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Cloning of Herpes Simplex Virus Type 1 Sequences Representing the Whole Genome

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Sequences representative of the whole genome of herpes simplex virus type 1 (HSV-1) strain KOS were cloned in the plasmid vector pBR325 in the form of *Eco*RI-generated DNA fragments. The cloned fragments were identified by digestion of the chimeric plasmid DNA with restriction enzymes *Eco*RI or *Eco*RI and *Bgl*II followed by comparison of their electrophoretic mobilities in agarose gels with that of similarly digested HSV-1 virion DNA. The cloned fragments showed the same migration patterns as the corresponding fragments from restricted virion DNA, indicating that no major insertions or deletions were present. The presence of HSV-1 sequences in the chimeric plasmids was confirmed by hybridization of plasmid DNA to HSV-1 virion DNA. Additionally, some of the cloned fragments were shown to be biologically active in that they efficiently rescued three HSV-1 temperature-sensitive mutants in cotransfection marker rescue experiments.

Herpes simplex virus type 1 (HSV-1) DNA is a double-stranded molecule of molecular weight about 100×10^6 (2, 13, 16). The genome consists of two covalently linked components which can invert relative to one another, resulting in four possible isomeric arrangements of the molecule (for recent reviews, see references 24-26). These isomers occur in equimolar amounts and are termed P (prototype), I_L (inversion of the long segment), I_S (inversion of the short segment), and I_{SL} (inversion of S and L) (9, 14). The long segment consists of a unique region (U_L) which is 67.2 megadaltons (Md) surrounded by inverted repeats termed ab and b'a', each 5.8 Md (25, 28). The short segment has a similar structure, with the unique region (U_S, 8.9 Md) being bordered by repeats termed a'c' and ca (4.1 Md each) (25, 28). The union of the two segments, consisting of the repeat sequences b'a'a'c', is termed the joint of the molecule (Fig. 1).

Because the HSV-1 DNA molecule exists in four equimolar isomers, restriction enzyme digestion of the DNA yields fragments of three different frequency classes (6, 29). Fragments derived entirely from within the unique regions of the molecule (U_L or U_S) appear in 1 M quantities relative to the molarity of intact viral DNA. Fragments consisting of the terminal sequences are present in only 0.5 M quantities, since each terminus is present in only two of the four isomers. Finally, fragments which consist of the joint sequences are present in 0.25 M quantities, since each joint arrangement is present in only one of the four isomers.

This complexity of the structure of the HSV-1 genome, along with its large size, presents difficulties in elucidating the mechanisms behind isomerization, replication, and other viral functions. To aid in the study of some of these problems, various investigators have begun to clone specific HSV-1 restriction fragments in plasmid or bacteriophage vectors. There have been numerous reports of the cloning of fragments containing the thymidine kinase gene of HSV-1 (8, 12, 36) or herpes simplex virus type 2 (20). In addition, Enquist et al. (11) have reported the cloning of 50% of the genome of HSV-1 (Patton) in the form of various *Eco*RI and *Eco*RI/*Hin*dIII restriction fragments in a bacteriophage lambda vector. Post et al. (23) have used the plasmid vector pBR322 to clone *Bam*HI fragments of HSV-1 (F) comprising over 95% of the genome. In this report, we describe the cloning of sequences representative of the whole genome of HSV-1 (KOS) in the form of *Eco*RI-generated restriction fragments in the plasmid vector pBR325. All of the internal 1 M fragments and the joint fragments were obtained as cloned inserts. The terminal fragments were represented within the joints. The cloned inserts were shown to be indistinguishable from *Eco*RI fragments of HSV-1 virion DNA, and some were

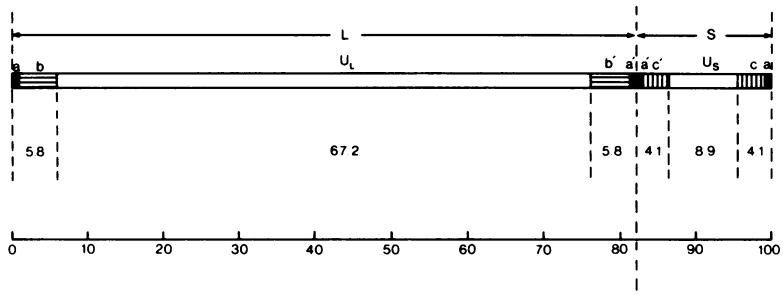

FIG. 1. *Diagram of the genome structure of HSV-1 (25). Molecular weights of the various regions are shown in megadaltons.*

demonstrated to be biologically active by rescuing HSV-1 temperature-sensitive (*ts*) mutants in cotransfection marker rescue experiments.

MATERIALS AND METHODS

Cells and virus. The propagation of primary rabbit kidney cells used in transfection experiments and African green monkey kidney (Vero) cells used for the preparation of virus stocks is described in the accompanying paper (27). Stocks of HSV-1 strain KOS and *ts* mutant virus were grown by low-multiplicity passage and titered by plaque assay as described previously (1).

