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The replicating intermediates of herpes simplex virus type 1 DNA are relatively short

Satish L Deshmane, Boonyos Raengsakulrach, Joanne F Berson and Nigel W Fraser

The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA

Herpes simplex virus type 1 (HSV-1) replication is thought to occur via a rolling circle type of mechanism, generating large DNA concatemers from which unit length genomes are subsequently cleaved. In this report, we have employed field inversion gel electrophoresis (FIGE), Southern blot hybridization, and endonuclease digestion, to identify and characterize these DNAs. Two species of HSV-1 DNA: (1) genome-length and (2) DNA that remained at the electrophoresis origin (referred to as well-associated DNA) were detected. To ascertain that the latter was large in size and not virion DNA trapped at the origin with high molecular weight cellular DNA, the infected cell DNA was digested with a restriction enzyme that does not cut the viral DNA. In order to do this HSV-1 strain 1702, lacking any XbaI sites in its genome, was utilized. After digestion of samples with XbaI, and FIGE, cellular DNA was seen to migrate into the gel; however, the viral DNA remained in the sample wells. Pulse labeling experiments showed that this large DNA was processed to 150 kb genome lengths. Endonuclease digestion of the well-associated DNA revealed that it contained a greater ratio of joint to terminal fragments than virion DNA — a characteristic of long concatemers. Quantitation of the terminal fragments revealed mainly L termini. Surprisingly, the ratio of joint to terminal fragments was 2.5 suggesting that the lengths of concatemers were short (in the order of 1–2 genome lengths) and that the well association was due to conformation rather than concatemeric length. Because one of these genome lengths is present as the replication intermediate, the growing tail must be less than genome length. Thus genome lengths must be processed from the replication intermediate soon after they are completed.

Keywords: HSV-1; replication-intermediate; pulse-field electrophoresis

Introduction

The genome of herpes simplex virus 1 (HSV-1) is a 150-kbp linear duplex of DNA that is divided into two segments, the L (long; approximately 125 kbp) and the S (short; approximately 25 kbp), which are bracketed by inverted repeats of 8.5 kbp and 6 kbp respectively(Sheldrick and Berthelot, 1975; Hayward *et al*, 1975; Roizman, 1979). The genome is flanked at each end by a direct repeat, the 'a' sequence, and an inverted copy of this sequence at the L and S junction. The genome structure is diagrammed in Figure 1. A remarkable property of the HSV-1 genome is that the L and S components can invert relative to each other (Hayward *et al*, 1975; Delius and Clements, 1976) creating four isomers. Although virion associated HSV-1 DNA is a linear molecule, after infecting and establishing long term latency in sensory neurons of humans and experimental animals, this DNA exists in the form of either a circular, a circular concatemer, or a large linear concatemer (Rock and Fraser, 1983, 1985; Efstathiou *et al*, 1986). The latent viral genome is episomal and associated with cellular histones forming nucleosome structures similar to those found in cellular chromatin (Mellerick and Fraser, 1987; Deshmane and Fraser, 1989). This structure may be crucial for establishment or maintenance of latent infection.

Circular and concatemeric genome forms are generated during normal lytic replication which proceeds via circularization of virion DNA followed by generation of newly replicated concatemeric DNA. From these concatemers, unit-length genomes are cleaved and packaged into pre-assembled empty capsids (Jacob *et al*, 1979; Vlazny and Frenkel, 1981; Ben-Porat, 1982; Varmuza and Smiley, 1985; Deiss and Frenkel, 1986; Sherman and Bachenheimer, 1987). Since during lytic infection

Correspondence: NW Fraser
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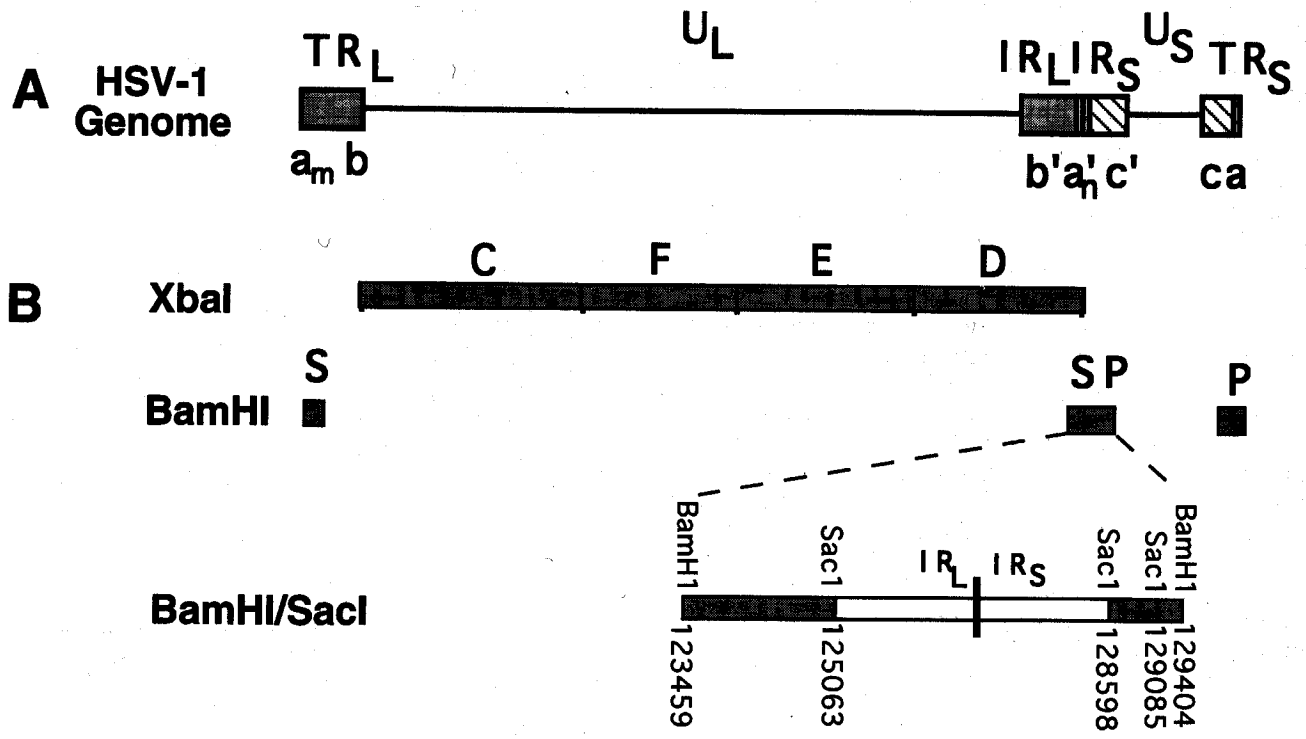

