Terminal Structure and Heterogeneity in Human Cytomegalovirus Strain AD169

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We have characterized the heterogeneity occurring at the junction of the long (L) and short (S) segments and at the termini of the strain AD169 human cytomegalovirus (HCMV) genome by restriction endonuclease mapping and nucleotide sequence analyses. The HCMV *a* sequence was identified by its position at both termini and inverted orientation at the L-S junction. Heterogeneity at both termini and the L-S junction was generated by the presence of fused and tandem *a* sequences. Some S termini lacked an *a* sequence. In addition, near the L terminus and at the L-S junction there were a variable number of 217-base-pair (bp) *Xho*I fragments arranged in tandem. The 217-bp fragments consisted of a portion of the *a* and adjacent *b* sequences (in the L-segment repeat) bounded by the same direct repeats (DR1) found at the boundaries of the *a* sequence. A model for the generation of these heterogeneous fragments is presented. We also determined the sequence of seven cloned terminal fragments, five from the L terminus and two from the S terminus. All L termini contained identical terminal sequences ending with base 32 of a 33-bp DR1. The S termini differed from each other and from the L-segment termini. One S terminus lacked an *a* sequence and terminated within S-segment repeat (*c*) sequences. The second S terminus contained an *a* sequence and terminated with bases 20 to 33 of a 33-bp DR1. A comparison of the cloned L and S terminal sequences with cloned L-S junction sequences suggested that the termini contained 3' single base extensions which were removed during the cloning. We also show that the herpesvirus conserved sequence is in a similar position relative to the termini of HCMV and several other herpesviruses, thus adding further support for the role of the sequence in the maturation of viral DNA.

Human cytomegalovirus (HCMV), a member of the herpesvirus group, has a double-stranded DNA genome 240 kilobase pairs (kbp) in length (7, 9, 13, 14, 28, 29). The genome consists of two covalently-linked segments, L (long) and S (short), each bounded by inverted repeats (8, 10, 13, 24, 28, 37). The region where the L and S segments meet is termed the L-S junction, and inversion of the L and S segments leads to the production of four genome isomers (7, 8, 10, 11, 13, 24, 28). Heterogeneity is observed at the L-S junction and both termini of the AD169, Towne, and Davis strains of HCMV, though the amount of L-terminal heterogeneity is variable (6, 13, 27, 28, 30, 31). The S termini of all three strains and the L termini of the Towne and Davis strains vary by increments of 500 to 750 base pairs (bp); the L termini of the AD169 strain are unique in that the fragments vary by increments of 220 bp.

In herpes simplex type 1 (HSV-1), which has a genome structure similar to that of HCMV (26), the inverted repeats have the form *ab-U_L-b'a'a'c'-U_S-ca* (35). The HSV-1 *a* sequence is bounded by direct repeats (DR1), and a variable number of tandem *a* sequences are observed at the L-S junction and L terminus (5, 15, 21, 36). The DNA sequence of the HSV-1 double-*a* junction revealed that the tandem *a*'s shared a single DR1 between them (21). The *a* sequence appears to be involved in genome segment inversion and cleavage or packaging of concatemeric progeny DNA (20, 22, 23, 32-34).

We previously described the cloning and characterization of heterogeneous junction fragments from the AD169 strain of HCMV (28, 30, 31) (Fig. 1). Analysis of these cloned fragments revealed three classes of heterogeneity as defined by *Xho*I endonucleolytic cleavage (Fig. 1). Class I heterogeneous fragments varied by increments of 420 bp from the minimal fragment size of 553 bp. Class II heterogeneity was defined as the presence of multiple class I fragments, and class III heterogeneity was defined as a variable copy number of a 217-bp *Xho*I fragment. The DNA sequence of one cloned junction (Fig. 1) showed that the 553-bp class I fragment was bounded by DR1 and contained a sequence conserved among the genomes of HCMV, HSV-1, and HSV-2 (30).

In this paper, we examine the heterogeneity occurring at both the L-S junction and termini and identify the HCMV strain AD169 *a* sequence. Further studies on the class I and II heterogeneous fragments are presented which show that these fragments consist of fused and tandem *a* sequences, respectively. Nucleotide sequences for the 217-bp class III heterogeneous fragment and the termini are presented. A model for the generation of the heterogeneous fragments and a possible role of the herpesvirus conserved sequence in the maturation of viral DNA will be discussed.

MATERIALS AND METHODS

Virus and cells. HCMV strain AD169 was obtained from the American Type Culture Collection. Human foreskin fibroblasts were a gift from Stephen Spector. Methods for cell culture, viral infections, and the harvesting of viral DNA have been described (31).

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Restriction digests, gel electrophoresis, and hybridization methods. Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc., and Boehringer Mannheim Biochemicals and were used in the buffers recommended by the suppliers. To visualize the restriction fragments generated from individual *Xho*I class I heterogeneous fragments, the digested fragments were labeled with the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories) and [α - 32 P]deoxyribonucleoside triphosphates as described by Maniatis et al. (16). After polyacrylamide gel electrophoresis, the gels were dried and the labeled fragments were visualized by autoradiography. The procedure for ordering fragments by partial digestion with *Xho*I has been described (30).

Agarose gel electrophoresis was performed as previously described (31). Acrylamide gel electrophoresis was carried out with 5, 6, and 20% gels (19:1 acrylamide:bisacrylamide) with Tris-borate-EDTA as the electrophoresis buffer (16). Individual restriction fragments were isolated from low-melting-point agarose (Bethesda Research Laboratories) by benzoyl-naphthol-DEAE (BND)-cellulose chromatography as previously described (31). Alternatively, nucleic acid chromatography systems (NACS-52 columns; Bethesda Research Laboratories) were used with the same loading and elution buffers used for BND-cellulose chromatography. DNA fragments were isolated from acrylamide gels by incubation of the gel slice in 1 M NaCl-10 mM Tris hydrochloride-10 mM EDTA (pH 8) at 37°C with shaking for 16 h.

For hybridization studies, DNA was transferred from agarose gels to nitrocellulose filters or diazobenzyloxymethyl (DBM)-paper (Schleicher & Schuell, Inc.) as recommended by the supplier. Prehybridization (37°C for 2 to 16 h) and hybridization (37°C for 10 to 48 h) conditions were as follows for nitrocellulose filters: 50% formamide, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.0), 5 $\times$ Denhardt solution (1 $\times$ Denhardt solution is 0.02% each polyvinylpyrrolidone, Ficoll [Pharmacia Fine Chemicals], and bovine serum albumin), 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1 mg of denatured salmon DNA per ml, 5 mM EDTA, and 0.1% sodium dodecyl sulfate. Conditions for hybridization with DBM filters were identical except that 0.25 mg of denatured salmon DNA per ml was used and the prehybridization buffer contained 1% glycine.

