digestion with *Eco*RI yielded a cluster of fragments 5.4 to 5.7 megadaltons (Mdal) in size. Cleavage with *Bam*HI gave a cluster of fragments 2.6 to 3.2 Mdal in size, plus two homogeneous, comigrating 1-Mdal fragments. One of the latter fragments contained the single *Eco*RI site approximately 65 base pairs from one end. Hybridization of in vitro labeled dDNA probe to *Eco*RI, *Hin*dIII, *Bam*HI, and *Hpa* I digests of nondefective HSV-1 DNA demonstrated that, in addition to the *S*-region terminal repeat, only one end of the *S* region was involved in the generation of this class of dDNA. Thus, the dDNA probe did not hybridize to either the *S* region 3.0-Mdal *Hin*dIII N fragment or a 3.0-Mdal *Bam*HI fragment of the adjacent 8.7-Mdal *Hin*dIII G fragment, but did hybridize to four *Bam*HI fragments of *Hin*dIII G (~5.7 Mdal). The cluster of 2.6- to 3.2-Mdal fragments obtained with *Bam*HI digestion of dDNA appears to represent a novel junction between the termination of dDNA adjacent to the 3.0-Mdal *Bam*HI fragment in *Hin*dIII G and the 2.0- to 2.3-Mdal *Bam*HI fragment terminal in HSV-1 DNA.

The DNA of herpes simplex virus type 1 (HSV-1) is a linear, double-stranded molecule with a mass of approximately 100 megadaltons (Mdal) (1, 6, 9). Evidence that the structural arrangement of the HSV genome consists of two unique segments, *L* and *S*, inverted about and separated by distinct terminally and internally inverted repetitions, was obtained from electron microscopy studies of intact self-annealed single-stranded DNA (14, 21, 22). Additional evidence for this structure was obtained from restriction endonuclease analyses of native DNA employing *Eco*RI, *Hin*dIII, *Hpa* I, *Bam*, and *Xba* I (3, 8, 16, 25). The presence of submolar amounts of restricted DNA fragments led to the conclusion that four possible permutations of the molecule occurred as a result of *L* and *S* inversion around the two sets of terminally and internally repeated sequences.

We have examined a portion of the HSV-1 *S* region and its adjacent repeated sequences, which are related to defective DNA (dDNA). The dDNA of HSV-1, generated by virus passaged at high multiplicites of infection (MOI), has been well characterized (2, 4, 23). For the Justin strain of HSV-1 the dDNA has been shown to consist of tandem repetitions of *S*-region sequences greater than 5 Mdal in complexity (4, 5). This dDNA is resistant to cleavage with *Hsu* I and *Hin*dIII, suggesting that it originates from a portion of the parental genome lacking these restriction sites. *Eco*RI cleavage yields two major cleavage fragments, 5.1 and 5.4 Mdal, each arising apparently from two different classes of defective molecules (4, 5). In contrast, dDNA from the KOS strain of HSV-1 yields a large number of fragments when cleaved with *Eco*RI (23), suggestive of multiple classes of dDNA. In our studies the dDNA of the Patton strain of HSV-1 is shown to be similarly sensitive to cleavage with *Eco*RI while resistant to *Hin*dIII cleavage. Moreover, by using in vitro labeled *Eco*RI-cleaved dDNA as a probe, sequences related to dDNA are physically mapped in one end of the *S* region 8.7-Mdal *Hin*dIII G fragment (16, 24) with *Bam*HI.

MATERIALS AND METHODS

Cells and viruses. The growth and maintenance of Vero cells (ATCC CCL81) have been described elsewhere (12). HSV-1 strain Patton (obtained from Electronucleonics Inc., Silver Spring, Md.) was propagated in Vero cells at low (0.10 to 0.03 PFU/cell) and at high (greater than 0.1 PFU/cell) MOI. For virus passages, Vero cells growing in T75 flasks (Falcon Plastics, Oxnard, Calif.) were infected and incubated at 37°C until 90 to 100% cytopathic effect occurred. The infected cells were frozen and thawed three times

and used as inoculum for one roller bottle (690 cm^2, Bellco, Vineland, N.J.) of Vero cells (approximately 10^8 cells per roller bottle). For subsequent virus passages, the harvest of one roller bottle was used as inoculum for 10 roller bottles. PO is used to designate HSV-1 continually passaged at low MOI, whereas a number indicates the appropriate passage at high MOI.

To isotopically label virus, [^{3}H]thymidine (20 μCi/ml) (6 Ci/mMol, New England Nuclear, Boston, Mass.) was added at 12 h postinfection, and the virus was harvested when the cells exhibited 90 to 100% cytopathic effect.

Isolation and purification of virus and DNA. The virus was concentrated from the supernatant fluids by polyethylene glycol precipitation and purified by either isopycnic banding in a linear Renografin gradient (12, 15) or sedimentation through a discontinuous sucrose gradient (6). The viral DNA was extracted and purified from HSV as described previously (12). Briefly, virus was lysed with 1% sodium dodecyl sulfate, incubated with 2 mg of autodigested Pronase (Calbiochem, La Jolla, Calif.) per ml for 3 h at 37°C, and then deproteinized with chloroform-isoamyl alcohol (24:1). The aqueous phase containing the DNA was adjusted to a density of 1.717 g/cm with crystalline cesium chloride (Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.), and the DNA was centrifuged to equilibrium in a Beckman type 30 or type 65 rotor at 24,000 rpm for 84 h at 20°C. The gradient was harvested by puncturing the tube with a 4 mm intradermic tube, and the DNA was located by radioactivity and/or UV absorption at 260 nm. The DNA-containing fractions were pooled and dialyzed at 4°C against 0.1 × SSC (SSC contained 0.15 M NaCl, 0.015 M sodium citrate, and 1 mM ethylenediaminetetraacetic acid [EDTA]).

