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Amundsen, Susan Kim

ANALYSIS OF HIGH FREQUENCY RECOMBINATION BETWEEN HERPES
SIMPLEX VIRUS TYPES 1 AND 2

The Ohio State University

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ANALYSIS OF HIGH FREQUENCY RECOMBINATION BETWEEN
HERPES SIMPLEX VIRUS TYPES 1 AND 2

A DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Susan K. Amundsen, B.S., M.S.

* * * * *

The Ohio State University
1983

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INTRODUCTION

The herpesviruses are a family of deoxyribonucleic acid (DNA) containing viruses (Russell et al., 1964; Ben-Porat and Kaplan, 1962) isolated from a wide variety of eukaryotic species. Human herpesviruses, including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), Epstein-Barr virus, varicella zoster virus, and cytomegalovirus, are structurally similar but have markedly different biological properties. Virus particles range in size from 180 (HSV) to 200 (cytomegalovirus) nanometers in diameter and contain linear double-stranded DNA with a molecular weight (MW) of $6 \times 10^6$ daltons (HSV) to $150 \times 10^6$ daltons (cytomegalovirus). The viral DNA is found in a core probably associated with protein (Furlong et al., 1972) and is further enclosed in an icosahedral capsid composed of 162 capsomers (Wildy et al., 1960). The capsid is surrounded by the amorphous tegument (Roizman and Furlong, 1974), and a lipid bilayer membrane (Epstein, 1962) composed of altered cellular membranes (Morgan et al., 1959; Armstrong et al., 1961; Darlington and Moss, 1969) and associated spikes (Wildy and Watson, 1963). Of all the human herpesviruses HSV-1 and HSV-2 have been the most extensively
studied, and will be the primary subject of this review. The two strains of HSV differ in a variety of properties, but were first separated on the basis of serological differences (Plummer, 1964). The immunological characterization of the viruses has demonstrated a series of type common and type specific antigens (Sims and Watson, 1973; Honess and Watson, 1974). Subsequent studies have demonstrated differences in the host ranges of the viruses (Figueroa and Rawls, 1969), and their replicative cycles (Schwartz and Roizman, 1969a). Despite these differences, the viral genomes of HSV-1 and HSV-2 are approximately colinear (Spear and Roizman, 1981), share a common structure, and have a 50% base sequence homology (Kieff et al., 1971; Kieff et al., 1972). Much of the interest in herpesviruses is related particularly to their medical significance as well as to complexities in the molecular biology of the group. Both HSV-1 and HSV-2 cause a variety of clinical syndromes including encephalitis, keratitis, labialis, and genitalis. The infections are often characterized by a cycle of primary infection, establishment of a latent state, and recurrent localized infection. These infections are generally not life threatening except in newborns or immunocompromised individuals.
Following a primary HSV infection, the virus travels through nerve cells to sensory ganglia and becomes latent in neurons or ganglia (Stevens and Cook, 1971). The source of recurrent infections may be the latently infected ganglia, as viral DNA has been detected by hybridization (Stevens and Cook, 1974) and DNA renaturation kinetics (Puga et al., 1978). Furthermore, virus has been isolated from homogenates of ganglion and by cocultivation of periperal (Stevens and Cook, 1971; Baringer, 1974; Walz et al., 1974), but not central nervous system tissue (Rock and Fraser, 1983). The inability to isolate infectious virus from the central nervous system of latently infected animals was thought to be due to the presence of incomplete or defective viral genomes (Kastrukoff et al., 1981). Current evidence suggests, however, that most of the viral genome is present in latently infected brain tissue (Rock and Fraser, 1983). Furthermore, the terminal fragments of the HSV-1 genome are increased in size by 300 to 500 base pairs and are present in submolar quantities. This suggests that the genome may be in a closed circular form, or altered by integration (Rock and Fraser, 1983). Although the mechanism by which HSV initiates and maintains the latent state is not understood, there is evidence that viral DNA replication is not required (Lofgren et al., 1977;
Watson et al., 1980). Analysis of HSV ribonucleic acid (RNA) from latently infected ganglia suggests that transcription occurs (Galloway et al., 1979), and is generally but not specifically restricted to certain regions of the genome (Galloway et al., 1982). Further work is required to determine the state of the viral DNA during latency, as well as how the virus regulates its transcription during this period and subsequent reactivation.

A second intriguing aspect of the molecular biology of HSV is its possible role in the etiology of certain forms of human cancer. The role of HSV in oncogenesis has been the subject of much research and speculation. Transformation of hamster cells has been obtained using ultraviolet irradiated HSV-1 and HSV-2 (Duff and Rapp, 1971; Duff and Rapp, 1973). Transformed cells express viral antigens (Duff and Rapp, 1971) and contain regions of the viral genome (Copple and McDougall, 1976; Frenkel et al., 1976b). Attempts to identify similar viral antigens or DNA in tissue from human tumor cells followed a number of serological and epidemiological studies that linked HSV-2 with cervical carcinoma (Nahmias et al., 1970; Rawls et al., 1973). Although some tumor cells have been identified that express viral antigens (Aurelian, 1973; Dreesman et al., 1980), DNA (Frenkel et al., 1972) or RNA (McDougall et al., 1980; Eglin, 1981), a clear understanding of the role of HSV
in the etiology of human forms of cancer is lacking (Galloway and McDougall, 1983).

Although the interaction of HSV with host cells during latency and transformation is not understood, significant progress has been made in determining some of the basic characteristics of the events that surround productive replication including viral adsorption, penetration and DNA replication. The viral envelope and associated glycoproteins appear to mediate the adsorption of the virions to the cell (Spring and Roizman, 1968; Stein et al., 1970; Rubenstein et al., 1972; Sarmiento and Spear, 1979; Spear and Roizman, 1981). There is evidence to suggest that glycoprotein B (Sarmiento and Spear, 1979) mediates fusion of the virus envelope and cell surface membrane (Spear and Roizman, 1981).

The mechanism involved in entry of the virus into the host cell has not been defined thoroughly, but probably results in the release of the nucleocapsid into the cytoplasm (Morgan et al., 1968; Abodeely et al., 1970; Para et al., 1980). Although it is known that viral DNA replicates in the nucleus of the infected cell (Newton and Stoker, 1958; Ben-Porat and Kaplan, 1963; Munk and Sauer, 1964) events associated with the release of DNA from the nucleocapsid and movement of the DNA into the nucleus are unknown (Spear and Roizman, 1981). The relative proportion of HSV DNA that enters the cell and reaches the nucleus for
replication ranges from 30 to 90% (Ben-Porat, 1981). Once in the nucleus, HSV DNA, unlike that of other DNA-containing viruses, does not form nucleosomal structures (Leinbach and Summers, 1980; Sinden et al., 1982). The DNA may, however, be associated with cellular DNA (Biegeleisen et al., 1977).

Following DNA replication (to be discussed later), nucleocapsids are assembled in the nucleus (Morgan et al., 1954; Reissig and Melnick, 1955). This phase is characterized by the accumulation of empty capsids (Watson et al., 1964; Tognon et al., 1981) prior to encapsidation. Electron microscopic evidence suggests that nucleocapsids are enveloped at the inner membrane (Morgan et al., 1959) and enter the cytoplasm and endoplasmic reticulum (Spear and Roizman, 1981) for transport through the cytoplasm (Darlington, 1968; Schwartz and Roizman, 1969b; Roizman et al., 1974). The virus particles remain cell associated and are released from the cell surface or from cytoplasmic vacuoles. The viral envelope consists of viral specific glycoproteins and cellular membrane material. The plasma membranes of infected cells contain new antigens (Roizman, 1963; Roane and Roizman, 1964) and viral specific glycoproteins (Schwartz et al., 1969b; Heine et al., 1972).

One component of the viral replicative cycle which is not understood well is the mechanism utilized by HSV for DNA
replication. However, considerable progress has been made in determining physical characteristics of the HSV genome. The molecular weight of the viral genome as determined by contour length, cosedimentation, or renaturation kinetics is approximately 100 x 10^6 daltons (Becker et al., 1968; Kieff et al., 1971; Frenkel and Roizman, 1971; Wadsworth et al., 1975). HSV-1 and HSV-2 differ in their buoyant densities at 1.726 and 1.728 grams/cm^3, representing 67 and 69 guanine-cytosine moles, respectively (Kieff et al., 1971).

Further characterization of HSV DNA demonstrated uniform sedimentation at 55 S in neutral sucrose gradients, but marked heterogeneity in denaturing alkaline sucrose gradients in which only 50% of the denatured DNA sedimented as full length single-stranded molecules (Kieff et al., 1971; Frenkel and Roizman, 1972; Wilkie, 1973). Although the cause of this phenomenon had been attributed to the presence of ribonucleotides in the genome (Gordin et al., 1973; Hirsch and Vonka, 1974), a recent investigation (Ecker and Hyman, 1981) confirmed another hypothesis that nicks and/or gaps in the DNA are the cause of heterogeneity (Kieff et al., 1971; Wilkie et al., 1975). It remains to be determined if the nicks or gaps are found at random or unique locations on the genome, although evidence for both exists (Frenkel and Roizman, 1972; Wilkie, 1973).
Electron microscopic examination of intact denatured HSV DNA by Sheldrick and Berthelot (1974) demonstrated a series of unique structural characteristics that led to a more complete understanding of the genome. The composition of the HSV genome is summarized in Figure 1. Examination of single-stranded DNA by electron microscopy demonstrated that the two ends of the HSV genome were capable of folding back, pairing with internal regions. This formation is indicative of the presence of complementary inverted nucleotide sequences within the strand. The inverted repeats promote the formation of fold back structures. The DNA contains direct terminal repeats as well as internal inverted sequence repetitions that divide the molecule into five regions; a long (L) and short (S) region of unique sequences, each bounded by terminal repeat (TR) sequences, and an internal region of inverted repeat (IR) sequences at least partially homologous to the termini (Figure 1). Analysis of partial denaturation mapping and reannealing studies by Wadsworth et al. (1975) suggests that the reiterated sequences around L and S represent 6% and 4.3% of the genome, respectively. Circularization of the DNA following processive exonuclease digestion of the ends (Grafstrom et al., 1975; Sheldrick and Berthelot, 1975) demonstrated that the termini of the genome were comprised
Figure 1. Structure of the HSV genome. The top line shows the fractional distance, and the second line the boundaries of the long (L) and short (S) regions, divided by the joint (broken line). The third line shows the sequence complexity of the unique (U), terminal repeat (TR), and internal repeat (IR) regions. Also shown is the sequence composition of each region (a, a', b, b', c, c'). The last four lines show the four isomers of the genome in the prototype (P), inverted short (I), inverted long (I), and inverted long and short (I) arrangements.
FIGURE 1
of direct terminal repeats. The length of the sequence responsible for circularization, referred to as the "a" sequence, has been estimated to be from 230 to 1600 base pairs (Wadsworth et al., 1975; Wadsworth et al., 1976; Wagner and Summers, 1978). This sequence is also present in its inverted form at the junction of L and S. The remaining sequences bracketing the L component are represented by "b", while those bracketing S are "c" sequences. Those sequences present in the inverted orientation are noted by the addition of the symbol for prime (') (Figure 1).

