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Genetics

Cloning of reiterated and nonreiterated herpes simplex virus 1 sequences as *Bam*HI fragments

(DNA cloning/herpesviruses/marker transfer/restriction enzyme mapping)

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ABSTRACT **Over 95% of the herpes simplex virus type 1 (strain F) DNA sequences have been cloned as *Bam*HI fragments in the pBR322 plasmid. With one exception, all of the cloned fragments have the same electrophoretic mobilities and restriction enzyme cleavage sites as do the authentic fragments derived from the *Bam*HI digests of the viral genome. The exception is the *Bam*HI B fragment mapping at the right end of L component in the prototype arrangement of the DNA. Thus, a small deletion mapping near the left end of the fragment was present in two independently derived plasmids. Included in the collection of plasmids are several clones containing DNA sequences that span the junction between the L and S components of the virus DNA. Several plasmids containing the junction fragment were found to be sufficiently stable to permit the preparation of large amounts of the DNA fragment for fine-structure mapping of the restriction enzyme cleavage sites. Preliminary studies on one cloned fragment (*Bam*HI G) have shown that it is biologically active in marker rescue of a temperature-sensitive mutation and in transfer of a plaque morphology marker.**

The herpes simplex virus (HSV) genome is a linear, double-stranded DNA molecule of molecular weight 96×10^6. Two features of the HSV genome are of particular interest. First, it consists of two covalently linked components, L and S, comprising 83% and 17% of the total DNA, respectively. Each of the components consists of unique sequences bracketed by inverted repeats (1). Each of the reiterated sequences of the L component, designated *ab* and *b'a'*, contains 6% of the DNA whereas each of those of the S component, designated *a'c'* and *ca*, contains 4.3% of the DNA (2). The terminal *a* sequences are arranged in the same orientation at both ends of the molecule and contain less than 400 base pairs each (3, 4). The *a* sequences at the junction of the L and S components and at the termini may be present in one to several copies (4). An unusual property of HSV DNA is that the L and S components can invert relative to each other; thus, DNA extracted from virions consists of four populations, differing in the orientation of L and S components (5). Although a model accounting for the isomerization of the DNA has been proposed, the molecular events underlying the inversion of L and S components are unknown (6). Another interesting feature of HSV DNA is that certain regions of the genome defined by restriction endonuclease cleavage sites are heterogeneous; thus, specific *Bam*HI and *Kpn* I fragments derived from progeny of plaque-purified stock and mapping outside the *ab* and *ca* reiterated sequences appear to vary in size (ref. 7; unpublished data).

Central to the understanding of the mechanism of isomerization of HSV DNA is the fine structure of the DNA sequences at or near the junction of the L and S components. Similar arguments can be made for the variable regions of the genome. Although various regions of the genomes have been cloned (8–10), most of the genome and especially DNA fragments spanning the junction of L and S components have not been cloned preparatory to a fine structure analysis. In this paper we report on the cloning, in the pBR322 plasmid, of most of the genome including the fragments spanning the junction.

MATERIALS AND METHODS

Cells and Viruses. HEp-2 and Vero cells were used for the preparation and titration of HSV-1 stocks, respectively. Rabbit skin cells originally obtained from J. McLaren were used for transfection of cells with viral DNA. The procedures for the cultivation of virus and cells were published elsewhere (11).

The source and properties of HSV-1(F) and HSV-1(mP) strains have been published elsewhere (12, 13). HSV-1(mP)-tsHA1 is a temperature-sensitive mutant produced by cotransfection of cells with the intact HSV-1(mP) DNA and a restriction endonuclease fragment mutagenized *in vitro*. The efficiency of plating at 39°C relative to that at 33.5°C is 5×10^{-6}. Marker rescue experiments have shown that the mutation maps between 0.384 and 0.388 on the physical map of HSV-1 DNA in the prototype management (unpublished data). HSV-1(mP)J7 *syn*− is a plaque morphology mutant; in contrast to HSV-1(F), which causes cells to clump (*syn*+), this mutant fuses cells into polykaryocyte (*syn*−). HSV-1(mP)J7 *syn*− was obtained by cotransfection of cells with intact HSV-1(mP) DNA and an *in vitro* mutagenized fragment carrying the thymine arabinoside (1-β-D-arabinofuranosylthymine) resistance marker ($AraT^r$). Marker transfer experiments have shown that the J7 *syn*− mutation maps between 0.337 and 0.388 on the physical map of HSV-1 DNA in the prototype arrangement (unpublished data).