Restriction enzyme analyses. The amount of *Eco*RI (Miles Laboratory, Elkhart, Ind.), *Hin*dIII, *Hpa*I, or *Bam*HI (Bethesda Research Laboratories, Bethesda, Md.) needed for complete digestion of 1 to 2 μg of DNA was predetermined by enzyme titration. The reaction mixtures contained: for *Eco*RI, 100 mM Tris-hydrochloride (pH 7.5)-50 mM NaCl-5 mM $MgCl_2$; for *Hin*dIII, 20 mM Tris-hydrochloride (pH 7.5)-60 mM NaCl-7 mM $MgCl_2$; for *Hpa* I, 30 mM Tris-hydrochloride (pH 7.5)-10 mM $MgCl_2$-6 mM KCl-5 mM 2-mercaptoethanol; for *Bam*HI, 20 mM Tris-hydrochloride (pH 7.5)-7 mM $MgCl_2$-2 mM 2-mercaptoethanol. Each reaction was stopped by the addition of EDTA (10 mM). The DNA was deproteinized with chloroform-isoamyl alcohol (24:1), precipitated with 2 to 3 volumes of ethanol in the presence of 0.3 M sodium acetate, and pelleted at 100,000 × *g* for 30 min. The pellet was suspended in 0.1× E buffer (E buffer contained 40 mM Tris, 10 mM sodium acetate, and 1 mM EDTA adjusted to pH 7.8 with acetic acid).

Gel electrophoresis and isolation of DNA restriction fragments. Gel electrophoresis was performed as described by Mulder et al. (11) in 30 by 15 by 0.4-cm gel slabs with 0.7% agarose. The DNA fragments were separated by electrophoresis on agarose at 60 V for 40 h at 4°C. The DNA bands were stained with ethidium bromide (0.5 μg/ml) and photographed by using a short-wave UV light source (Mineral Light model C51, Ultra Violet Product, Inc., San Gabriel, Calif.). Molecular weights were estimated relative to adenovirus, HSV-1, or simian virus 40 markers.

Large quantities (100 to 150 μg) of specific HSV-1 or dDNA fragments were obtained after endonuclease restriction and electrophoretic separation on vertical agarose gels. The stained bands were excised from the gel, and the DNA fragments were isolated by the freeze-squeeze method (20). By using this technique, 50 to 70% of the DNA was recovered intact.

Transfer of fragments from agarose gel to nitrocellulose membranes. The procedure for transferring DNA fragments to nitrocellulose membranes has been described by Southern (18). The DNA fragments in agarose gels were denatured by soaking the gel in 0.5 M NaOH-1.5 M NaCl for 30 min at room temperature. This was followed by a distilled water rinse and neutralization with 1.0 M Tris-hydrochloride (pH 7.2)-1.5 M NaCl for 40 min at room temperature. The DNA was transferred onto nitrocellulose membranes using 20× SSC as the eluting buffer. The efficiency of fragment transfer was monitored by UV examination of the agarose gel after restaining with ethidium bromide. The DNA containing nitrocellulose membrane was heated for 2 h at 80°C and pretreated as described (12). Hybridization was performed by placing the membrane in a chamber (described below) and adding the labeled probe in 3× SSC containing 0.5% sodium dodecyl sulfate and 20 μg of yeast tRNA per ml in a minimal volume sufficient to wet the membrane. The conditions for hybridization were 66°C for 20 to 24 h. After hybridization, the membrane was washed exhaustively in 3× SSC, dried, and exposed to Kodak SB54 X-ray film.

A chamber was custom made for each hybridization mixture. Three layers of parafilm were cut 1 mm longer and wider than the nitrocellulose membrane and placed on a siliconized glass plate. The glass surface, in contact with the parafilm, was coated with a thin layer of silicone vacuum grease. The chamber, containing the membrane and hybridization mixture, was covered with another siliconized glass plate and clamped with binder clips. The glass plates were sealed air tight by the action of heat (66°C) on the parafilm and silicone grease.

In vitro labeling of restriction fragments. The conditions for labeling restriction fragments with ^{32}P-deoxynucleotide triphosphates have been described by Maniatis et al. (10). Briefly, the reaction mixture contained in 100 μl: 50 mM Tris-hydrochloride (pH 7.8), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg of bovine serum albumin per ml, 0.5 to 1 μg of DNA, 180 pmol of two [^{32}P]dNTP, and 180 pmol of two unlabeled dNTP. The reaction was started by the addition of 2.8 U of *Escherichia coli* polymerase 1 (Boehringer Mannheim Biochemicals) and 10^{-8} μg of DNase I (DPFF, Worthington Biochemical Corp.). After 1 h at 15°C, the reaction was terminated by chloroform-isoamyl alcohol (24:1) extraction. The unincorporated nucleotides were removed by gel electrophoresis or ethanol precipitation. The specific activity of the product ranged from 1-10 × 10^7 cpm/μg.