Analysis of the structure of the HSV genome was complicated further by the suggestion that recombination between terminal and internal repeat sequences could occur and lead to isomerization of the DNA molecule. As first proposed by Sheldrick and Berthelot (1974) this could lead to the formation of a variety of isomers composed of linear rearrangements of L and S (Figure 1). This possibility was confirmed by partial denaturation mapping (Hayward et al., 1975; Delius and Clements, 1976) as well as by examination of restriction endonuclease fragments of HSV-1 and HSV-2 (Roizman and Furlong, 1974; Hayward et al., 1975). The identification of terminal restriction fragments, as well as the analysis of their molar ratios demonstrated that some fragments were present in quarter and half molar amounts.
when compared with the fragments representing only the unique regions. Genome rearrangement results in this underrepresentation as the fragments forming the termini and junction of the genome change. Restriction enzymes such as Bgl II (Figure 2) that cleave HSV genomes outside of the inverted repeats produce three classes of fragments. Two copies of each terminal fragment is present for every set of four isomers, or at a 0.5 M ratio. The junction fragments are present in 0.25 M amounts, because each of the four possible fragments are represented once for every four isomers. The third class of fragments are present in 1.0 M amounts and represent the unique sequences of L and S. Those enzymes such as Eco R I or Hpa I that cleave within the terminal repeat sequences of S produce three possible terminal fragments, but only 2 junction fragments from the four genome isomers. The established nomenclature for the four structural isomers of HSV DNA are prototype (P), inversion of L (I), inversion of S (I), and inversion of L and S (I) (Roizman and Furlong, 1974; Hayward et al., 1975). These isomers differ only in the relative orientation of the two covalently linked regions with respect to one another (Figure 1).

The unique structure of the HSV genome suggests that the mechanism used by the virus to replicate may be complex. Although a number of models for DNA replication have been proposed (Sheldrick and Berthelot, 1974; Becker, 1978;
Figure 2. Map of restriction endonuclease cleavage sites on the physical map of HSV-1 (strain KOS) for Eco R I, Bgl II, and Hpa I. The molecular weights of the terminal and junction restriction fragments are shown for all four isomers of the genome.
Terminal Fragments: 3, 8.2, 10.2
Junction Fragments: 11.5, 13.5

Terminal Fragments: 6.1, 9.7, 10.8, 13.0
Junction Fragments: 15.8, 19.1, 20.5, 23.8

Terminal Fragments: 3.8, 6.6, 10.4
Junction Fragments: 10.4, 14.2

FIGURE 2
relatively little is known about the specific mechanism of replication or if replication is responsible for genome isomerization.

One approach to the analysis of events associated with HSV DNA replication within the nucleus has been to identify the physical conformation of HSV DNA during replication. This has been accomplished using sedimentation and pulse labeling techniques (Hirsch et al., 1976; Ben-Porat et al., 1976; Hirsch et al., 1977; Ben-Porat and Tokazewski, 1977), electron microscopy (Jacob and Roizman, 1977; Jean and Ben-Porat, 1976; Jean et al., 1977), and restriction endonuclease analysis (Jacob et al., 1979). Replicating HSV DNA sediments heterogeneously as compared to mature viral DNA (Hirsch et al., 1976; Hirsch et al., 1977). Replicating pseudorabies virus DNA (another herpesvirus), is composed of molecules having S values approximately twice that of mature DNA at early times after infection. The S value increases approximately 100 fold at later times during the course of infection (Ben-Porat and Tokazewski, 1977). Replication appears to be at least partially discontinuous (Ben-Porat, 1981) as single stranded gaps can be detected by electron microscopy (Jacob and Roizman, 1977) and velocity sedimentation analysis (Frenkel and Roizman, 1972; Ben-Porat et al., 1976).
Extensive examination of replicating HSV DNA by electron microscopy has lead to the identification of replicative intermediates, and has suggested that replication occurs in two phases. The first phase is characterized by the presence of unit-sized, linear molecules with gaps at the L-S junction and single-stranded ends (Jacob and Roizman, 1977). These molecules appear to form circles of varying size (Hirsch et al., 1976; Jean and Ben-Porat, 1976; Hirsch et al., 1977; Jacob and Roizman, 1977) with tails, loops or branches, (Ben-Porat et al., 1980). During the final phase of replication a variety of structures are observed including unit-size or large linear molecules with internal eyes and forks (Friedman et al., 1977; Jacob and Roizman, 1977; Hirsch et al., 1977), circular molecules of unit size or smaller, and structures containing various sized linear and circular components (Friedman and Becker, 1977; Ben-Porat et al., 1980). Jacob and Roizman (1977) also observed concatameric tangles, while Becker (1978) detected molecules in figure eight configurations.

Characteristics of the rolling circle mechanism of DNA replication are consistent with many of the observations made of replicating HSV DNA (Roizman, 1979). Analysis of
restriction endonuclease cleavage patterns of replicative intermediates during the second phase of DNA replication has suggested that DNA is in the form of circles or linear concatemers linked head to tail (Jacob et al., 1979). This hypothesis is supported by results of pulse chase experiments in which label was first associated with molecules of greater than 230 S and chased into unit sized molecules (Jacob et al., 1979). The processing of genomic concatemers to unit length molecules involves viral sequence specific recognition signals (Jacob et al., 1979; Roizman, 1979). Packaging and processing signals appear to exist in the S region because defective HSV DNA molecules consisting of reiterations of the S termini sequences are packaged and processed normally (Vlazny et al., 1982). Cleavage of DNA at specific recognition sequences may suggest that genomes require a mechanism to repair or complete sequences lost during cleavage (Roizman, 1979). This process was also thought to be necessary to regenerate sequences lost during the initial events of viral replication and genome circularization. Watson (1972) suggested that a free 3' end would be produced if concatemers were separated by a staggered cut, and would be repaired by a DNA polymerase. A more complex model has been proposed by Roizman (1979) which requires the use of the internal inverted repeat sequences as a template for end repair. Recent investigations which
have determined the sequences of the "a" and "a" regions suggest, however, that such regeneration may not be required since each terminal sequence contains only a portion of the inverted internal repeat (Mocarski and Roizman, 1982).

Although some general features of HSV DNA replication have been characterized, major questions remain on the mechanism of genome isomerization. It is likely that isomerization is an important event in the replicative cycle, but little is known about the process, and investigators have speculated that roles may exist for both replicative and recombinational mechanisms (Sheldrick and Berthelot, 1974; Skare and Summers, 1977; Becker, 1978; Roizman, 1979; Ben-Porat, 1981; Davison and Wilkie, 1983).

The possibility that isomerization occurs by a replicative mechanism was raised by Skare and Summers (1977), but more completely proposed by Roizman (1979). The Skare and Summers model suggests that single-stranded DNA produced during replication will pair with the internal inverted sequences, eventually displacing the template strand. Cleavage of the DNA molecule by a nuclease would then allow the molecule to isomerize, and replication to be completed. A second replicative mechanism also involving intramolecular recombination was proposed by Roizman (1979). In this model single stranded DNA produced by cleavage of concatemers,
will pair with and displace junction sequences. The cleavage of one of the two DNA stands would result in isomerization or the maintenance of the original form. An important prediction of this model is that end repair will occur after DNA replication, and that it requires that free single stranded DNA be repaired following isomerization, essentially by copying the junction sequences. The primary evidence cited in support of this model was a series of observations made during marker rescue experiments of three temperature-sensitive (ts) mutants of HSV-1 mapping within the reiterated region of S (Frenkel et al., 1975; Frenkel et al., 1976; Marsden et al., 1976; Morse et al., 1977; Knipe et al., 1979). The inability of mutants to rescue themselves by recombination between junction and termini sequences suggested that sequences in the region of the mutation were identical at both ends of S (Roizman, 1979). This suggested that a copying mechanism existed which could maintain the homology of sequences in the junction and termini (Knipe et al., 1979), and could support the mechanism proposed by Roizman (1979). Since this model was proposed, new mapping evidence places the gene responsible for the ts defects outside of the "a" sequence (Davison and Wilkie, 1983) and severely reduces data in support of a replicative mechanism for genome isomerization (Mocarski and Roizman, 1982).
The Roizman model is refuted further by the work of Smiley et al. (1981) in which genomes containing additional inversions were produced by the insertion of joint sequences within L. Genome length molecules containing deletions of one or two unique regions were identified and could be produced only if isomerization was mediated by recombination events that occurred prior to the formation of concatemers.

The remaining models of HSV replication rely on recombination to support the isomerization process. Sheldrick and Berthelot (1974) first proposed that isomerization could be mediated by recombination events between terminal and internal inverted repeats. Others have speculated specifically on the roles of inter- or intramolecular recombination events (Skare and Summers, 1977; Becker et al., 1978; Ben-Porat, 1981; Smiley et al., 1981; Ihara et al., 1982; Davison and Wilkie, 1983). Intramolecular recombination events between linear or circularized molecules could result in the production of molecules with a single or double inversion (Ben-Porat, 1981). Moreover, isomerization by intermolecular recombination events could produce genomes in the expected orientations as well as in repeating units of the L or S component (Sheldrick and Berthelot, 1974).
A variety of recombination-isomerization models involving both linear and circular molecules were proposed by Skare and Summers (1977). Problems associated with the maintenance of genome integrity and conversion of molecules from linear to circular forms were reviewed, but were unable to exclude the possible existence of any single isomerization mechanism.

The model proposed by Davison and Wilkie (1983) hypothesizes that isomerization would occur after genomes are circularized by direct ligation of the termini. Following restricted amounts of DNA synthesis, inversion of one or both segments would occur by site-specific recombination. According to their model, isomerization occurs prior to the period of rapid DNA synthesis or concatemer formation.

The role of recombination in the isomerization of the HSV genome has been supported further by a variety of studies detailing both the role and sequence composition of the terminal and junction regions (Wagner and Summers, 1978; Locker and Frenkel, 1979; Mocarski et al., 1980; Mocarski and Roizman, 1982; Smiley et al., 1981; Davison and Wilkie, 1981, 1983). The importance of the "a" sequence specifically has been demonstrated by the lack of isomerization of genomes demonstrating only partial homology of terminal and
internal "a" sequences (Preston et al., 1978; Wagner and Summers, 1978; Wilkie et al., 1978). More importantly, intertypic recombinants of HSV types 1 and 2 that have homologous "a" sequences but contain other heterologous sequences demonstrate normal inversion patterns (Davison and Wilkie, 1981).

The crucial importance of the "a" sequence to isomerization is best suggested by experiments in which additional isomers of the HSV genome were produced after insertion of a DNA restriction fragment containing the L-S junction into the unique region of L (Mocarski et al., 1980; Smiley et al., 1981; Mocarski and Roizman, 1981). These experiments demonstrated that sequences will invert when bounded by inverted but not direct repeats of "a". The "a" sequence then appears to be involved in site-specific recombination events that lead to genome isomerization.

It is clear that recombination plays an important role in the isomerization of the HSV genome, but any relationship between DNA replication and genome isomerization has yet to be established. The probability that recombination is important in the establishment of viral latency and transformation places additional emphasis on understanding both the mechanisms involved in and the viral functions that mediate recombination.
The strategies that have been employed in the past to investigate the process of recombination have involved both physical and genetic studies aimed at determining the relationship between DNA replication and isomerization. Density labeling studies conducted by Ben-Porat et al. (1982) have supported the view that recombination events precede DNA replication. This work with pseudorabies virus has shown that the two segments of DNA do not replicate independently, and then isomerize. Furthermore, although the parental genomes recombined and could be followed by buoyant density changes, they did not recombine readily with progeny genomes. This information, coupled with the observation that parental genomes maintained their integrity during replication suggests that isomerization and replication are either separate events or overlap in sequence only slightly.