Bacterial strains. *Escherichia coli* K-12 strain 1100 derivative DH-1 (*recA1 hsdR hsdM*$^+$ *nalA96*^{r} *thi-1 endA1 supE44*), which was used as the host for transformation with chimeric plasmids, and *E. coli* K-12 strain GM31 (*dcm-6 thr-1 leu-6 thi-1 his lacY galK2 galT22 ara-14 tonA31 tsx-78 supE44*) (31), containing the plasmid pBR325 (4), were obtained from Rex Chisholm.

Purification of viral DNA. HSV-1 DNA used in marker rescue mapping experiments was purified from HSV-1 mutant-infected cell lysates as described in the accompanying paper (27).

Viral DNA to be used for cloning and as a hybridization probe was purified from cell-free virus as follows. Vero cell monolayers in roller bottles were infected with HSV-1 (KOS) at a multiplicity of infection of 0.1. When cytopathic effect was generalized, cells were scraped into the medium and pelleted by centrifugation at 3,000 × *g* for 15 min. The supernatant was removed and centrifuged at 7,500 × *g* for 4 h at 4°C to pellet extracellular virus. The viral pellet was suspended in a small volume of TE buffer (0.01 M Tris-hydrochloride, pH 8.0, and 0.001 M EDTA). DNA was extracted from the virus pellet, and the viral DNA was banded in CsCl as described previously (27). Fractions comprising the viral DNA peak (densities of 1.735 to 1.720) were pooled, banded in CsCl two additional times, dialyzed extensively against TE buffer, and precipitated with ethanol.

Restriction enzyme digestion and agarose gel electrophoresis. Restriction enzymes *Eco*RI, *Bgl*II, *Bam*HI, and *Hpa*I (Bethesda Research Laboratories) were all used in reaction mixtures consisting of 100 mM Tris-hydrochloride, pH 7.2, 5 mM $MgCl_2$, 50 mM NaCl, and 2 mM 2-mercaptoethanol at 37°C for at least 2 h. Restricted DNA was electrophoresed in horizontal agarose slab gels (27 cm by 13 cm by 3 mm) completely submerged in buffer consisting of 30 mM NaH_2PO_4–36 mM Tris–1 mM EDTA (pH 8.0) at a constant voltage of 50 V for 20 h.

Extraction of plasmid DNA. Plasmid DNA was extracted from cleared lysates of spheroplasts prepared by the procedure of Clewell and Helinski (7), as modified by Chisholm et al. (5). Bacteria containing the plasmid were grown in 1 liter of M9 medium (20) plus 0.5% Casamino Acids (Difco Laboratories), 0.4% glucose, 0.012% $MgSO_4$, 5 μg of thiamine per ml, and 50 μg of ampicillin per ml to an absorbancy at 600 nm of 0.6 to 0.8. Chloramphenicol or spectinomycin was then added to 250 μg/ml, and the culture was incubated for an additional 12 to 16 h at 37°C to amplify the plasmid copy number (15). The plasmid DNA was then extracted as described by Chisholm et al. (5).

Construction of chimeric plasmids. *Eco*RI restriction fragments of HSV-1 (KOS) DNA were inserted into the single *Eco*RI site of pBR325 by the following procedure. HSV-1 DNA was digested to completion with *Eco*RI, extracted twice with phenol and then twice with ether, and precipitated with ethanol. A 250-ng amount of similarly treated pBR325 DNA was combined with 1 or 4 μg of digested HSV-1 DNA in 200 μl of ligation buffer (66 mM Tris-hydrochloride, pH 7.6, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 40 μM ATP). One unit of T4 ligase (Bethesda Research Laboratories) was added, and the reaction mixture was incubated for 12 to 16 h at 4°C.

When size-fractionated HSV-1 DNA was used as the substrate for cloning, HSV-1 DNA was digested and extracted as above and then layered onto linear 10 to 40% sucrose gradients in 20 mM Tris-hydrochloride, pH 8.0, 10 mM EDTA, and 1 M NaCl (18). The gradients were centrifuged at 25,000 rpm in an SW27 rotor for 24 h at 20°C and collected into 0.5-ml fractions by pumping through an ISCO UV flow cell and measuring the absorbancy at 260 nm. Fractions containing the appropriate HSV-1 *Eco*RI fragments were identified by electrophoresing 20-μl aliquots on 0.5% agarose slab gels at 40 V for 20 h. DNA in each appropriate fraction was precipitated twice with ethanol and combined with 250 ng of *Eco*RI-restricted pBR325 DNA in 100 μl of ligation buffer and ligated as described above.

