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# Class I Defective Herpes Simplex Virus DNA as a Molecular Cloning Vehicle in Eucaryotic Cells†

JIM W. BARNETT, DEBORAH A. EPPSTEIN, AND HARDY W. CHAN*
*Institute of Bio-Organic Chemistry, Syntex Research, Palo Alto, California 94304*

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Defective herpes simplex virus type 1 genomes are composed of head-to-tail tandem repeats of small regions of the nondefective genome. Monomeric repeat units of class I defective herpes simplex virus genomes were cloned into bacterial plasmids. The repeat units functioned as replicons since both viral and convalently linked bacterial plasmid DNA replicated (with the help of DNA from nondefective virus) when transfected into rabbit skin cells. Recombinant plasmids were packaged into virions and were propagated from culture to culture by infection with progeny virus. Replication was evidently by a rolling circle mechanism since plasmid DNA was present in a high-molecular-weight form in transfected cells. Circular recombinant plasmid DNA replicated with a high degree of fidelity. In contrast, linear plasmid DNA underwent extensive deletions of both viral and bacterial sequences when transfected into rabbit skin cells. Derivative plasmids, a fraction of the size of the parental plasmid, were rescued by transforming *Escherichia coli* with DNA from the transfected rabbit skin cells. These plasmids functioned as shuttle vectors since they replicated faithfully in both eucaryotic and procaryotic cells.

The herpes simplex virus type 1 (HSV-1) genome, a double-stranded linear DNA molecule of approximately 160 kilobases (kb), is composed of two covalently joined DNA segments, L and S (34, 35). Both segments contain unique sequences, $U_L$ and $U_S$, which are flanked by inverted repeats designated $TR_L$ and $IR_L$ or $TR_S$ and $IR_S$, respectively. All four of the repeats terminate with a single copy or multiple copies of an approximately 500-base-pair repeat, the *a* sequence, which mediates inversions of L and S to form four isomers of the genome (27, 28).

Serial undiluted passage of HSV-1 in cultured cells at a high multiplicity of infection generates defective viruses which can replicate only with the help of nondefective viruses (3). DNA isolated from defective viruses is similar in size to that of nondefective viruses (1, 12, 44) but is far less complex genetically (2, 32). Two classes of defective HSV (dHSV) which have markedly different genomes have been described (36, 40, 45). Class I dHSV genomes are derived mostly or entirely from the S segment of viral DNA and typically include all the repeat sequences and a variable amount of contiguous $U_S$ sequences (11, 13, 17, 22, 23, 25). The genomes of class II dHSV, on the other hand, contain a stretch of $U_L$ sequences covalently linked to a small portion of repeat sequences, including the *a* sequence, from the S segment (6, 22). In both class I and class II dHSV, head-to-tail concatemers of monomeric repeat units form genome-length molecules which are encapsidated into infectious virions (14). Digestion of defective genomes with suitable restriction endonucleases releases the monomers from the concatemeric structure.

Vlazny and Frenkel (43) recently demonstrated that monomers from class I dHSV genomes contain all *cis* functions required for DNA replication and encapsidation. Cotransfection of cultured cells with these monomers and helper DNA from nondefective HSV-1 resulted in the generation of concatemers of the seed DNA which was encapsidated into infectious virions (43, 44). Subsequent reports show that foreign DNA covalently linked to class II dHSV genome monomers (15, 39) or DNA fragments from the S component of nondefective genomes (41, 42) are amplified along with viral DNA in eucaryotic cells which are cotransfected with DNA from nondefective virus. We now report that class I dHSV genome monomers can also be utilized as a molecular cloning vehicle for amplifying foreign DNA in eucaryotic cells. In addition, we

† Contribution no. 164 from the Institute of Bio-Organic Chemistry, Syntex Research, Palo Alto, Calif.

have compared the stability of circular recombinant plasmids and linearized plasmids in transfected cells.

Our results demonstrate that circular chimeric plasmid DNA, in the presence of helper viral DNA, replicated with good fidelity in eucaryotic cells to form concatemers of the original chimeric structures. The high-molecular-weight concatemers were in turn encapsidated into infectious virions. Linear recombinant plasmids, generated by restriction enzyme cleavage, also replicated in the presence of helper DNA. However, extensive deletions (and possibly rearrangements) occurred. Deletions and rearrangements were much less evident when smaller derivative plasmids were cleaved at sites which are probably close to important *cis* functions for replication and encapsidation. The structure of monomeric plasmids retrieved from eucaryotic cells has shed light on the mechanism by which the linear molecules circularize before replication.

## MATERIALS AND METHODS

**Cells and virus.** Human epidermoid cells (HEp-2) and monkey Vero cells were purchased from the American Type Culture Collection. Rabbit skin cells were a gift from L. McLaren (University of New Mexico). All cell lines were grown in Eagle minimal essential medium (Flow Laboratories, Inc.) supplemented with calf or fetal calf serum and maintained by biweekly passages.

Stock HSV-1 (strain F) was grown in HEp-2 cells and titrated in Vero cells (9). Defective virus was obtained by serially passaging HSV-1(F) in HEp-2 cells at 1:2 dilutions. The initial infection was a 10 PFU per cell. Virus inocula were prepared by disrupting infected cells (2 days postinfection) mechanically with a type B Dounce homogenizer.

**Isolation of virion and infected cell DNA.** HSV-1(F) virion DNA was purified from cytoplasmic nucleocapsids as described by Kieff et al. (24). Infected cell DNA was prepared by lysing whole cells in 0.5% sodium dodecyl sulfate–2% sarkosyl at 60°C and extracting with phenol and chloroform.

**Preparation of radioactively labeled probes.** HSV-1 virion DNA (from the ninth serial passage) containing a high proportion of defective genomes, plasmid pBR328 DNA, and recombinant plasmid pDHD-6 DNA were radioactively labeled in vitro with [$^{32}$P]dCTP by a modification of the nick translation method described by Rigby et al. (33). Specific activities of the probes ranged from $20 \times 10^6$ to $30 \times 10^6$ cpm/μg of DNA.