Recombinant DNA techniques. The cloning of the *Eco*RI junction fragments, *Pvu*II-*Eco*RI subfragments containing the region of heterogeneity, and the *Xho*I 1,390-, 970-, 553-, and 217-bp heterogeneous fragments in plasmid vectors has been previously described (30, 31). The M13 vectors mp8, mp18, and mp19 were used to generate constructs for dideoxy sequencing, and the plasmid pUC13 was used to clone genomic termini. All vectors were treated with calf intestinal alkaline phosphatase before ligation with inserts. Ligation reactions were carried out with T4 DNA ligase (Bethesda Research Laboratories or P-L Biochemicals, Inc.) in ligase buffer (50 mM Tris hydrochloride [pH 7.2], 10 mM

MgCl₂, 10 mM dithiothreitol, 1 mM spermidine, 1 mM ATP, 100 μ g of bovine serum albumin) at 22°C for 1 h or at 14 to 16°C for 6 to 16 h. Transformation of pUC13 into the host *Escherichia coli* JM83 was accomplished by the method of Kushner (12); M13 transformations and preparation of single-stranded templates used *E. coli* JM103 and the method of Messing et al. (18).

The strategies used to clone junction subfragments and genomic termini were as follows. The *Xho*I 217-bp fragment was gel purified on 2% agarose gels and ligated into the *Sal*I site of the vector M13mp8. The internal fusion regions of the *Xho*I 970- and 1,390-bp class I fragments were isolated as 80-bp *Sma*I-*Hae*III fragments on 5% acrylamide gels and cloned into the *Sma*I site of M13mp18. In some of the junction regions that we wanted to sequence, no convenient restriction sites were found. In these cases, the recombinant plasmids containing the *Pvu*II-*Eco*RI heterogeneous fragment were linearized with *Pvu*II, and BAL 31 exonuclease (Bethesda Research Laboratories) in buffer containing 20 mM Tris, 12 mM CaCl₂, 12 mM MgCl₂, 200 mM NaCl, and 1 mM EDTA (pH 8) was used to produce deletions through the region of interest. The resulting fragments were treated with T4 DNA polymerase to produce blunt ends and cleaved with the restriction endonuclease *Bam*HI. The deletion fragments in the desired size range were selected by gel purification and ligated to *Bam*HI-*Sma*I-cleaved M13mp19. This construction placed the deletion adjacent to the primer binding site for sequencing.

Two approaches were used to clone the *Eco*RI terminal fragments of the genome. In each instance, HCMV virion DNA was treated with T4 polymerase plus deoxyribonucleoside triphosphates to produce blunt ends. In the first cloning attempt, *Sma*I-*Eco*RI-cleaved pUC13 was ligated to uncleaved virion DNA. This mixture was cleaved with *Eco*RI, and a second ligation reaction was performed to circularize the construct. After transformation, clones were screened by colony hybridization (31) with fragments unique to each of the termini as probes. A few termini were cloned in this manner, but the background levels were high, perhaps due to the presence of fragmented genomes which were cloned in the same manner as authentic termini. In the second approach, the virion DNA was cleaved with *Eco*RI and the terminal fragments were isolated by gel purification before ligation to the vector.

DNA sequencing. Inserts cloned into M13 vectors were sequenced by the dideoxy chain termination method (25). The method of Maxam and Gilbert (17) was used to sequence regions of the *Xho*I 970- and 1,390-bp fragments closest to the L genome segment. The gel-purified *Xho*I fragments were treated with calf intestinal alkaline phosphatase, labeled with T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ - 32 P]ATP, and cleaved with *Hinf*I. The 400-bp *Xho*I-*Hinf*I fragment was gel purified for sequencing. The virion termini were also sequenced by the method of Maxam and Gilbert (17). Recombinant plasmids containing the terminal fragments were linearized and labeled (as de-

FIG. 1. The L-S junction region of the HCMV genome. At the top is shown the *Eco*RI restriction endonuclease map of the HCMV genome strain AD169 in one orientation (28). ///, Inverted repeats bounding the L and S segments of the genome; ~~~, L-S junction. Below this map is shown an expanded restriction endonuclease map of the *Bam*HI-*Pvu*II fragment containing the region of heterogeneity at the L-S junction (30). Roman numerals indicate the location of the three types of heterogeneity. At the bottom is shown the nucleotide sequence of a *Hae*II fragment containing the region of heterogeneity in one cloned L-S junction fragment (30). This L-S junction fragment contained one copy of the 553-bp *Xho*I class I fragment and no copies of the 217-bp *Xho*I class III fragment. Direct repeats of 15 bp or more are indicated by DR. The arrows mark inverted repeats near DR1. The conserved region X is also indicated. The nucleotide numbers correspond to those presented previously for the entire 2,538-bp *Bam*HI-*Pvu*II fragment.

FIG. 3. Relationships of the class I heterogeneous fragments. The *XhoI* 553-, 970- and 1,390-bp fragments are diagrammed schematically. The 970- and 1,390-bp fragments are composed of head-to-tail fused 553-bp fragments. □, Portions of the DR1 which lie within the *XhoI* sites. The *SmaI* (S)-*HaeIII* (H) fragments spanning the fusion regions of the 970- and 1,390-bp fragments were isolated and sequenced. The nucleotide numbers are from the L-S junction sequence (Fig. 1). Horizontal numbers indicate the positions of the *SmaI* and *HaeIII* sites within the junction; the vertical numbers indicate the boundaries of the deletion. The fusion points are marked with arrowheads. The sequence of the *SmaI*-*HaeIII* fusion fragment is given. Not all *SmaI* and *HaeIII* sites are shown.

scribed above for the *XhoI* heterogeneous fragments) at the *HindIII* or *BamHI* site in the polylinker adjacent to the virion terminus. *EcoRI* or *HindIII* cleavage produced uniquely labeled fragments, and the fragment containing the terminus was isolated by gel purification.

RESULTS

Relationships of the class I heterogeneous fragments. The previously described class I heterogeneous fragments varied in increments of 420 bp and mapped to identical locations within the junction (30; Fig. 1). Three representatives of this group of heterogeneous fragments (*XhoI* fragments of 553, 970, and 1,390 bp) were present in our cloned junction fragments. The sizes used previously to describe these fragments were 553, 950, and 1,350 bp, but the restriction endonuclease analyses described below indicated that 970 and 1,390 bp were more accurate sizes for the two larger fragments.

The relationships of the class I fragments were examined by restriction endonuclease digestion. Gel-purified class I *XhoI* fragments were cleaved with a single restriction endonuclease (*SmaI*, *HaeIII*, *AvaII*, *HinfI*, *BstNI*, or *SstII*) and end labeled with the Klenow fragment of DNA polymerase I and [α - 32 P]deoxyribonucleoside triphosphates. The resulting

fragments were compared on 6 and 20% nondenaturing acrylamide gels. For each of the enzymes, the restriction patterns generated for the *XhoI* 970- and 1,390-bp fragments were almost identical to those of the 553-bp fragment, except that an extra band was observed for the 970- and 1,390-bp fragments (Fig. 2A). For example, *HinfI*, *BstNI*, and *SstII* each had a single recognition site within the *XhoI* 553-bp fragment and produced two fragments from each of the class I fragments (*HinfI*, 398 and 155 bp; *BstNI*, 350 and 203 bp; and *SstII*, 295 and 258 bp). In addition, all three enzymes cleaved the 970- and 1,390-bp fragments to produce a band of approximately 420 bp. The *SmaI*-digested class I fragments all contained bands of 303, 161, 47, 22, and 20 bp. The 970- and 1,390-bp fragments also yielded a 330-bp fragment. *HaeIII* digests gave similar results (data not shown). In each restriction digest (except for *AvaII*, which will be discussed below), the higher-molecular-weight class I fragments contained all the bands appearing in the 553-bp fragment plus a single additional band equal in size to the sum of the terminal fragments minus 130 to 135 bp. These results suggested that the higher-molecular-weight class I fragments were generated by head-to-tail fusions of the 553-bp fragment, with the loss of approximately 135 bp at the fusion point (Fig. 2B).