Transformation of bacteria. Ligated chimeric plasmids were introduced into *E. coli* K-12 strain DH-1 by the procedure of Chisholm et al. (5). Cells were grown in 50 ml of L broth plus 0.1% glucose (21) to an absorbancy at 600 nm of 0.3 to 0.4 and then chilled and centrifuged at 3,000 × *g* for 5 min at 4°C. The pellet was suspended in 25 ml of cold CTG (50 mM $CaCl_2$, 50 μg of thymidine per ml, and 10% glycerol) and held on ice for 20 min. The cells were pelleted as before and resuspended in 2.5 ml of CTG. The shocked cells remained competent for up to 24 h when kept on ice. To transform the cells, 0.1 ml of ligation reaction mix was combined with 0.2 ml of shocked cells at 4°C. After 15 min on ice, the mixture was placed at 42°C for 2 min and then returned to ice for 15 min. Next, 0.8 ml of L broth without antibiotics was added, and the cells were incubated at 37°C for 30 to 60 min. Aliquots (0.1 ml) were plated onto L agar (LA) plates containing ampicillin (50 μg/ml) to select for cells containing pBR325. Since the single *Eco*RI site in pBR325 is located in the chloramphenicol resistance gene, insertion of DNA at that site inactivated the gene. Cells containing chimeric plasmids were therefore sensitive to chloramphenicol; these were selected by picking colonies onto LA plates containing ampicillin (50 μg/ml) and LA plates containing chloramphenicol (250 μg/ml) and choosing those colonies which grew only on the ampicillin plates.

Screening of chimeric plasmids for HSV-1 sequences. Chimeric plasmids were analyzed by a rapid-lysis extraction of the plasmid DNA followed by restriction enzyme digestion and agarose gel electrophoresis. The plasmid DNA was extracted by a modification of the alkaline rapid-lysis technique of Birnboim and Doly (3). This procedure involves the selective alkaline degradation of high-molecular-weight bacterial chromosomal DNA, leaving the covalently closed circular plasmid DNA intact. The chromosomal DNA was then renatured and precipitated, and the plasmid DNA remaining in the supernatant was purified by three successive precipitations with ethanol. The DNA pellet, after the final ethanol precipitation, was suspended in 32 μl of TE buffer. After the addition of 4 μl of 10× *Eco*RI restriction enzyme buffer, the sample was divided into two aliquots. One aliquot was digested with *Eco*RI alone (2 to 4 U), and the other was digested with *Eco*RI and *Bgl*II (2 to 4 U of each). The reactions were incubated at 37°C for at least 2 h and then loaded onto 0.8% agarose slab gels and electrophoresed at 50 V for 20 h. Specific *Eco*RI restriction fragments were identified by comparing their mobilities with that of the fragments resulting from an *Eco*RI or *Eco*RI/*Bgl*II digestion of HSV-1 virion DNA electrophoresed in the same gel.

Labeling of HSV-1 DNA by nick translation. HSV-1 DNA was labeled with ^{32}P to 1×10^8 to 2×10^8 cpm/μg by a modification of the procedure of Maniatis et al. (19). HSV-1 DNA (0.9 μg) was first nicked for 15 min at 37°C with 1 ng of DNase I (Worthington Biochemicals Corp.). The DNA was then incubated at 15°C in a 50-μl reaction mixture consisting of 100 μCi each of α-^{32}P-labeled dATP and dCTP (Amersham Radiochemicals), 750 pmol each of dGTP and dTTP, and 4.5 U of *E. coli* DNA polymerase I (Boehringer Mannheim Corp.) in 50 mM Tris-hydrochloride, pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.05% bovine serum albumin. After 30 min, the reaction was quenched with 5 μl of 0.5 M EDTA and then further diluted with 450 μl of TE buffer containing 50 μg of *E. coli* DNA as carrier. The DNA was extracted once with phenol and precipitated by the addition of an equal volume of 4 M ammonium acetate and 3 volumes of ethanol followed by centrifugation in an SW50.1 rotor at 45,000 rpm for 4 h at 0°C. This procedure precipitates less than 5% of the unincorporated counts and between 40 to 50% of the incorporated label.

Transfer of chimeric plasmid DNA to nitrocellulose and hybridization with HSV-1 DNA. DNA from the chimeric plasmids was digested to completion with *Eco*RI, electrophoresed in a 1% agarose slab gel, and transferred to nitrocellulose paper (Schleicher & Schuell Co.) as described by Wahl et al. (34). Before transfer, the DNA in the gel was depurinated by immersion in 0.25 N HCl twice for 5 min each time, followed by denaturation by soaking in 0.5 M NaOH–1.5 M NaCl twice for 10 min each time. The gel was then neutralized by immersion in 0.5 M Tris-hydrochloride, pH 7.4, and 3 M NaCl twice for 15 min each time, after which the DNA was transferred for 2 h. After transfer, the nitrocellulose sheet was rinsed in 2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then baked under vacuum at 80°C for 3 h. Before the hybridization, the filter was pretreated for 3 h at 65°C in 6× SSC, 1× Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin) (10), and 25 μg of denatured, sonicated salmon sperm DNA per ml. The ^{32}P-labeled HSV-1 probe DNA was denatured by heating to 117°C for 7 min, and then 4×10^6 cpm was added to the filter in 20 ml of hybridization solution (6× SSC, 1× Denhardt solution, 10% dextran sulfate, 0.5% sodium dodecyl sulfate [SDS], 1 mM EDTA, and 100 μg of denatured, sonicated salmon sperm DNA per ml). Hybridization was carried out in a sealed plastic bag for 18 h while shaking at 65°C. After this, the filter was rinsed three times in 2× SSC–0.1% SDS at room temperature and washed twice in 0.1× SSC–0.1% SDS while shaking at 65°C for 30 min each time. The filter was then rinsed in 2× SSC–0.1% SDS at room temperature, dried, and exposed for 48 h to Kodak X-Omat R film, using Ilford fast tungstate intensifying screens.