Figure 1 Structure of HSV-1 genome and maps of restriction endonuclease fragments used in this paper. (A) The HSV-1 genome consists of two covalently joined segmens—the long (125 kbp) and the short (25 kbp) segment—which can isomerize relative to each other. The long segment consists of a unique region bounded by terminal and internal repeat regions (TR_L and IR_L). The short segment also consists of a unique region bounded by terminal and internal repeat regions (IR_S and TR_S). (B) Restriction enzyme fragments used as probes. The location of the 1.6 kb *BamHI-SacI* fragment specific for *BamHI* S, and 0.8 kb *SacI-SacI-BamHI* fragment specific for *BamHi* P used as probes in the termini analysis are shown.

the linear genome circularizes and acts as a rolling cirle, with a long concatemeric tail of newly replicated DNA, it is possible that latent DNA may have this structure.

The 200–500 bp 'a' sequence which occurs at the termini and the L-S segment junction has been shown to be responsible for segment inversion as well as providing cleavage and packaging signals for newly replicated concatemeric DNA (Mocarski *et al*, 1980; Smiley *et al*, 1981; Mocarski and Roizman, 1982a). The 'a' sequence has been shown to be present in a variable number of copies within the L-S junction and at the L termini of all four isomers (Locker and Frenkel, 1979). The termini of mature viral DNA are generated by a process involving two separate DNA cleavages at the site distal to cleavage signals located within the 'a' sequence (Varmuza and Smiley, 1985). Some of these cleavage events generate L and S termini ending with a 3' protruding nucleotide (Mocarski and Roizman, 1982b). Studies have demonstrated that the signals for cleavage and packaging of DNA, designated as pac1 and pac2, are located in the 'a' sequence (Varmuza and Smiley, 1985; Deiss *et al*, 1986). It is interesting to note that these pac sequences have recently been shown to play a critical role in genome isomerization (Sarisky and Weber, 1994). DNA-protein interaction essential for cleavage occurs within the pac2 sequence. Specific polypeptides, one of which has been identified as a HSV-1 specific DNase, bind to these sequences to mediate cleavage and packaging (Chou and Roizman, 1989).

Viral DNA genome numbers in latently infected neurons have been estimated in the range of 20 genomes per cell (Stevens 1989) indicating that there could be limited viral DNA replication without killing of the host cell resulting in establishment of latency. During a lytic herpes virus infection some of the early events such as circularization of the linear viral genome are known to be mediated in presence of inhibitors that would arrest protein and viral DNA synthesis, indicating that either cellular factors present before infection or factors associated with the virion may be involved (Poffenberger and Roizman 1975; Garber *et al*, 1993; McVoy and Alder 1994). Presumably such viral DNA replication, if it occurs in cells destined to become latently infected, would not result in neuronal death, at least in the establishment phase of latency, and suggests that limited viral replication may take place via a cellular mechanism. In order to account for this increase in HSV DNA levels in the

infected cells, it has been proposed that circular DNA may be replicated like 2μ plasmid of Saccharomyces cerevisiae, which has a genomic organization strikingly similar to that of a herpes viruses (Hartley and Donelson, 1980; Zhang *et al*, 1994). It seems that in a neuron destined to become a latent reservoir initial 2μ plasmid like amplification of herpes DNA may be allowed, however these amplified circular templates are somehow prevented from entering into further lytic cycle rolling-circle replication.

Development of field inversion gel electrophoresis has offered new opportunities in the structural analysis of large species of DNA (Schwartz and Cantor, 1984; Carle and Olson, 1984). We have used this technique to analyze HSV-1 DNA generated during replication in culture cells. Analysis of well associated DNA showed that long-arm termini were prevalent, occurring at a ratio of about one terminus per 2.4 L-S joints. Thus replication intermediate HSV DNA consists of a circular unit length genome (containing 2 joint regions) with a short tail of newly replicated DNA (containing on average 0.4 joint regions) and predominantly an L termini.

Results

FIGE analysis of replicating HSV-1 DNA

We have attempted to separate, identify, and characterize the replicative intermediates of the HSV-1 genome by FIGE and blot hybridization. Briefly, CV-1 cells were mocked-infected or infected with HSV-1 strain F. At various times post infection (p.i.), the infected cells were harvested and gel blocks were prepared for FIGE analysis as described in the methods.

By studying the separation of yeast chromosomal DNA markers, it was clear that FIGE was capable of resolving linear fragments up to 2200 kb in size (Figure 2a, lane 8). However resolution in the lower size range (20 kb to 150 kb) was compromised, since the largest fragment of a HindIII digest of λDNA (23.1 kb), and first three concatemers of a λDNA ladder (50 kb, 100 kb, 150 kb) were found to migrate very close to each other (Figure 2a, lanes 9 and 10).

Southern blotting and hybridization to a HSV-1 genomic probe was used to detect the viral DNA (Figure 2b). Two species were observed. One migrated at 150-kbp, was seen at 2 h p.i. and was probably unit length virion DNA from the inoculum. The other species remained at the electrophoresis origin. Since this DNA did not migrate from the sample well, it might consist of DNAs of variable length but too large in size to be resolved with our FIGE conditions. We named these DNAs collectively as well-associated DNA and conclude that they were either larger than 2.2 mb (approximately 14 times the size of the unit-length HSV genome) or had a conformation unresolvable by FIGE.