**Analysis of restriction endonuclease fragments.** All restriction endonucleases used in these experiments were purchased from Bethesda Research Laboratories, Inc. Reaction conditions were as specified by the supplier. Restriction fragments were separated by electrophoresis on 0.7% agarose gels (20). Restriction fragments were then denatured in situ (19) and blotted onto nitrocellulose filters by the method of Southern (38) as modified by Jeffreys and Flavell (21). Blotted nitrocellulose filters were soaked for 2 h at 60°C in 3×

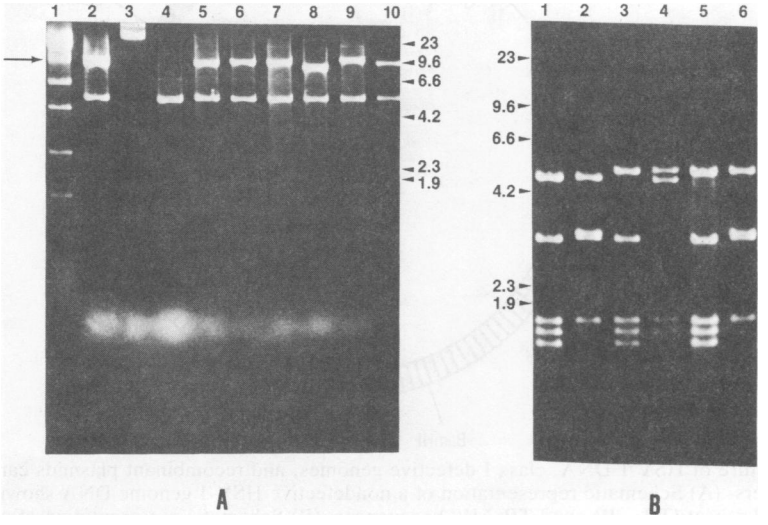


FIG. 1. Restriction endonuclease analysis of recombinant plasmids which carry HSV defective genome monomers. (A) *Eco*RI-digested recombinant plasmids (lanes 2, 5 through 10), virion DNA containing a high proportion of defective genomes (lane 1), an uncleaved recombinant plasmid (lane 3), and *Eco*RI-cleaved pBR328 (lane 4). The arrow marks the position of resulting monomers. Numbers on the right designate positions and size (in kb) of *Hin*dIII-cleaved λ DNA fragments. (B) *Eco*RI-*Bam*HI double digests of selected recombinant plasmids (lanes 1, 3, and 5) juxtaposed with *Bam*HI single digests of the same clones (lanes 2, 4, and 6). DNA preparations analyzed in lanes 1 and 2, 3 and 4, 5 and 6 of panel B are the same as those in lanes 5, 6, and 7 of panel A, respectively. Sizes (in kb) of molecular weight markers are shown to the left.

SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) containing 10× Denhardt solution (7) and denatured calf thymus DNA (100 μg/ml). The filters were hybridized against radiolabeled probes (0.5 μg per filter) as previously described (29). Filters were washed (10 min each wash) at 60°C once in 3× SSC, three times in 3× SSC containing 10× Denhardt solution, twice in 1× SSC containing 0.1% sodium dodecyl sulfate and 100 μg of denatured calf thymus DNA per ml, and once in 0.1× SSC. After air drying, filters were exposed to X-ray film (Kodak XAR-2) to detect hybridized probe.

**Cloning of defective genome monomers.** HSV-1 virion DNA from the ninth serial passage containing a high proportion of defective genomes was digested with *Eco*RI. The digest was ligated to plasmid pBR328 (37) which had been cleaved with *Eco*RI and treated with calf intestine alkaline phosphatase. Competent *Escherichia coli* HB101 cells were transformed with the ligation mixture by standard calcium chloride-heat