Genetic studies of herpesviruses were initially directed at determining the genetic organization of the genome, but have also suggested the importance of recombination to the viral replicative cycle, and have been interpreted in light of what is known about genome isomerization. These studies have demonstrated that HSV genetic recombination occurs at an extremely high frequency as compared to other DNA viruses (Wildy, 1955; Brown et al., 1973; Dubbs et al., 1974; Schaffer et al., 1974;
Young and Silverstein, 1980). Traditionally, HSV recombination has been analyzed in biological assays using nonselectable temperature-sensitive (ts) mutants of HSV-1 and HSV-2 in two- or three-factor crosses. The relative amount of recombinant (ts) progeny as determined by plaque assay at the permissive and nonpermissive temperature has been used to obtain a recombination frequency (RF) which approaches 50% between some ts mutants (Schaffer, 1975; Dixon et al., 1983). Recombination frequencies have been determined between ts mutants of HSV-1 (Brown et al., 1973; Schaffer et al., 1974; Dixon and Schaffer, 1980), HSV-2 (Dixon et al., 1983) and HSV-1 and HSV-2 (Timbury and Subak-Sharpe, 1973; Esparza et al., 1976; Wilkie et al., 1978).

One of the major goals of genetic recombination studies has been to determine if RF's reflect the participation of all isomers of the HSV genome, or if they suggest that only a single form participates in recombination. The rationale of these studies is that if all isomers participate in recombination, genetic markers will map equidistant from each other (Subak-Sharpe and Timbury, 1977). Recombination frequencies reflecting participation of a single isomer could suggest that recombination occurs early in the replicative cycle when all four isomers are not present.
This may be the case as Parris et al. (1980) observed that RF's favored the participation of the \( L \) and or \( S \) S conjugomers in recombination. In the case of pseudorabies virus, the genetic map appears to be circular, suggesting that recombination occurs early in the replicative cycle when the molecule is circularized (Ben-Porat et al., 1981; Ihara et al., 1982).

Although the high frequency of recombination observed in genetic studies can be used as evidence to support the possibility that recombination plays an important role in the replicative cycle of HSV, and that exchanges occur early in the cycle, this approach has yielded little direct evidence to support its role in genome isomerization. This is in part because such studies are limited in their ability to detect early recombination events because they rely on the analysis of complete infectious progeny virus in a biological assay. A second disadvantage to this approach is that substantial variability in RF's are observed in repeated experiments in the same laboratory (Honess et al., 1980), making absolute determination of recombinational activity difficult.

In an effort to begin to dissect the process of recombination between HSV genomes in terms of both frequency and the time at which these events occur in the viral replicative cycle, it is necessary to utilize a different
approach to detect and quantify recombination. The technique must allow recombinants to be detected early in the viral replicative cycle, before either progeny virions or infectious DNA are produced. Such an option is possible by the analysis of restriction endonuclease digests of DNA from viruses possessing markedly different cleavage patterns (Young and Silverstein, 1980). Recombination events can be detected by the appearance of novel DNA fragments produced by the gain or loss of a characteristic restriction endonuclease cleavage site in the area of a crossover event. Such fragments would not be present in digests of parental or input genomes. This approach has been used to identify the portion of the recombinant genome originating from HSV-1 and HSV-2 parental molecules in HSV intertypic recombinants (Morse et al., 1977), and to physically map adenovirus mutations (Sambrook et al., 1975; Williams et al., 1975). Other applications have allowed the kinetics of adenovirus heterotypic recombination to be determined (Young and Silverstein, 1980), as well as the identification of viral polypeptides required for bacteriophage T7 recombination (Lee and Sadowski, 1981). Among the advantages of this technique is that biological activity is not required to detect recombinants so that even very early recombination events may be detected.
The detection of recombination between the genomes of HSV-1 and HSV-2 by the analysis of restriction endonuclease cleavage patterns is possible and may be a useful tool in the elucidation of the role of recombination in the HSV replicative cycle. The two serotypes recombine efficiently (Halliburton, 1980) because their genomes are roughly colinear (Spear and Roizman, 1981) and they share a 50% base sequence homology (Kieff et al., 1971; Wilkie et al., 1978). Despite these similarities, the two genomes have markedly different restriction endonuclease cleavage patterns (Skare et al., 1975; Skare and Summers, 1977; Cortini and Wilkie, 1978). Consequently, the cleavage patterns of intertypic recombinant genomes can be used to determine the parental origin of each DNA fragment (Sambrook et al., 1975; Williams et al., 1975), and may reveal the presence of novel DNA fragments formed by crossover events.

The analysis of intertypic recombination events has proven useful in a variety of studies. Recombination between ts mutants of HSV-1 and HSV-2 has demonstrated that viable ts+ progeny can be obtained (Timbury and Subak-Sharpe, 1973; Esparza et al., 1976; Preston et al., 1978; Wilkie et al., 1978). Furthermore, intertypic recombination has been detected in all regions of the genome, and the RF's found to be relatively constant except in the terminal regions (Halliburton, 1981). Although RF's are often lower than in
similar intratypic crosses (Esparza et al., 1976), analysis of genome structure and viral polypeptides produced by intertypic recombinants has demonstrated stable expression of both HSV-1 and HSV-2 characteristics (Halliburton et al., 1977; Morse et al., 1977; Morse et al., 1978).

The objective of this study was to analyze HSV intertypic recombination in order to begin to dissect the process of recombination between HSV genomes. This was accomplished by examining restriction endonuclease cleavage patterns for the presence of novel DNA fragments produced by crossover events. The results of these experiments may help to elucidate the relationship between recombination and genome isomerization by the detection of early recombination events. In order to accomplish this study it was necessary to optimize conditions for detecting and quantifying recombination in mixed infections of HSV-1 and HSV-2. Consequently, it was possible to determine the effect of input multiplicity on the recombination frequency after a single round of viral replication. The kinetics of recombination were determined throughout productive replication in order to determine if genome isomerization could be supported by recombination events observed in this system.
The analysis of recombination between genomes may help to elucidate the role of recombination in genome isomerization, as well as to understand some of the mechanisms that may mediate transformation and the establishment of latency. In addition, this approach may serve as an important tool to be used toward understanding eucaryotic recombination in general. Because recombination is involved in such diverse processes as genetic variation, regulation of gene expression, integration of viral genomes, and the possible promotion of base sequence mismatch and their subsequent repair, it is important to understand both the mechanisms and enzymes that support these events.
MATERIALS AND METHODS

Preparation of cells and viruses. Primary rabbit kidney (RK) cells were prepared from 7 to 10 day old rabbits and passaged no more than three times in minimum essential medium (Flow Laboratories) containing 5% newborn calf serum (Flow Laboratories), 2.5% fetal bovine serum (Biolabs, Inc.), 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.075% NaHCO$_3$ for closed vessels or 0.225% NaHCO$_3$ for open vessels. African green monkey kidney cells (VERO) were passaged in Dulbecco's modified minimum essential medium (Flow Laboratories) supplemented as above.

Stocks of HSV-1 (KOS strain; Smith, 1964) and HSV-2 (HG 52 strain) were prepared by inoculation of VERO cell monolayers in 100 mm tissue culture dishes (Lux). The multiplicity of infection (m.o.i.) for growth of all viral stocks was 0.01 to 0.1 plaque forming units (PFU) per cell. Only plaque purified stocks were used as the inoculum.

The viral inoculum was prepared by dilution in Tris buffered saline (0.05 M Tris-HCl, pH 7.4; 0.13 M NaCl; 0.005 M KCl; 0.07 mM NaHPO$_4$; 0.9 mM MgCl$_2$; 0.004 M CaCl$_2$; 0.1% glucose) containing 1% fetal bovine serum. Monolayers of cells were infected at 37°C, and after 60 minutes the inoculum was removed and medium added.
Infected cultures were incubated at 34 C until 80 to 90% of the cells exhibited cytopathology. This usually required three days for HSV-1 and two days for HSV-2. Stocks were harvested by scraping cells into the medium with a rubber policeman* and freezing at -70 C until processing. Stocks were processed by disruption of cells for 45 seconds with a Branson sonifier (20 kHz)*, and cell debris was removed by low speed centrifugation. Aliquots of the supernatant were frozen at -70 C until use.

Stocks of [32P]-labeled HSV were prepared by inoculation of VERO cells preincubated in phosphate-free Dulbecco's medium supplemented with 2.5% newborn calf serum and 0.035 M KPO₄. The inoculum was removed after one hour at 37 C* and medium containing 10 uCi of carrier free [32P] orthophosphate (Schwartz/Mann) per ml was added. At 18 hours postinfection (p.i.) the cells were scraped into the medium, pelleted by centrifugation, resuspended in reticulocyte standard buffer (RSB; 0.01 M Tris-HCl, pH 7.4; 0.01 M NaCl, 0.003 M MgCl₂) and placed on ice. After 10 minutes, the cells were broken by dounce homogenization and the nuclei separated from the cytoplasm by centrifugation (11,400 x g for 15 minutes). Virions were purified as described under the preparation of viral DNA. Each viral pellet was resuspended in minimum essential medium, dispersed by sonication for 30 seconds and the concentration adjusted to
5 x 10^8 PFU per ml. Stocks of [³H]-thymidine labeled HSV were prepared in the same manner except that during labeling Dulbecco's medium contained 10 uCi of [³H]-thymidine (specific activity 70 Ci/mM) per ml.

The infectivity of all virus preparations was determined by plaque assay on monolayers of VERO cells in 35 mm culture dishes. Several 10-fold dilutions were made of the stock, and 0.1 ml aliquots inoculated on to monolayers. The virus was adsorbed for 60 minutes at 37 C, and then the plates were overlayed with methylcellulose (2% in Dulbecco's medium supplemented as described previously). Culture dishes were incubated at 34 C for 5 days prior to staining with 0.01% neutral red in Tris-buffered saline. Plaques were enumerated 24 hours after adding the neutral red overlay with the aid of a fluorescent light box, and the titer of the stock was determined.

Preparation of DNA. Bottles (32 oz.) of RK cells passaged two to three times were inoculated with HSV-1 and/or HSV-2 at the m.o.i.'s indicated in the results. The virus was allowed to adsorb for 60 minutes at 37 C, the monolayers were washed twice with Tris-buffered saline, and medium added. The cells were incubated at 34 C for various times as indicated in the results.

Total infected-cell DNA was prepared at various times postinfection by washing the monolayers twice with Tris-
buffered saline, followed by lysis of the cells with 0.25% sodium dodecyl sulfate (SDS), and 0.4 M disodium ethylenediaminetetraacetic acid (EDTA) at 37 C. The bottles were rotated occasionally and incubated for three to four hours when cell debris was no longer detectable by microscopic analysis.