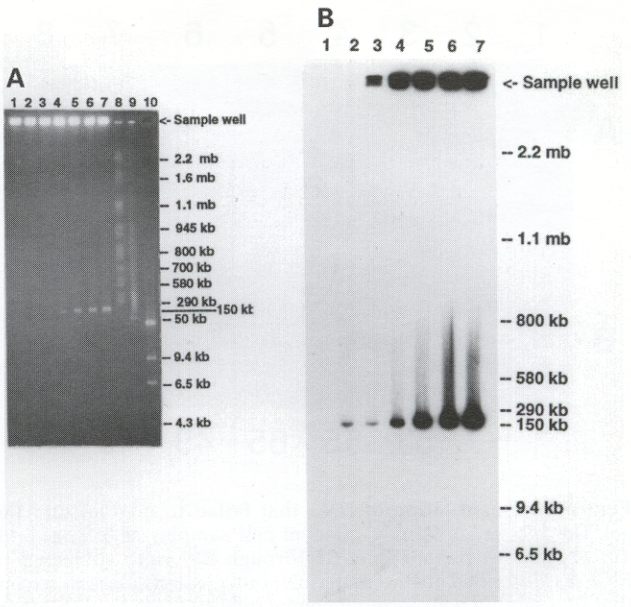

Figure 2 FIGE of HSV-1 DNA during productive infection. (A) Ethidium bromide-stained gel. DNA from mock-infected cells (lane 1) or from HSV-1 infected cells at 2, 4, 6, 8, 10, 12 h p.i. (lanes 2 to 7) was analyzed by FIGE. Size of DNA was estimated from location on the gel of molecular weight markers: yeast chromosomal DNA (lane 8), lambda DNA ladder (lane 9), and lambda DNA/HindIII fragments (lane 10). The electrophoresis origin is marked 'sample well'. The gel was run for approximately 48 h, at 6 V cm^{-1}, 15°C, with time ramp from 0.1 to 61 second reverse and 0.3 to 185 second forward. (B) Southern blot hybridization of gel in (A) with HSV-1 genomic probe.

The amount of well-associated DNA increased between 2–4 h p.i., suggesting that the viral replication was initiated at about this time. An increase in the amount of 150-kbp DNA species at 8 h p.i. indicated cleavage and packaging of new genome length DNA. The difference between these two times probably reflects the time required for expression of significant amounts of late viral proteins, which are required for packaging of the viral DNA.

The well associated DNA appeared by 4 h p.i. and increased in amount as the infection progressed. By comparing the amount of HSV DNA remaining in the sample well after FIGE with the amount of DNA in the gel blocks prior to FIGE, it was estimated that the DNA which could not migrate into the gel accounted for approximately 50% of the total HSV-1 specific DNA (Figure 3). Since FIGE can resolve linear molecules up to 2.2 mb in size, it appeared that well associated DNA was either greater than 2.2 mb in size, a linear structure shorter than 2.2 mb which is trapped with the cellular chromatin (due to tangling), or has a novel structure which may not enter the gel.

The well associated DNA is not trapped with cellular DNA

In order to rule out the tangled molecule possibility

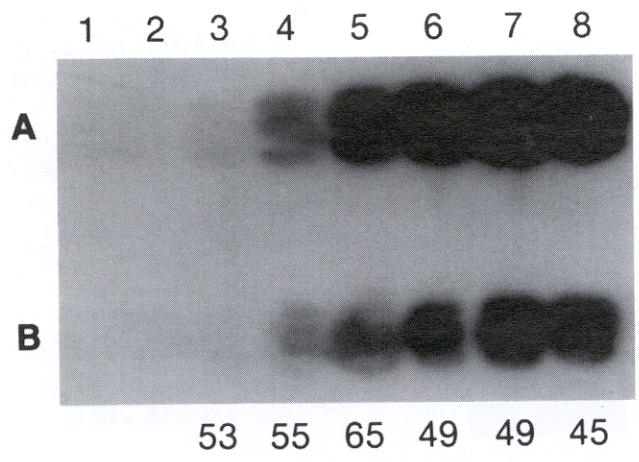

Figure 3 Quantitation of DNA that failed to migrate into the gel. Gel blocks containing infected cell samples at 0, 2, 4, 6, 8, 10, 12, and 14 h p.i. (Slots B1 through B8) were subjected to FIGE as described in the materials and methods section except that two sample well forming combs (placed close to each other) were used in casting the gel. At the end of FIGE, original agarose blocks which were not subjected to FIGE (Slots A1 through A8) were inserted into replicate loading wells. The part of the gel containing both sets of samples was cut off from the rest of the gel, depurinated, neutralized and blotted onto Nytran nylon membranes by capillary blotting (Southern, 1975). The blot was hybridized with HSV-1 genomic probe, washed and processed for auto radiography, the percent of radioactivity (numbers shown at the bottom of panel B) remaining in the sample blocks at the end of FIGE, was determined by counting corresponding slots in a liquid scintillation counter.

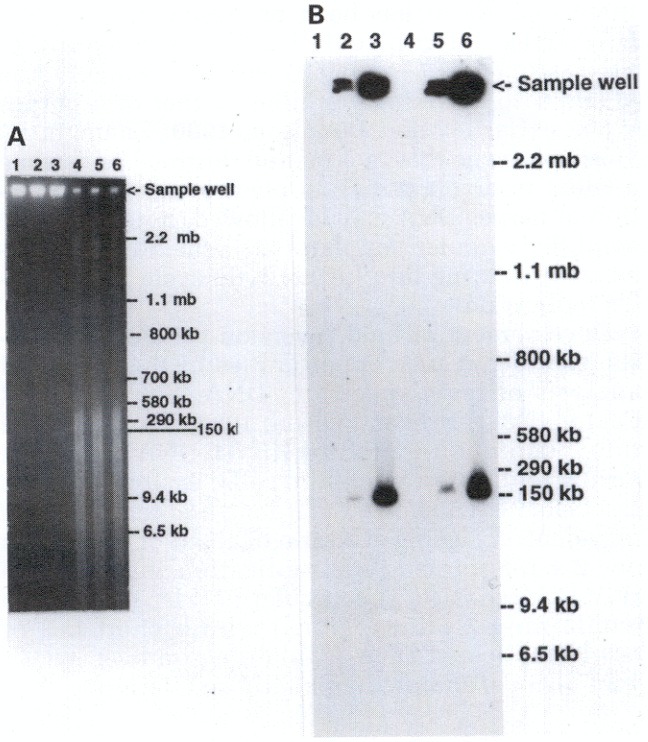

Figure 4 FIGE of HSV-1 strain 1702 DNA after XbaI digestion. (A) Ethidium bromide-stained gel. Gel blocks containing infected DNA were treated with XbaI (lane 4 to 6) to reduce the size of cellular DNA as described in materials and methods. Lanes 1 to 3 were samples that had been processed for XbaI digestion except no enzyme was added. The gel blocks were analyzed by FIGE according to condition in Figure 1. Lanes 1 and 4, DNA from mock-infected cells; lanes 2 and 5, DNA from 1702-infected cells at 8 h p.i.; lanes 3 and 6, DNA from 1702-infected cells at 12 h p.i. (B) Southern hybridization of blot in (A) with HSV-1 genomic probe.