The *AvaII* restriction pattern of the *XhoI* 970-bp fragment

FIG. 2. (A) Restriction digestions of class I *XhoI* heterogeneous fragments. Subcloned class I heterogeneous fragments, labeled at the top of each lane, were cleaved with the restriction endonucleases indicated. All samples were electrophoresed on 6% polyacrylamide gels. The *SmaI* and *AvaII* digested samples were also run on 20% gels (short panels). The fragment sizes (indicated next to the bands) were obtained from the L-S junction sequence. The minor bands in the 1,390-bp lanes were probably due to exonucleolytic nibbling of the restriction fragments in this sample. (B) Restriction maps of the class I heterogeneous fragments. Restriction sites are shown for the endonucleases *XhoI* (X), *AvaII* (A), *HaeIII* (H), *HinfI* (Hf), *SstII* (St), *SmaI* (S), and *BstNI* (B). The *XhoI* 553-bp fragment is diagrammed within the junction. The DR1 are indicated by the bars. The 970- and 1,390-bp fragments are shown as isolated *XhoI* fragments (*XhoI* cleaves within the DR1). Nucleotide sequences are given for the L-S junction regions at the left (No. 1) and right (No. 2) borders of the *XhoI* 553-bp heterogeneous fragment and for the left borders of the *XhoI* 970 (No. 3)- and 1,390 (No. 4)-bp fragments. The nucleotide numbers for sequences correspond to the numbering in the L-S junction sequence (Fig. 1). The 25-bp DR1 sequences are underlined. Asterisks indicate the variant sequence found in the *XhoI* 970-bp fragment which corresponds to the DR1 S-segment border of a previously sequenced junction (30). The repetition of this sequence extends the DR1 sequence to 33 bp.

FIG. 4. Comparison of cloned class I heterogeneous fragments with *XhoI*-cleaved virion DNA isolated subsequent to the original cloning. The sizes (bp) of the cloned *XhoI* heterogeneous fragments are indicated at the top of the lane. V, Virion DNA cleaved with *XhoI*. Samples were run on a 1.5% agarose gel, blotted to DBM paper, and hybridized with a ^{32}P -labeled *XhoI* 553-bp fragment. Sizes for the cloned fragments and observed viral bands are indicated. (A) Virion DNA (designated 83A) isolated 3 years after the L-S junction fragments were cloned; (B) virion DNA (designated 83B) isolated from a plaque-purified viral stock derived from 83A.

differed slightly from the patterns of both the 553-bp and 1,390-bp fragments (Fig. 2A). The restriction fragments generated from the 553-bp fragment were 294, 199, and 55 bp in size (an additional 5-bp fragment was predicted from the sequence). The 1,390-bp fragment contained these *AvaII* fragments plus the additional 360-bp fusion fragment. The 970-bp fragment contained the 199- and 55-bp *AvaII* fragments and the 360-bp fusion fragment but had fragments of 275 and 23 bp replacing the 294-bp fragment. These restric-

FIG. 5. Nucleotide sequence and diagram of the border between tandem class I fragments. Clone 355/4, containing two tandemly repeated *XhoI* 553-bp fragments, was treated with BAL 31 exonuclease to create deletions through the border region. The deletion fragments were cloned into M13mp19 and sequenced by the dideoxy method. The underlined sequence and open boxes in the diagram below correspond to the 25-bp DR1. Asterisks indicate the variant sequence found in the *XhoI* 970-bp fragment which corresponds to the DR1 S-segment border of a previously sequenced junction (30). The repetition of this sequence extends the DR1 sequence to 33 bp. ■, Location of the sequence shown. The nucleotide numbers correspond to the numbering in the L-S junction sequence (Fig. 1).

tion fragment differences occurred near the left border of the 970-bp fragment adjoining the L segment of the viral genome. DNA sequencing revealed that all three class I fragments had identical sequences from the *XhoI* site through the first DR1 (Fig. 2B). The differences occurred immediately after the DR1: the 970-bp fragment had an 8-bp sequence (5'-GGAATGGA-3') replacing 4 bp (5'-CGCA-3') found in the 553- and 1,390-bp fragments. The variant sequence appearing in the 970-bp fragment corresponded to the DR1-S segment border in the previously sequenced junction (Fig. 1; nucleotides 2353-2359). The repetition of this sequence extended the DR1 to 33 bp rather than 25 bp. We will refer to 25-bp and 33-bp DR1 sequences to simplify later references to this variant sequence.

To characterize the point of fusion generating the *XhoI* 970- and 1,390-bp fragments, *SmaI*-*HaeIII* fragments spanning the fusion region were isolated, cloned, and sequenced. Three clones generated from the *XhoI* 970-bp fusion region were sequenced, and these results indicated that the 970-bp

FIG. 6. Relationship of the *XhoI* 217-bp class III fragment to the L-S junction. The *EcoRI* (R)-*PvuII* (P) L-S junction-spanning fragment from clone 310 is diagrammed at the top of the figure, with *Bam*HI (B) and *XhoI* (X) restriction sites and the restriction fragment sizes (kbp) indicated. The 553-bp fragment and border regions are shown in the expanded section along with the 217-bp *XhoI* class III fragment. The boxes indicate the 25-bp DR1; 1, 2, and 3 indicate regions of identity between the L-S junction and class III fragment. The class III fragment was sequenced as a subcloned *XhoI* fragment and also was used with BAL 31 exonuclease deletions through a cloned L-S junction. The DR1 sequences and *XhoI* (X) cleavage sites are indicated. *, Nucleotide region 1; =, region 2. Unmarked bases correspond to region 3.

FIG. 7. Terminal heterogeneity in HCMV virion DNA. The restriction endonucleases *PvuII*, *HindIII*, and *EcoRI* were used to study heterogeneity occurring at both S-segment termini, the S-segment terminus corresponding to *EcoRI*-L, and both L-segment termini, respectively. The digested DNA was separated by agarose gel electrophoresis and transferred to filters for hybridization with the probes indicated below each panel. The origin of each probe within the L-S junction WN fragment is indicated. The L-segment and S-segment repeats are indicated by the heavy line. The region of heterogeneity (het) lies between the L- and S-segment repeats. Restriction sites for *EcoRI* (R), *PvuII* (P), *XhoI* (X), and *AvaII* (A) are indicated. Not all *XhoI* and *AvaII* sites are shown. The sizes of *HindIII*-cleaved lambda DNA and *HaeIII*-cleaved ϕ X174 DNA markers are indicated.

fragment was derived from a head-to-tail arrangement of 553-bp fragments, with the loss of sequences spanning the DR1, including the herpesvirus conserved sequence. If a single DR1 was shared between the 553-bp fragments, the deletion would cover 134 bp. The fusion of the class I fragments is diagrammed in Fig. 3. Using the nucleotide numbers for the L-S junction sequence (Fig. 1), we found that the breakpoints occurred at nucleotide 2227 in the first 553-bp fragment and at 1809 in the second 553-bp fragment to generate the 970-bp fragment. There was no obvious homology between the two sequences in this region.