RESULTS

Generation and characterization of defective HSV DNA. Defective HSV-1 Patton

was generated by passage of the PO virus stock at high MOI. The generation of dDNA was monitored at every fourth passage both by isopycnic centrifugation in CsCl and digestion of extracted viral DNA. The centrifugation analyses showed that with high MOI passages (P8, P12, and P16), new, higher density classes of DNA were detected relative to HSV-1 DNA marker (not shown). The *Eco*RI cleavage of PO DNA gave the expected profile for HSV-1 passaged at low MOI (Fig. 1) (7, 17). However, the *Eco*RI analyses of DNA from HSV passaged at high MOI (P4 to P16) showed additional fragments: two distinct minor fragments with molecular weights of 4.3 and 6.5 Mdal (Fig. 1) and a cluster of fragments ranging from 5.4 to 5.7 Mdal. The 4.3- and 6.5-Mdal fragments were not further characterized. Whereas PO *Hin*dIII-cleaved fragments were detected in P8 DNA, some of the DNA was resistant to cleavage with *Hin*dIII (Fig. 1), as has been found for Justin dDNA (4, 5). The concurrent appearance of new HSV-1 DNA density classes and *Eco*RI restriction fragments (Fig. 1) after virus passage at high MOI are characteristics of dHSV DNA (4, 23).

The structure of dDNA was examined further by digestion with *Bam*HI (Fig. 2). The disappearance of the 5.4- to 5.7-Mdal *Eco*RI dDNA fragments after digestion with *Bam*HI and the appearance of a new cluster of fragments, 2.6 to 3.2 Mdal, plus two 1-Mdal fragments (B_2 and B_3-R), indicated that several *Bam*HI cleavage sites existed in dDNA. In a separate experiment, *Bam*HI cleavage of isolated 5.4- to 5.7-Mdal *Eco*RI fragments yielded B_2, B_3-R, and the 2.6- to 3.2-Mdal cluster of fragments, indicating that the latter fragments were responsible for the

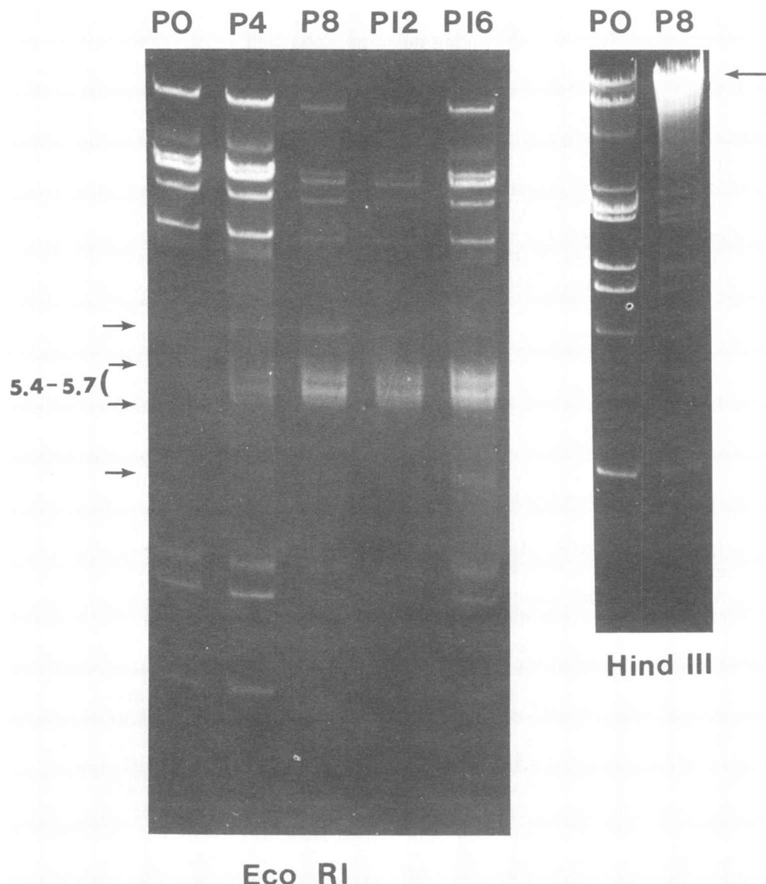

FIG. 1. *EcoRI restriction enzyme analysis of HSV DNA. DNA from HSV passaged at low (PO) and high (P4, P8, P12, P16) MOI purified by isopycnic centrifugation in cesium chloride was digested with EcoRI or HindIII. The molecular weights of minor, new DNA species (arrows) in the EcoRI digests are 4.3 and 6.5 Mdal, respectively.*