for the trapping of viral DNA with cellular DNA in the gel well during FIGE, DNA sample blocks were digested with XbaI- a restriction endonuclease for which no cut site is found in HSV-1 strain 1702 (Brown *et al*, 1984). Cells were infected with HSV-1 strain 1702, and gel blocks prepared as described in the materials and methods section. Gel blocks were treated with *XbaI* before FIGE to reduce the size, and hence the trapping ability of the cellular DNA. After digestion with *XbaI*, gel blocks were run on FIGE, Southern blotted, and hybridized with an HSV specific probe as described in the materials and methods section. Ethidium bromide staining revealed that the cellular DNA migrated into the gel as a uniform smear (Figure 4a lanes 4, 5, and 6). Although *XbaI* treatment removed most of the cellular DNA, the intensity of the viral specific signal in the region of the gel blocks was not different in treated and untreated samples (Figure 4b). We have tried two other strategies to facilitate the migration of DNA species from the sample wells during FIGE: (1) reduction of the percentage of agarose in the FIGE gel (0.5%), (2) the number of cells put in each gel sample was reduced (from 10^6 to 10^5). In both cases, no change in migration was noted.

From these results we conclude that trapping of viral DNA within the cellular DNA was not the reason for the retention of viral DNA in the sample wells during FIGE.

Is the well-associated DNA the HSV-1 replicative intermediate?

It is possible that the DNA remaining in the gel blocks at the origin of the gel may represent large replicating intermediates from which linear genomic DNA may be cleaved and packaged. Electron-microscopic examination of replicating HSV-1 DNA has revealed many configurations such as linear, genome length and larger, molecules with 'eye' or 'D' loops at or near one end of the DNA; and, large, entangled masses of DNA (Friedmann *et al*, 1977; Jacob and Roizman, 1977).

The precursor-product relationship of the well and 150 kb DNA was examined by pulse labeling cells with [methyl-^{3}H] thymidine. Labeling of infected cell cultures, for a short period with [^{3}H] thymidine, should result in the labeling and detection of replicating HSV-1 DNA, but not input genomic DNA. Detection of label in new genomic

DNA will eventually occur in those cultures which are pulse labeled and chased. Figure 5 shows a pulse chase experiment. Sample gel blocks, each containing 10^6 infected cells, were subjected to FIGE using parameters designed to resolve DNA molecules in the 100–500 kb range, and the gel treated and autoradiographed as described in the materials and methods section. The radioactivity at the gel origin of the mock infected samples, was probably due to [^{3}H] incorporation into cellular DNA (Figure 5, lanes 1 and 2). Four hours post infection, lanes 3 and 4 reveal that the initial incorporation of [^{3}H] thymidine occurs exclusively in the band at the origin of the FIGE. Thus this is the replicating HSV-1 DNA and it is detected from 4–12 h p.i. (Figure 5, lanes 3, 5, 7, 9, and 11). From 6 h p.i. onwards, the incorporated thymidine could be chased into radioactivity that migrated in the gel (Figure 5, lanes 6, 8, 10, and 12). The incorporated radioactivity could be identified as a wide band (100 kb to about 300 kb). The wide band may be the result of slow intermittent elution of virion DNA from the gel blocks. It is interesting to note that the wide band is decreased in strength by 12 h p.i., suggesting that processing of newly replicated concatemeric to virion DNA is decreased by this time.

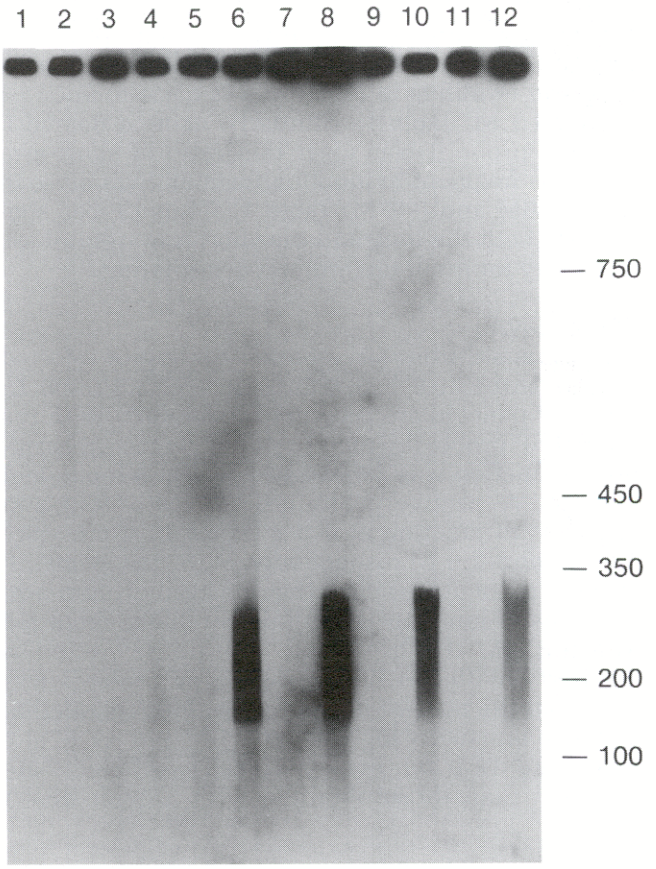

Figure 5 Pulse-chase experiment of [^{3}H] thymine incorporation in HSV-1 DNA separated by FIGE. One set of infected cell cultures was labelled with 40 μCi ml^{-1} of [^{3}H] by thymidine for 20 min and thereafter harvested immediately, while another set was chased for 2 h by incubating them in medium containing excess of cold thymidine (300 mg ml^{-1}) before analyzing by FIGE. Samples prepared from pulse-labeled infected cultures at 0, 4, 6, 8, 10, and 12 h p.i. are shown in lanes 1, 3, 5, 7, 9, and 11 respectively while lanes 2, 4, 6, 8, 10, and 12 represent similar set of cultures harvested after chasing them in cold thymidine for 2 h. Sample gel blocks, each containing approximately one million labeled cells were analyzed. The selection of separation parameters was such that DNA molecules in the range of 100 kb to 500 kb would be resolved more efficiently. The gel was processed for fluorographic detection of ^{3}H-labeled DNA as described by Bonner and Laskey (1974).