DNA was extracted by the addition of 5.0 M sodium perchlorate to a final concentration of 1.0 M. After extensive, gentle mixing, 2 volumes of chloroform:isoamyl alcohol (24:1 v/v) were added to the suspension. The aqueous and organic layers were separated by low speed centrifugation. The upper aqueous layer was removed with a wide-bore Pasteur pipet without disturbing the protein interface. The chloroform:isoamyl alcohol extractions were repeated until there was no evidence of protein at the interface (three to four times). The DNA was dialysed for 48 hours against 6 changes of 1 L each of 0.1 x standard saline citrate (SSC; 0.15 M NaCl, 0.015 sodium citrate, pH 7.4) at room temperature to remove the large quantity of SDS present. The dialysis tubing was prepared by boiling in distilled water for 20 minutes, cooled to room temperature and washed 3 or 4 times with sterile distilled water. The DNA was concentrated with polyethylene glycol (molecular weight 6000, Fisher Scientific Co.) and dialysed for an additional day at 4 C. Uninfected RK DNA was prepared in the same manner.
The concentration of total infected-cell DNA was determined by measuring the absorbance of DNA diluted in 0.1 x SSC at 260, 280 and 320 nanometers in a Zeiss spectrophotometer. The absorbance at 260 nanometers is a reflection of the concentration of DNA, as an absorbance of 1.0 is obtained for 50 ug per ml of DNA. Contamination of the DNA by protein causes absorbance at 280 and 320 nanometers, so that the ratio of absorbance at 260 and 280 nanometers is a reflection of DNA purity. Typically, DNA preparations had a 260/280 ratio of 1.8 to 2.0. Any absorbance of the DNA preparations at 320 nanometers was subtracted from the reading at 260 nanometers before calculation of DNA concentration. Alternatively, DNA samples in a total volume of 0.1 ml of 0.1 x SSC were added to 2.0 ml of fluorescence buffer (0.05 M Tris-HCl, pH 8.0; 0.5 mM EDTA; 0.5 ug/ml ethidium bromide) and the concentration determined in a Gilson Spectra/Glo fluorometer by comparison to a standard curve for dilutions of calf thymus DNA. Approximately 60 to 75 ug of DNA were obtained from two bottles of cells.

Virion DNA was prepared from partially purified KOS virions essentially as described by Parris et al. (1978). RK cells in 100 mm culture dishes were inoculated with an m.o.i. of 0.01 to 0.1 PFU per cell. Virus was adsorbed for one hour at 37 C, removed, medium added and incubation
continued at 34°C. When 80% of the cells showed cytopathology, the cells were scraped into the medium and pelleted by low speed centrifugation at 4°C. The supernatant containing extracellular virions was pooled and placed on ice. The cell pellets were suspended in RSB and placed into a 40 ml Dounce homogenizer. After the cells were allowed to swell on ice for 10 min, they were broken by five up and down strokes with a tight fitting pestle. The suspension was poured into a 50 ml conical tube, and cell debris and nuclei removed by centrifugation. The supernatant was added to the medium containing extracellular virions collected earlier, and all virions were collected by centrifugation in a JA-14 rotor (Beckman) for 1 hour at 14,000 x g.

The pelleted virions were resuspended in Tris-NaCl-EDTA buffer (TNE; 10 mM Tris-HCl, pH 7.4; 100 mM NaCl, 1mM EDTA), and layered onto a 20-60% sucrose (W/W in TNE buffer) gradient. Gradients were prepared on the day prior to use by placing 5.0 ml of each sucrose solution (20%, 30%, 40%, 50%, 60%; W/W) into a SW27 cellulose nitrate centrifuge tube (Beckman). The sucrose gradients were refrigerated overnight to obtain linear gradients.

The virions were separated from cellular cytoplasmic contaminants by centrifugation in a SW 27 rotor (Beckman)
for 1 hour at 80,000 x g. The virions were collected by puncturing the bottom of the tube and collecting the viral band. The viral band was diluted in TNE, and then concentrated by centrifugation for 15 minutes at 40,000 x g in the same rotor. The viral pellet was resuspended in 9.5 ml of TNE.

The virions were lysed by the addition of 0.5 ml of 10% SDS, and suspended further in 5.0 ml of TNE. After adding 5.0 ml of 5.0 M sodium perchlorate and equal volumes of phenol and chloroform:isoamyl alcohol (24:1 v/v), the DNA was extracted. The phenol was redistilled prior to use and frozen at -20 C. Before addition to lysates it was saturated with water and the pH adjusted to 7.5 with Tris-HCl. The phases of the mixture were separated by low speed centrifugation. The upper phase containing phenol was discarded, and the aqueous layer containing viral DNA was removed with a wide bore pipet. Two to three additional chloroform:isoamyl alcohol extractions were required to remove all contaminating protein. The DNA was dialysed at room temperature for 24 hours against 4 changes (1 L each) of 0.1 x SSC, and concentrated with polyethylene glycol. Dialysis was continued for 24 hours in the cold, and then DNA concentrations were determined as described previously. Yields of KOS DNA were 75 to 100 ug/ml from 80, 100 mm culture plates. The DNA was stored in aliquots at -70 C until use.
Recombinant plasmid pDG 305 (Galloway and Swain, 1980) composed of pBR 322 containing HSV-2 (strain 333) Bgl II fragment G was a gift of Denise Galloway (Seattle, Washington), and pSG 16 (Goldin et al., 1981) composed of pBR 325 containing HSV-1 (strain KOS) Eco R 1 fragment G was a gift of Myron Levine (Ann Arbor, Michigan). The plasmids were propagated in *Escherichia coli* K12 strain HB101 and isolated as covalently closed circular DNA from ethidium bromide, cesium chloride equilibrium gradients by the method of Wensink et al. (1974).

For large scale preparation of plasmid DNA, 1 liter of L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose) containing 50 ug/ml ampicillin (in 0.1 M sodium phosphate buffer, pH 8.0; adjusted to pH 6.0 with HCl) was inoculated with 10 ml of an overnight culture of the stock organism. The culture was incubated with shaking at 37 C until it reached mid-log phase as monitored at 590 nanometers in a Zeiss spectrophotometer. Chloramphenicol (in absolute ethanol) was then added to a final concentration of 150 ug/ml, and incubation continued overnight. The addition of chloramphenicol resulted in the amplification of the plasmid while inhibiting bacterial protein synthesis and cell division.
The bacteria were harvested by centrifugation at 15,000 x g for 10 minutes in a JA-14 rotor (Beckman). Cell pellets were combined and washed twice in cold Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). Spheroplasts were prepared by resuspending the cell pellet in 5.0 ml of cold 40% (w/v) sucrose in 0.2 M Tris-HCl (pH 8.0), and by adding 30 ul of 0.25 M EDTA (pH 8.0), and 30 mg of lysozyme (in 0.25 M Tris-HCl, pH 8.0). The weakening of the bacterial cell wall by lysozyme was monitored by making a series of 1:100 dilutions of the mixture in water, and observing the loss of turbidity. A marked reduction in turbidity was usually obtained after 10 minutes. The mixture was swirled on ice for 15 minutes after the addition of 7.0 ml of cold water. An additional 3.0 ml of 0.25 M EDTA (pH 8.0) was added and the mixture placed on ice for 5 minutes.

Lysis of the bacterial cell was accomplished by the addition of 17 ml of lysis buffer (1% BRIJ 58; 0.4% sodium deoxycholate; 0.0625 M EDTA; 0.05 M Tris-HCl, pH 8.0) while swirling on ice for 20 minutes. The suspension was centrifuged for 30 minutes at 48,000 x g to separate the chromosomal DNA and cellular debris from the plasmid DNA. The resulting supernatant was poured into a 100 ml graduated cylinder to determine the total volume, and 0.87 g of cesium chloride (Accurate Chemical Co.) and 1.0 ml of ethidium
bromide (20 mg/ml in water) were added per ml. The solution was kept in the dark to avoid nicking the DNA. After adjusting the refractive index of the solution to 1.3965, it was added to a 1 inch x 3.5 inch cellulose nitrate tube, capped, and centrifuged at 110,000 x g in a 60 Ti rotor (Beckman) for 48 hours. After centrifugation, four bands could be visualized with a 320 nm ultraviolet light. These contained (from top to bottom) protein, nicked plasmid and chromosomal DNA, supercoiled plasmid DNA, and RNA. The intensities of the supercoiled plasmid band and the nicked plasmid-chromosomal band were similar. The bands containing protein and nicked plasmid DNA were removed from the gradient with a Pasteur pipet and discarded. The supercoiled plasmid DNA band was harvested and the ethidium bromide removed by three to four extractions with water saturated isoamyl alcohol. After dialysis for 18 hours against 0.1 x SSC, the DNA was deproteinized by two to three chloroform:isoamyl alcohol (24:1 v/v) extractions and concentrated by ethanol precipitation. This was accomplished by adjusting the salt concentration of the solution to 0.5 M sodium acetate with a 5.0 M stock (pH 7.5), and adding three volumes of cold absolute ethanol. The DNA was precipitated overnight at -20 C, and collected by centrifugation at 25,700 x g for 30 minutes at -20 C. The
supernatant was discarded and the pellet dried under nitrogen gas. The plasmid DNA was dissolved in 1.0 to 2.0 ml of 0.1 x SSC, and the concentration determined as described previously. Typically 100 to 200 ug of DNA were obtained from 1.0 liter of bacterial cells. Aliquots of DNA were stored at -70 C until use.

**Assay of DNA infectivity by transfection.** The infectivity of HSV-1 DNA isolated throughout productive replication was determined according to a modification of the technique of Graham and van der Eb (1973) as described by Parris *et al.* (1980). Serial dilutions of DNA were made in HEPES-buffered saline (HBS; 0.5% HEPES, 0.0125% Na HPO4, 0.8% NaCl, 0.037% KCl; 0.1% dextrose, pH 7.05) and adjusted to a final concentration of 20 ug per ml with RK DNA. The DNA was precipitated for 20 minutes at room temperature by the addition of CaCl2 to 125 mM.

Cells were prepared for transfection as follows. Primary RK cells were trypsinized, counted, and resuspended in MEM, and aliquots representing 1 x 106 cells placed in tubes. The cells were pelleted by low speed centrifugation, the supernatant discarded, and the pellet disrupted by vortexing. A 0.5 ml aliquot of precipitated DNA was added to each tube, and then the tubes were placed at 37 C in a shaking water bath for 45 minutes.
The transfected cells (3.0 ml) were plated in duplicate 35 mm culture dishes after adding 6.0 ml of MEM containing 2 x 10^6 uninfected RK cells. Cells were incubated at 34 C, until 4 hours p.i., when the medium was changed. The following morning, the medium was removed, and the cells overlayed with methylcellulose. After 5 days of incubation at 34 C, the monolayers were stained with neutral red. Plaques were counted 18 hours later to determine the number of PFU per ug of DNA.

Cleavage of DNA and electrophoresis. Total infected-cell DNA was digested for 6 hours at 37 C with 3 units of the restriction enzyme Bgl II (Bethesda Research Laboratories) per ug of DNA in buffer (50 mM Tris-HCl, pH 7.5; 10 mM MgCl₂; 50 mM NaCl; 1 mM dithiothreitol). The reaction was terminated by the addition of one quarter volume of stop buffer (1% SDS; 0.05 M EDTA; 1% bromophenol blue in 60% glycerol). The digested DNA was incubated at 65 C for 10 minutes prior to electrophoresis.