For the 1,390-bp *XhoI* class I fragment, only a single-sized fusion fragment was seen in each of the digests. This suggested that the two fusion events that were needed to generate a 1,390-bp fragment from three 553-bp fragments occurred at identical or nearly identical positions. Three clones were obtained and sequenced for this fusion fragment, and in each case, the sequence obtained was identical to the fusion of the 970-bp fragment.

Variation of class I heterogeneous fragments with time. The viral DNA used to clone the heterogeneous junction fragments described above was harvested in 1980. In 1983, another preparation of virion DNA designated 83A was used to study junction heterogeneity. In addition, this virus (83A) was used to establish another plaque-purified viral stock from which further DNA preparations were made (designated 83B). *XhoI* cleavage of these DNA preparations and hybridization with a 32 P-labeled class I fragment revealed the presence of altered class I heterogeneous fragments (Fig. 4). The smallest class I fragment was consistently 550 to 560 bp in size, but the larger fragments varied by increments of approximately 520 bp rather than the 420-bp increments previously observed. The 83A viral DNA (Fig. 4A) also contained a low level of fragments 1,390 and 1,810 bp in size, though no 970-bp fragments were detectable. Plaque purification of the 83A virus resulted in a viral stock (83B) containing only the new series of class I fragments (Fig. 4B).

Further characterization of class II heterogeneity. The

FIG. 8. Restriction endonuclease analysis of cloned terminal fragments from 83B viral DNA. Cloned *EcoRI* terminal fragments *EcoRI*-L (lane 1), -N (lane 6), and -W (lanes 2 to 5, 7 to 18) were cleaved with *HindIII* plus *EcoRI* plus *XhoI*. The fragments were separated by gel electrophoresis, transferred to DBM paper, and hybridized with a probe consisting of WN and WL junction fragments (A). The filters were dehybridized and hybridized with the *XhoI* 970-bp class I fragment (B). Lanes: 1, clone 393; 2, clone 394; 3, clone 396; 4, clone 400; 5, clone 402; 6, clone 407; 7, clone 419; 8, clone 436; 9, clone 445; 10, clone 464; 11, clone 484; 12, clone 510; 13, clone 535; 14, clone 553; 15, clone 570; 16, clone 607; 17, clone 629; 18, clone 648. The sizes (kbp) of the hybridizing bands are indicated.

region between tandem class I fragments was investigated to determine whether the DR1 was present as a single shared copy (as in HSV-1) (21) or as repeated copies. The clone 355/4, which contained two tandem 553-bp *XhoI* fragments (30), was used for these studies. The nucleotide sequence of the region between the two *XhoI* 553-bp fragments was determined with BAL 31 exonuclease to create deletions through the border region. The region between the two 553-bp fragments was found to contain neither a single, shared DR1 nor tandemly repeated DR1 (Fig. 5). Instead, the first 553-bp fragment contained a complete 33-bp DR1, followed by a partial DR1 consisting of the terminal 14 bp of a second 33-bp DR1. After this, the sequence matched the 553-bp *XhoI* fragment (starting at position 1804, as occurs at the left border of the 970-bp fragment).

Sequence of the class III fragment. The *XhoI* 217-bp class

III fragment was sequenced both as an isolated, subcloned *XhoI* fragment and, with BAL 31 deletions, as part of an intact L-S junction. The class III fragment was bounded by 25-bp DR1 and was composed entirely of sequences found in the previously sequenced junction fragment (Fig. 6): DR1, the 8-bp variant sequence from the 970-bp sequence, 35 bp (nucleotides 1804-1838) of the *XhoI* 553-bp fragment, 149 bp (nucleotides 1626-1774; *b* sequence) of the *XhoI* 1,039-bp fragment, followed by DR1. The DR1 bounding the class III fragment lay in the same orientation as those bounding the class I heterogeneous fragments. The breakpoints in the sequence did not show regions of homology.

Heterogeneous termini detected in virion DNA. Heterogeneity at the genomic termini was first observed during the construction of physical maps of the genome. Cloned L-S junctions or junction subfragments, hybridized to restriction

FIG. 9. *Xho*I partial digestions of cloned *Eco*RI terminal fragments. Recombinant plasmids were 32 P-labeled at a site in the pUC13 polylinker adjacent to the terminus and cleaved with restriction endonucleases to produce fragments labeled at one end. These fragments were subjected to partial *Xho*I digestion and electrophoresed on agarose gels. The gels were dried, and the fragments were visualized by autoradiography. Clone numbers are indicated at the top of each lane. *Hind*III-cleaved lambda DNA and *Alu*I- or *Hae*III-cleaved ϕ X174 replicative-form DNA were used as molecular weight markers. Lambda: 9.4, 6.7, 4.36, 2.3, 2.0, and 0.59 kbp. *Alu*I- ϕ X174 (middle panel): 1.01, 0.85, 0.66, and 0.35 kbp. *Hae*III- ϕ X174 (right panel): 1.35, 1.08, 0.87, 0.6, and 0.3 kbp.

digests of virion DNA, showed a laddering of bands corresponding to terminal and junction fragments (28). The smallest heterogeneous region subfragment that was used as a probe in these studies was the *Eco*RI-*Pvu*II fragment which contained the total heterogeneous region, 208 bp of S-segment repeat, and approximately 3,300 bp of L-segment repeat. To determine whether the class I heterogeneous fragments were present at both termini and might be analogous to the HSV-1 *a* sequence, virion DNA (83A) was cleaved with restriction endonucleases which allowed one terminus to be distinguished from the other terminus and

junction fragments. Probes representing various portions of repeated sequence were hybridized to Southern blots of the digested virion DNA.

*Pvu*II cleaves within the S-segment repeat to produce identical S termini, independent of S-segment orientation. The two probes used, the *Xho*I 553-bp class I heterogeneous fragment (probe A in Fig. 7) and the 5.3-kbp *Pvu*II-*Eco*RI heterogeneous fragment (designated probe B), gave identical hybridization results. The smallest hybridizing fragment was approximately 760 bp in size, equivalent to a 553-bp class I fragment plus the 208-bp *Xho*I-*Pvu*II fragment from the S-segment repeat. A ladder of hybridizing fragments varying by increments of approximately 550 bp was seen. As *Pvu*II cleaves outside the region of heterogeneity, this ladder would have been produced by both class I and II heterogeneity (both fused 553-bp fragments and tandem arrays of class I fragments). A less intense band containing the 1,390-bp class I fragment was also observed. With probe B, there was no 208-bp fragment visible. This result might suggest that all S termini contain a class I heterogeneous fragment, but a more likely explanation was that the 208-bp fragment did not form a large enough proportion of the 5.3-kbp probe to be visualized. As shown below, this proved to be the case.