DNA. HSV-1(F) DNA was prepared from virions accumulating in the cytoplasm of infected HEp-2 cells and purified by equilibrium density centrifugation in sodium iodide gradients (5, 14). Plasmid pBR322 (15) was extracted from bacteria by the technique of Guerry *et al.* (16) and centrifuged to equilibrium in cesium chloride/ethidium bromide gradients (17). Candidate recombinant plasmids were analyzed after phenol extraction of a lysate (18).

Construction of Recombinant Plasmids. HSV-1(F) and pBR322 DNAs were digested to completion with *Bam*HI in 10 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/50 mM NaCl/1 mM di-

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Abbreviations: HSV, herpes simplex virus; NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate (standard saline citrate).

thiothreitol. To ensure complete inactivation of the *Bam*HI, the reaction mixtures were heated at 65°C for 10 min and then treated with diethyl pyrocarbonate (19). A 20-μl ligation reaction mixture contained 0.2 μg of digested pBR322 DNA, 0.5 μg of digested HSV-1(F) DNA, and 1.5 units of T4 DNA ligase (Pabst Laboratories) in 66 mM Tris·HCl, pH 7.6/6.6 mM $MgCl_2$/10 mM dithiothreitol/0.5 mM ATP (15). The ligation reaction was carried out at 12°C for 16 hr.

The recipient strain C600SF8 [C600 rk− mk− *rec*B⁻ C⁻ *lop*-11 *lig*⁺ (20)] was transformed with ligated DNA by the calcium shock method (21). Ampicillin-resistant bacteria were selected and screened for sensitivity to tetracycline.

Colony Hybridization. Colony hybridization was done by a modification of the method of Grunstein and Hogness (22). The colonies were transferred from agar plates onto Schliecher & Schuell BA85 nitrocellulose paper (23). The filters were placed on Whatman 3MM paper soaked in 0.5 M NaOH. After 7 min, the filters were placed for 1 min each successively on two stacks of 3MM paper soaked in 1 M Tris·HCl (pH 7.4). The filters were then placed for 5 min on a stack of Whatman paper soaked in 1.5 M NaCl/0.5 M Tris·HCl, pH 7.4, and then rinsed in 2× standard saline citrate (NaCl/Cit; 0.15 M NaCl/0.015 M sodium citrate), dried, and baked at 80°C for 2 hr. Before hybridization, the filters were incubated at 66°C for 3–12 hr in modified Denhardt's solution (0.025% bovine serum albumin, 0.025% Ficoll/0.025% polyvinylpyrrolidone, and 6× NaCl/Cit). For hybridization, the filters were incubated at 66°C for 24 hr in sealed plastic bags containing the radioactive probe, 30% formamide, 0.5% sodium dodecyl sulfate, and 50 μg of calf thymus DNA per ml in modified Denhardt's solution. The hybridization probe consisted of a HSV-1 restriction endonuclease DNA fragment extracted from agarose gels as described (24). The fragments were sonicated extensively and labeled with ^{32}P by the exchange reaction (25) of T4 polynucleotide kinase (Pabst Laboratories). Unreacted [γ-^{32}P]ATP was removed by chromatography on Sephadex G-50. After hybridization, the filters were rinsed three times in 2× NaCl/Cit at room temperature, once in 60% hybridization buffer at 63°C for 2 hr, and again three times with 2× NaCl/Cit at room temperature. Autoradiographic images of the dried filters were made on Dupont Cronex film with the aid of intensifying screens.