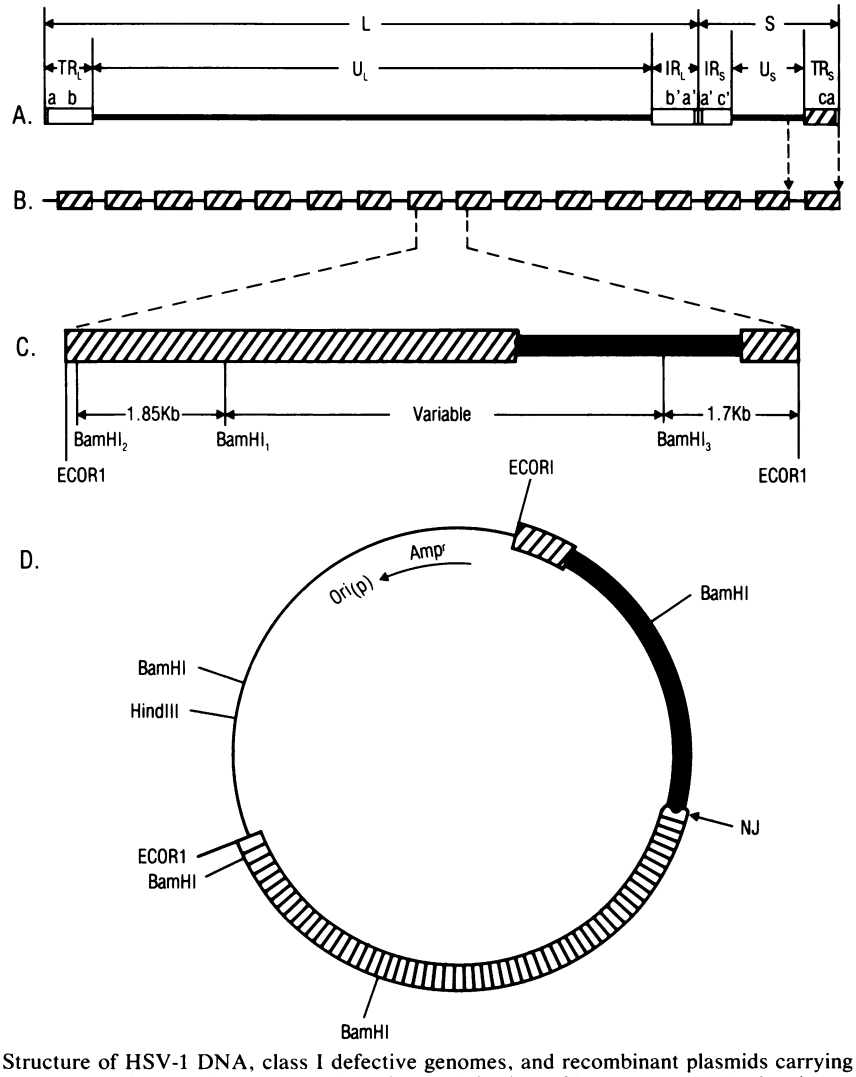


FIG. 2. Structure of HSV-1 DNA, class I defective genomes, and recombinant plasmids carrying defective genome monomers. (A) Schematic representation of a nondefective HSV-1 genome DNA showing unique ($U_L$, $U_S$) and inverted repeat ($TR_L$, $IR_L$ and $TR_S$, $IR_S$) sequences. (B) Schematic representation of class I defective genomes. Repeat (crosshatched) and unique (solid line) sequences from which defective genomes originated are indicated. (C) Monomers generated by *Eco*RI cleavage of class I defective genomes. *Bam*HI cleavage sites are shown. Closely spaced *Bam*HI sites are represented by a single line at *Bam*$HI_1$ and *Bam*$HI_2$ (8). DNA which originated from $TR_S$ (crosshatched area) and $U_S$ (thick solid line) are represented. (D) Structure of recombinant plasmid carrying defective genome monomers. *Bam*HI, *Hin*dIII, and *Eco*RI restriction sites are shown. DNA originating from $TR_S$ (crosshatched) and $U_S$ (thick solid line) are represented. $Amp^r$ and Ori(p) mark the bacterial plasmid β-lactamase gene and origin of replication, respectively. NJ designates the novel junction formed by the fusion of $TR_S$ and $U_S$ in the formation of class I defective genomes.

shock procedures (26). Ampicillin-resistant, chloramphenicol-sensitive colonies were selected, and supercoiled recombinant plasmid DNAs were prepared (4, 5).

**Transfection of rabbit skin cells.** Rabbit skin cells (50 to 80% confluent in 25-$cm^2$ flasks) were cotransfected with DNA from nondefective HSV-1 (F) virions (0.5 to 1 μg) and recombinant plasmid DNAs (10 to 100 molar excess) by the calcium phosphate method first described by Graham and Van der Eb (18) and subsequently modified by Wigler et al. (46). Briefly, DNA was precipitated overnight with calcium phosphate at room temperature. Salmon sperm DNA was included as carrier DNA during precipitation. The concentration of 10 μg/ml resulted in maximal transfection efficiency as measured by plaque formation (unpublished observation). The calcium phosphate-DNA precipitate was placed on cell monolayers for 30 min at room temperature. An equal volume of Eagle minimal essential medium (supplemented with 10% calf serum) was then added to each culture. After incubation at 37°C for 5 h, the monolayers were washed with Eagle minimal essential medium, and fresh medium supplemented with 10% calf serum was added to each culture. When herpesvirus cytopathic effect had spread throughout the culture (4 to 6 days after transfection), cells were harvested by scraping and were either lysed (by Dounce homogenization) to prepare virus inocula for infecting cultures or saved for DNA extractions.

## RESULTS

**Molecular cloning of HSV-1 defective genome monomers.** Defective HSV-1 genomes were generated by serially passaging HSV-1(F) in human HEp-2 cells at high multiplicities of infection. After three passages, defective genomes were detected by the emergence of novel 9 to 10-kb *Eco*RI restriction fragments which are characteristic of class I dHSV (14). The overabundance of *Bam*HI fragments that map within $IR_S$ and $TR_S$ confirmed that the novel DNA fragments were from class I defective genomes (data not shown). The proportion of viral genomes that were defective increased in subsequent passages (data not shown).

Virion DNA containing a high proportion of dHSV genomes was cleaved with restriction endonuclease *Eco*RI and ligated to *Eco*RI-cleaved plasmid pBR328. *E. coli* HB101 was transformed by the ligated mixture, and recombinant clones were selected. Several dHSV genome monomers, which differed slightly in size, were obtained (Fig. 1A). This heterogeneity was attributed to a single *Bam*HI fragment that ranged in size from 5 to 6 kb in the various clones (Fig. 1B, lanes 1, 3, and 5). Since this fragment spanned the novel junction formed by the fusion of $TR_S$ and $U_S$ sequences (Fig. 2), variations in size among the different monomers is probably due to different locations of the novel joints. Restriction endonuclease site mapping (see Fig. 8) confirmed that the cloned fragments were from class I dHSV genomes which were derived from the right end (prototype orientation) of the S component of the viral genome. Therefore, these monomers are similar to those previously described by Denniston et al. (8).

**Amplification of recombinant plasmids in eucaryotic cells.** Vlazny and Frenkel (43) previously demonstrated that class I dHSV genome monomers replicate, with the help of DNA from nondefective virus, in eucaryotic cells forming concatemers which are packaged into infectious virions. This implies that class I dHSV genome monomers, which contain all *cis* functions necessary for DNA replication and packaging, could potentially be developed into a eucaryotic cloning vehicle.

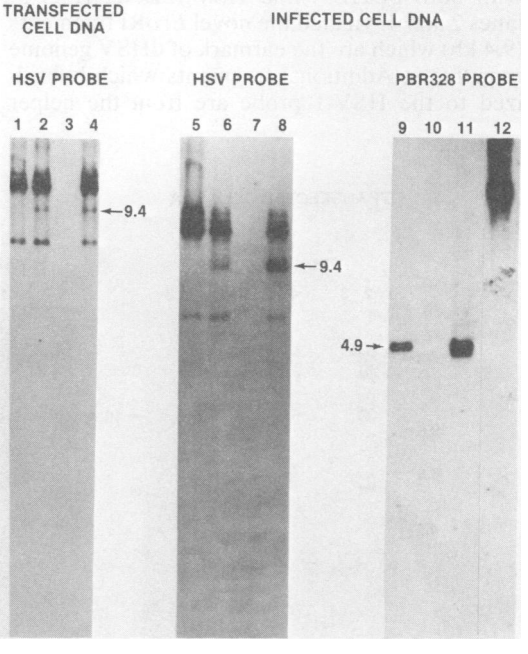


FIG. 3. Amplification of supercoiled plasmid pDHD-6 in rabbit skin cells. DNA preparations from rabbit skin cells which had been transfected with pDHD-6 and helper DNA (lanes 2 and 4) or from cells infected with virus harvested from these transfected cells (lanes 6, 8, 9, and 11) were digested with *Eco*RI and analyzed by Southern blot hybridization. Uncleaved DNA from these infected cells is also shown for comparison (lane 12). Control cultures were transfected with helper virion DNA only (lane 1) or infected with virus originating from these control cells (lane 5). In addition, *Eco*RI-cleaved DNAs from cells transfected with pDHD-6 only (lane 3) or mock infected with lysates from these cells (lanes 7 and 10) are shown. Labeled probes were either HSV-1 DNA (lanes 1 through 8) or pBR328 (lanes 9 through 12). Arrows mark the positions of the 9.4-kb dDNA monomer and the 4.9-kb pBR328.