Transfection procedure. Transfections for mapping mutants by marker rescue were carried out by a modification of the procedure described by Wigler et al. (35). Chimeric plasmids containing HSV-1 *Eco*RI fragments were digested with *Eco*RI as described earlier at 37°C for 2 h. The DNA was precipitated with ethanol and suspended in TE buffer. Three to 5 μg of cloned specific *Eco*RI fragment DNA and 5 to 10 μg of intact HSV-1 mutant DNA and cellular DNA (see purification of viral DNA) were added to 0.5 ml of 2× HBS buffer (0.28 M NaCl–0.05 M HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]–1.5 mM Na_2HPO_4, pH 7.0). One-half milliliter of 250 mM $CaCl_2$ was added dropwise with gentle bubbling (35). The DNA solutions were allowed to stand at room temperature for 30 min to allow precipitates to form. One milliliter of each DNA solution was added to a 25-cm^2 flask containing 1×10^6 to 2×10^6 rabbit kidney

cells, which had been seeded into the flasks 4 to 5 h earlier. The medium (minimal essential medium containing 10% fetal calf serum) was not removed from the cells before the addition of the transfection solution. The flasks were incubated at 34°C for 18 to 20 h, after which the DNA solution was removed. Fresh medium containing 5% fetal calf serum was added, and incubation at 34°C was continued until cytopathic effect was generalized. Flasks were then frozen until assayed.

RESULTS

Isolation and identification of chimeric plasmids. *Eco*RI restriction fragments of HSV-1 (KOS) DNA were inserted into the single *Eco*RI site in pBR325 by digesting both types of DNA to completion with *Eco*RI and then mixing them for ligation with T4 ligase. The resulting mix was used to transform competent *E. coli* strain DH-1 bacteria, and about 3,000 transformed cells were selected by plating onto LA plates containing ampicillin (50 μg/ml). The colonies were picked onto replicate LA plates containing ampicillin or chloramphenicol (250 μg/ml). This procedure identified 92 chloramphenicol-sensitive colonies. These bacteria presumably contained chimeric plasmids with the chloramphenicol resistance gene of pBR325 being inactivated by the insertion of exogenous DNA at the *Eco*RI site in the gene. The chloramphenicol-sensitive colonies were picked, grown in L broth containing ampicillin, and analyzed further by rapid-lysis extraction of plasmid DNA followed by restriction enzyme digestion and agarose gel electrophoresis.

Three molar classes of restriction fragments were generated upon digestion of the HSV-1 genome with *Eco*RI. Figure 2 shows a map of the KOS genome on which the restriction sites for *Eco*RI and *Bgl*II have been marked. The two terminal *Eco*RI fragments flanking the S segment (K_1 and K_2) have the same molecular weight and are therefore indistinguishable. These two fragments are both termed K, and together they will be considered a 1 M terminus. Because of this, the two joint fragments EK (EK_1 and EK_2) and JK (JK_1 and JK_2) will each be considered a 0.5 M joint fragment. This map of *Eco*RI restriction sites is identical to that of Morse et al. (22) with the exception of the molecular weights of fragments K and L. We find that with our KOS strain of HSV-1, obtained from W. Munyon, these two fragments have very similar electrophoretic mobilities, with fragment L migrating slightly slower than fragment K. This agrees with the results of Skare and Summers (29). Skare et al. (29, 30), in order to be consistent among the different strains of HSV-1, called the slower-migrating fragment of strain KOS L and called the faster-migrating fragment K. This nomenclature has been retained here for clarity, and the molecular weights of fragments EK, JK, K, and L have been modified to reflect the differences in electrophoretic mobility.

Since many of the *Eco*RI fragments are very similar in size, such as A and EK or D, E, F, and G, they could not be resolved by agarose gel electrophoresis. However, all of these comigrating fragments were clearly distinguishable upon double digestion with *Eco*RI and *Bgl*II. For example, fragment A was cut by *Bgl*II into two fragments of 9.0 and 5.2 Md, whereas fragment EK was not cut at all by *Bgl*II. Similarly, fragments D, E, F, and G were all cut into distinctively different patterns upon digestion with *Bgl*II. This allowed the unique identification of all *Eco*RI fragments of HSV-1 (KOS) DNA.