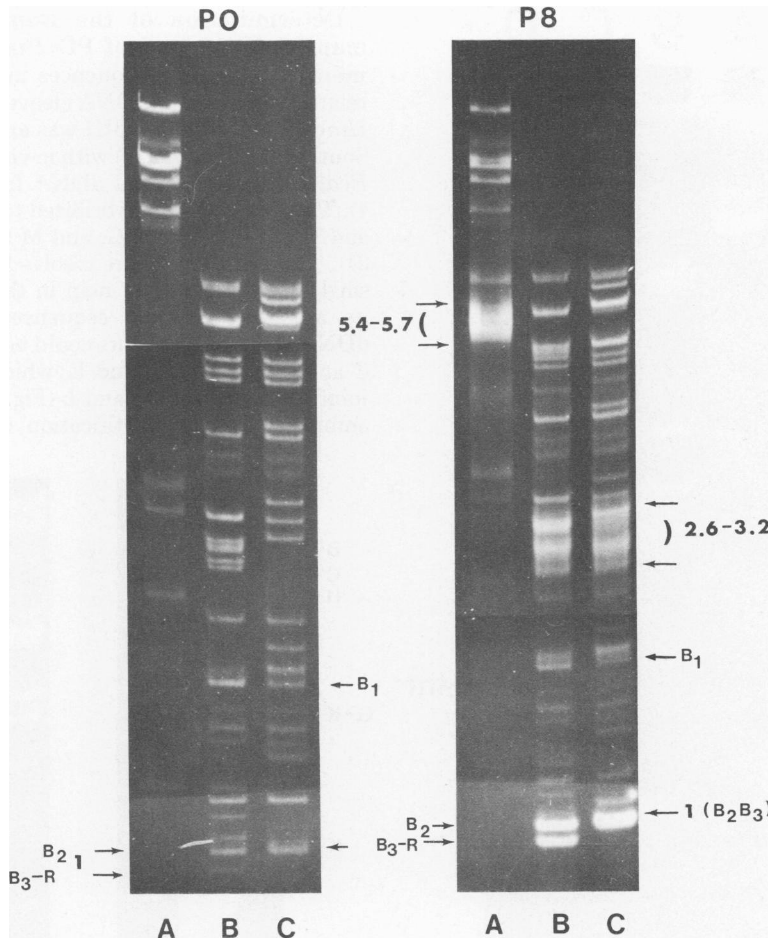

FIG. 2. *Endonuclease restriction analyses of PO and P8 HSV DNA. DNA from PO and P8 was restricted with endonucleases. (A) EcoRI; (B) EcoRI-BamHI; and (C) BamHI. The molecular weights of the dDNA species are indicated. A numerical convention was used for the BamHI fragments of HSV-1 because of the complexity of the digest (more than 26 fragments). B_2 and B_3 are comigrating BamHI fragments, one of which contains an EcoRI site and, when doubly cleaved, is designated B_3-R. From this and other analyses, fragments in PO which have electrophoretic mobilities of dDNA B_2 and B_3 (or B_3-R) are so indicated. The differences in the appearance of the 2.6- to 3.2-Mdal cluster between BamHI-EcoRI (B) and BamHI (C) cleavages of P8 is due to the contribution of PO fragments.*

heterogeneity in the *Eco*RI dDNA. Moreover, the two 1-Mdal dDNA fragments were equivalent in mobility and related by hybridization to two *Bam*HI fragments of PO DNA (B_2 and B_3-R of PO; Fig. 3) (data not shown), indicating that they were normal *Eco*RI-*Bam*HI cleavage products of PO DNA. Cleavage of the *S*-region terminal *Eco*RI K fragment (8, 16, 19, 25) with *Bam*HI (see Fig. 6) yielded the B_1 fragment indicated in PO and P8 DNA (Fig. 2). *Bam*HI digestion of P8 DNA produced a single 1-Mdal fragment (B_2, B_3), while two fragments were detected in the doubly cleaved (*Eco*RI-*Bam*HI) P8 DNA, suggesting that a single *Eco*RI site was present on only one of two comigrating 1-Mdal fragments. This was clearly demonstrated using high resolution polyacrylamide gels (Fig. 3) where the mobility of the *Bam*HI B_3 fragment increased after *Eco*RI digestion (B_3-R) and a new fragment (R) was detected in 20% acrylamide with an approximate size of 65 base pairs (based on the difference in mobility between B_3 and B_3-R). The two 1-Mdal *Bam*HI fragments, which comigrated in agarose (Fig. 3), were separated and inverted in mobility with respect to the *Eco*RI-sensitive fragment (i.e., $B_3 > B_2$) in 5% polyacrylamide. This inversion may be due to an effect of polyacrylamide on high guanine-cytidine (G-C)-containing DNA fragments, as has been observed for adenovirus DNA frag-