Thus, the first set of experiments was designed to determine whether the recombinant plasmids containing both dHSV genome monomers and pBR328 sequences would replicate and be packaged into virions. One of these chimeric plasmids, designated pDHD-6, was selected for these studies. The pBR328 DNA, covalently linked to class I dHSV genome monomer in plasmid pDHD-6, served as a convenient marker for monitoring the fate of foreign DNA linked to the viral replicon.

Rabbit skin cells were cotransfected with HSV-1(F) DNA and plasmid pDHD-6. Control cells received either pDHD-6 DNA or helper DNA alone. DNA from the transfected cells was cleaved with appropriate restriction endonucleases and analyzed by Southern blot hybridization using either $^{32}$P-labeled HSV-1 DNA or plasmid pBR328 as probe. Only cells transfected with both pDHD-6 and HSV-1 DNA (Fig. 3, lanes 2 and 4) yielded the novel *Eco*RI fragments (9.4 kb) which are the earmark of dHSV genome monomers. Additional fragments which hybridized to the HSV-1 probe are from the helper DNA since cells transfected with helper alone yielded the identical fragments (Fig. 3, lane 1). As expected, pDHD-6 DNA, in the absence of helper DNA, failed to replicate in rabbit skin cells (Fig. 3, lane 3). These results are consistent with the hypothesis that the replication of dHSV genome monomers require *trans* acting factors (e.g., DNA polymerase, etc.) which must be supplied by a nondefective genome. Furthermore, these results affirm that the detected *Eco*RI 9.4-kb fragments shown in Fig. 3, lanes 2 and 4, were due to recombinant plasmid DNA replication and not to residual input DNA.

To determine whether recombinant DNA was encapsidated into infectious virions, uninfected rabbit skin cells were infected with cell lysates from transfected cells. Since naked DNA molecules have extremely low infectivity, infection resulting from cell lysates indirectly implies that the replicated DNA molecules were encapsidated into virions. *Eco*RI-digested DNA from the infected cells was analyzed by Southern blot hybridization. When $^{32}$P-labeled HSV-1 DNA was used as the hybridization probe, the novel 9

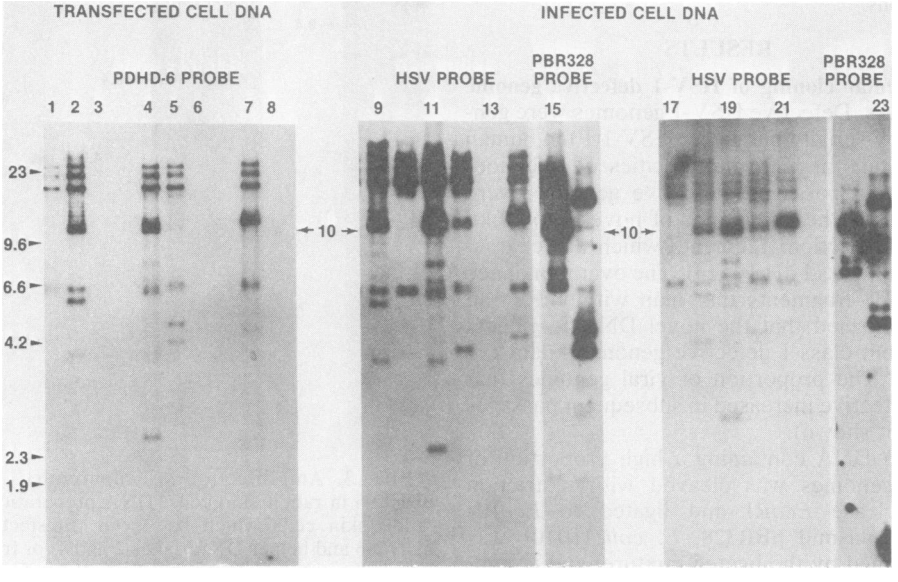


FIG. 4. Amplification of linear plasmid pDHD-6 in rabbit skin cells. DNAs isolated from rabbit skin cell cultures were cleaved with *Eco*RI and analyzed by Southern blot hybridization with radiolabeled hybridization probes as indicated. Autoradiograms of these blots are shown. The rabbit skin cells were cotransfected with helper HSV-1 DNA and *Eco*RI-cleaved pDHD-6 (lane 2), *Hin*dIII-cleaved pDHD-6 (lanes 4 and 5), or *Eco*RI-digested virion DNA containing a high proportion of class I defective genomes (lane 7). Control cells were transfected with the same preparations but without helper DNA (lanes 3, 6, and 8) or with helper DNA only (lane 1). Viruses from transfected cells were passaged twice in fresh rabbit skin cells (first passage, lanes 9 through 16; second passage, lanes 17 through 23). Virus inocula were from cells transfected with helper DNA only (lanes 10 and 17) or helper DNA and *Eco*RI-digested pDHD-6 (lanes 9 and 18), *Hin*dIII-cleaved pDHD-6 (lanes 11, 12, 15, 16, 19, 20, 22, and 23), or *Eco*RI-cleaved defective genomes (lanes 14 and 21). Control cells were mock infected with lysates from cells transfected with *Hin*dIII-cleaved pDHD-6 but no helper DNA (lane 13). Arrows mark the positions of novel *Eco*RI fragments of approximately 10 kb. Numbers to the left mark positions and sizes of *Hin*dIII-cleaved λ DNA fragments used as molecular size markers.