Horizontal gels (20 x 25 x 1 cm) of 0.4% agarose (Type 1, low EEO, Sigma Chemical Company) in 400 ml of Tris-acetate buffer (TA buffer; 40 mM Tris-HCl, pH 7.8; 5 mM sodium acetate; 1 mM EDTA) were used for electrophoresis in a horizontal gel apparatus (model H1, Bethesda Research Laboratories). Each well was loaded with 2 ug of DNA.
Electrophoresis was conducted at 3 volts per cm for 18 to 20 hours in TA buffer. DNA was visualized with ethidium bromide (1 ug/ml) in TA buffer using a 302 nanometer transilluminator (UV Products). Photographs of agarose gels were taken using Kodak Tri-X Pan film, and developed using D-76 developer (Kodak).

The molecular weights of novel DNA fragments were determined by comparison to the relative mobilities and molecular weights of parental DNA fragments on the same gel (Southern, 1979).

**Gel blotting and hybridization.** DNA fragments in agarose gels were nicked with 260 nanometer ultraviolet light for 40 minutes in preparation for transfer to nitrocellulose paper (Schleicher and Schuell) by the technique of Southern (1975). The DNA was denatured by soaking the gel in 0.4 N sodium hydroxide and 1.5 M NaCl for 40 minutes with occasional shaking. The gel was neutralized for the same length of time in 1.0 M Tris-HCl (pH 7.6) and 1.5 M NaCl. The DNA was transferred to nitrocellulose with 10 x SSC over a period of 48 hours. After blotting, the gel was removed and the filter washed for 5 minutes in 3 x SSC with gentle rubbing. The filter was dried and then baked at 70 C for 2 hours in a vacuum oven.
Nitrocellulose filters were soaked in 3 x SSC for 30 minutes and prehybridized at 56 C for 4 to 6 hours in 3 x SSC, 2 x Denhardt's solution (0.4% bovine serum albumin, 0.4% polyvinyl-pyrrolidone-MW 4,000, 0.04% Ficoll; Denhardt, 1966), 0.5% SDS and 25 ug/ml sheared and denatured salmon sperm DNA.

Probes for hybridization were prepared by nick translation (Rigby et al., 1977) of partially purified KOS DNA or plasmid pDG 305 and pSG 16 DNA. The conditions and reagents were those provided in the Bethesda Research Laboratory nick translation kit. For every 5 lanes of DNA to be hybridized, 0.5 ug of DNA was nick translated. The reaction mixture had a total volume of 25 ul and contained 17.0 uM dATP, dGTP and dTTP in 50 mM Tris-HCl (pH 7.8), 5mM MgCl2, 10 mM 2-mercaptoethanol and 100 ug/ml 32 bovine serum albumin; and 40 uCi of 5'-[32P]dCTP (specific activity >800 Ci/mM; Amersham Corp.). The solutions were mixed, and 2.5 ul of an enzyme mixture was added containing 1 unit of DNA polymerase I, 10 pg of DNase I in 50 mM Tris-HCl (pH 7.5), 5mM magnesium acetate, 1mM 2-mercaptoethanol and 100 ug/ml bovine serum albumin in 60% glycerol. After incubation for 60 minutes at 15 C in an ethylene glycol bath (Forma), the reaction was terminated by the addition of 2.5 ul of 300 mM EDTA (pH 8.0). The percentage of label incorporated was determined by spotting 1 ul of the
reaction mixture on two 3 mM filter paper discs (2.5 cm; Whatman). One filter was precipitated in a 5% trichloroacetic acid solution containing 1% sodium pyrophosphate. After several washes the radioactivity on the filter was determined in an LS 8100 liquid scintillation counter (Beckman). Approximately 50-60% of the labeled dCTP was incorporated into TCA precipitable material and yielded probes with a specific activity of 5 to 10 x 10^7 cpm/ug of plasmid or 2 to 4 x 10^7 cpm/ug of HSV-1 DNA. Unincorporated nucleotides were separated from the nick translated probe by chromatography through a 6.5 cm column (Isolab) of Sephadex G-50 (Pharmacia) equilibrated in column buffer (10 mM Tris-HCl, pH 8.0; 50 mM NaCl; 0.1 mM EDTA). The sample was applied to the column and 20, 80 ul fractions were collected. The number of TCA precipitable and total radioactivities was determined and those fractions containing greater than 90% TCA precipitable counts were pooled. A recovery of 80 to 90% of the TCA precipitable counts applied to the column was obtained. The pooled probe was sheared by passage through a 21 guage needle, and denatured by boiling for 10 minutes. The probe, in 3 x SSC, 0.5% SDS and 2 x Denhardts solution was added to the nitrocellulose filter for hybridization at 65 C for 48 hours.
Following hybridization, the filters were washed for 30 minutes at room temperature in 3 x SSC and then at 65°C for 30 minutes in each of 3 x, 2 x, 1 x, 0.5 x, and 0.1 x SSC containing 0.1% SDS and 0.1% sodium pyrophosphate. The filters were autoradiographed at -70°C for various lengths of time using Kodak X-Omat film in cassettes with intensifying screens (Cronex, DuPont Chemical Company).

**Densitometry.** Autoradiograms were scanned with a Transidyne scanning densitometer which calculated peak areas by integration. For all calculations, the area of each peak was normalized for its molecular weight. All quantitative measurements of exposed areas were made using two or three exposure times for which the increase in area compared to exposure time was a linear function.

The percentage of HSV-1 genomes that participated in recombination (participation index; PI) was determined by comparing the molar amount of DNA in the recombinant fragment to the total molar amount of DNA available for recombination which included the recombinant fragment as well as parental HSV-1 fragment N. For all calculations recombinant fragments R<sub>1</sub> and R<sub>2</sub> were compared because they were formed by reciprocal crossover events.

**Separation of nuclear and cytoplasmic fractions.** The distribution of HSV-1 DNA in the nucleus and cytoplasm of
singly and mixedly infected cells was determined in an effort to characterize the interference phenomenon. Primary RK cells on the third passage were seeded into 100 mm culture dishes. After the cells were confluent, replicate cultures were infected singly with 15 PFU of \[^{32}\text{P}\]-labeled HSV-1 per cell or mixedly with 15 PFU of \[^{32}\text{P}\]-labeled HSV-1 and 3 PFU of nonlabeled HSV-2 per cell. The virus was allowed to adsorb for one hour at 37 C and after extensive washing with cold Tris buffered saline medium was added. The cultures were incubated at 34 C until 1, 2, 4, or 10 hours p.i. when duplicate plates were processed and harvested. The cells were harvested by scraping into the medium, pelleting with low speed centrifugation and washing the cell pellets. Each cell pellet was resuspended in 5.0 ml of RSB if dounce homogenization was being used, or 5.0 ml of TNE if cells were being lysed in buffer. The cells were lysed by incubation in cold lysis buffer (0.15 M NaCl; 0.01 M Tris-HCl, pH 7.2; 0.0015 M MgCl; 0.1% NP-40) or by dounce homogenization. The cells were monitored throughout the lysis procedure by staining with a 2% solution of crystal violet, and observing microscopically for the presence of unlysed cells or damaged nuclei. The lysis procedure was carried out until no intact cells were seen, but nuclei remained intact. This generally required six strokes with
a tight fitting pestle by Dounce homogenization, or 10 minutes incubation in lysis buffer. The cytoplasmic fraction was separated from the intact nuclei by centrifugation at 7,130 x g for 10 minutes at 4 C. The nuclei were resuspended in 2.0 ml of lysis buffer and centrifuged an additional time to remove any contaminating cytoplasmic material. Both the nuclear and cytoplasmic fractions were observed microscopi-
cally to determine if the nuclei were intact.

The total volume present at each step was determined, and aliquots were taken to assay for infectious virions. The total amount of radioactivity associated with the nuclear and cytoplasmic fractions was determined by trichloroacetic acid precipitation of samples on glass fiber filters.
RESULTS

Experimental rationale. The detection of recombination events between HSV-1 and HSV-2 by the analysis of restriction endonuclease cleavage patterns is possible because the two genomes have markedly different restriction endonuclease cleavage patterns, despite the fact that they share a 50% base sequence homology. These differences occur along the entire length of the genome as evidenced by the Bal II restriction endonuclease cleavage maps of HSV-1 (strain KOS) and HSV-2 (strain HG 52) shown in Figure 3. The complex restriction pattern of the HSV genome makes it impossible to detect novel DNA fragments among parental DNA fragments after electrophoretic separation on agarose gels (Figure 4). Hybridization of a whole genomic HSV probe increased the sensitivity of detection, but still made it difficult to visualize novel DNA fragments. However, hybridization of an HSV-1 or HSV-2 subgenomic probe to viral DNA allowed specific regions of the DNA to be visualized, and simplified the detection of novel DNA fragments. The successful utilization of this technique required the alignment of the HSV-1 and HSV-2 restriction enzyme cleavage maps, and the selection of a region in which the cleavage sites were markedly different. In addition the cleavage sites of one of
Figure 3. Map of restriction endonuclease cleavage sites and the fragments generated by cleavage of HSV-1 (strain KOS) and HSV-2 (strain HG 52) with Bgl II.
FRACT. DIST. 0 0.2 0.4 0.6 0.8 1.0

HSV-1: JKQOPNM'I D G F H L

HSV-2: DHPGJOCN'I H MQLK

FIGURE 3
Figure 4. Diagramatic representation of the fragments generated by cleavage of HSV-1 (strain KOS) and HSV-2 (strain HG 52) DNA with the restriction enzyme Bgl II, as separated by agarose electrophoresis. The $-6$ molecular weights ($x 10^{-6}$ daltons) are shown.
FIGURE 4
the serotypes had to fall within the boundaries of one restriction fragment of the other, so that a recombination event could be detected by the loss of a restriction site. Furthermore, in order to detect recombinants in a mixed pool of parental and recombinant molecules, the molecular weights of the novel fragments predicted to be formed by recombination had to be unlike those of any parental fragment.

In this study a region from 0.21 to 0.32 on the physical map within the unique region of L was selected for analysis (Figure 5). This region contains approximately $11 \times 10^6$ daltons or 16 kilobases (kb) of DNA and allows a sufficient area for recombination to occur, in a region of very high base sequence homology (Kudler et al., 1983). The pDG 305 probe selected to be hybridized to viral DNA was composed of plasmid pBR 322 and the HSV-2 Bgl II G fragment $11.1 \times 10^6$ daltons). The probe would be expected to hybridize to HSV-2 fragment G, as well as to homologous HSV-1 sequences represented by fragments I, M, N, and P within the same region (Figure 5). These fragments had molecular weights of $10.2, 4.0, 3.7,$ and $3.0 \times 10^6$ daltons, respectively.

Recombination events which occur within the region delineated by HSV-2 fragment G could result in the formation
Figure 5. Formation of six novel DNA fragments by crossover events predicted to occur within the region defined by HSV-2 Bgl II fragment G, in cells mixedly infected with HSV-1 and HSV-2. The Bgl II restriction endonuclease cleavage sites and fragments with their molecular weights ( x 10^-6 daltons) are shown. The crossover events predicted to form intertypic recombinant fragments R through R could occur at any site within the region delineated by the sigmoidal line connecting the HSV-1 (top) and HSV-2 (bottom) genomes. The predicted molecular weights ( x 10^-6 daltons) of the novel fragments are shown in parentheses.
FIGURE 5
of 6 recombinant fragments designated R through R (Figure 5).