*Hind*III cleaves outside the repeated regions of the genome to produce four terminal and four junction fragments (28). The S-segment terminus *Hind*III-Q (contained in *Eco*RI-L) is smaller (the predominant species is 7.1 kbp) and easily distinguished from the other *Hind*III termini (K, 13 kbp; I, 17 kbp; and H, 18 kbp) and junction fragments.

TABLE 1. Order of *Xho*I fragments in cloned *Eco*RI termini

Clone	<i>Eco</i> RI terminus	<i>Xho</i> I fragments (kbp)
393	L	3.8, ^a 2.8
407	N	0.55, ^{a,b} 6.45
402	W	1.08, ^{a,b} 1.6, ^b 1.08, ^b 0.22, ^c 1.04, ^c 2.3
388	W	0.56, ^{a,b} 0.22, ^c 1.04, 2.3
553	W	0.56, ^{a,b} 0.22, ^c 1.04, 2.3
464	W	0.54, ^{a,b} 0.22, ^c 0.22, ^c 0.22, ^c 1.04, 2.3
629	W	0.54, ^{a,b} 0.22, ^c 0.22, ^c 0.22, ^c 1.04, 2.3

^a Terminal fragment.

^b Class I fragment.

^c Class III fragment.

FIG. 10. Comparison of HCMV terminal and junction sequences. The DNA sequences (written 5' to 3') for termini W (clones 388, 402, 464, 553, and 629), L (clone 393), and N (clone 407) were placed end to end and aligned with L-S junction sequences from clones 310 (top) and 355/4 (bottom). The underlined sequence marked X is the herpesvirus conserved sequence. The unmarked underlined sequence indicates complete or partial 25-bp DR1 sequences. Asterisks indicate the variant sequence found in the *Xho*I 970-bp fragment that extends the DR1 sequence to 33 bp. The nucleotide numbers correspond to the numbering in the L-S junction sequence (Fig. 1).

*Hind*III-digested virion DNA was hybridized with the *Xho*I 553-bp fragment or with a 960-bp *Ava*II fragment from the S-segment repeat (probe C, Fig. 7). Different results were obtained with each probe. Probe C from the S-segment repeat hybridized predominantly to fragments of 6.55, 7.1, and 7.65 kbp. The 553-bp fragment (probe A) hybridized to the 7.1- and 7.65-kbp fragments but not to the 6.55-kbp fragment. This result suggested that a population of S termini does not contain a class I-hybridizing fragment and that the 6.55-kbp fragment represents an S-terminal fragment lacking a class I fragment. The increments between this and the higher-molecular-weight forms were approximately 550 bp. The higher-molecular-weight fragments were the result of the hybridization of probe C to the S terminus, *Hind*III-H, and junction fragments, as well as the hybridization of probe A to L and S terminal and junction fragments.

*Eco*RI cleaves within the L-segment repeat to produce terminal fragment *Eco*RI-W, whose size is distinct from S-segment termini *Eco*RI-L and -N and the junction fragments WL and WN. Both probes A (*Xho*I 553-bp fragment) and B (*Pvu*II-*Eco*RI heterogeneous fragment) hybridized to the same pattern of fragments. The smallest fragment in the series was 3.9 kbp, equivalent to an L-segment terminus with a single class I *Xho*I 553-bp heterogeneous fragment and no class III fragments. Distinct bands appeared at 4.12 and 4.42 kbp, most likely corresponding to termini with one *Xho*I 553-bp fragment plus one 217-bp fragment and fused or tandem 553-bp *Xho*I fragments, respectively. Other bands were apparent, but the banding pattern was not distinct, probably due to overlapping series of fragments resulting from multiples of 550-bp (combined class I and class II heterogeneity) and 217-bp (class III) fragments. Hybridization of the probes to other terminal and junction fragments was responsible for the bands larger than 6.7 kbp.

Studies on cloned *Eco*RI termini. Terminal fragments were cloned to determine the sequence of the terminal fragments and to examine heterogeneity within individual termini. Uncleaved virion DNA (83B) was treated with T4 polymerase to produce blunt ends for cloning. Two approaches for cloning *Eco*RI terminal fragments were used. In one, the uncleaved blunt-ended virion DNA was ligated to *Sma*I-*Eco*RI-cleaved pUC13, then cleaved with *Eco*RI, and circularized by ligation. Clones were screened by colony hybridization. Only three clones were obtained by this method. With the other approach, the blunt-ended high-molecular-

weight virion DNA was cleaved with *Eco*RI and the fragments were separated by gel electrophoresis. Gel slices corresponding to the predominant forms of each terminal fragment (*Eco*RI-W, -N, and -L) were taken. These gel-purified fragments were ligated to *Sma*I-*Eco*RI-cleaved pUC13. From the *Eco*RI-W gel slice, 274 putative *Eco*RI-W clones were obtained. The *Eco*RI-N fragment gel slice yielded single clones for *Eco*RI-N and -L and 13 clones for *Eco*RI-W. The reason for the low cloning efficiency of the S termini from this region of the gel is unknown.

Eighteen clones were analyzed by restriction digestion and hybridization. The cleavage with *Eco*RI plus *Hind*III plus *Xho*I, transfer to DBM paper, and hybridization with *Eco*RI junction fragments WL and WN yielded the results shown in Fig. 8A. Sixteen (lanes 2 to 5 and 7 to 18) of the clones yielded the characteristic *Xho*I pattern for the L-segment terminus corresponding to *Eco*RI-W: constant fragments of 2.3 and 1.04 kbp and class I and III heterogeneous fragments. Rehybridization of the same filters with the *Xho*I 970-bp class I fragment (Fig. 8B) confirmed the identity of the class I fragments. Eight of the *Eco*RI-W clones (lanes 7 to 9, 11 to 14, and 16) contained the 553-bp *Xho*I fragment, and two others (lanes 10 and 17) contained a slightly smaller fragment approximately 540 bp in size. Two clones (lanes 15 and 18) contained a 1,080-bp class I fragment (these heterogeneous fragments were from 83B DNA). The *Eco*RI clones shown in lanes 2 to 5 were obtained from the *Eco*RI-N gel slice and thus represented higher-molecular-weight forms. Two of these contained both 1,080- and 1,600-bp class I fragments (lanes 3 and 5), a third clone (lane 4) contained only the 1,600-bp fragment, and the fourth clone (lane 2) contained the 1,600- and 2,120-bp class I fragments. These results indicated that L-segment termini contained class I heterogeneity and that some termini could contain more than a single class I fragment (class II heterogeneity). Although fewer higher-molecular-weight *Eco*RI-W fragments (13 clones) were obtained compared with the lower-molecular-weight forms (274 clones), it was not possible to calculate the frequency of termini exhibiting class II heterogeneity, since the fragments used to clone the termini were from discontinuous gel slices and some size classes of terminal fragments would not have been represented.