to 10-kb *Eco*RI fragments characteristic of class I dHSV genome monomers were detected in DNA isolated from cells that were infected with virus produced in cells previously cotransfected with both HSV-1 helper and pDHD-6 DNAs (Fig. 3, lanes 6, 8). As expected, these DNA fragments were absent from cells infected with virus from lysates of cells transfected with helper DNA alone (Fig. 3, lane 5) and from cells mock infected with lysates from cells transfected with pDHD-6 alone (Fig. 3, lane 7). Southern blot hybridization with radiolabeled pBR328 as the hybridization probe yielded consistent results. No hybridization was evident in cells infected with the lysate of cells transfected with only pDHD-6 DNA (Fig. 3, lane 10). A single *Eco*RI DNA fragment of 4.9 kb was detected in cells infected with virus obtained from cells cotransfected with helper and pDHD-6 DNAs (Fig. 3, lanes 9 and 11). The 4.9-kb fragment agreed with the expected size of intact linear pBR328, indicating that these foreign sequences, covalently joined to the HSV-1 DNA, replicated and were packaged into virions without undergoing deletions or recombination. Vlazny and Frankel (43) previously demonstrated that transfection with dHSV genome monomers resulted in the generation of high-molecular-weight concatemers of monomeric units. To ascertain whether pDHD-6 also formed high-molecular-weight molecules in infected cells, uncleaved cellular DNA was also analyzed. The hybridization results indicate that all pBR328-related sequences were associated with the high-molecular-weight DNA (Fig. 3, lane 12). These results are consistent with replication by a rolling circle mechanism which results in the formation of head-to-tail concatemers of pDHD-6 DNA. The concatemeric DNA is subsequently encapsidated into viral particles.

**Replication of linear pDHD-6.** Supercoiled pDHD-6 replicated efficiently to form high-molecular-weight concatemeric DNA in eucaryotic cells, presumably by a rolling circle mechanism. To determine whether linearized pDHD-6 could also replicate by the same mechanism, transfection experiments similar to those described above were performed with *Hin*dIII linearized pDHD-6 DNA. The linearized molecules did indeed replicate since DNA fragments that hybridized to the recombinant plasmid probe were observed in cells transfected with linear pDHD-6 and helper DNA (Fig. 4, lanes 4 and 5), but not in control cells transfected with helper DNA or linearized pDHD-6 alone (Fig. 4, lanes 1 and 6, respectively). In contrast to the results obtained with supercoiled pDHD-6 DNA, replication of *Hin*dIII-cleaved chimeric DNA (Fig. 4, lanes 4 and 5) resulted in the generation of multiple molecular species which were different from input DNA. In fact, two basically identical but independent experiments involving transfection with linear pDHD-6 DNA resulted in dissimilar results (Fig. 4, lane 4 versus lane 5). The various species of progeny DNA molecules were encapsidated into virions since cells infected with virus from those transfected cells yielded DNA restriction fragments (Fig. 4, lanes 11 and 12, 15 and 16, 19 and 20, 22 and 23) that were identical to those obtained in the initial transfection experiment (Fig. 4, lanes 4 and 5). Restriction enzyme analysis of first-passage progeny DNAs with *Bgl*II, an enzyme that does not cleave pDHD-6 DNA, yielded a number of DNA fragments that hybridized to the $^{32}$P-labeled pBR328 probe (Fig. 5, lanes 1 and 5). The very-high-molecular-weight chimeric plasmid DNA is probably concatemers of linearized pDHD-6, resulting from replication by a rolling circle mechanism. The origin of the smaller *Bgl*II fragments which were absent from undigested DNA samples (data not shown) remains unclear. Acquisition of *Bgl*II restriction sites indicates that recombination between linear pDHD-6

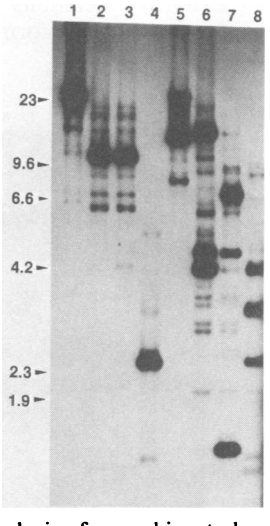


FIG. 5. Analysis of recombinant plasmid DNA after replication in rabbit skin cells. Rabbit skin cells were infected with HSV that originated from cells transfected with *Hin*dIII-cleaved plasmid pDHD-6 and helper DNA. DNA was prepared from these cells (lanes 1 through 4 are from one experiment; lanes 5 through 8 are from an independent but similar experiment). The DNA preparations were cleaved with *Bgl*II (lanes 1 and 5), or with *Eco*RI (lanes 2 and 6) or were double digested with *Eco*RI and *Hin*dIII (lanes 3 and 7) or *Eco*RI and *Bam*HI (lanes 4 and 8). These digests were analyzed by Southern blot hybridization with plasmid pBR328 as the radiolabeled hybridization probe. Numbers to the right designate positions and sizes (in kb) of *Hin*dIII restriction fragments of λ DNA used as molecular weight markers.

DNA and some other species of DNA (e.g., helper or carrier DNA) had occurred.

Cleavage of infected cell DNA with *Eco*RI endonuclease also generated many DNA fragments (Fig. 5, lanes 2, 6) with sizes ranging from 20 to 2 kb. Only one of the prominent DNA fragments corresponded to the size of linear plasmid pBR328 (4.9 kb) (Fig. 5, lane 6). Formation of heterogeneous molecular species of a different size from the input DNA implies that multiple alterations of the original recombinant plasmid had occurred without affecting the capability of the molecules to replicate and become encapsidated. Formation of prominant *Eco*RI fragments of approximately 10 kb (versus linear pDHD-6 DNA of 14.5 kb) which remained hybridizable to both pBR328 (Fig. 5, lane 2) and HSV-specific probes (data not shown), but no longer contained a *Hin*dIII cleavage site (Fig. 5, lane 3), indicates that sequences proximal to the site of linearization (i.e., the *Hin*dIII site) were deleted. Double-enzyme digestion with *Eco*RI and *Bam*HI yielded a single prominent fragment of 2.4 kb (Fig. 5, lane 4) which hybridized to pBR328. This fragment, which did not appear to hybridize to the viral probe (data not shown), confirms that extensive deletions of bacterial plasmid DNA occurred during formation of the major replicating form of the chimeric plasmid. The number and sizes of DNA fragments generated by cleavage with single- or double-restriction enzyme digestions with *Eco*RI, *Hin*dIII, and *Bam*HI (Fig. 5, lanes 6 through 8) implies that a combination of deletions, rearrangements, and recombinations occurred. Nonetheless, formation of a limited number of discrete DNA bands suggests that there are a few preferred forms of rearranged molecules which are still competent for replication.