Hybridization of Plasmids. Radioactive plasmid DNA was prepared from the crude small-scale preparations of DNA (18). The plasmids were digested with *Hpa* II. The magnesium in the reaction mixture was removed by adding EDTA, and NaOH was added to 1 M. The alkaline reaction mixture was boiled for 10 min to degrade the large amounts of RNA in the crude preparations. The solution was neutralized with acetic acid and diluted 1:3 with water. The DNA was then precipitated with ethanol and end-labeled by the phosphate exchange reaction of T4 polynucleotide kinase (25). After chromatography on Sephadex G-50, the labeled DNA was hybridized to DNA immobilized on nitrocellulose by the method of Southern (26), as described above.

Marker Transfer and Marker Rescue with HSV-1 DNA Fragments. The procedure involves cotransfection of rabbit skin cells with intact DNA (which serves as a recipient) and individual restriction endonuclease fragments (which serve as donors of specific sequences or markers). Details of the experimental procedures were published elsewhere (24).

RESULTS

Digestion of HSV-1(F) DNA with *Bam*HI produces approximately 40 fragments (7). The unfractionated *Bam*HI fragments were ligated to *Bam*HI-digested pBR322 and transformed into recipient bacteria. Of 1300 ampicillin-resistant transformants tested, 450 were sensitive to tetracycline. These candidates were replica plated and hybridized individually with ^{32}P-labeled restriction endonuclease DNA fragments *Xba* I C, *Xba* I F, *Eco*RI AL, *Bgl* II G, *Hsu* I DG, *Hpa* I DL, and *Bgl* II L (6), which span all of the DNA sequences of the genome. Most of the tetracycline-sensitive transformants hybridized with one or more HSV-1 fragments. The results of the hybridizations allowed systematic screening of the colonies for recombinant plasmids.

Individual colonies were grown in small liquid cultures for DNA preparation by the method of Meagher *et al.* (18). The DNAs from such preparations were digested with *Bam*HI and analyzed by gel electrophoresis along with *Bam*HI-digested HSV-1(F) DNA (Fig. 1).

Although in most cases the electrophoretic mobility of a cloned fragment along with the colony hybridization data clearly identified it, digestion with another restriction enzyme was generally done for an unambiguous identification. Analyses of the recombinant plasmids showed that all of the *Bam*HI fragments of HSV-1(F) were cloned except *Bam*HI V, *Bam*HI I′, and *Bam*HI K′. Several plasmids contained either *Bam*HI G′ or *Bam*HI H′, but no attempts were made to discriminate between these fragments of identical electrophoretic mobility. In addition, several fragments smaller than molecular weight