Plasmid DNA was therefore analyzed both by digestion with *Eco*RI alone and by double digestion with *Eco*RI and *Bgl*II. The digests were

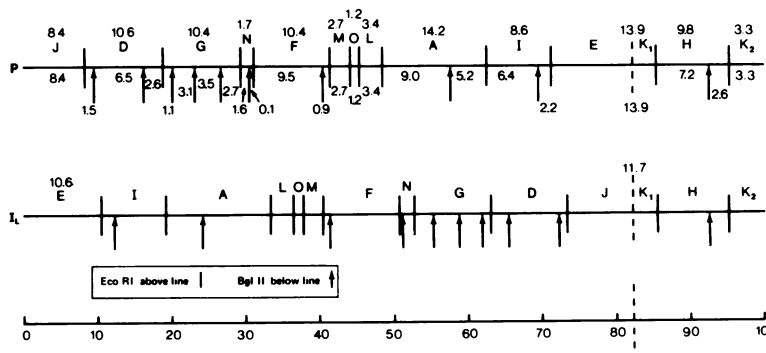

FIG. 2. *EcoRI restriction map of HSV-1 (KOS) showing BglII restriction sites (22). The map represents the P and I_L orientations of the molecule. Molecular weights (in megadaltons) of EcoRI fragments are shown above the line, and those of EcoRI/BglII fragments are shown below the line.*

electrophoresed on 0.8% agarose slab gels with *Eco*RI- or *Eco*RI/*Bgl*II-fractionated HSV-1 virion DNA in adjoining slots as markers. Figure 3 shows the resulting electrophoretic patterns for the distinct clones that were isolated. Only 11 of the 12 *Eco*RI fragments that were possible to clone by this procedure were initially isolated. Fragment A was not obtained by using unfractionated HSV-1 DNA. As can be seen, the *Eco*RI/*Bgl*II digestion patterns for plasmids containing fragments EK, JK, D, F, G, H, I, L, M, N, and O were all consistent with the data in Fig. 2. The terminal *Eco*RI fragments E, J, and K were not cloned by this procedure because they had only one sticky end and one blunt end, but they were represented within the joint fragments EK and JK.

To obtain a chimeric plasmid containing *Eco*RI fragment A, *Eco*RI-digested HSV-1 DNA was size fractionated on linear 10 to 40% sucrose gradients. Fractions containing fragment A were identified by electrophoresing equal aliquots on 0.5% agarose slab gels, and the DNA in these aliquots was then ligated to restricted pBR325 DNA. The ligated mixture was used to transform competent DH-1 bacteria, and 12 colonies consisting of bacteria containing chimeric plasmids were identified on the basis of resistance to ampicillin and sensitivity to chloramphenicol as described above. Chimeric plasmid DNA was extracted by the rapid-lysis procedure and analyzed on 0.8% agarose slab gels.

Figure 3A shows the electrophoretic profile of the DNA from pSG124, the one chimeric plasmid that contained *Eco*RI fragment A. As can be seen, the inserted fragment comigrated with fragment A from an *Eco*RI digest of HSV-1 virion DNA. Upon double digestion of the DNA with *Eco*RI and *Bgl*II, this insert yielded two fragments of 9.0 and 5.2 Md, consistent with the data in Fig. 2 for *Eco*RI fragment A. Therefore, all of the 1 M internal fragments and the 0.5 M joint fragments of *Eco*RI-digested HSV-1 DNA were represented as chimeric plasmids cloned in *E. coli* DH-1. In addition, all of the cloned inserts and *Eco*RI/*Bgl*II-digested fragments of the inserts comigrated with fragments from *Eco*RI- or *Eco*RI/*Bgl*II-digested HSV-1 virion DNA, indicating that no large deletions or insertions were present in any of the cloned inserts.