**Rescue of chimeric plasmid DNA.** To examine the structure of replicated linear pDHD-6 in greater detail, we utilized the plasmid pBR328 sequences within the progeny DNA molecules to transform *E. coli* and retrieve chimeric plasmids. This requires only that the origin of replication for the plasmid molecule, as well as the gene coding for antibiotic resistance, are not deleted. DNA from cells infected with virus, which originated from rabbit skin cells transfected with linearized pDHD-6 and helper DNA, was cleaved with *Eco*RI or *Kpn*I. After ligation, the recircularized DNA molecules were used to transform *E. coli* HB101 cells. Ampicillin-resistant clones were selected. Several recombinant plasmids were retrieved in spite of the overall low efficiency of transformation. The retrieved

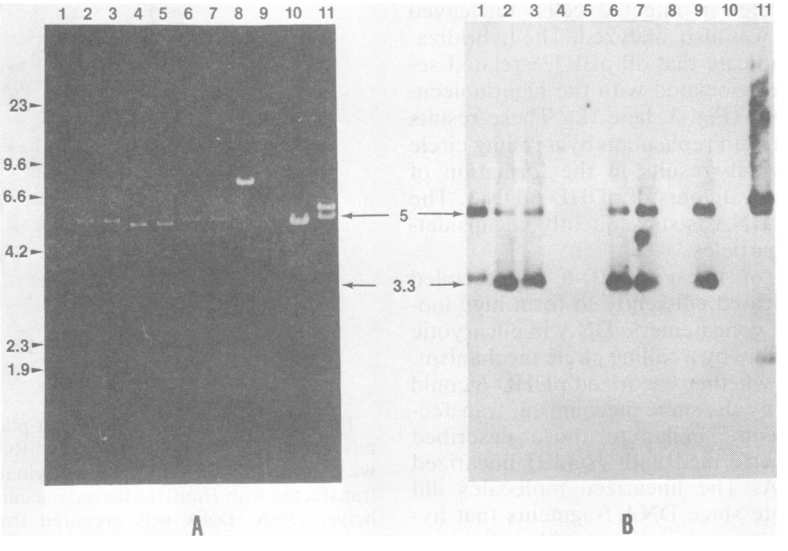


FIG. 6. Rescue of recombinant plasmids from rabbit skin cells. DNA from cells infected with virus from cell transfected with *Hin*dIII-cleaved pDHD-6 and helper DNA (the same DNA preparation analyzed in Fig. 5, lanes 1 through 4) was cleaved with *Eco*RI or *Kpn*I. The DNA was treated with DNA ligase and used to transform *E. coli* cells to ampicillin resistance. (A) *Bam*HI restriction fragments of plasmid DNA from these transformants were separated by electrophoresis on agarose gels and visualized by ethidium bromide staining. Plasmids rescued from DNA cleaved with *Eco*RI are shown in lanes 1 through 3 and 6. Plasmids from *Kpn*I-cleaved DNA are shown in lanes 7 and 9. *Bam*HI-digested pBR328 (lanes 4, 5, and 10) and pDHD-6 (lane 11) are included for comparison. A recombinant plasmid which did not contain viral DNA is shown in lane 8. Arrows mark the 5- and 3.3-kb fragments. Numbers to the left show the position and size (in kb) of molecular weight markers. (B) The electrophoresis gel was analyzed by Southern blot hybridization with labeled HSV DNA as a probe.

plasmids were characterized and found to be quite similar in structure (Fig. 6, lanes 1, 2, 3, 6, 7, and 9). Each of the plasmids contained two *Bam*HI restriction sites. The larger *Bam*HI fragment coelectrophoresed with the 5-kb *Bam*HI fragment of the parental pDHD-6 DNA (Fig. 6, lanes 11). Both of the *Bam*HI fragments from the retrieved plasmids hybridized to both viral (Fig. 6B, lanes 1, 2, 3, 6, 7, and 9) and the pBR328 probes (data not shown). However, the 3.3-kb fragments hybridized to the pBR328 probe only slightly (data not shown). Results of *Hin*fI digestion (Fig. 7) confirmed the structural similarities among the retrieved plasmids and showed unequivocally that both viral and bacterial plasmid sequences were lost from the original pDHD-6 DNA (Fig. 7, lanes 1 through 5 and 7 versus lanes 8 and 9).

One of the retrieved plasmids, designated $pCR_6$, was cleaved with a combination of restriction enzymes. A preliminary restriction enzyme map consistent with this data was constructed. Comparison of the restriction map of $pCR_6$ with the equivalent map of parental pDHD-6 (Fig. 8) reveals that a large proportion of HSV $TR_S$ sequences and a substantial part of

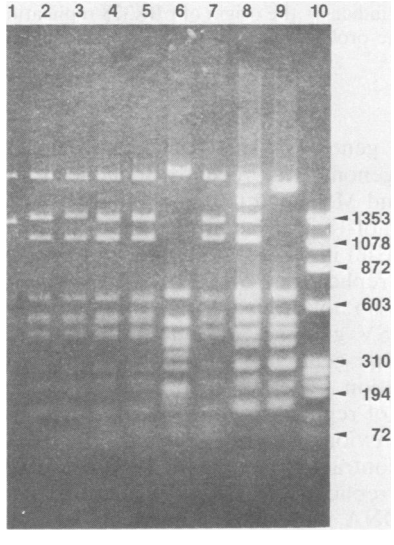


FIG. 7. Analysis of rescued plasmids by *Hin*fI restriction endonuclease cleavage. Plasmids rescued from rabbit skin cells (Fig. 6) were digested with *Hin*fI. Restriction fragments were separated by electrophoresis on a 1.4% agarose gel and visualized by ethidium bromide staining (lanes 1 through 5 and 7). For comparison, *Hin*fI digests of plasmids pDHD-6 (lane 8) and pBR328 (lane 9) are included. A recombinant plasmid which did not contain viral DNA is shown in lane 6. *Hae*II digest of $\phi$X 174 phage DNA was run as molecular weight markers (lane 10), and the numbers to the right show the sizes (in kb) of several of these fragments.

plasmid pBR328 sequences on the pDHD-6 molecule were lost during the formation of $pCR_6$. The original novel junction created by the fusion of the *a* sequence with $U_S$ sequences was, however, unaltered. It is evident, therefore, that the *a* sequence was not involved in the collapse of the parental plasmid to form $pCR_6$.