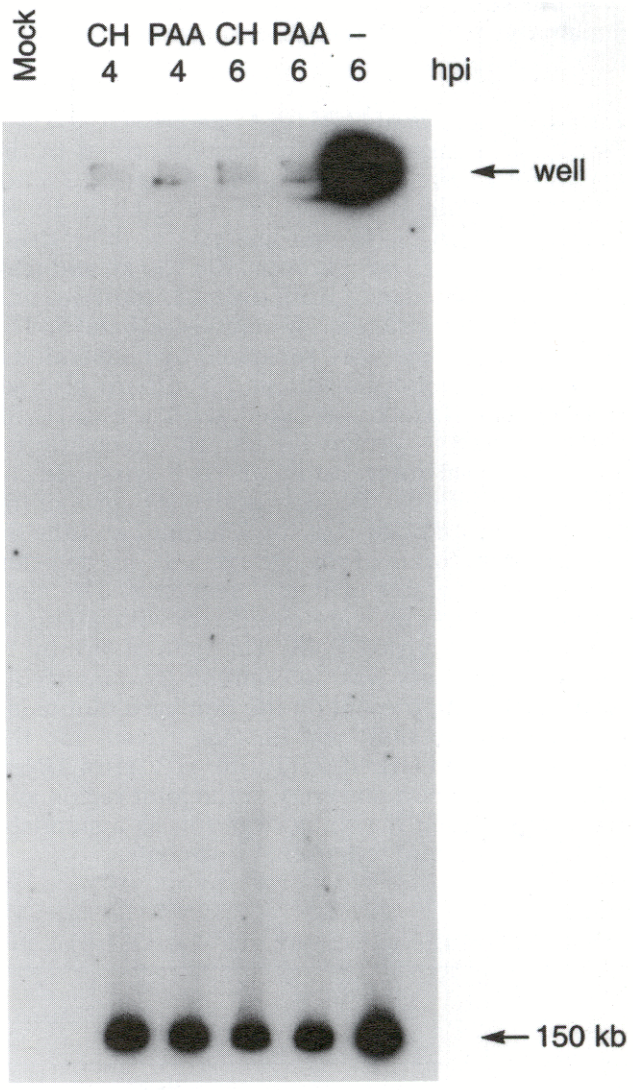

Figure 6 HSV-1 DNA in cycloheximide or phosphonoacetic acid treated cells. Cells were preincubated for 1 h before infection and maintained throughout the infection in media containing 100 μg ml^{-1} cycloheximide or 200 μg ml^{-1} phosphonoacetic acid. Lane 1, DNA from mocked infected cells; lanes 2 and 4, DNA from cycloheximide-treated infected cells at 4 and 6 h p.i., respectively; lanes 3 and 5, DNA from phophonoacetic acid-treated infected cells at 4 and 6 h p.i., respectively; lane 6, DNA from infected cells at 6 h p.i.

In the second experiment to determine if the well associated DNA was large replicative intermediate DNA, viral replication was blocked using cycloheximide (CH) or phosphonoacetic acid (PAA). CH inhibits the translation of immediate early RNAs, whereas PAA specifically inhibits HSV-1 DNA replication. Thus, in the presence of these two drugs, replicative intermediates of HSV-1 should not be detected. As seen in Figure 6, this is the case. The amount of well associated DNA did not increase in cells treated with CH or PAA as compared to untreated cells. This result suggested that the well associated DNA was the replicative intermediate of HSV-1.

From these studies we conclude that DNA remaining in the gel blocks represents replicating HSV-1 DNA.

Terminal analysis of HSV DNA species

Using the separation parameters described in the legend for Figure 1, and samples of about 0.5×10^6 cells per gel block, we separated 150 kb and well associated DNA for further analysis of their structure. Following electrophoresis, the 150 kb and well associated DNA bands were excised, purified, and digested with restriction endonuclease BamHI. The resulting fragments were separated by conventional agarose gel electrophoresis and Southern blotted. These blots were hybridized with a BamHI SP probe, as well as probes for BamHI S (specific for long-arm terminus), and BamHI P (specific for short-arm terminus). Because multiple copies of the 'a' sequence are present at the L termini and the SP junction, two forms of these fragments are detected differing by the size of the 'a' sequence. The electrophoretic patterns were compared with a BamHI digest of HSV-1 virion DNA. The ratio of termini to junction fragments was determined by quantifying the radioactivity present on the Southern blot using an AMBIS radio analytical imaging system and the data are summarized in Table 1. Virion DNA, assumed to contain one copy of the joint region and one copy of each terminus, was run as a standard for the ratio of joint to terminal fragments.

Data obtained from the analysis of virion DNA (lanes V of Figure 7a and b) are presented in Table 1. With the BamHI SP probe the molar ratio of short-arm termini to joint fragment was close to one (0.97 M and 1.0 M); however, the molar ratio of long-arm termini to joint was less than one (0.39 M and 0.46 M). The analysis of terminal band molarity is complicated by the existence of multiple 'a' repeat elements in the long termini. Amplification of the 'a' sequence (approximately 500 bp in HSV-1 strain F) in some of the long termini results in an increase in the size of the fragment BamHI S (originally 3.1 kb to 3.6 kb); consequently a portion of long-arm terminus fragment (BamHI S) comigrates with the fragment derived from the short-arm terminus (BamHI P, 3.6 kb) of the virion DNA.

To avoid these ambiguous results probes specific for BamHI S and BamHI P were used. With the BamHI S specific probe it was clear that the ratio of long-arm terminus to junction was close to one (1.4 M and 1.1 M; Table 1). Similarly, using a BamHI P specific probe it was clear that ratio of short-arm terminus fragment to junction fragment was close to one (1.26M and 0.97M, Table 1). Thus probes specific for both the long and the short arm termini are necessary for accurate terminal quantitation.