Single clones for the S-segment termini corresponding to *Eco*RI-L (Fig. 8A, lane 1) and *Eco*RI-N (lane 6) were obtained. Cleavage of the clone for *Eco*RI-L with the en-

zymes *EcoRI* plus *HindIII* plus *XhoI* produced fragments of 3.8, 2.8, and 1.65 kbp. Hybridization with a class I fragment (Fig. 8B) indicated that no class I-related sequences were present in this terminus. This was consistent with the results shown in Fig. 7, in which some *HindIII* fragments from the S-segment terminus did not hybridize to a class I fragment. The *EcoRI*-N clone yielded a 6.45-kbp band and a 0.56-kbp fragment which hybridized with the class I fragment probe. The identities of the putative *EcoRI*-N and -L clones were confirmed by digestion with *PvuII*, which produces a characteristic restriction pattern for the S-segment termini (data not shown).

The *EcoRI*-L, *EcoRI*-N, and five *EcoRI*-W clones were

TABLE 2. Regions of DNA sequence homology between two 553-bp class I fragments^a

Region and nucleotide no. ^b	No. of bases	No. of matching bases	Avg % homology	No. of bases shifted ^c
1				
1852–2015	163	134	83	35
1817–1977	160			
2				
1852–2015	163	128	79	11
1841–2003	162			
3				
1864–2014	150	127	84	58
1806–1956	150			
4				
1863–2009	146	116	80	22
1841–1983	142			
5				
1908–2014	106	93	89	91
1817–1920	103			
6				
1885–1993	108	84	79	44
1841–1946	105			
7				
1908–2008	100	78	80	67
1841–1937	96			
8				
1919–1987	68	54	82	78
1841–1905	64			
9				
1944–2008	64	52	81	127
1817–1881	64			
10				
1944–2009	65	50	80	103
1841–1902	61			
11				
1955–2009	54	44	85	114
1841–1891	50			
12				
1959–2005	46	39	86	140
1819–1864	45			

^a Nucleotides 1775 to 2360. Region analyzed includes both copies of DR1.

^b Nucleotide numbers correspond to the sequenced L-S junction (30).

^c Number of bases by which one sequence is shifted relative to the other.

FIG. 11. Model for the generation of class I heterogeneity. The 553-, 970-, and 1,390-bp class I heterogeneous fragments are diagrammed. □, DR1; the numbers represent identical regions within the fragments. Apparently nonhomologous recombination between two *XhoI* 553-bp fragments (top) generates the 970-bp fragment (middle). This process may have been aided by homologous pairing between the single 553-bp fragment and the second tandem 553-bp fragment (see Table 2), leading to generation of the fusion fragment. The 1,390-bp class I fragment (bottom) can be generated by homologous recombination between two 970-bp fragments (middle).

examined by partial digestion analysis and DNA sequencing. Partial digestion with *XhoI* was used to determine the order of the *XhoI* fragments (Fig. 9 and Table 1). All clones except *EcoRI*-L (clone 393) contained one or more class I fragments at the genome terminus. *EcoRI*-W clones 388, 553, 464, and 629 contained single class I fragments, followed by one (clones 388 and 553) or three class III fragments (clones 464 and 629) and the 1.04- and 2.3-kbp constant fragments. *EcoRI*-W clone 402 contained three class I fragments (1,080, 1,600, and 1,080 bp), followed by a single class III fragment and the constant fragments. *EcoRI*-N contained a single class I fragment, followed by the 6.45-kbp fragment. *EcoRI*-L contained no class I fragment; the terminal fragment was the 3.8-kbp *XhoI* fragment, followed by the 2.8-kbp *XhoI*-*HindIII* fragment (since *HindIII* was used as the second enzyme for this sample, the 1.65-kbp *HindIII*-*EcoRI* fragment did not appear).

The terminal sequences are shown in Fig. 10, along with matching regions of two L-S junction sequences for comparison. All *EcoRI*-W clones contained identical terminal sequences ending with base 32 of a 33-bp DR1. The S-segment termini differed from each other and from the L-segment termini. The *EcoRI*-L terminus (5'-TGTCGGG-3') lay in S-segment repeat (c) sequences. If aligned end to end, the *EcoRI*-W and -L termini would form an L-S junction with a single 553-bp class I fragment. The aligned terminal sequences, however, were missing 1 bp relative to the L-S junction sequence of clone 310. This result was consistent with the presence of terminal, single base 3' extensions which were removed during the cloning.

The *EcoRI*-N terminus occurred within a DR1 sequence and consisted of the last 14 bp of a 33-bp DR1, followed by the 553-bp class I sequence (starting 5 bp past the end of the 25-bp DR1 at nucleotide 1804 of the clone 310 L-S junction sequence). When the *EcoRI*-W and -N sequences were aligned end to end, the sequence matched that of clone 355/4, which contained tandem 553-bp class I fragments. Again, one base from the junction was absent between the

HCMV
CCCCCGCTAAAAACACCCCCCGCCCTCGAGGACCCACACGCGCCCGGAATGGA

MCMV
CCCCCCCATCAAAATACCCCCCGGGGGGCCGCGCACTCAGACGCCGGGGGGG

HSV-1
CCCCCGGAAACACACCCCCCGGGGGTCGCGCGCGGCCCTTTAAAGCGCGCGCGCG

VZV
CCCCCGGAAATACCCCCCGCGTTTCTGGGCGCCGCGGACCCCGGAGAGG

EBV
CCCCCGAGAAAGACCCCCCGGGGGGCATCGGGGGGGTGTGGCGGGGCATGGGGG

H. SAIMIRI
CCCCACTGATTTTCCCCCGGGGGATAACTTGGTAGGCAAGAGGGGAAGGGG

FIG. 12. Comparison of herpesvirus termini. Terminal sequences (5' to 3') from HCMV, murine cytomegalovirus (J. R. Marks and D. H. Spector, manuscript in preparation), varicella-zoster virus (4), herpesvirus saimiri (2), and HSV-1 (23) and an inferred terminus from the Epstein-Barr virus (1) are compared. The conserved putative cleavage-packaging sequences are underlined.

terminal sequences, consistent with the existence of 3' single base extensions.

DISCUSSION

In a previous study of heterogeneous L-S junction fragments (30), we described three types of heterogeneity (classes I, II, and III) occurring within the L-S junction region. The structure and location of the class I heterogeneous fragments made them likely candidates for the HCMV *a* sequence. To examine this possibility and to gain information about the involvement of the heterogeneous fragments in the viral life cycle, we characterized further the three classes of heterogeneity and examined the structure of the genome termini.

The HCMV *a* sequence was identified by its position at both termini and inverted orientation at the L-S junction. The smallest class I heterogeneous fragment corresponded to a single *a* sequence, and the larger class I fragments were shown to consist of head-to-tail fusions of single *a*'s. The fusion event that formed the 970-bp fragment deleted both the DR1 and conserved sequence. This may be a frequent result of fusion events, since the conserved sequence and DR1 occur at the *a* sequence border. If either or both of these entities have a functional significance, the higher-molecular-weight class I fragments may be functionally equivalent to a single *a* sequence.