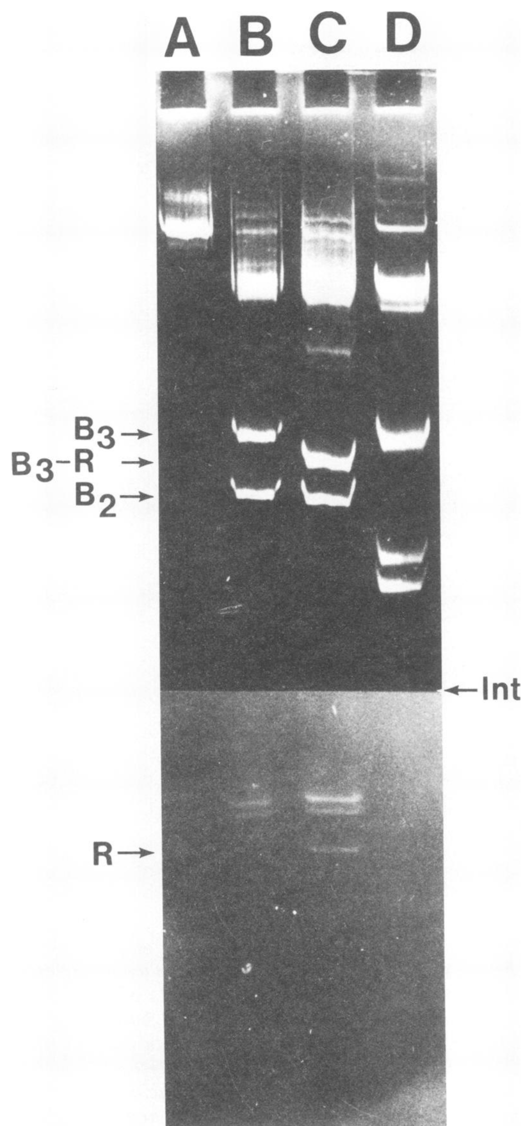

FIG. 3. *Polyacrylamide gel electrophoresis of endonuclease-restricted P8 dDNA. High MOI passaged viral DNA was restricted with (A) EcoRI, (B) BamHI, or (C) EcoRI-BamHI and subjected to electrophoresis on a discontinuous 5 and 20% polyacrylamide gel (arrow indicates the interphase). The EcoRI-HpaI cleavage products of SV40 (D) were used as molecular weight standards. The B_3 (or B_3-R) is identified by its EcoRI sensitivity (Fig. 2).*

ments (Carel Mulder, personal communication). At least two additional *Bam*HI fragments, approximately 0.1 Mdal in size, were also observed in the 20% acrylamide by ethidium bromide staining. These analyses (Fig. 3) were not used for absolute molecular weight determination because of possible G-C effects on the high G-C HSV-1 DNA versus the low G-C SV40 DNA markers.

Determination of the *Bam*HI physical map of dDNA and of PO *Hin*dIII G fragment. To map the sequences in HSV-1 DNA related to dDNA, PO DNA cleaved with *Eco*RI, *Hin*dIII or *Eco*RI-*Hin*dIII was annealed by the Southern technique (18) with in vitro ^{32}P-labeled *Eco*RI 5.4- to 5.7-Mdal dDNA fragments (Fig. 4). The dDNA probe hybridized to the *Eco*RI H and K and *Hin*dIII F, G, and M fragments (16, 24). These fragments are resolved in agarose as single components and map in the *S* region or its adjacent repeated sequences. The other dDNA positive fragments could be *Eco*RI B and C and *Hin*dIII B, C, and E, which map in the joint region between *S* and *L* (Fig. 5). A possible ambiguity in the identification of latter frag-

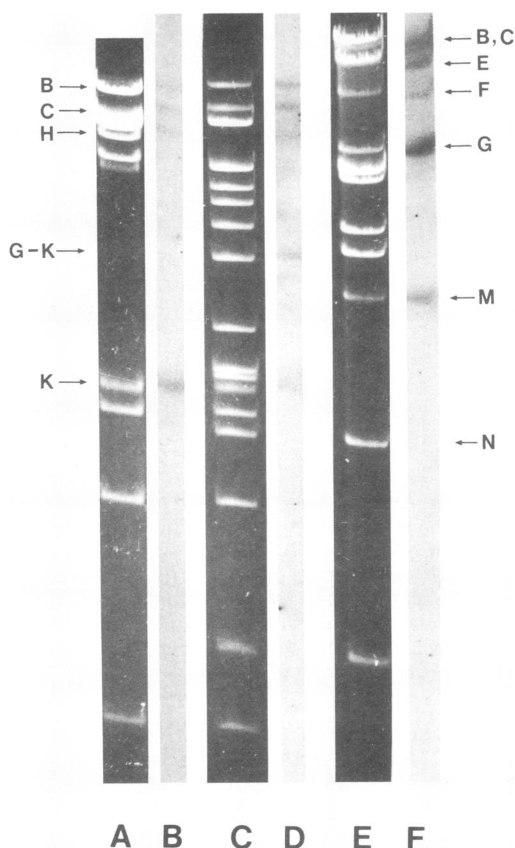

FIG. 4. *Determination of the PO HSV restriction fragments related to dHSV DNA. PO DNA was digested with EcoRI, EcoRI-HindIII and HindIII, fractionated by agarose gel electrophoresis (A, C, and E, respectively), and subjected to the Southern technique (18) using ^{32}P in vitro labeled 5.4- to 5.7-Mdal EcoRI-cleaved dDNA fragments as probe (B, D, and F, respectively). The fragments hybridizing with the dDNA probe are indicated and occur in the S region or in its adjacent repeated units as diagramatically presented in Fig. 5.*