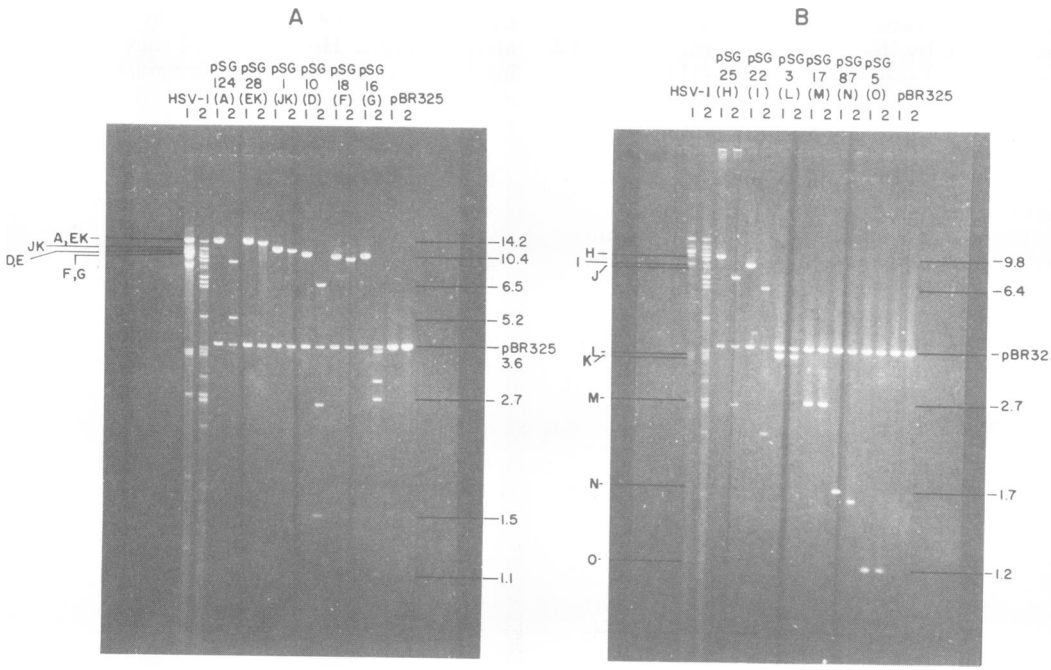

FIG. 3. *Identification of chimeric plasmid inserts. DNA of each of the distinctive chimeric plasmids was isolated and digested to completion with either EcoRI alone (1) or EcoRI and BglII (2) as described in Materials and Methods. The digests were then electrophoresed on 0.8% agarose slab gels at 50 V for 20 h. (A) Plasmids containing EcoRI fragments A, EK, JK, D, F, and G. (B) Plasmids containing EcoRI fragments H, I, L, M, N, and O. HSV-1 (KOS) virion DNA and pBR325 DNA were treated similarly as controls. The fragment inserted in each chimeric plasmid is indicated in parentheses after the plasmid name. EcoRI fragments of HSV-1 (KOS) are indicated on the left, and molecular weights (in megadaltons) are shown on the right.*

All of the chimeric plasmids have been stored in bacterial cultures in 50% glycerol at −20°C with no obvious changes in restriction profiles.

Although the joint fragments were only present in 0.5 M quantities, clones containing them were found with a high frequency. Six clones containing EK and seven containing JK were identified. In contrast, fragment A was obtainable only by using size-fractionated HSV-1 DNA, and only one clone was found. Since fragment A was only 0.3 Md larger than EK, size alone could not account for this difference. Between 5 and 10 clones were found for each of the other fragments with the exception of N and O, the two smallest. Only three clones of fragment O and only one clone of fragment N were found.

Confirmation of HSV-1 sequences in the chimeric plasmids. In the previous section, the cloned *Eco*RI restriction fragments were identified by the presence and location of *Bgl*II restriction sites within the fragments, and their identity was confirmed in some cases by using the restriction enzymes *Hpa*I and *Bam*HI (data not shown). To further demonstrate that the fragments contain HSV-1 sequences, they were analyzed by the transfer and hybridization procedure of Southern (32). DNA was extracted from each of the chimeric plasmids and digested to completion with *Eco*RI, electrophoresed on a 1.0% agarose slab gel, and stained with ethidium bromide (Fig. 4A). The DNA was then transferred to nitrocellulose, hybridized with ^{32}P-labeled HSV-1 DNA, washed, and autoradiographed (Fig. 4B). All of the *Eco*RI restriction fragments hybridized with the HSV-1 probe and were apparent in the autoradiogram. The bands representing pBR325, however, showed no hybridization to HSV-1 DNA and could be seen only in the gel. This demonstrated that the cloned fragments did contain HSV-1-specific sequences. In some cases, the chimeric plasmid DNA was also labeled with ^{32}P and hybridized