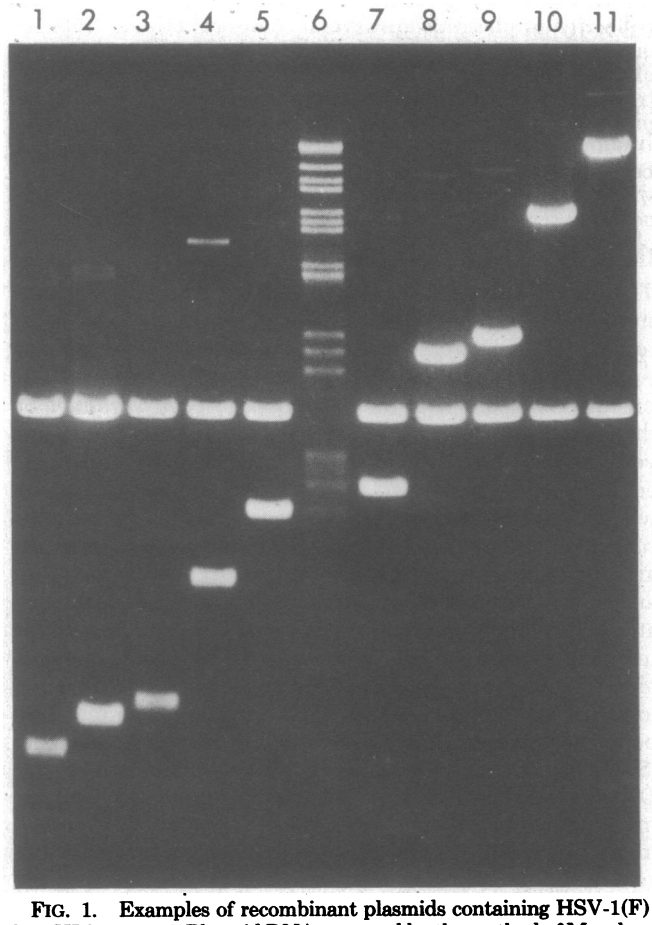

FIG. 1. Examples of recombinant plasmids containing HSV-1(F) *Bam*HI fragments. Plasmid DNA prepared by the method of Meagher *et al.* (18) was digested with *Bam*HI and subjected to electrophoresis on a 0.8% agarose gel. Lane 6 contains *Bam*HI-digested HSV-1(F) DNA. All other lanes contain digests of plasmids containing HSV-1 fragments as follows: 1, pRB122, *Bam*HI Z; 2, pRB124, *Bam*HI X; 3, pRB116, *Bam*HI W; 4, pRB119, *Bam*HI T; 5, pRB118, *Bam*HI R; 7, pRB103, *Bam*HI Q; 8, pRB125, *Bam*HI M; 9, pRB126, *Bam*HI L; 10, pRB128, *Bam*HI F; 11, pRB131, *Bam*HI A. The band common to all of the digested plasmids is linear pBR322. The large submolar bands are partial digestion products and represent linear plasmid.

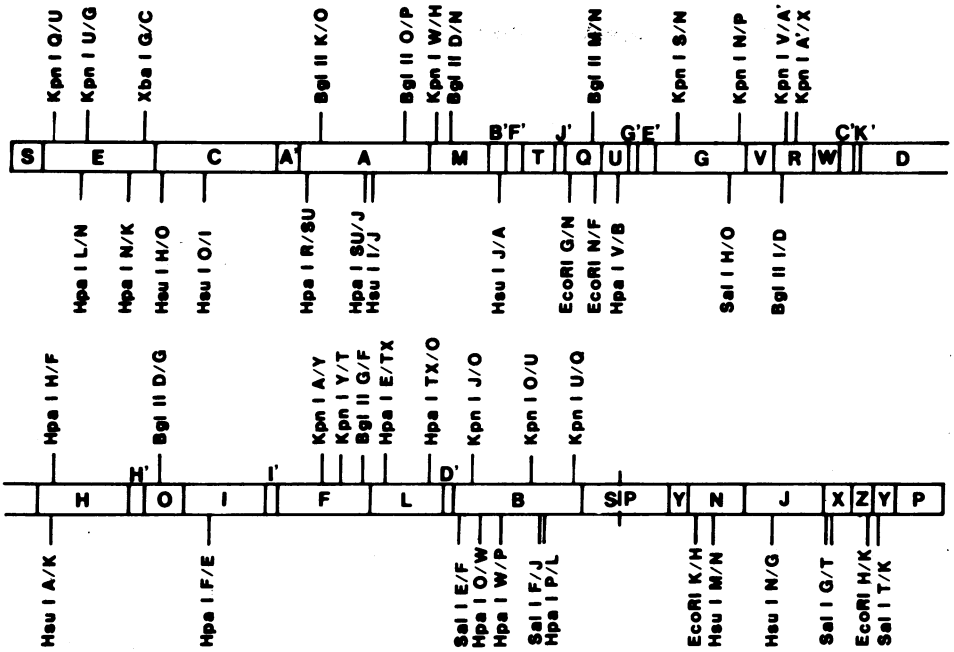

FIG. 2. *Bam*HI map of prototype arrangement of HSV-1(F) DNA. The *Hpa* I, *Hsu* I (isoschizomer of *Hind*III), *Eco*RI, *Sal* I, *Bgl* II, and *Kpn* I cleavage sites shown discriminate between fragments of similar size (6, 7) and were used to authenticate the cloned *Bam*HI fragments. The *Bam*HI map of HSV-(F) DNA is that of Locker and Frenkel (7) except that the locations of the *Bam*HI X and Z fragments were interchanged on the basis of hybridization experiments which showed that *Bam*HI Z contains a portion of the reiterated sequences of the S component whereas *Bam*HI X is derived entirely from the unique sequences. The junction between the L and S components is represented by the broken line through the SP fragment. (*Upper*) Map positions 0.0 to 0.50; (*Lower*) map positions 0.50 to 1.00.