**Replication of retrieved plasmids in eucaryotic cells.** To determine whether the retrieved plasmids, which had lost a substantial portion of the viral DNA sequences, were still competent for DNA replication and encapsidation in eucaryotic cells, they were cleaved with either *Eco*RI or *Kpn*I and cotransfected with helper HSV-1 DNA into rabbit skin cells. The virus was passaged by inoculating cell lysates from transfected cells into fresh rabbit skin cell cultures. Southern blot analysis of DNA isolated from these infected cells verified that all the retrieved plasmids retained the *cis* functions required for both DNA replication and encapsidation (Fig. 9, lanes, 1, 2, and 4 through 11). Moreover, the bulk of progeny DNA retained the structure of input DNA. It is of interest to note, however, that anomalous fragments were more abundant in cells transfected with *Kpn*I-cleaved plasmids (Fig. 9, lanes 1, 4, 6, 8, and 10) than in cells transfected with *Eco*RI-cleaved DNA (Fig. 9, lanes 2, 5, 7, 9 and 11). Overall, the smaller linear DNA molecules (e.g., $pCR_6$) replicated with a higher degree of fidelity than did the larger linear pDHD-6. This, however, may be because the *Eco*RI and *Kpn*I cleavage sites are closer to the essential *cis* functions for replication and encapsidation in the smaller molecules than the *Hin*dIII cleavage site in pDHD-6 is. Deletions and rearrangements of these sites in $pCR_6$ are, therefore, likely to result in molecules incapable of undergoing amplification. This may also explain the greater heterogeneity of progeny DNA from *Kpn*I-cleaved plasmids since the *Eco*RI cleavage site is closer to the origin of replication for dHSV DNA molecules (41).

## DISCUSSION

The results presented here demonstrate that a chimeric plasmid composed of bacterial plasmid pBR328 DNA covalently linked to a class I defective HSV-1 genome monomer can replicate and be encapsidated into virus particles when cotransfected with nondefective helper DNA into eucaryotic cells. Both plasmid and HSV-1 DNA sequences were found in a high-molecular-weight form which, upon cleavage with appropriate restriction endonucleases, yielded fragments identical in size to the original input DNA. The formation of head-to-tail concatemers by the recombinant plasmid DNA is consistent with a rolling circle DNA replication mechanism. The encapsidated DNA can be

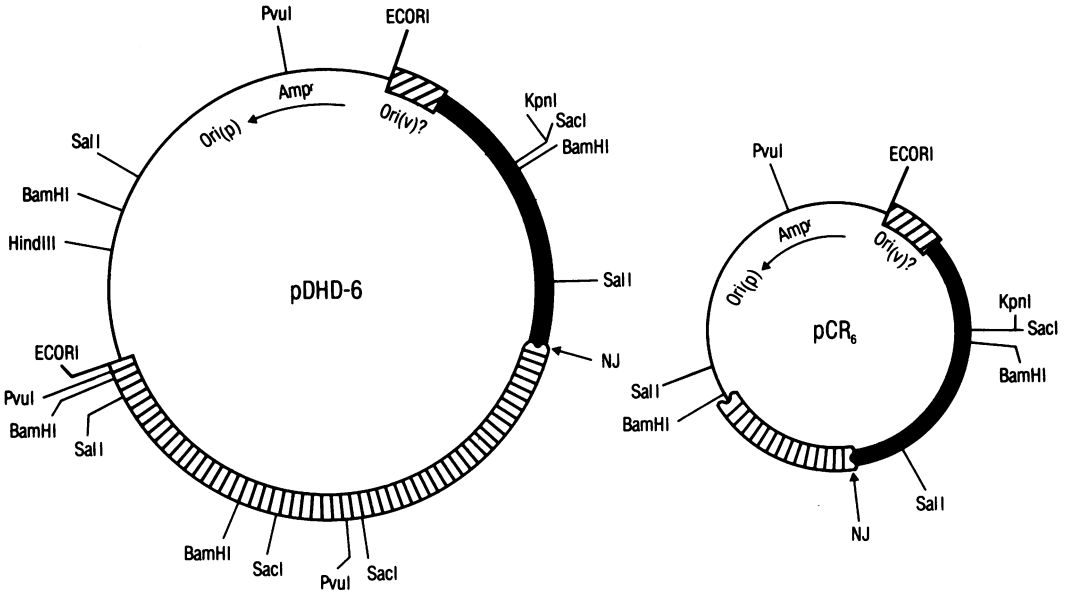


FIG. 8. Physical maps of plasmids pDHD-6 and $pCR_6$. Restriction sites of recombinant plasmids were located by determining the sizes of restriction endonuclease cleavage products of end-labeled restriction fragments isolated from the plasmids. The parental recombinant plasmid, pDHD-6, was constructed by ligation of the class I defective genome monomer into the *Eco*RI site of plasmid pBR328. A derivative plasmid, $pCR_6$, was rescued from infected rabbit skin cells by transforming *E. coli* and selecting ampicillin-resistant colonies. Crosshatched areas and thick lines indicate HSV DNA from $TR_S$ and $U_S$ regions, respectively. Thin lines represent pBR328 DNA. NJ marks the novel junction of defective genomes. Ori(p) indicates the origin of pBR328 replication, and $Amp^r$ designates the plasmid β-lactamase gene. Ori(v) shows the probable region of viral DNA containing an HSV-1 origin of replication.

propagated by serial passage of progeny virus. Thus, class I dHSV genome monomers are suitable for use as molecular cloning vehicles for the introduction of foreign DNA into eucaryotic cells as well as for the subsequent amplification of the foreign DNA in the absence of selection.

Herpesvirus replicons may have advantages over some of the existing eucaryotic vectors such as those derived from papovavirus replicons (10) which are limited in that only relatively small foreign DNA inserts can be encapsidated and therefore amplified. Much larger inserts would be expected to be encapsidated into HSV virions which can package the 160-kb genome. The vector itself would be only a small fraction of this size. Moreover, since genes carried by dHSV genomes are overexpressed in infected cells (12, 14, 31), genes spliced onto the recombinant vector may also be overexpressed and, therefore, easily monitored.