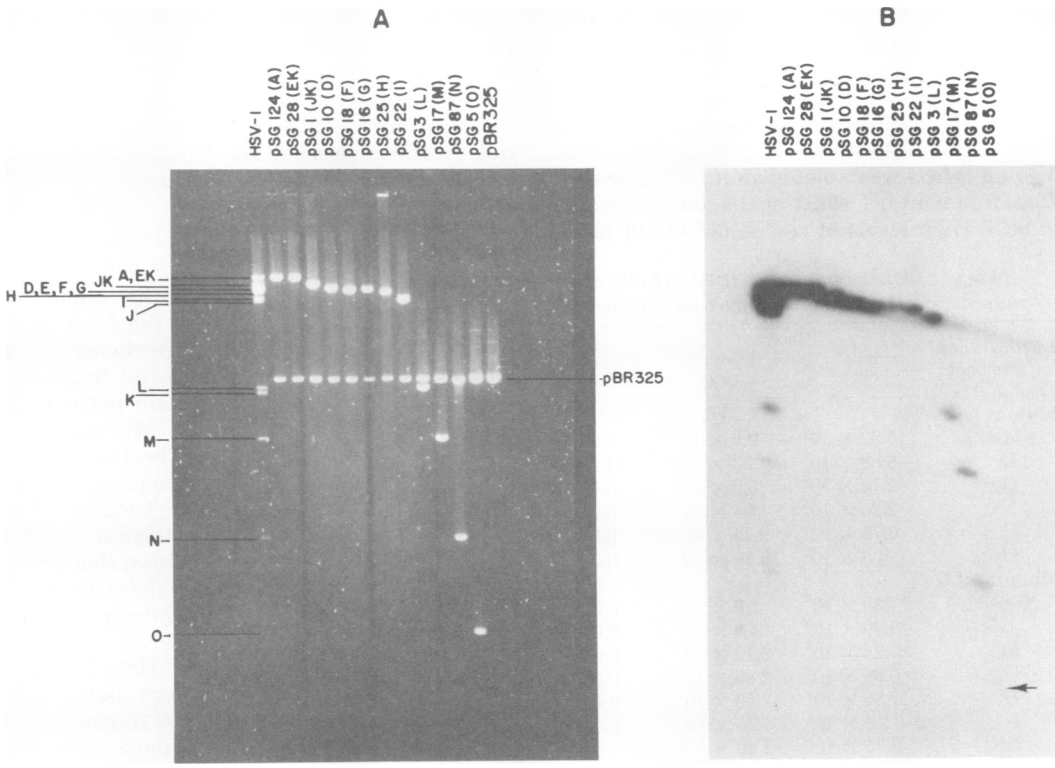

FIG. 4. *Demonstration of HSV-1 sequences in the chimeric plasmids. (A) DNA of each of the chimeric plasmids was isolated, digested to completion with EcoRI, and electrophoresed on a 1% agarose slab gel at 50 V for 20 h as described in Materials and Methods. The DNA bands were visualized by staining with ethidium bromide. (B) The DNA in the gel was transferred to nitrocellulose and hybridized to ^{32}P-labeled HSV-1 (KOS) virion DNA, and the filter was autoradiographed as described in Materials and Methods. HSV-1 (KOS) virion DNA and pBR325 DNA were treated similarly as controls. The fragment inserted in each chimeric plasmid is indicated in parentheses after the plasmid name. EcoRI fragments of HSV-1 (KOS) are indicated on the left. The arrow indicates the location of EcoRI fragment O from pSG5.*

to nitrocellulose blots of *Eco*RI digested HSV-1 DNA, confirming the identity of those cloned inserts (data not shown).

Marker rescue of HSV-1 *ts* mutants by cloned DNA fragments. Hybridization analysis demonstrated that the cloned *Eco*RI restriction fragments contained HSV-1 sequences (Fig. 4). To assess the biological activity of these cloned inserts, they were tested for their ability to rescue HSV-1 *ts* mutants in cotransfection experiments. The accompanying paper (27) describes the isolation of HSV-1 *ts* mutants after mutagenesis of specific *Hpa*I fragments and the subsequent transfer of the mutations to the viral genome by recombination during cotransfection with fragments and viral DNA. Three of these mutants were selected for marker rescue experiments with cloned *Eco*RI fragments. Table 1 shows the mapping data for mutants *tsJ*176, *tsLG*4, and *tsB*84. *TsJ*176 was isolated from a transfection in which *Hpa*I fragment J was mutagenized. *Hpa*I-J maps between coordinates 18.2 and 22.8 on the HSV-1 physical map (Fig. 5). *Eco*RI fragment D, which spans coordinates 8.4 to 19.0, efficiently rescued *tsJ*176 (Table 1), confirming the location of the mutation in *Hpa*I fragment J and further defining the position of the mutation to between 18.2 and 19.0 map units. Mutant *tsLG*4 was isolated after mutagenesis of *Hpa*I fragment LG, which spans coordinates 77.9 to 88.4. High levels of rescue (33.5%) of *tsLG*4 were achieved with cloned *Eco*RI fragment EK, which maps between coordinates 71.6 and 85.5, again confirming that the mutation in *tsLG*4 lies in the region of LG. The position of the mutation in *tsLG*4 can be placed between coordinates 77.9 and 85.5.

TABLE 1. *Marker rescue of HSV-1 (KOS) ts mutants by cloned EcoRI restriction fragments*

*Eco*RI-cloned fragment	Titer		% Rescue[a]
	34°C	39°C	
Mutant *tsJ*176			
None	5.22×10^6	2.3×10^3	0.04
D	3.88×10^6	1.33×10^6	34.0
G	2.70×10^5	5.0×10^1	0.02
N	2.25×10^6	1.03×10^3	0.05
F	6.75×10^6	9.75×10^2	0.01
H	4.5×10^6	9.70×10^2	0.02
Mutant *tsLG*4			
None	7.39×10^5	1.8×10^2	0.02
JK	9.40×10^5	4.6×10^2	0.04
M	5.28×10^5	2.12×10^2	0.04
F	6.88×10^5	2.86×10^2	0.04
H	7.85×10^5	7.50×10^1	0.01
I	5.0×10^5	5.75×10^1	0.01
EK	6.86×10^5	2.30×10^5	33.5
N	7.19×10^5	2.35×10^2	0.03
Mutant *tsB*84			
None	1.79×10^7	5.0×10^3	0.03
JK	1.18×10^7	2.02×10^4	0.2
F	1.49×10^7	8.0×10^5	5.4
H	1.5×10^7	5.18×10^3	0.03
EK	9.0×10^6	3.6×10^4	0.4

[a] Determined as (titer at 39°C/titer at 34°C) × 100.