One possible mechanism for the generation of the fused class I species relies on the presence of homology in other regions of the sequence. A comparison of the sequences of the 553-bp *Xho*I fragment revealed that there were regions 45 to 160 bp in length with greater than 75% homology (see Table 2). Most of these imperfect repeats were located within the left half of the 553-bp fragment, a region which is enriched for multiple, short direct repeats and alternating GT dinucleotides (30). Table 2 shows that the alignments repre-

sented would shift one sequence 11 to 140 bases with respect to the other. A model for the generation of a fused *a* fragment is shown in Fig. 11 (top panel). The fused *a* fragment could have arisen through a recombinational event between a single *a* sequence and tandem *a* sequences. No homology exists at the actual fusion point, but regional homologous pairing between the single *a* sequence and the second tandem *a* could promote recombination between the single *a* sequence and the first tandem *a* in the region indicated. The imperfect direct repeats described above for the *a* sequence may promote unequal crossing over and lead to the generation of expanded or truncated *a* sequences (such as the 540-bp fragments observed in two of the cloned termini) or to different series of class I fragments whose members vary by different size increments. Such series of class I fragments were observed in different preparations of viral DNA. The formation of the 970-bp series of class I fragments by this mechanism would require an unequal crossover, shifting two *a* sequences by 134 bases. Table 2 lists two regions of homology which would cause shifts of 127 and 140 bases. Generation of the 1,080-bp series of fragments through unequal crossing over would require that the pairing segments be staggered by approximately 35 bases. A region of homology meeting this requirement is also listed in Table 2 (region 1). After the initial generation of a fused *a* fragment through unequal crossover (such as the 970- or 1,080-bp fragments), the higher-molecular-weight class I fragments might arise through homologous recombination between two staggered fused *a* fragments (Fig. 11, middle and lower panels).

Class II heterogeneity was generated by tandem *a* sequences. The border between two *a* sequences was sequenced and was found to consist of 1.5 copies of a 33-bp DR1. Since the partial DR1 in this sequence was shifted 14 bp relative to the complete DR1, it is possible that this structure may have been generated by unequal crossover in a manner similar to that proposed for the generation of the fused *a* fragments. Table 2 shows a 160-bp region with 79% homology which would shift the two *a* sequences 11 bases relative to one another. This result differed from the single DR1 observed between double *a*'s in HSV-1 (21). However, we only sequenced one double *a* junction, and it is possible that other borders between tandem *a* sequences would differ.

The class III fragment was located within the L-segment repeat (*b* sequence) adjacent to the *a* sequence. This 217-bp fragment consisted of sequences from the *a* and *b* regions bounded by DR1s and probably also arose through nonhomologous recombination. This event may have been promoted by unequal pairing as described above for the generation of the class I heterogeneous fragments, and there exists a 19-bp region with 79% homology which would shift the two *a* sequences 225 bases relative to one another. It is interesting that this same recombination event could have generated an *a* sequence lacking the DR1 adjacent to the *b* region, which has been observed in the Towne strain. The Towne *a* sequence is not bounded by DR1 (27), but comparison of the Towne and AD169 L-S junction sequences shows that a sequence homologous to the AD169 DR1 does exist in the Towne strain at the *a-c* border. The Towne strain also appears to lack class III heterogeneity.

The structure of the HCMV AD169 *a* sequence is similar to that of the HSV-1 *a* sequence (5, 21). For both viruses, the *a* sequences are bounded by DR1 and contain an internal region where a variable number of direct repeats occur. The *a* sequences of both viruses may also play an important role in recombination events. It has been proposed that defective

HSV genomes evolved as a consequence of a recombination process involving an *a* sequence (19). For HSV-1, there is also direct evidence that the *a* sequence is involved in inversion of the L and S segments and that the region containing the internal direct repeats in particular is needed to promote the inversion (3). Unequal crossover events during inversion may be responsible for generating the variability in the number of internal repeats. For the HCMV *a* sequence, the evidence is less direct. However, as described above, the heterogeneity at the L-S junction and termini can be most easily explained by recombination involving the *a* sequence.

A large number of studies have provided evidence that herpesvirus DNA replication proceeds through circular or concatemeric intermediates, which are subsequently cleaved and packaged as unit length molecules. Our studies suggest that like the termini of HSV-1 (23), varicella-zoster virus (4), and murine cytomegalovirus (Marks and Spector, in preparation), the HCMV AD169 termini have single base 3' extensions. Furthermore, analyses of the junction sequences and termini indicate that circularization of the genome could occur by end-to-end ligation of the termini. The mechanisms involved in the subsequent cleavage and packaging of unit length DNA molecules are not well understood. However, a comparison of the HCMV terminal sequences with those of murine cytomegalovirus (Marks and Spector, in preparation), HSV-1 (23), varicella-zoster virus (4), Epstein-Barr virus (1), and herpesvirus saimiri (2) reveals that the terminal cleavage site occurs 55 to 58 bp from the 5' end of the conserved sequence previously identified (30) (Fig. 12). Since the terminal nucleotide sequences of these viruses differ (and for HCMV, the S-segment terminal sequence can vary), it seems likely that the conserved sequence serves as a recognition site for a cleavage-packaging enzyme and that the actual site of cleavage is more dependent on distance from the signal sequence. The observation that the Towne strain *a* sequence contains a cleavage-packaging signal which is functional in the generation of HSV-1 defective genomes (27) adds further support to this hypothesis.

Recently Varmuza and Smiley (32) have presented evidence which suggests that the HSV-1 genome contains two cleavage sites, one at each end of the *a* sequence. In their experiments, a portion of the HSV-1 *a* sequence was placed within the thymidine kinase gene of the HSV-1 genome. This partial *a* sequence contained the proper signals for cleavage-packaging, but the actual cleavage event occurred outside the *a* sequence and within the thymidine kinase sequences. Since in the unsubstituted HSV-1 genome these cleavages normally occur within the DR1 of the intact *a* sequence, it was previously assumed that only one cleavage had occurred. Varmuza and Smiley postulate the existence of two signals, one on either side of the variable tandem repeat region, which dictate cleavages that are a specific distance from the signals but are not sequence specific. The conserved sequence is a likely candidate for one of these signals, but we have not found in HCMV a sequence equivalent to the second signal. Sequence comparison of the HCMV and HSV-1 junction sequences indicated a region of homology within the HSV-1 region containing the second signal, but it is not the correct distance from the HCMV terminus (unpublished results). Varmuza and Smiley also found that the single partial *a* sequence was duplicated at both termini. This is consistent with the observation that all HSV-1 termini contain at least one *a* sequence (15, 36) but differs from our finding that some HCMV S-segment termini lack an *a* sequence. Thus, while HSV-1 and HCMV are very similar in

their genome structure, it is likely that the two viruses differ in some aspects of viral DNA maturation.