0.3×10^6 were cloned, but their locations within the genome are not known (7).

Each of the cloned fragments, except *Bam*HI B and *Bam*HI SP had the same electrophoretic mobility as the corresponding fragment from viral DNA. All except *Bam*HI B were found to contain the expected restriction enzyme cleavage sites (Fig. 2). The exceptional fragments are discussed below.

***Bam*HI SP.** The *Bam*HI SP fragment spans the junction of the L and S components of the genome and contains the terminal portion of the reiterated *b* and *a* sequences separated by one or more *a* sequences. Because of the variability in the number of *a* sequences, at least four forms of the *Bam*HI junction fragments, designated as SP_1, SP_2, SP_3, and SP_4 in the order of decreasing electrophoretic mobility, have been described (7). Several plasmids containing the *Bam*HI SP fragment have been characterized. The structure of three independently isolated plasmids containing the *Bam*HI SP_1 fragment appears to be stable, at least in the C600SF8 background

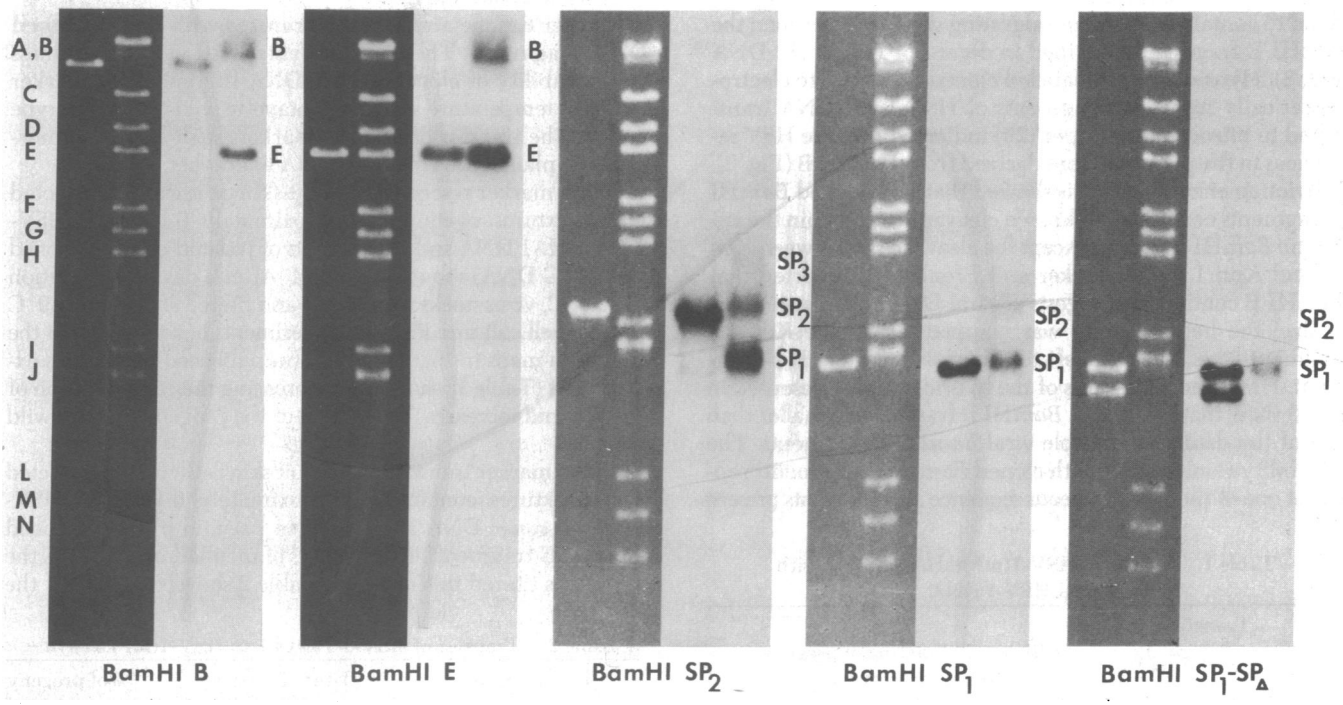

FIG. 3. Hybridizations of recombinant plasmids to HSV-1(F) DNA: ethidium bromide-stained gels and autoradiograms of the gels after hybridization with the labeled probes. For each of the five experiments, *Bam*HI-digested HSV-1(F) and plasmid DNAs were subjected to electrophoresis in adjacent lanes on a 0.5% agarose gel. The DNA in two lanes was transferred