1 6

1 2

3 4

5 6

Fragments R and R were predicted to have molecular weights of 6.8 and 8.5 x 10^6 daltons and were formed by reciprocal crossovers within the region defined by HSV-1 fragment M (5.5 kb). Both R and R would be formed by reciprocal crossovers within the region defined by HSV-1 fragment M (6.0 kb), while R and R would be formed by crossovers in the small region of overlap between HSV-1 fragment I and HSV-2 fragment G (approximately 1.3 kb).

Detection of intertypic recombinants. One of the major goals of this research was to detect and quantify recombination events that occur early in the viral replicative cycle. It was necessary, therefore, to isolate replicating DNA rather than progeny genomes. This was accomplished by the isolation of total infected-cell DNA at various times postinfection. The ability to detect the recombinant DNA fragments predicted in Figure 5 was first tested using DNA isolated from infected cells after a single round of virus replication. Primary RK cells were infected singly with 10 PFU of HSV-1 or HSV-2 per cell, or mixedly with 10 PFU of each per cell, and incubated at 34 C for 18 hours. The approach relied on the digestion of DNA with Bgl II, transfer of the DNA to nitrocellulose, and hybridization of the DNA to a nick translated, subgenomic HSV-2 Bgl II fragment G probe for detection of homologous HSV-1, HSV-2 and novel recombinant
fragments. As shown in Figure 6, the fragment probe hybridized to HSV-1 fragments P, M, N, and I, HSV-2 fragment G, as well as to three novel recombinant fragments. The calculated molecular weights of 7.0, 8.7, and 22.3 x 10^6 daltons for the novel fragments are in good agreement with predicted values for R1, R2, and R5 (Figure 5). Recombinant fragments R1 and R2 were predicted to have molecular weights of 11.0 and 4.1 x 10^6 daltons, respectively (Figure 5). However, since the sizes of the parental HSV-2 G and HSV-1 M fragment are similar to these fragments, lack of resolution of R3 and R4 on these gels is not unexpected. Furthermore, this gel would not have been capable of resolving the smallest recombinant fragment (R6) under the conditions of electrophoresis which included a low percentage agarose gel.

Before the novel DNA fragments could be identified as recombinants, it was necessary to determine that they were not partial restriction digestion products. The fact that the three novel fragments were derived by recombination is supported by their novel molecular weights in as much as they could not be accounted for by simple fusion, or incomplete digestion products. In addition, incompletely cleaved DNA could not be detected by hybridization to the pDG 305 or whole viral DNA probes (Figures 6 and 7). The role of recombination in the formation of fragments R
Figure 6. Southern blot of total infected-cell DNA isolated at 18 hours p.i. Primary RK cells were infected with 10 PFU HSV-1 per cell (lane 1), 10 PFU HSV-2 per cell (lane 2) or 10 PFU of each per cell (1 x 2) and the DNA extracted as indicated in Materials and Methods. The DNA, cleaved with Bgl II, was electrophoresed on a 0.4% agarose gel, blotted and hybridized to a [\( ^{32}P \)] nick translated HSV-2 Bgl II fragment G probe. The molecular weights of the parental fragments (x 10^6 daltons) are indicated on the left and novel recombinant bands are shown on the right by arrows.
Figure 7. Southern blot of total infected-cell DNA isolated at 18 hours p.i. Primary RK cells were infected with 5 PFU HSV-1 per cell (lane 1), 5 PFU HSV-2 per cell (lane 2) or 5 PFU of each per cell (1 x 2) and the DNA extracted as indicated in Materials and Methods. The DNA, cleaved with Bgl II, was electrophoresed on a 0.4% agarose 32 gel, blotted and hybridized to a [32P] nick translated HSV-1 whole virion DNA probe.
and R is also supported by the fact that they were predicted to be formed from reciprocal crossovers and were present in approximately equimolar amounts (Figure 6).

Analysis of the autoradiograms by densitometry (Figure 8) made it possible to quantify the amount of parental and recombinant fragments based on the intensity of bands following hybridization to the HSV-2 Bgl II fragment G probe. A comparison of the area of the fragments normalized for MW demonstrated a 66% reduction in the relative intensity of hybridization to parental HSV-1 fragments in DNA from cells mixedly infected compared to levels in singly infected cells, and suggested that HSV-2 interfered with the replication of HSV-1. In order to determine if the interference was the result of a large number of defective particles in HSV-2 stocks, the particle to PFU ratio for each HSV-1 and HSV-2 stock used was determined (Table 1). These results demonstrate no detectable difference between the particle to PFU ratio of HSV-1 and HSV-2 suggesting that defective interfering particles were not the cause of the interference. Furthermore, 84% of the HSV-1, and 82% of the HSV-2 particles counted were enveloped, and only 2% of capsids of both types appeared to lack DNA.

Two of the major technical problems that could have affected the relative amounts of DNA detected by hybridization, and consequently the ability to detect
Figure 8. Detection of parental and recombinant fragments in DNA from RK cells infected with 15 PPU of HSV-1 per cell and 3 PPU of HSV-2 per cell by densitometry. The locations of the origin and each parental HSV-1, HSV-2, and recombinant fragment are indicated by arrows.
FIGURE 8
### TABLE 1
PARTICLE TO PLAQUE FORMING UNIT RATIO OF HSV-1 AND HSV-2 STOCKS

<table>
<thead>
<tr>
<th>STOCK</th>
<th>PARTICLES/ML</th>
<th>PFU/ML</th>
<th>PARTICLE/PFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 (9/15/81)</td>
<td>1.5 x 10^3</td>
<td>3.9 x 10^8</td>
<td>38/1</td>
</tr>
<tr>
<td>HSV-1 (6/8/82)</td>
<td>2.1 x 10^3</td>
<td>4.1 x 10^8</td>
<td>52/1</td>
</tr>
<tr>
<td>HSV-2 (10/3/81)</td>
<td>3.1 x 10^3</td>
<td>7.0 x 10^7</td>
<td>44/1</td>
</tr>
<tr>
<td>HSV-2 (7/10/82)</td>
<td>2.9 x 10^3</td>
<td>6.1 x 10^7</td>
<td>48/1</td>
</tr>
</tbody>
</table>

*a* Viral stocks and Dow uniform latex beads (0.176 um diameter; Fullum, Inc.) were diluted 1/10,000 in Tris-buffered saline, stained with 2% phosphotungstic acid for 2 minutes, and applied to carbon coated, nitrocellulose grids. Grids were dried with filter paper and particles enumerated under a Hatachi electron microscope as empty or full capsids. Particles/ml represents the total number of particles counted (sum of empty and full capsids).

*b* Determined by serial dilution of stocks for plaque assay as described in Material and Methods.
recombinant fragments, were the conditions of hybridization and filter washing. The stringency of hybridization and washing are determined by the ionic strength of the solutions and the temperature at which they are used. To determine the optimum conditions for the detection of recombinants and limitation of nonspecific hybridization, the hybridization and washing conditions were varied for replicate samples on nitrocellulose filters. As shown in Table 2, the level of DNA in HSV-1 and HSV-2 fragments were not appreciably different if 3X, 2X, or 1X SSC were included in hybridization solutions. The most notable effects on the quality of the autoradiograms were apparent when washing conditions were adjusted to include 1x, 0.5x, and 0.1x SSC washes at 65 C. Other combinations of washes from 3.0 to 0.1x SSC at room temperature, 37 C, or 56 C resulted in increased non-specific hybridization, and were not used.

In an effort to reduce background hybridization further, the concentration of RK cellular DNA used in the prehybridization mixture was increased from 5 to 100 ug/ml. As indicated in Table 2, prehybridization with 25 to 100 ug/ml of RK DNA resulted in the least background hybridization, but 5 ug/ml allowed best resolution of the restriction fragments. Further manipulation of hybridization conditions failed to decrease the background hybridization, perhaps due to non-specific hybridization to
TABLE 2

EFFECT OF HYBRIDIZATION AND WASHING CONDITIONS ON THE APPEARANCE OF PARENTAL AND RECOMBINANT DNA FRAGMENTS

<table>
<thead>
<tr>
<th>CONDITIONS</th>
<th>INTENSITY OF FRAGMENTS</th>
<th>BACKGROUND</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prehybridization</strong> (ug/ml RK DNA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>4+</td>
<td>2+</td>
</tr>
<tr>
<td>10.0</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>25.0</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>50.0</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>100.0</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td><strong>Hybridization</strong> (ionic strength)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 x SSC</td>
<td>4+</td>
<td>1+</td>
</tr>
<tr>
<td>2 X SSC</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td>1 X SSC</td>
<td>3+</td>
<td>1+</td>
</tr>
</tbody>
</table>

a Replicate lanes of a Southern blot of Bgl II cleaved total infected-cell DNA prepared as described in Figure 6 were prehybridized in 2 x Denhardt's solution, 0.5% SDS, 3 x SSC plus various amounts of RK DNA.

b Replicate lanes of a Southern blot of Bgl II cleaved total infected-cell DNA prepared as described in Figure 6 were prehybridized in 2 x Denhardt's solution, 0.5% SDS, 3 x SSC, plus 5 ug/ml RK DNA. The filters were then hybridized to the HSV-2 Bgl II fragment G probe in the same solution but at the ionic strengths indicated.

c Score is based on the comparison of all lanes. The lane determined to have the best resolution of all fragments was scored as 4+.

d Score is based on the comparison of all lanes. The lane determined to have the least background was scored as 1+. 
the large quantity of cellular DNA present in each well. Decreasing the total amount of DNA in each well, resulted in a decrease in the amount of viral sequences to be detected. Therefore, all hybridizations were conducted at 65 C, in 3 x SSC, 2 x Denhardt's solution, 0.5% SDS, with 5 ug per ml of RK DNA. All washes were done at 65 C in decreasing ionic strength solutions of SSC.

It was also possible that the stringency of the hybridization and washing conditions reduced the ability to detect consistently the total amount of HSV-1 fragments present with the HSV-2 probe. For this reason an HSV-1 Eco R I fragment G probe (pSG 16; MW $10,2 \times 10^6$ daltons; Goldin et al., 1981) was used for hybridization and the relative intensity of hybridization to both types of fragments compared. The intensity of hybridization of the HSV-1 fragments was 7.7% that of the HSV-2 fragment using the HSV-2 Bgl II fragment G probe. With the HSV-1 probe, HSV-1 fragments were present at 6.7% the intensity of the HSV-2 fragment suggesting that the reduced level of HSV-1 DNA was not an artifact of sequence homology.

**Effect of HSV-1 input multiplicity on the frequency of recombination.** The interference demonstrated in mixedly infected cells could be overcome either by superinfection of cells with HSV-2 at various times following infection of HSV-1, or by increasing the relative input multiplicity of
HSV-1 as compared to HSV-2. However the observation that HSV recombination is predominantly an early event in pseudorabies virus (Ben-Porat et al., 1982) suggested that superinfection might not be appropriate. For example, the use of HSV-2 superinfection to overcome interference could reduce the apparent recombination frequency (RF) because the HSV-1 and HSV-2 genomes would not replicate simultaneously. Because the most accurate comparison to homotypic recombination could be made if both genomes replicated synchronously, compensation for interference was attempted first by adjusting the HSV-1 input multiplicity.