Recently, Stow (41) as well as Mocarski and Roizman (28) localized an origin of HSV replication to a region of the genome that maps entirely within $TR_S$-$IR_S$ sequences. These same sequences are contained within both pDHD-6 and $pCR_6$ [designated as Ori(v) in Fig. 8] and may function as the origin of replication for class I dHSV genomes. This particular region of the HSV genome was recently sequenced by Murchie and McGeoch (30) and shown to contain a stretch of 18 adenine or thymine residues which may be an important component of the origin of DNA replication. Since class II defective genomes do not originate from this same region of the HSV genome, it is doubtful that class II defective genomes utilize the same origin of replication. The existence of more than one origin of replication in HSV DNA is consistent with previous electron microscopic studies (16).

In contrast to supercoiled plasmid pDHD-6, which replicated without loss of integrity, linearized DNA molecules underwent deletions, recombinations, and rearrangements. It is likely that only molecules which circularized and retained intact essential *cis* functions could be amplified. Once formed, these rearranged molecules replicate stably and can be propagated from culture to culture by infecting with progeny virus. Linear pDHD-6 (14.5 kb) gave rise predominantly to progeny DNA with *Eco*RI restriction fragments that were approximately the same size as defective genome monomers generated by serial passages of HSV at a high multiplicity of infection. This suggests that 9 to 10 kb

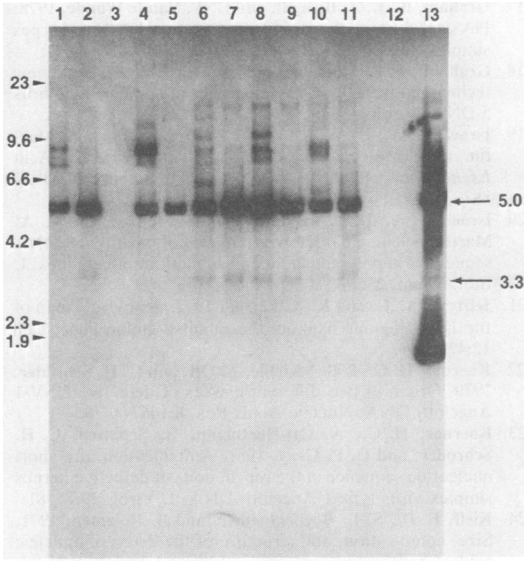


FIG. 9. Replication of rescued plasmids in rabbit skin cells. Rabbit skin cells were cotransfected with linearized plasmids that had been retrieved from infected cells (Fig. 6) and with helper virion DNA. Control cultures were transfected with plasmid only or with helper and plasmid pBR328. Virus preparations harvested from these cells were inoculated into fresh rabbit skin cells, and the resulting DNA was digested with *Bam*HI and analyzed by Southern blot hybridization with pBR328 as the labeled hybridization probe. Autoradiograms of DNAs from cells infected with lysates of cells transfected with helper DNA and recombinant plasmids cleaved with *Kpn*I (lanes 1, 4, 6, 8, and 10) or *Eco*RI (lanes 2, 5, 7, 9, and 11) are shown. Control cells infected with lysates of cells transfected with helper DNA and pBR328 (lane 12) or with rescued plasmid only (lane 3) are also shown. *Bam*HI-digested pDHD-6 (lane 13) was included for comparison. Numbers to the left mark the positions and sizes (in kb) of molecular weight markers. Arrows to the right designate the 5- and 3.3-kb fragments of recovered plasmids.

is a preferred size for class I monomers. Nevertheless, smaller chimeric molecules, such as the 8.3-kb recombinant plasmid, $pCR_6$, were also amplified. The larger 10-kb monomer was not retrieved, presumably because either the bacterial plasmid origin of replication or the ampicillin resistance gene had been deleted.

The retrieved plasmids retained the sequences expected of a molecule with their biological properties, (i.e., the viral and bacterial plasmid origins of replication, the ampicillin resistance gene, and the *a* sequences which are probably essential for viral packaging of replicated molecules [44]). The novel junction (referring to the junction formed by the fusion of *a* sequence to $U_S$ sequences) in the deleted DNA molecules apparently was not different from that of the original found in defective genomes (Fig. 8), indicating that the *a* sequences were not involved in the in vivo deletion event which formed circular plasmid $pCR_6$. Formation of the linkage between viral and bacterial plasmid DNA probably did not require a high degree of sequence homology for the recombination event to take place. This observation is similar to those of Wilson et al. (47). These investigators constructed chimeric molecules containing the simian virus 40 genome and bacterial plasmid DNA that was too large to be packaged into virions. Efficient replication and packaging of these molecules did occur, however, owing to deletions of dispensible sequences within transfected cells. By sequencing the novel junctions created by the deletion, these authors concluded that only a minimal degree (1 to 4 base pairs) of homology or none at all was required for DNA joining. Eucaryotic cells, evidently, possess the enzymatic machinery to join DNA sequences efficiently even if they possess no or very little homology. A similar mechanism was probably involved in the formation of circular molecules from linearized pDHD-6 plasmid.

Since $pCR_6$ and the other retrieved chimeric plasmids are shown to retain the capacity to replicate in eucaryotic cells, they can be used as shuttle vectors to amplify foreign DNA in both eucaryotic and procaryotic cells.

The stability of linear molecules can be affected by the length of the DNA and the location of the ends with respect to HSV-1 sequences. The smaller plasmid $pCR_6$ replicated with fewer alterations than larger DNAs. The greater tendency of *Kpn*I-cleaved $pCR_6$ DNA molecules, compared with the *Eco*RI cleaved molecules, to form anomalous fragments is probably a result of the *Eco*RI cleavage site being closer to the viral origin of replication than the *Kpn*I site (Fig. 8). Deletions or recombinations near the termini of *Eco*RI-cleaved molecules are, therefore, less likely to result in molecules that are capable of replication than are comparable alterations of *Kpn*I-cleaved molecules.

Since larger linear molecules are unstable in transfection experiments, circular cloning vectors are more useful when cloning large DNA fragments. Nevertheless, linear cloning vehicles could still be useful, particularly if the termini are near important *cis* functions so that only molecules that are minimally altered would be amplified.

The ability to alter dHSV genome monomers by using molecular cloning techniques to insert, delete, or mutate specific viral sequences provides an approach to understanding the replication and packaging of the HSV genome. In addition, cloning of HSV viral genes in these

vectors will yield new information concerning HSV gene structure, function, and regulation.


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