to one piece of nitrocellulose filter and hybridized to ^{32}P-labeled plasmid. In each case, the labeled plasmid was the same as that immobilized on the filter. The plasmids used for these experiments were pRB106 (*Bam*HI B), pRB111 (*Bam*HI E), pRB104 (*Bam*HI SP_2), pRB115 (*Bam*HI SP_1), and pRB133 (*Bam*HI SP_1 and SP_Δ); SP_Δ represents a deletion in SP_1, as described in the text.

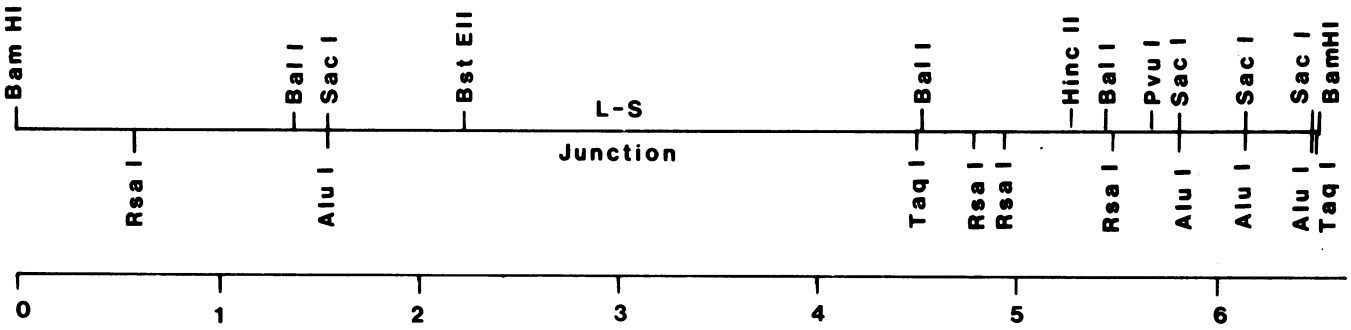

FIG. 4. Restriction endonuclease fragment map of the cloned *Bam*HI SP_2 fragment. All mapping was completed by using agarose gel-purified *Bam*HI SP_2 fragment end-labeled with ^{32}P by using polynucleotide kinase. The map position of the *Pvu* I site was determined by digesting end-labeled SP_2 fragment with *Pvu* I and isolating the two fragments from an agarose gel. The smaller fragment was hybridized to a Southern blot of *Bam*HI-digested HSV-1 (F) DNA to determine whether it contained *c* or *b* sequences. The positions of all other restriction enzyme sites were mapped relative to the *Pvu* I site. The *Hin*cII and *Bst*EII sites were mapped by double digestion of end-labeled *Bam*HI SP_2 fragment with *Pvu* I. The positions of the *Bal* I, *Alu* I, *Sac* I, *Rsa* I, and *Taq* I sites were determined by partial digestion of end-labeled *Bam*HI SP_2 fragment and double digestion with *Pvu* I. The molecular size of all restriction fragments is indicated by the scale expressed in 10^3 base pairs.