The HSV-1 m.o.i. was varied from 5 to 25 PFU per cell, while the HSV-2 input multiplicity was held at 5 PFU per cell in order to overcome HSV-2 interference and to optimize the ability to detect recombinants. The autoradiogram of a Southern blot of Bgl II cleaved total infected-cell DNA isolated from each culture at 18 hours p.i. and hybridized to the HSV-2 probe is shown in Figure 9. It is apparent that recombinant fragments $R_1$ and $R_2$ were present at each input multiplicity, although $R_3$ could not be visualized with ease at this exposure. However, densitometry of this and other exposures revealed the presence of all three fragments as distinguishable peaks. Based on the area of each peak, the relative intensities of hybridization of the probe to the parental and recombinant fragments were compared. In Figure
Figure 9. Effect of HSV-1 m.o.i. on the appearance of recombinant fragments R\textsubscript{1} and R\textsubscript{2}. Primary RK cells were infected with 5 PFU per cell of HSV-2 and 5, 10, 15, 20, or 25 PFU per cell of HSV-1 and total infected-cell DNA isolated at 18 hours p.i. The DNA was cleaved, electrophoresed, blotted, and hybridized to HSV-2 Bgl II fragment G probe as described in Materials and Methods. The arrows denote the positions of recombinant fragments R\textsubscript{1} and R\textsubscript{2}. 
FIGURE 9
the relative intensity of HSV-1 to HSV-2 DNA as well as the area of the fragment R is shown for each multiplicity. A similar relationship was found for R and R. The intensity of hybridization to HSV-1 parental fragments indicates that the availability of HSV-1 genomes increased from 2.7 to 10.2% of the HSV-2 parental genomes with increases in HSV-1 multiplicities from 5 to 25 PFU per cell. The increase in availability of HSV-1 genomes up to 15 PFU per cell was accompanied by a severe increase in the area of R. However, the area increased only slightly when m.o.i.'s of 20 and 25 PFU per cell were used, indicating that the amount of HSV-1 DNA available was not the major factor limiting recombination at these multiplicities.

The intensity of hybridization to recombinant fragments was compared to that of the HSV-1 fragments in order to determine the percentage of HSV-1 genomes participating in the formation of R and R at HSV-1 input multiplicities of 5 to 15 PFU per cell (Table 3). The HSV-1 parental fragments were chosen for this calculation because they were present in the lowest amount and increased exposure of autoradiograms resulted in linear increases in fragment intensities over prolonged periods of time. The results demonstrate that even at the most limiting HSV-1 m.o.i.'s, approximately 10% of the available genomes participated in recombination within this region by 18 hours p.i.
Figure 10. Effect of increasing HSV-1 input multiplicities on availability of HSV-1 genomes and area of recombinant fragment R. Primary RK cells were mixedly infected with 5 PFU per cell of HSV-2 and 5, 10, 15, 20, or 25 PFU per cell of HSV-1 and the DNA isolated at 18 hours p.i. The area of $R_1$ normalized for molecular weight (■—■) is shown, as is the relative intensity of hybridization of HSV-1 parental fragments as compared to HSV-2 Bgl II fragment G (○—○).
FIGURE 10
# TABLE 3

**PARTICIPATION OF PARENTAL GENOMES IN THE FORMATION OF RECOMBINANT FRAGMENTS R₁ AND R₂**

<table>
<thead>
<tr>
<th>HSV-1:HSV-2</th>
<th>EXP</th>
<th>PARTICIPATION INDEX</th>
<th>RF (% PER Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R⁻¹⁰.² Kb</td>
<td></td>
</tr>
<tr>
<td>5:5</td>
<td>1</td>
<td>8.5</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.3</td>
<td>9.2</td>
</tr>
<tr>
<td>10:5</td>
<td>1</td>
<td>9.6</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9.5</td>
<td>9.2</td>
</tr>
<tr>
<td>15:5</td>
<td>1</td>
<td>30.8</td>
<td>34.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>37.8</td>
<td>39.0</td>
</tr>
</tbody>
</table>

a

**PARTICIPATION INDEX**

\[
\text{PARTICIPATION INDEX} = \left(\frac{\text{AREA}_{R₁}}{\text{MW}_{R₁}}\right) \times \left(\frac{\text{AREA}_{R₂}}{\text{MW}_{R₂}}\right) \times 100
\]

\[
\left(\frac{\text{AREA}_{N₁}}{\text{MW}_{N₁}} + \frac{\text{AREA}_{N₂}}{\text{MW}_{N₂}}\right)
\]

b

**RECOMBINATION FREQUENCY**

\[
\text{RECOMBINATION FREQUENCY} = \frac{\text{PI}_{R₁} + \text{PI}_{R₂}}{5.5 \text{ Kb}}
\]
Furthermore, participation of genomes in recombination increased dramatically when the availability of DNA was no longer limiting (Figure 10) such that 37% of the available HSV-1 genomes recombined (participation index, P.I.) at HSV-1 input multiplicities of 15 PFU per cell (Table 3). At HSV-1 m.o.i.'s of 20 and 25 PFU per cell the increased background in the autoradiogram made it impossible to make an accurate direct comparison between the HSV-1 and recombinant fragments to determine participation indices.

The analysis of similar data for recombinant fragment R is complicated by two factors. The first is that the fragment is formed by a crossover within a very small region of overlap between HSV-1 fragment I and HSV-2 fragment G (1.3 kb; Figure 5). Secondly, because of the similarity in size between HSV-1 parental fragment I (10.1 x 10^6 daltons) and HSV-2 parental fragment G (11.1 x 10^6 daltons), it was difficult to obtain accurate measurements of fragment area for the calculation of PIs. Therefore, R was not used for further analysis.

Calculations of RF's were performed by making assumptions similar to those made in biological assays of recombination. Specifically, RF's in biological assays are obtained by determination of the ratio of PFU at the nonpermissive and permissive temperatures for any pair of mutants. The titers of virus at the nonpermissive temperature are doubled on the
assumption that any double ts virus could not be detected, but would arise due to reciprocal crossover events. In the system used in this study, calculation of RF's were made by adding the PI's for the formation of recombinant fragments $R_1$ and $R_2$ in as much as they are formed by reciprocal crossover events and account for all odd numbered physical exchanges within the 5.5 Kb region delineated by HSV-1 fragment N. Thus Table 3 demonstrates that HSV-1 recombines with HSV-2 at a rate of 1.2 to 1.8% per Kb when input multiplicities of HSV-1 were 5 and 10 PFU per cell, respectively, but increased to 6.5% per Kb when the number of available HSV-1 genomes was no longer limiting.

**Kinetics of recombination.** It is apparent that HSV-1 and HSV-2 recombine at a high frequency as measured at the end of a single cycle of viral replication. However, it was anticipated that even earlier events might be expected to occur if recombination was responsible for genome isomerization. Conventional techniques for the analysis of recombination rely on the production of progeny virions or infectious DNA. In order to determine the time at which these techniques could potentially detect recombinants, replicate plates of RK cells were infected with 5 PFU per cell of HSV-1. This input multiplicity was representative of that used in conventional approaches. At various times post-infection, cultures were harvested and assayed for infectious
virions as well as DNA. Figure 11 demonstrates that progeny virions and infectious DNA could be detected after 7 hours p.i.. This time period would represent the first point in the replicative cycle that biological assays of recombination could be expected to detect recombinants.

If genome isomerization is supported by recombination, it is possible that exchanges occur even earlier than could be detected by biological assays. Because biological activity is not required to detect recombinants by hybridization analysis, earlier time points were assayed for the presence of novel fragments. Therefore, primary RK cells were infected with 15 PFU of HSV-1 and 5 PFU of HSV-2 and total infected-cell DNA isolated at 2, 4, 6, 8, and 18 hours postinfection. The DNA was cleaved with Bgl II, electrophoretically separated, transferred to nitrocellulose and hybridized to the HSV-2 Bgl II fragment G probe. The autoradiograms were analyzed for the appearance of novel DNA fragments detected in the previous experiments. As shown in Figure 12, recombinant fragments $R_1$ and $R_2$ could be detected beginning at 4 hours p.i., although fragment $R_5$ could not be detected. The absence of $R_5$ could have been due to a lower frequency of recombination within the very limited area of overlap between HSV-1 fragment I and HSV-2 fragment G. The autoradiograms were analyzed by densitometry to determine the percentage of HSV-1 genomes that participated in
Figure 11. Kinetics of synthesis of infectious viral DNA and progeny virions. Replicate plates of primary RK cells were infected with HSV-1 (strain KOS) at an m.o.i. of 5 PFU per cell. At various times postinfection, cultures were removed and the cells scraped into the medium. A portion was removed, sonified and titrated for the production of plaques in VERO cell monolayers (○—○) while the DNA from the remainder of cells was extracted. Infectivity of total infected-cell DNA (▲—▲) was determined by transfection assay on RK cells in suspension as described in Materials and Methods.
Figure 12. Southern blot of total infected-cell DNA isolated from cells mixedly infected with 15 PFU of HSV-1 and 5 PFU of HSV-2 per cell. The DNA was isolated at the times indicated and hybridized to the HSV-2 Bgl II fragment G probe. Recombinant fragments are indicated by arrows.
recombination throughout the cycle (Table 4). Recombination occurred primarily early in the cycle as the PI for the formation of R increased from 12% at 4 hours p.i. to 21% at 6 hours p.i. Subsequent time points demonstrate that the percentage of genomes involved in recombination increased to 29% at 8 hours p.i., but at 18 hours had increased to only 32%. Because R was formed by a reciprocal crossover, the results were similar (Table 4).

**Kinetics of HSV-1 and HSV-2 DNA replication.** The sensitivity of hybridization of subgenomic probes to whole infected-cell DNA for the detection and quantitation of recombination events was demonstrated by the ability to analyze recombination events that occur early in the replicative cycle. Several proposed mechanisms for genome isomerization required that recombination occur prior to HSV DNA replication, and the detection of recombinant fragments by 4 hours p.i. suggested that these events did occur early enough to support this contention. The relationship between viral DNA replication and recombination in mixedly infected cells was determined by hybridization of pulse-labeled viral DNA isolated throughout the replicative cycle, to filter immobilized HSV-1 DNA. Nonlabeled, denatured HSV-1 DNA (12 ug) was applied to replicate 2.5 cm nitrocellulose filters. Primary RK cells were mixedly infected with 25 PFU of HSV-1 and 5 PFU of HSV-2 and pulse labeled with 10 uCi
**TABLE 4**

**PARTICIPATION OF HSV-1 GENOMES IN RECOMBINATION IN CELLS INFECTED WITH HSV-1 (15 PFU/CELL) AND HSV-2 (5 PFU/CELL)**

<table>
<thead>
<tr>
<th>TIME POSTINFECTION (HR)</th>
<th>% OF HSV-1 GENOMES PARTICIPATING IN RECOMBINATION&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R R</td>
</tr>
<tr>
<td>0</td>
<td>0 0</td>
</tr>
<tr>
<td>2</td>
<td>0 0</td>
</tr>
<tr>
<td>4</td>
<td>12 11</td>
</tr>
<tr>
<td>6</td>
<td>21 19</td>
</tr>
<tr>
<td>8</td>
<td>29 28</td>
</tr>
<tr>
<td>18</td>
<td>32 33</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on densitometric tracings of autoradiograms and calculated as:

\[
\frac{\text{AREA}_{/MW}}{\text{AREA}_{/MW} + \text{AREA}_{/MW}} \times 100
\]

Where R is the novel recombinant fragment and N is the HSV-1 parental fragment participating in the formation of the recombinant fragment.
of $^{3}H$-thymidine per ml for 30 minutes prior to DNA isolation at 2, 4, 6, 8, and 18 hours p.i. The labeled DNA was hybridized to the filters for 48 hours, and the filters were washed and counted as described in Materials and Methods. As demonstrated in Table 5, the maximum period of viral DNA synthesis occurred between 2 and 4 hours p.i. However, further interpretation of this data is complicated by the low number of counts that hybridized to the filters, and the inability to resolve differences that might have existed between the timing of HSV-1 and HSV-2 DNA replication because of base sequence homology.