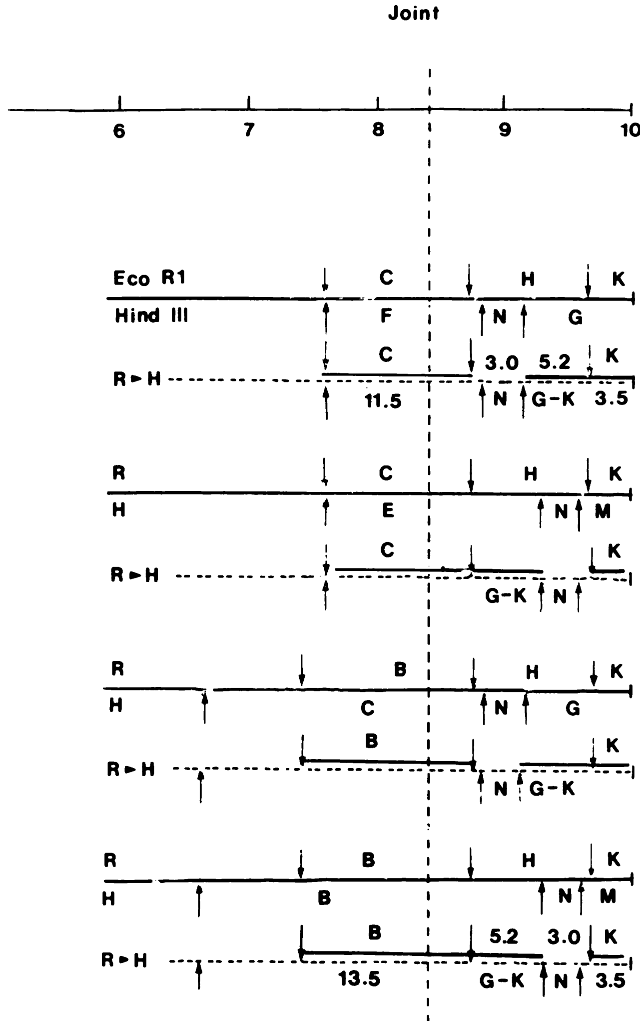

FIG. 5. *Schematic presentation of the S region EcoRI, HindIII, and double digest physical maps (according to Skare et al., 16). The EcoRI and HindIII fragments associated with the "joint" and S regions are shown with the four permutations of HSV-1 DNA (—). The doubly cleaved EcoRI-HindIII (R-H) permutations (-----) are shown beneath their corresponding permutation. The solid bars above the dotted lines correspond to the fragments that hybridize with the dDNA probe (Fig. 4).*

ments could arise because each comigrates with fragments from other regions of the genome (16, 24). However, evidence for dDNA hybridization with only *S*-region fragments was indicated from the *Eco*RI-*Hin*dIII double digest. The restriction fragment maps of this region (Fig. 5) show the fragments, which hybridized to dDNA in the double digest (R-H), as solid bars above the dotted line map. The largest fragments obtained with the *Eco*RI-*Hin*dIII digest corresponded to *Eco*RI B (13.5 Mdal) and C (11.5 Mdal) (16) and contained sequences corresponding to the terminal *Eco*RI K fragment plus a portion of the *L* region and the *L*-region repeated sequences. Since none of the *L*-region *Eco*RI-*Hin*dIII fragments hybridized to the dDNA probe (e.g., *Eco*RI E and F; 10.2 Mdal through the *Hin*dIII K 6-Mdal fragment), the dDNA-related sequences of the Patton strain originated only from the *S* region. The Justin strain dDNA has also been shown to map in the *S* region (5). These results also showed that the *S* region 3.0-Mdal *Hin*dIII N fragment did not hybridize with the dDNA probe (as it did with PO probe; data not shown), whereas both the adjacent *Hin*dIII G (8.7 Mdal) and the 5.2-Mdal *Hin*dIII G minus *Eco*RI K (G-K) fragment did. *Hin*dIII M (4.5 Mdal), containing the 3.5-Mdal *Eco*RI K fragment (16), also hybridized with the dDNA probe. It was not determined whether the small 1.0-

Mdal fragment generated by *Eco*RI digestion of *Hin*dIII M hybridized with dDNA since this fragment would still contain a portion of the *S*-region repeated sequence (9, 16, 24).

The hybridization of the 5.4- to 5.7-Mdal dDNA probe with the G-K fragment, but not with *Hin*dIII N, suggested that the dDNA was derived from only one end of the *S* region. To determine the extent of dDNA sequences in the *Hin*dIII G fragment, preparative amounts of the latter in the form of *Eco*RI K (K) and G-K were digested with either *Bam*HI for comparison to the dDNA cleavage (Fig. 2) or *Hpa* I for the location of the single *S* region *Hpa* I site (24). The digests were analyzed by the Southern technique (18) (Fig. 6) by using either in vitro ^{32}P-labeled *Eco*RI dDNA or in vitro ^{32}P-labeled PO DNA as probes. Partial digestion of G-K with *Bam*HI yielded five fragments (4.4, 3.0, 2.4, 1.2, and 1.0 Mdal) (Fig. 6C and D), whereas complete digestion yielded only the 3.0-, 1.2-, and 1.0-Mdal fragments (PO hybridization pattern Fig. 6E). Partial digestion of K with *Bam*HI yielded 2.0- to 2.3-Mdal fragments, which correspond in mobility to the B1 fragment indicated in the *Bam*HI digests of PO and P8 DNA (Fig. 2) and three fragments 1.0 to 1.2 Mdal in size. The 2.0- to 2.3-Mdal fragment and the two smaller 1.0-Mdal fragments were resistant to further digestion with *Bam*HI (Fig. 6E). By agarose electrophoretic analysis, B_2 in K and B_3-R in G-K had the same mobilities as the B_2 and B_3-R fragments in *Eco*RI-*Bam*HI-digested P8 DNA (Fig. 2). The K fragment was resistant to *Hpa* I digestion, whereas G-K yielded 3.2- and 2.0-Mdal fragments. The hybridization showed that the uncleaved G-K and K fragments hybridized to the dDNA probe and that all of the G-K and K *Bam*HI and *Hpa* I cleaved fragments hybridized with the PO DNA probe. In contrast, the G-K 3.0-Mdal *Bam*HI fragment and the 2.0-Mdal *Hpa* I fragment did not hybridize with the