150 kb DNA

Data obtained from lanes corresponding to infected cell cultures at 8 h p.i. and 12 h p.i., shown in Figure 7 panel a, are presented in Table 1. With the BamHI SP probe, the molar ratio of short-arm terminus to junction was 2.8 M and 3.6 M at 8 h p.i. and 12 h p.i. respectively. Similarly, ratios of long-arm terminus to junction at 8 h p.i. and 12 h p.i. were 1.30 M and 2.0 M respectively. With the BamHI P specific probe it was clear that the ratio of short-arm terminus to junction was unusually high; about 5.5 M and 5.1 M at 8 h p.i. and 12 h p.i. respectively. Similarly, with the BamHI S specific probe it was clear that the ratio of long-arm terminus to junction was also unusually high; about 8.1 M and 8.7 M at 8 h p.i. and 12 h p.i. respectively. These results are different than those obtained with 150 kb-HSV-1

Table 1 Ratio of terminal fragments with respect to L-S junction

DNA	*Panel/lane*	*Junction probe (Bam HI SP)*		*L terminal probe (Bam-Sac)*	*S terminal probe (Sac-Bam)*
Band		P+S2	S1	S1+S2	P
Virion	7a/V	0.97 M	0.39 M	1.40 M	1.26M
	7b/V	1.00 M	0.46 M	1.10 M	0.97M
~150kb	7a/8 h	2.78 M	1.30 M	8.10 M	5.50 M
	7a/12 h	3.60 M	2.00 M	8.70 M	5.10 M
Well	7b/8 h	0.17 M	0.18 M	0.43 M	0.03 M
	7b/12 h	0.15 M	0.14 M	0.40 M	0.09 M

Individual lanes from blots similar to that shown in Figure 7 were analyzed by an Ambis radioanalytic imaging system (Amois Inc, San Diego, CA 92123). Radioactive counts in each band were normalized by dividing them by the molecular weight of the band. The ratio of termini to junction was derived by dividing normalized counts of termini by those of junction fragments.

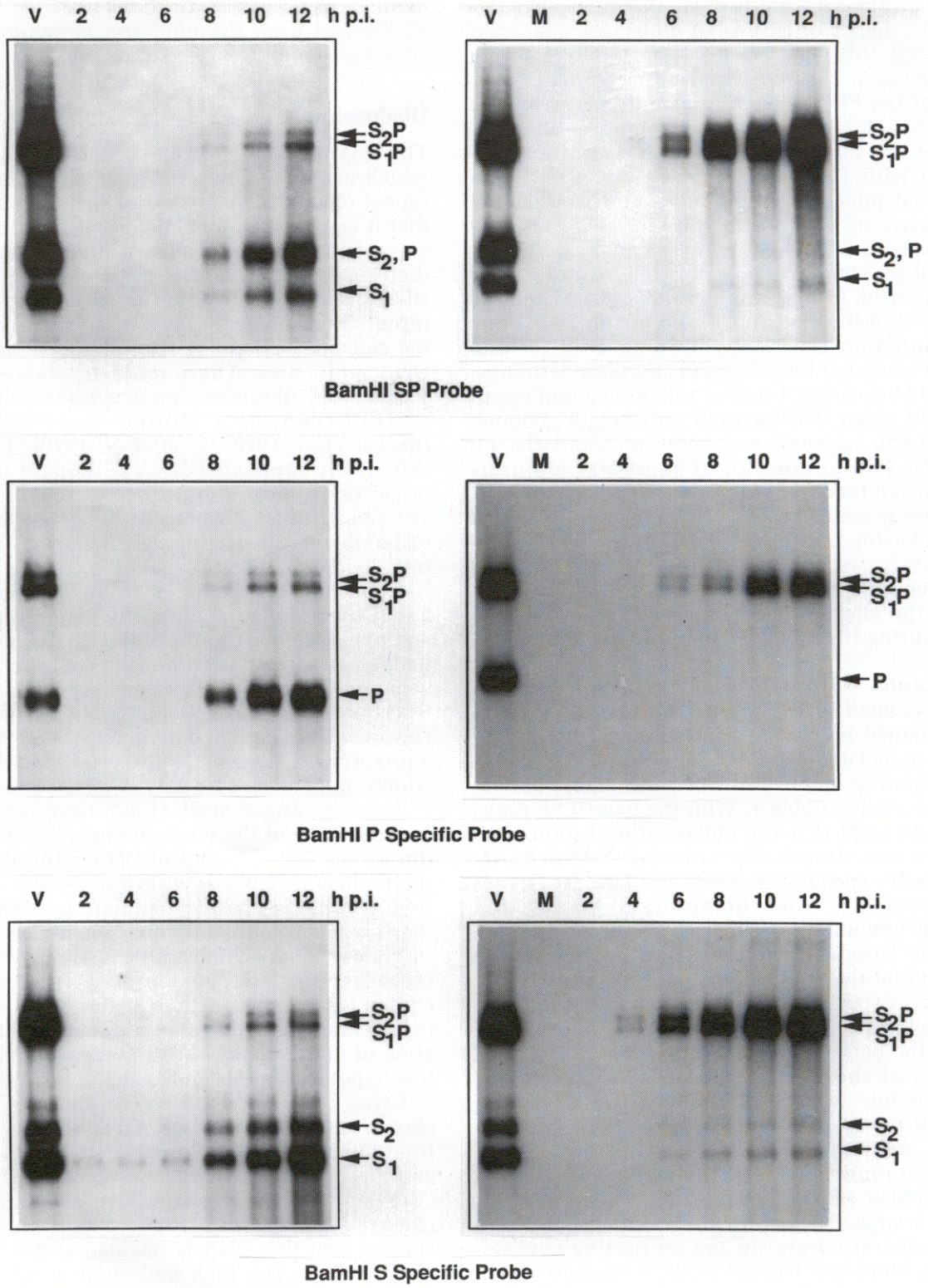

Figure 7 Terminal analysis of HSV-1 DNA. Southern blot hybridization of BamHI SP, P-specific, or S-specific probes to the Bam HI digests of 150-kb DNA (left hand panel) and well associated DNA (right hand panel). HSV-1 infected cells were harvested at 2, 4, 6, 8, 10, 12 h p.i. and the gel blocks were prepared for FIGE. Following the FIGE, the 150-kb bands and remaining gel blocks (containing well-associated DNA) were excised and purified using a Gene Clean kit. The purified DNA was subjected to BamHI digestion and the resulting fragments were separated by regular gel electrophoresis. The DNA was transferred to Nytran membranes and probed with BamHI SP, P-specific, or S-specific probes. Lane V contains BamHI digests of HSV-1 virion DNA. Lane M contains BamHI digests of mock-infected, well associated DNA. The nomenclature of the DNA bands are given to the right of each panel.

virion DNA and it would appear that there are a significant number of HSV DNA molecules generated containing only long or short-arm termini, during cleavage and packaging of replicating HSV-1 DNA.