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LITERATURE CITED

1. Bankier, A. T., P. L. Deininger, S. C. Satchwell, R. Baer, P. J. Farrell, and B. G. Barrell. 1983. DNA sequence analysis of the *EcoRI* Dhet fragment of B95-8 Epstein-Barr virus containing the terminal repeat sequences. *Mol. Biol. Med.* 1:425-445.
2. Bankier, A. T., W. Dietrich, R. Baer, B. G. Barrell, F. Colbère-Garapin, B. Fleckenstein, and W. Bodemer. 1985. Terminal repetitive sequences in herpesvirus saimiri virion DNA. *J. Virol.* 55:133-139.
3. Chou, J., and B. Roizman. 1985. Isomerization of herpes simplex virus 1 genome: identification of the *cis*-acting and recombination sites within the domain of the *a* sequence. *Cell* 41:803-811.
4. Davison, A. J. 1984. Structure of the genome termini of varicella-zoster virus. *J. Gen. Virol.* 65:1969-1977.
5. Davison, A. J., and N. M. Wilkie. 1981. Nucleotide sequences of the joint between the L and S segments of herpes simplex virus types 1 and 2. *J. Gen. Virol.* 55:315-331.
6. DeMarchi, J. M. 1981. Human cytomegalovirus DNA: restriction enzyme cleavage maps and map locations for immediately, early, and late RNA's. *Virology* 114:23-38.
7. DeMarchi, J. M., M. L. Blankenship, G. D. Brown, and A. S. Kaplan. 1978. Size and complexity of human cytomegalovirus DNA. *Virology* 89:643-646.
8. Fleckenstein, B., I. Muller, and J. Collins. 1982. Cloning of the complete human cytomegalovirus genome in cosmids. *Gene* 18:39-46.
9. Geelen, J. L. M. C., C. Walig, P. Wertheim, and J. van der Noordaa. 1978. Human cytomegalovirus DNA. I. Molecular weight and infectivity. *J. Virol.* 26:813-816.
10. Greenaway, P. J., J. D. Oram, R. G. Downing, and K. Patel. 1982. Human cytomegalovirus DNA: *BamHI*, *EcoRI*, and *PstI* restriction endonuclease cleavage maps. *Gene* 18:355-360.
11. Kilpatrick, B. A., and E.-S. Huang. 1977. Human cytomegalovirus genome: partial denaturation map and organization of genome sequences. *J. Virol.* 24:261-276.
12. Kushner, S. R. 1978. An improved method for transformation of *Escherichia coli* with ColE1 derived plasmids, p. 17-23. In H. W. Boyer and S. Nicosia (ed.), *Genetic engineering*. Elsevier/North-Holland Biomedical Press, Amsterdam.
13. LaFemina, R. L., and G. S. Hayward. 1980. Structural organization of the DNA molecules from human cytomegalovirus. ICN-UCLA Symp. Mol. Cell. Biol. 18:39-55.
14. Lakeman, A. D., and J. E. Osborn. 1979. Size of infectious DNA from human and murine cytomegaloviruses. *J. Virol.* 30:414-416.
15. Locker, H., and N. Frenkel. 1979. *BamHI*, *KpnI*, and *SalI* restriction enzyme maps of the DNAs of herpes simplex virus strains Justin and F: occurrence of heterogeneities in defined regions of the viral DNA. *J. Virol.* 32:429-441.
16. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*, p. 466-467. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65:499-560.
18. Messing, J., R. Crea, and P. H. Seeberg. 1981. A system for shotgun DNA sequencing. *Nucleic Acids Res.* 9:309-321.
19. Mocarski, E. S., L. P. Deiss, and N. Frenkel. 1985. Nucleotide

- sequence and structural features of a novel U_s - a junction present in a defective herpes simplex virus genome. *J. Virol.* **55**:140–146.
20. Mocarski, E. S., L. E. Post, and B. Roizman. 1980. Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. *Cell* **22**:243–255.
 21. Mocarski, E. S., and B. Roizman. 1981. Site-specific inversion sequence of the herpes simplex virus genome: domain and structural features. *Proc. Natl. Acad. Sci. USA* **78**:7047–7051.
 22. Mocarski, E. S., and B. Roizman. 1982. Herpesvirus-dependent amplification and inversion of cell-associated viral thymidine kinase gene flanked by viral a sequences and linked to an origin of viral DNA replication. *Proc. Natl. Acad. Sci. USA* **79**:5626–5630.
 23. Mocarski, E. S., and B. Roizman. 1982. Structure and the role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. *Cell* **31**:89–97.
 24. Oram, J. D., R. G. Downing, A. Akrigg, A. A. Dollery, C. J. Duggleby, G. W. G. Wilkinson, and P. J. Greenaway. 1982. Use of recombinant plasmids to investigate the structure of the human cytomegalovirus genome. *J. Gen. Virol.* **59**:111–129.
 25. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 26. Sheldrick, P., and N. Berthelot. 1975. Inverted repetitions in the chromosome of herpes simplex virus. *Cold Spring Harbor Symp. Quant. Biol.* **39**:667–678.
 27. Spaete, R. R., and E. S. Mocarski. 1985. The a sequence of the cytomegalovirus genome functions as a cleavage/packaging signal for herpes simplex virus defective genomes. *J. Virol.* **54**:817–824.
 28. Spector, D. H., L. Hock, and J. C. Tamashiro. 1982. Cleavage maps for human cytomegalovirus DNA strain AD169 for restriction endonucleases *EcoRI*, *BglII*, and *HindIII*. *J. Virol.* **42**:558–582.
 29. Stinski, M. F., E. S. Mocarski, and D. R. Thomsen. 1979. DNA of human cytomegalovirus: size heterogeneity and defectiveness resulting from serial undiluted passage. *J. Virol.* **31**:231–239.
 30. Tamashiro, J. C., D. Filpula, T. Friedmann, and D. H. Spector. 1984. Structure of the heterogeneous L-S junction region of human cytomegalovirus strain AD169 DNA. *J. Virol.* **52**:541–548.
 31. Tamashiro, J. C., L. J. Hock, and D. H. Spector. 1982. Construction of a cloned library of the *EcoRI* fragments from the human cytomegalovirus genome (strain AD169). *J. Virol.* **42**:547–557.
 32. Varmuza, S. L., and J. R. Smiley. 1985. Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. *Cell* **41**:793–802.
 33. Vlazny, D. A., and N. Frenkel. 1981. Replication of herpes simplex virus DNA: localization of replication recognition signals within defective virus genomes. *Proc. Natl. Acad. Sci. USA* **78**:742–746.
 34. Vlazny, D. A., A. Kwong, and N. Frenkel. 1982. Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length viral DNA. *Proc. Natl. Acad. Sci. USA* **79**:1423–1427.
 35. Wadsworth, S., R. J. Jacob, and B. Roizman. 1975. Anatomy of herpes simplex virus DNA. II. Size, composition, and arrangement of inverted terminal repetitions. *J. Virol.* **15**:1487–1497.
 36. Wagner, M. J., and W. C. Summers. 1978. Structure of the joint region and the termini of the DNA of herpes simplex virus type 1. *J. Virol.* **27**:374–387.
 37. Weststrate, M. W., J. L. M. C. Geelen, and J. van der Noordaa. 1980. Human cytomegalovirus DNA: physical maps for the restriction endonucleases *BglII*, *HindIII*, and *XbaI*. *J. Gen. Virol.* **49**:1–21.