(Fig. 3). However, one other plasmid also containing a *Bam*HI SP_1 had apparently undergone some rearrangement in the C600SF8 background such that both the intact *Bam*HI SP_1 and a smaller SP-derived fragment were propagated as a mixed population.

One plasmid containing a *Bam*HI SP_2 fragment was identified. However, even after colony purification a small amount of fragments approximately the size of the SP_1 fragment were found in the plasmid population. Although the nature of this deletion event has not been investigated, it seems likely that the recombinational event which produced the smaller fragment involved the deletion of one or more *a* sequences.

The cloned SP_2 fragment was analyzed with several restriction enzymes (Fig. 4). The *Hin*cII and *Alu* I cleavage sites correspond to previously published maps of the region spanning the L–S junction of HSV-1 DNA (4).

***Bam*HI B.** The two independent clones of *Bam*HI B were found to contain a fragment migrating slightly faster than the *Bam*HI B fragment contained in digests of HSV-1 (F) DNA (Fig. 3). Hybridization of labeled cloned *Bam*HI B to electrophoretically separated fragments of HSV-1 (F) DNA transferred to nitrocellulose paper (26) indicated that the HSV sequences in the plasmids were derived from *Bam*HI B (Fig. 3). Restriction enzyme analysis showed that both cloned *Bam*HI B fragments contained all known cleavage sites within the authentic *Bam*HI fragment except the cleavage site between *Kpn* I J and *Kpn* I O (7). Locker and Frenkel (7) reported that *Bam*HI B contained in digests of viral DNA is heterogeneous in size; the heterogeneity was mapped within the *Kpn* I J fragment—i.e., near the left terminus of the *Bam*HI B fragment. However, the results of the hybridizations presented in Fig. 3 show that the cloned *Bam*HI B fragment is smaller than any of the detected multiple viral *Bam*HI B fragments. The possibility remains that both cloned *Bam*HI B fragments represent one of the heterogeneous sequence arrangements present in uncloned DNA in amounts too small to be detected in the experiment shown in Fig. 3. Alternatively, the cloned fragment could be the result of some specific modification of the fragment after cloning.

It is likely that the reiterated sequences in the cloned *Bam*HI B fragments are not modified. This conclusion is based on the following. *Bam*HI B and *Bam*HI E share a set of sequences derived from the *b* sequence of the inverted repeats bracketing the L component but differ with respect to the unique sequences adjacent to the reiterated *b* sequence. The cloned *Bam*HI E fragment could not be differentiated from the authentic *Bam*HI E fragment in size (Fig. 3) and known restriction endonuclease cleavage sites (Fig. 2). Comparison of the *Sal* I/*Bam*HI and *Kpn* I digests of *Bam*HI B and *Bam*HI E fragments showed that the small fragments derived from the portion of the cloned DNAs containing the shared reiterated sequences were identical.

Marker Rescue and Marker Transfer with HSV-1 Cloned DNA Fragments. These experiments illustrate the usefulness and suitability of cloned HSV-1 DNA fragments for marker rescue of temperature-sensitive mutants to wild-type phenotype and for the transfer of specific markers such as plaque morphology phenotype from one DNA to another.

In the marker rescue test, rabbit skin cells were transfected with mixtures containing approximately 0.1 μg of HSV-1(mP)tsHA1 DNA and the amounts of plasmid pBR102 (cloned *Bam*HI G DNA) shown in Table 1. After 4 days of incubation at 33.5°C, virus stocks were made and titered at 33.5 and 39°C in Vero cell cultures. Previous experiments have shown that the mutation maps in that fragment (unpublished data). This experiment (Table 1) shows that increasing the concentration of the plasmid increases the fraction of the progeny that are wild type.