Under the FIGE separation conditions used it is likely that HSV DNA molecules representing fractions of the viral genome length would migrate together with 150 kb DNA. Therefore, if not all, a significant portion of these molecules would have been recovered along with 150 kb DNA. This may be the reason why slightly lower molar ratios were obtained for short-arm terminus (5.5 M and 5.1 M) in comparison to long-arm terminus (8.1 M and 8.7 M). Considering that in 150 kb virion DNA, one molar junction fragment would account for one molar fragment of long-arm and short-arm terminus, then 7.0 M excess (8.1 minus 1.0) of long-arm termini would mean that for each unit length genome, 3.5 long-arm segments are produced. Similarly, 4.0 M excess (5.0 minus 1.0) of short-arm terminus would mean that approximately two short-arm segments are produced during the process of cleavage and packaging of a copy of HSV-1 genome. From these results we conclude that significant amounts of subgenomic segments consisting of either long-arm or the short-arm of the genome may be produced during HSV-1 replication, cleavage and packaging.

Well associated DNA

Data obtained from analysis of lanes corresponding to well associated DNA from infected cells at 8 h p.i. and 12 h p.i. are shown in panel B of Figure 7, and presented in Table 1. With the BamHI SP probe it was apparent that the molar ratio of termini to junction was drastically reduced. However, by using probes specific for short- and long-arm termini, it was clear from the autoradiographs that although low amounts of restriction fragment specific for long-arm terminus (BamHI S) were detectable in the well associated DNA, the amount of fragment specific for the short-arm terminus was below detectable levels (Figure 7b).

With the BamHI P specific probe it was clear that the ratio of short-arm terminus to junction was unusually low; about 0.03 M and 0.09 M at 8 h p.i. and 12 h p.i. respectively. However, with BamHI S specific probe, relatively higher ratios of long-arm terminus to junction were obtained; about 0.43 M and 0.40 M at 8 h p.i. and 12 h p.i. respectively. At 8 h p.i. it appeared that there were almost 14 times more long-arm termini in the replicating concatemer than short-arm termini (0.43 M *versus* 0.03 M). In later time point (12 h p.i.) in the infection, however, it appeared that concentration of short-arm termini increased significantly such that the long-arm termini were only four times (0.40M *versus* 0.09M) more abundant than short-arm termini. From these results, we conclude that DNA associated with the sample well is indeed a large concatemer predominantly containing L-S junctions and very few termini derived from the long-arm component of the HSV-1 genome.

Discussion

The HSV genome is a 150 kb linear DNA molecule which consists of two unique regions bounded by repeat elements. A feature of the viral genome is that it can exist in four distinct isometric forms by way of inversion of the two unique regions and their repeat elements. Our current understanding of HSV DNA replication is that the termini of each input viral genome fuse together, soon after entry of the cell nucleus, giving rise to monomeric circular DNA molecules. These molecules serve as templates for synthesis of concatemeric newly replicated HSV DNA, by a 'rolling circle' mechanism (Becker *et al*, 1978; Jacob *et al*, 1979). The size of this newly replicated DNA is thought to be very large, representing multiples of the 150 kb HSV genome. Unit-length progeny genomes are thought to be cleaved from the concatemeric replication intermediates and packaged by a head full λ phage like mechanism (Jacob *et al*, 1979; Vlazny *et al*, 1982). In order to explain the generation of four equimolar isomeric forms of the HSV genome, alternative cleavage sites within the concatemeric DNA sequence during replication has been proposed (Hayward *et al*, 1975; Delius and Clements, 1976). However a single monomeric template arising from a prototype HSV genome gives rise to a concatemer which could acount for only two isomeric forms.

Recently, Zhang *et al*, (1994) have reported that isomerization of the viral genome takes place early during the initial round of DNA replication while the replicating DNA is still in a circular configuration. A dimeric circular template in which one of the L segment is inverted has potential to give rise to concatemers which upon utilizing alternative cleavage sites are capable of giving rise to four equimolar isomers. Although this proposed selection of alternative cleavage sites explains the generation of four isomers, direct evidence in support of this hypothesis has been lacking.

Using pulse field gel electrophoresis, we have shown that approximately 50% of the viral DNA from infected tissue culture cells failed to enter the gel. These data are similar to those of Zhang *et al* (1994). With XbaI digestion of HSV-1 strain 1702 (lacking XbaI sites) infected cell samples we showed that this DNA is retained at the top of the gel because it is a high molecular weight concatemeric viral DNA, and not because of non-specific trapping of virion DNA in high molecular weight cellular DNA. By analyzing a mixture of crude virions with uninfected cells Garber *et al* (1993) reached similar conclusions. Moreover we could establish a precursor–product relationship between this high molecular weight replicative intermediate

and unit length virion DNA by undertaking analysis of *H*3-thymidine labeled infected cells (Figure 5). By utilizing probes specific for the long and short arm of the genome we could demonstrate that the well associated concatemeric DNA had termini mainly derived from the terminal repeat long component of the genome. Only now are reasons for this rather unexpected and interesting observation becoming clear.

Zhang *et al* (1994) have made similar observations and hypothesized that the exclusive linkage of the L segment to the termini could either represent unique initiation or termination sites of DNA replication or may result from a unique cleavage/packaging mechanism (Varmuza and Smiley, 1985; Deiss *et al*, 1986). From our analysis of infected cells at various times post infection it was clear that appearance of termini coincided with appearance of 150 kb DNA in the gel, therefore unique cleavage/packaging seems to be the more likely cause of this observation.

Latent DNA has been shown to be devoid of any termini (Rock and Fraser, 1983; Efstathiou *et al*, 1986) indicating that it could either be a very long linear concatemer or a circular form of DNA. Since long-arm termini were present at detectable levels in replicating concatemeric DNA it seems unlikely that replicating DNA simply becomes latent DNA by a change in transcriptional events in the cell. However, if the pre replication dimer hypothesized by Zhang *et al* (1994) exists, it would be an endless molecule like latent DNA.

Studies by Bataille and Epstein (1994) and Severini *et al* (1994) have also identified high molecular weight intermediates of DNA replication by FIGE. Interestingly they found adjacent genomes with L segments in the same orientation or with inverted segments in similar amounts. They suggested that this was due to a high rate of isomerization. However, Severini *et al* (1994) suggested that the replicative intermediates from relatively early times in the replicative cycle were present in large networks of molecules that were linked together by recombinational events.