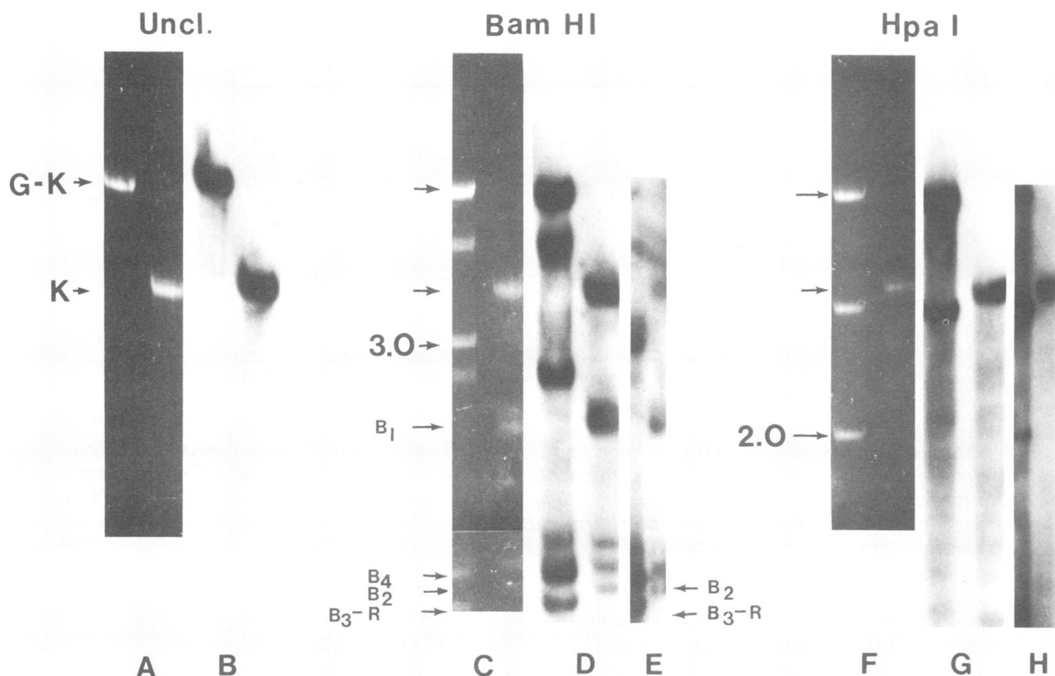

FIG. 6. *Characterization of dDNA sequences related to the BamHI and Hpa I cleavage fragments of the HindIII G-EcoRI K (G-K) and EcoRI K fragments of PO DNA. Purified PO DNA K and G-K fragments (A) were cleaved with BamHI (C) or Hpa I (F) restriction enzymes, separated electrophoretically on agarose gels, stained with ethidium bromide, and by Southern technique (18), hybridized either to ^{32}P-labeled 5.4- to 5.7-Mdal EcoRI dDNA (D and G) or ^{32}P in vitro labeled PO DNA (E and H). G-K (upper arrows in C and F) and its BamHI and Hpa I digests are on the left of each column, whereas the corresponding digests of K (lower arrow in C and F) are to the right. The fragments obtained with Bam HI (3.0 Mdal) and Hpa I (2.0), which hybridized to PO probe but not to dDNA probe, are indicated. B_1 and B_2 comigrate with B_1 of PO and P8 DNA and B_2 of dDNA respectively (Fig. 2), and are obtained with BamHI digestion of EcoRI K. B_3-R is the fastest migrating fragment obtained with BamHI digestion of G-K and corresponds in mobility to B_3-R of EcoRI-BamHI-digested dDNA (Fig. 2 and B_3-R in Fig. 3). B_1 of K and B_4 of G-K are not found in dDNA, but hybridize with dDNA probe.*

dDNA probe. Thus, 3.0 Mdal of the 8.7-Mdal *Hin*dIII G fragment was not present in dDNA sequences, whereas the remaining *Bam*HI fragments (~5.7 Mdal) all hybridized to dDNA.

DISCUSSION

The Patton strain dDNA fragments obtained after *Eco*RI digestion are heterogeneous in size with apparent mass ranging from 4.3 to 6.5 Mdal. However, the majority of the molecules clustered between 5.4 and 5.7 Mdal (Fig. 1). The origin of the 4.3- and 6.5-Mdal fragments is unknown. KOS strain dDNA appears to be more heterogeneous with respect to both the number and size of *Eco*RI fragments obtained and its distribution in CsCl (23). However, some similarity exists between both strains in the cluster of fragments observed between 5 and 6 Mdal. In contrast to the heterogeneity of both the Patton and KOS *Eco*RI dDNA digests, only two fragments (5.1 and 5.4 Mdal) are obtained from *Eco*RI digestion of the Justin dDNA. Each fragment appears to be derived from a distinct species of dDNA (4, 5). The heterogeneity differences of *Eco*RI-digested dDNA derived from the three HSV-1 strains could be a strain-dependant phenomenon; however, it is also possible that other factors could influence heterogeneity (e.g., host cell or MOI).