In the marker transfer test, rabbit skin cells were transfected with mixtures containing approximately 0.1 μg of HSV-1(mP)J7 *syn*− DNA and 0.1 μg of plasmid pBR102 (cloned *Bam*HI G fragment). After 4 days of incubation at 33.5°C the virus was titered in Vero cells. Table 2 shows that 15% of the

Table 1. Rescue of HSV-1(mP)tsHA1 mutant with cloned HSV-1 DNA

Transfection mixture, μg		Efficiency of plating of progeny after transfection, ratio of titers at 39 and 33.5°C
ts HA1 DNA	pRB102 DNA	
0.1	0	5×10^{-6}
0.1	0.1	3×10^{-3}
0.1	0.2	4×10^{-3}
0.1	0.4	3.2×10^{-2}

Table 2. Transfer of HSV-1(F) *syn*+ to HSV-1(mP) J7 *syn*−

Transfection mixture, μg		Titer of progeny exhibiting:		% of progeny exhibiting syn+ phenotype
J7syn− DNA	pRB102 DNA	syn− phenotype	syn+ phenotype	
0.1	0	1.5×10^7	$<10^3$	<0.01
0.1	0.1	1.2×10^7	1.8×10^6	15

progeny virus exhibited the syn+ phenotype of the donor [HSV-1(F)] virus.

DISCUSSION

We anticipated that, of all of the sequences of the HSV-1 genome, the *Bam*HI SP fragments that span the junction between L and S components might be the most difficult to clone inasmuch as these fragments may contain several copies of the *a* sequence in the same orientation as well as reiterations of at least a portion of the *a* sequence in inverted orientation (3, 27). In actual practice, several clones containing the *Bam*HI SP fragment were isolated and at least some of these clones appear to contain *bona fide* copies of the authentic *Bam*HI SP_1 and SP_2 fragments. On the other hand the failure to isolate clones of *Bam*HI V fragments may be of more than marginal interest. *Bam*HI V fragment encodes, at least in part, the viral DNA polymerase (28); this fragment is also contained in a subset of defective genomes, and current studies favor the hypothesis that these defective genomes contain an origin of DNA synthesis within or in the immediate vicinity of the *Bam*HI V fragment (29).

Earlier studies have shown that the restriction endonuclease fragments containing the terminal DNA sequences as well as the sequences spanning the junction between the L and S components vary in size presumably because of the variation in the number of tandemly reiterated *a* sequences. More recent studies on viral DNA populations derived from plaque-purified stocks have shown that fragments adjacent to the inverted repeats of the L and S component (*Bam*HI E, B, N, and J) may also fluctuate in size (ref. 7; unpublished data). With the exception of the *Bam*HI B, the cloned fragments appear to resemble the predominant authentic fragment in size. The cloned *Bam*HI B fragments are appreciably different from the authentic *Bam*HI B fragments, but the significance of this finding, particularly with respect to information content of this fragment, remains obscure. It is of interest to note that the L component of HSV-1 (HFEM) DNA contains a deletion of molecular weight 2×10^6. This deletion appears to be contained entirely within the *Bam*HI B fragment (unpublished data). Assuming that all HSV-1 strains are heterogeneous in that region, it would appear that the left portion of the *Bam*HI B fragment does not contain genetic information such that its loss significantly impairs the function of the virus.

Experiments described in this paper show that cloned DNA can be used to transfer genetic information to the HSV genome. Also, a plasmid containing *Bam*HI Q has been used to transform thymidine kinase-negative L cells as done previously by others (9, 10, 30). Thus, in addition to its role in fine-structure physical analysis of the HSV genome, recombinant DNA appears to be a useful tool for genetic studies.

We thank Susan Mackem for screening several of the plasmids and for aligning the *Bam*HI Z and X fragments in HSV-1(F) DNA. The studies on the heterogeneity of *Bam*HI fragments E, B, N, and J by M. Tognon and B. Roizman will be published elsewhere. These studies were aided by grants from the National Cancer Institute (CA98494 and CA19264) and the American Cancer Society (MV-0-2). L.E.P., A.J.C., and E.S.M. are U.S. Public Health Service Postdoctoral Trainees (AI-07182-01, GM-07543-03, and CA-09241-02, respectively).

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