The work presented here clearly indicated that the replicating intermediate of HSV is not a large molecule consisting of many newly replicated viral genomes, linked end to end, in a large polymer streaming from a circular replication template around which the DNA polymerase races. Rather, from the analysis of the ratio of termini to joints, it is clear that only a short length of newly replicated DNA is attached to the circular replication template — in the order of half of a genome length. This indicates that the packaging occurs efficiently between 8 and 12 h p.i. and the average molecular weight of a replicating intermediate is 225 kb. Thus viral replication intermediates are retained at the well in FIGE because of their conformation rather than their molecular weight.

The finding of mainly L termini on replicating intermediates suggests that the L terminus is packaged first. The models of Deiss and Frankel (1986) and Varmuza and Smiley (1985), propose that the packaged DNA is cleaved out of the replicating intermediate after recognition of the pac1 and pac2 sequences in the 'a' sequence. It is possible that the repeat 'a' sequences found at the L termini are sufficient for this packaging mechanism to work. Certainly the long concameter of newly replicated DNA from which genomes were cleaved endolytically does not appear to be present.

Many facets of HSV-1 DNA replication are still not clearly understood. The exclusive association of long-arm termini with concatemeric DNA is unexpected and puzzling. However, the use of FIGE to separate circular molecules and accurate quantitation of terminal and joint fragments of the viral genome can be used to reveal interesting information not only of lytic cycle replication intermediates but also of latent viral DNA. Further work with non-inverting mutant viruses, such as those described by Poffenberger and Roizman (1985) may reveal further insight into the formation of herpes simplex virus genomes.

Materials and methods

Virus and cell culture

HSV-1 strain F (obtained from B Roizman, U Chicago) and 1702 (obtained from M Brown, MRC Virology Unit, Glasgow, Scotland) was propagated and titered on CV-1 cells (African green monkey kidney cells) as described previously (Deatly *et al*, 1988). CV-1 cells were routinely cultured in Eagle minimum essential medium (Gibco, Gaithersberg, MD) plus 10% new born calf serum and antibiotics (penicillin 250 units ml^{-1} and streptomycin 125 µg ml^{-1}). In experiments, confluent cell monolayer (approximately 4–5 × 10^6 cells 75 cm^2 $flasks^{-1}$) were infected with HSV-1 at a multiplicity of infection (m.o.i.) of 5–10 pfu $cell^{-1}$. The virus was adsorbed for 1 h at 37°C, after which cell monolayers were washed and maintained in medium containing 5% newborn calf serum and antibiotics. Labeling of infected cultures was carried out by adding 40µCi ml^{-1} of [Methyl-^{3}H] thymine (specific activity 85Ci ml^{-1}, Amersham, Il.). At the end of the labeling, cultures were washed with and incubated in medium containing 300 µg ml^{-1} of unlabeled thymidine.

Cycloheximide was made freshly and stock of phosphonoacetic acid (PAA) was prepared as previously described (Spivack and Fraser, 1988). Cycloheximide (100 µg ml^{-1}) and PAA (200 µg ml^{-1}) were added to the cell monolayer at 1 h before infection and maintained until cells were harvested.

Field inversion gel electrophoresis (FIGE)

For FIGE, agarose gel blocks containing DNA samples were prepared according to the method of

Kenwrick *et al* (1987). Briefly, infected cell monolayers were washed with phosphate buffer saline (PBS), scraped into PBS, and collected by centrifugation at 500 g for 10 min at 4°C. The cell pellets were then resuspended in PBS at a concentration of 3×10^6 cells ml^{-1}. A 1% solution of low melting agarose (GTG grade, FMC, Rockland, ME) was melted in PBS, cooled to 50°C, and added to the equal volume of cell suspension. The mixture was poured into a glass tube (4 mm in diameter) and allowed to solidify at 4°C. When set, the gel was removed from the tube and cut into 4–5 mm wide cylindrical blocks. The gel blocks were then placed in 3–5 volume of a solution containing 0.5 M EDTA (pH 8.0), 1% SDS, 0.5 mg ml^{-1} proteinase K, and incubated overnight at 50°C with constant, gentle rocking. The blocks were washed several times in 10 volume of TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 7.5]) for 2 h at room temperature and stored at 4°C in 0.5 M EDTA (pH 8.0). Gel blocks, each containing DNA from 1×10^5 cells, were loaded into electrophoresis wells of the gel for FIGE.

XbaI digestion of DNA in gel blocks

Gel blocks, prepared with HSV strain 1702 infected cells, were washed extensively at room temperature in 50 volume of 1X TE, 0.1X TE, and XbaI digestion buffer, respectively (twice for each buffer, 20 min for each wash). XbaI digestion buffer was supplied by the manufacturer (Boehringer Mannheim, Indianapolis, IN). The sample blocks were then treated with XbaI (100 units $block^{-1}$) at 37°C. After 3 h of incubation, an additional 100 units of enzyme were added per block and the digestion continued for 3 more h. The XbaI-treated gel blocks were washed briefly with 1X TE and analyzed by FIGE.

FIGE running conditions

Sample blocks were loaded into 1% agarose gels prepared in 0.5X TBE (1X TBE was 89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.3). The running buffer (0.5X TBE) was circulated and maintained at 15°C through a cooling unit. A programmable power inverter (MJ Research PPI-200, MJ Research, Cambridge, MA) was used. Suitable ramping time for resolving a particular size range of DNA was calculated using a PPI-200 Gel Times software (MJ Research). Molecular weight markers were lambda DNA/HindIII fragments (BRL, Gaithersburg, MD), lamda ladder PGF marker (New England Biolabs, Beverly, MA), yeast chromosomal DNA (Beckman, Fullerton, CA).

Southern blot hybridization

DNA was purified from the gel blocks or DNA bands excised from the gels using a Gene Clean kit (Bio101, La Jolla, CA). After digestion and electrophoretical separation on the gels, DNA was depurinated, denatured, neutralized, and blotted onto Nytran membrane by capillary transfer according to the manufacturer's specification (Schleicher & Schuell, Keen, NH). DNA probes were labeled with ^{32}P by random priming (Feinberg and Vogelstein, 1983). Hybridization and auto radiography were carried out as described previously (Mellerick and Fraser, 1987). BamHI SP DNA clone of HSV-1 (pBR115) was obtained from B Roizman (University of Chicago). BamHI S-specific was 1.6-kb BamHI-SacI subfragment and BamHI P-specific was 0.3-kb SacI-SacI subfragment of BamHI SP clone (Post *et al*, 1980). Probe fragments are mapped on the viral genome in Figure 1. For detection of ^3H labeled DNA, gels were processed for fluorographic detection as described by Bonner and Lasky, 1974).

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