From these studies, DNA sequences of Patton HSV-1 related to dDNA were identified, and the indicated linear arrangement of the *Bam*HI fragments in both the PO *Hin*dIII G DNA fragment and dDNA is given in Fig. 7. These physical maps are based on: (i) the demonstration that *Bam*HI digestion of the 5.4- to 5.7-Mdal *Eco*RI cluster of fragments yields only B_2, B_3-R, and a 2.6- to 3.2-Mdal cluster of fragments and (ii) the likely assumption that the linear arrangement of sequences in a single unit of dDNA is the same as that in the related PO DNA sequences. The 3.5-Mdal *Eco*RI K fragment has been shown to be the *S* region terminal fragment in HSV-1 DNA (8, 16, 19, 25) and the terminal fragment of Justin dDNA (5). Cleavage of *Eco*RI K with *Bam*HI (Fig. 6) produced the B1, 2.0- to 2.3-Mdal fragment as well as two 1-Mdal fragments. The increase in amounts of both B_1 and *Eco*R1 K in P8 compared with that of PO DNA (Fig. 2A and C) suggests that B_1 is terminal in both PO and dDNA. The B_1 fragment is also considered to be part of a novel junction formed with the B_4 fragment (Fig. 7), giving rise to the 2.6- to 3.2-Mdal cluster of *Bam*HI dDNA fragments (Fig. 2). The fact that B_1 strongly hybridizes with the 5.4- to 5.7-Mdal *Eco*RI dDNA probe (Fig. 6D), but is absent from *Bam*HI digests of the latter, is suggestive evidence for its presence in the novel junction. The variability in the size of the B_1 fragment may be similar to the variability observed in the L-region terminal repeat (16) or may be due to incomplete cleavage between B_1 and one of the smaller (~0.1 Mdal) *Bam*HI fragments. Only B_2 is detected in dDNA, which also suggests that the larger 1-Mdal *Bam*HI fragment of *Eco*RI K (Fig. 6E) represents incomplete cleavage of B_2 and one of the ~0.1-Mdal *Bam*HI fragments. A fragment slightly larger than B_2, perhaps corresponding to this incomplete cleavage, has occasionally been detected in P8 DNA (P8, Fig. 2C). At least two 0.1-Mdal *Bam*HI fragments (Fig. 3) must be adjacent to B_2 to account for the 1.2-Mdal fragment detected in partial *Bam*HI digests of the *Eco*RI K fragments (Fig. 6), but their exact position is unknown. Complete *Bam*HI digestion of the G-K fragment yielded 3.0-, 1.2 (B_4)- and 1.0 (B_3-R)-Mdal fragments, whereas partial digestion gave two additional fragments, 4.4 and 2.4 Mdal, representing incom-

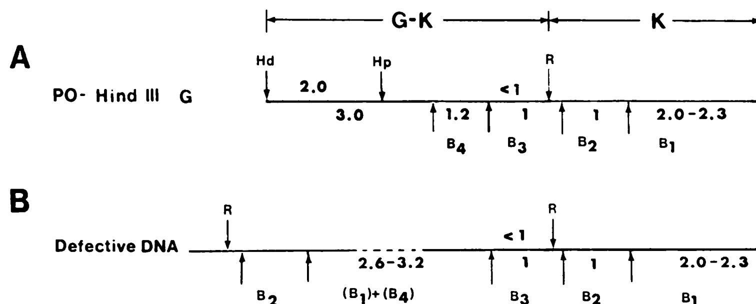

FIG. 7. *Diagrammatic presentation of BamHI sites in the HindIII G fragment (G-K and K) and dDNA of HSV-1. (A) Linear arrangement of BamHI (B) sites in the PO HindIII (Hd) G fragment are shown in relation to the published EcoRI (R) and Hpa I (Hp) sites (8, 16, 24, 25). (B) Arrangement of dDNA BamHI sites compared with those of the HindIII G fragment. (-----) Indicates the variable region in dHSV probably resulting from the fusion of the terminal B_1 fragment and the termination of the dDNA in the B_4 fragment. Parentheses indicate possible variable amounts of B_1 and B_4 fragments appearing in the joint.*

plete cleavages of 3.0 Mdal + B_4 and B_4 + B_3-R, respectively. The position of these fragments (Fig. 7) was based on the loss of the B_4 site in dDNA, the presence of the *Eco*RI K site in B_3-R, and the lack of dDNA-related sequences in the 3.0-Mdal fragment. The 3.0-Mdal fragment is thus adjacent to the 3.0-Mdal *S* region *Hin*dIII N fragment (16, 24), which also lacks dDNA related sequences (Fig. 4 and 6). Similarly, the 2.0-Mdal *Hpa* I fragment of G-K did not hybridize with the dDNA probe and was also placed terminal in G-K. This *Hpa* I site corresponds to the position assigned by Wilke (24) for the generation of *S*-region terminal 6.9-Mdal *Hpa* I G fragment.

The 2.6- to 3.2-Mdal cluster of *Bam*HI fragments in dDNA (Fig. 2) would therefore represent the termination of dDNA sequences within the B_4 fragment and the recombination of these sequences with all or part of the terminal B_1 fragment. The size distribution in the cluster could arise from either a variable termination point in the B_4 fragment or variations in the size of B_1. Electron microscopy studies of Justin dDNA suggest that the variability is derived from the *S* region (4, 5), and, as with Justin dDNA (5), the 5.4- to 5.7-Mdal *Eco*RI component of Patton-defective genomes also originates from the *S* region.

Virtually all of the Patton dDNA sequences can be accounted for in the related G-K and K sequences. This relationship does not preclude the possibility of other regions giving rise to defective genomes (see Schroeder et al. [13], HSV-1 ANG dDNA lacks *Eco*RI cleavage sites) but suggests that an origin of replication (4, 5, 16) and a structural predisposition to dDNA formation exists within fragments B_1 through B_4. It is possible that dDNA is generated in two steps. First a recombination event occurs between sequences in B_1 and B_4, which gives rise to a replicating circle. Second, linear repeating units are synthesized from the circle with their size selected for by virion packaging requirements. In any case the linear arrangement of these fragments together with their convenient 1- to 3-Mdal size should be useful for identifying the specific sequences associated with the recombination event and dDNA formation.

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