As described in the accompanying paper (27), mutant *tsB*84, which was originally called *tsLD*84, was isolated from a transfection using mutagenized DNA extracted from *Hpa*I band LD, which maps between 77.9 and 91.7. However, when *tsB*84 was mapped by marker rescue using *Hpa*I fragments which had undergone two-dimensional gel purification, it was found that *Hpa*I fragment B (32.8 to 45.2 map units) and not LD rescued the mutant (27). Since only one gel purification was used in the isolation of DNA fragments for the original mutagenesis and since *Hpa*I fragments LD and B migrate closely in agarose gels, it was concluded that the B fragment was mutagenized as a contaminant of the LD extract, resulting in the isolation of *tsB*84. To confirm the position of *tsB*84, marker rescue experiments with cloned *Eco*RI fragments were performed. *tsB*84 was rescued by *Eco*RI fragment F, which lies between 31.1 and 41.5 map units (Table 1). Therefore, the mutation in *tsB*84 does lie in *Hpa*I-B, and its position can be further defined to lie between 32.8 and 41.5 map units.

DISCUSSION

Sequences representative of the whole genome of HSV-1 strain KOS in the form of 12 *Eco*RI restriction fragments were cloned in the plasmid vector pBR325. The cloned fragments were identified by their migration patterns in agarose gels compared with *Eco*RI-restricted HSV-1 virion DNA (Fig. 3). The presence of HSV-1 sequences in the chimeric plasmids was shown by hybridization to HSV-1 virion DNA (Fig. 4). As a measure of the biological activity of the cloned fragments, it was shown that HSV-1 *ts* mutants could be rescued efficiently in cotransfection experiments with the cloned fragments (Table 1).

The availability of a library of HSV-1 cloned sequences should prove to be very useful both for in vitro mutagenesis of DNA fragments as described in the accompanying paper (27) and for mapping HSV-1 mutants by marker rescue. As previously discussed, the purification of fragments from gels often results in the contamination of one fragment with another (27). In addition, fragment yields after agarose purification are usually low. The use of cloned fragments circumvents both problems, and large quantities of pure fragments can be obtained readily.

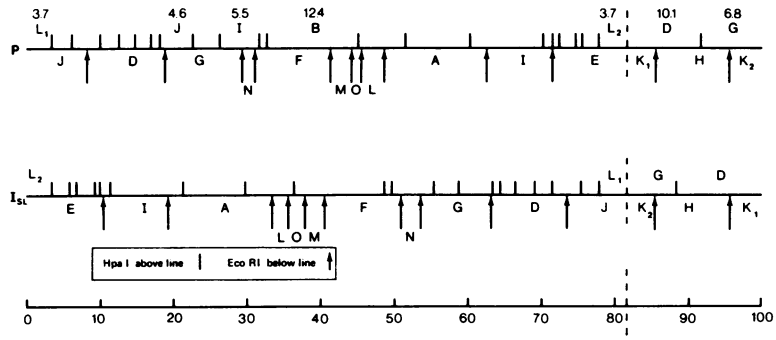

FIG. 5. *EcoRI restriction map of HSV-1 (KOS) DNA showing HpaI restriction sites (22). The map represents the P and I_{SL} orientations of the molecule. The HpaI fragments that were mutagenized in vitro, as described in the accompanying paper (27), are shown above the line.*

It was observed that the cloned fragments had migration patterns in agarose gels after *Eco*RI or *Eco*RI/*Bgl*II digestion that were indistinguishable from the patterns of the corresponding fragments generated by digestion of HSV-1 virion DNA. Unlike the results of Post et al. (23), no deletions or insertions were found in the cloned fragments. Because the majority of the cloned *Eco*RI inserts are relatively large (in the range of 10 Md), only large deletions or insertions would be detected by our procedures. For this same reason, it was not possible to determine the number of copies of the terminal redundancy (sequences indicated as "a" in Fig. 1) contained in each joint fragment. This number has been shown to be variable (17, 23, 33). Preliminary results from *Bam*HI digests of the cloned *Eco*RI joint fragments indicate that we have clones containing at least two differently migrating forms of the *Bam*HI joint fragment. This could be the consequence of different copy numbers of the "a" sequence. The exact number of copies of the "a" sequence in all of the cloned *Eco*RI joint fragments has not yet been determined, however.

Finally, the presence of "poison" sequences, that is, sequences of HSV-1 which cannot be cloned, seems unlikely since we have obtained clones for *Eco*RI fragments spanning the whole genome. Post et al. (23) were not able to obtain a *Bam*HI fragment (fragment V) in their cloning of HSV-1 strain F. This fragment is contained entirely within our cloned *Eco*RI fragment F. Therefore, it is unlikely that the presence of the coding sequence for the HSV-1 DNA polymerase molecule or a DNA synthesis initiation site within this fragment precludes its cloning, as suggested by Post et al. (23). As noted earlier, however, multiple clones of some fragments were obtained, whereas only one clone of fragment A was isolated, even after enriching for fragments of that size by fractionation on sucrose gradients. Since fragment A is only 0.3 Md larger than EK, a 0.5 M fragment for which we identified six clones, size alone cannot account for the differences in the isolation frequencies observed. It is not clear, therefore, why some sequences are cloned more readily than others.

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