The examination of individual DNA replication patterns was possible however, by quantifying the area of HSV-1 and HSV-2 specific restriction endonuclease cleavage fragments detected by hybridization. Therefore, the areas of the HSV-2 Bgl II fragment G, and HSV-1 Bgl II fragments P, M, and N were measured from autoradiograms of DNA isolated at 2, 4, 6, 8, and 18 hours p.i. All fragments were normalized for MW, and the area of the HSV-2 fragment compared to the mean normalized area of the HSV-1 fragments (Figure 13). It should be noted that the normalized areas of the HSV-1 fragments were within 2% of each other. In singly infected cells the level of HSV-2 DNA began to increase after 2 hours p.i., while HSV-1 DNA increased after 4 hours p.i. Despite
### TABLE 5

RATE OF HSV DNA SYNTHESIS IN MIXEDLY INFECTED CELLS

<table>
<thead>
<tr>
<th>Time P.I. (hr)</th>
<th>Hybridized 6 μg DNA/filter</th>
<th>Hybridized 12 μg DNA/filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>64</td>
<td>71</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>141</td>
<td>191</td>
</tr>
<tr>
<td>6</td>
<td>55</td>
<td>66</td>
</tr>
<tr>
<td>8</td>
<td>48</td>
<td>71</td>
</tr>
<tr>
<td>18</td>
<td>53</td>
<td>64</td>
</tr>
</tbody>
</table>

---

*RK cells were infected with HSV-1 (25 PFU/cell) and superinfected 30 min. later with HSV-2 (5 PFU/cell).*

*Time of infection was considered to be the time of HSV-2 addition.*

*DNA was obtained from partially purified HSV-1. Conditions of hybridization allowed HSV-1 and HSV-2 sequences to hybridize.*
Figure 13. Levels of HSV-1 and HSV-2 DNA during productive replication as detected by hybridization analysis. Primary RK cells were infected with 15 PFU of HSV-1 per cell, or 3 PFU of HSV-2 per cell and the DNA isolated throughout productive replication as described in Materials and Methods. The DNA, cleaved with Bgl II, was electrophoresed on a 0.4% agarose gel, blotted, and hybridized to a 32P-labeled nick translated HSV-2 Bgl II fragment G probe. The autoradiograms were analyzed by densitometry to determine the area of HSV-1 and HSV-2 fragments. The normalized area of HSV-2 Bgl II fragment G is shown (■—■), as is the mean normalized area of HSV-1 Bgl II fragments P, M, and N (●—●).
FIGURE 13
the higher input multiplicity, the level of HSV-1 DNA was only 10% greater than that of HSV-2 at 18 hours p.i., suggesting that the particle to PFU ratio of HSV-2 might have been slightly higher than that of HSV-1, although they were not detectably different by electron microscopic determination (Table 1). It is unlikely, however, that a difference of less than 10-fold could be detected by this technique.

The events associated with replication may be preceded by or may at least involve recombination as suggested by the data shown in Figure 13 and Table 4. Together, these data indicate that viral DNA replication begins as early as 2 hours p.i. (HSV-2), and recombination can be detected by 4 hours p.i. The apparent difference in the time at which HSV-1 and HSV-2 DNA replication are initiated complicate the interpretation of this data, but it appears that at least some recombination events occur early in the replicative cycle, and the majority do not occur after the period of maximal DNA replication.

**Interference of HSV-1 replication by HSV-2.** It is apparent from the analysis of autoradiograms that HSV-2 interferes with the replication of HSV-1 in mixedly infected cells despite the use of higher input multiplicities (Figure 10). One reason for interference might have been differences in the viral replicative cycle as reflected in the patterns of
DNA replication (Figure 13). The analysis of HSV recombination in this system relied on intertypic genetic exchanges, and it is apparent that interference complicated interpretation of the results. Consequently, a series of experiments were undertaken to understand the mechanism of interference.

**HSV-1 and HSV-2 growth curves.** If HSV-2 interference is caused by simple competition for adsorption sites or replicative enzymes, adjustment of the relative HSV-1 m.o.i. upward might be expected to result in marked increases of HSV-1 DNA in mixedly infected cells. However, as shown by Figure 11, increases in HSV-1 m.o.i.'s above 15 PFU per cell were unable to increase significantly the amount of HSV-1 DNA detected. Further increases in HSV-1 input to 100 PFU per cell did not increase the relative levels of HSV-1 DNA in mixedly infected cells, again confirming that simple competition alone could not account for the interference observed. A decrease in the HSV-2 input multiplicity from 5 to 3 PFU per cell also did not change the level of interference.

It also was possible that the relative duration of the HSV-1 and HSV-2 replicative cycles might have accounted for some differences in yield of DNA at 18 hours p.i. Therefore, the yield of infectious virions at various times p.i. from replicate 100 mm cultures of RK cells infected with 15 PFU
per cell of HSV-1 or 3 PFU of HSV-2 were compared. As shown in Figure 14, the HSV-1 eclipse period (the time between viral adsorption and production of infectious virions) was approximately 2 hours longer than that of HSV-2. In addition the continued maturation of HSV-1 virions in the later (non-exponential) phase of the cycle was protracted (12–20 hours p.i.), compared to HSV-2 (10–14 hours p.i.). These differences alone do not appear to account for the major differences in DNA levels of HSV-1 and HSV-2 in mixedly infected cells at 18 hours p.i. (Figure 10).

**Effect of HSV-2 on HSV-1 DNA replication.** Differences in the replicative cycles of HSV-1 and HSV-2 DNA were detected by the analysis of specific restriction fragments as discussed previously (Figure 13). In order to determine if interference could be characterized by changes in the levels of DNA synthesized in mixedly infected cells, similar experiments were conducted in RK cells infected with 15 PFU per cell of HSV-1 and 3 PFU per cell of HSV-2. DNA was isolated at 2, 4, 6, 8 and 18 hours p.i. and the area of HSV-1 and HSV-2 fragments determined by densitometry (Figure 15). As previously shown (Figure 13), in singly infected cells the level of HSV-2 DNA began to increase after 2 hours p.i. while HSV-1 DNA increased at 4 hours p.i. The level of HSV-2 DNA in mixedly infected cells was not significantly different than in singly infected cells, but
Figure 14. Production of infectious HSV-1 and HSV-2 virions as determined by plaque assay. Primary RK cells were infected with 15 PFU of HSV-1 per cell (●—●) or 3 PFU of HSV-2 per cell (■—■) and harvested, sonified and assayed as described in Materials and Methods. The titer of total infectious virions (intracellular + extracellular) is shown at various times postinfection.
FIGURE 14
Figure 15. Levels of HSV-1 and HSV-2 DNA during productive replication as detected by hybridization analysis. Primary RK cells were infected with 15 PFU of HSV-1 per cell, and 3 PFU of HSV-2 per cell and the DNA isolated throughout productive replication as described in Materials and Methods. The DNA, cleaved with Bgl II, was electrophoresed on a 0.4% agarose gel, blotted, and hybridized to a $^{32}$P-labeled nick translated HSV-2 Bgl II fragment G probe. The autoradiograms were analyzed by densitometry to determine the area of HSV-1 and HSV-2 fragments. The normalized area of HSV-2 Bgl II fragment G is shown (■——■), as is the mean normalized area of HSV-1 Bgl II fragments P, M, and N (●——●).
FIGURE 15
there was a striking change in the appearance of HSV-1 DNA in mixed infections (Figure 15). Interference was associated with an increase in the period prior to the initiation of replication (6 to 8 hours p.i.) and the level of HSV-1 DNA present at 18 hours p.i. was reduced by 68%.

**Effect of superinfection on interference.** Because at least one effect of HSV-2 on HSV-1 occurred very early, prolonging the period prior to HSV-1 DNA replication, it might have been possible to bypass it by superinfecting HSV-2 at 1, 2, 3, or 4 hours p.i. DNA was isolated at 8 hours p.i. and the levels of HSV-1 DNA in mixedly and singly infected cells compared. Figure 16 demonstrates that the relative level of HSV-1 DNA in cells superinfected with HSV-2 at 1 hour p.i. was the same as in cells infected simultaneously with both serotypes. When HSV-2 was added at 2 or 3 hours p.i., the amount of HSV-1 DNA in mixedly infected cells increased to 22 and 28% of that in singly infected cells. Comparable levels of HSV-1 DNA in singly and mixedly infected cells could be obtained only by superinfection of HSV-2 at 4 hours after the inoculation of HSV-1. These data suggested that the major cause of interference was not simple competition at the level of adsorption or penetration and that HSV-2 exerted its interference prior to HSV-1 DNA replication.

**Distribution of HSV-1 and HSV-2 DNA in the nuclei and cytoplasm of infected cells.** Among the early events
Figure 16. Effect of superinfection of HSV-2 on the level of HSV-1 DNA in mixedly infected cells. Primary RK cells were infected with 15 PFU of HSV-1 per cell, and at 1, 2, 3, or 4 hours later infected with 3 PFU of HSV-2 per cell. As a control, RK cells were infected singly with 15 PFU of HSV-1 per cell. Total infected-cell DNA was isolated at 8 hours p.i., cleaved, electrophoresed, blotted, and hybridized to the HSV-2 Bgl II fragment G probe. The mean normalized area of HSV-1 Bgl II fragments P, M, and N was determined by densitometry.
Figure 16

Relative level of HSV-1 DNA (% mixed infect/single infect) vs. time of HSV-2 superinfection (HRS).
preceding DNA replication that could be the target of interference is the transport of viral DNA through the cytoplasm and entry of viral DNA into the nucleus of the infected cell. Therefore, the distribution of \(^{32}\)P-labeled HSV-1 DNA in the nuclear and cytoplasmic fractions of singly and mixedly infected cells was monitored at 1, 2, 4, and 10 hours p.i. The cells were infected with 15 PFU \(^{32}\)P-labeled HSV-1 per cell and/or 3 PFU of HSV-2 per cell. Similar experiments were done with HSV-2 labeled with \(^{3}\)H-thymidine, except that cells were fractionated at 1, 2, and 3 hours p.i. Virus was adsorbed to the cells for 1 hour at 37 °C, and unadsorbed virus was removed by extensive washing and incubation continued at 34 °C. Duplicate cultures were harvested at each time point, washed and nuclear and cytoplasmic fractions separated as described in Materials and Methods. Radioactivity in the cytoplasmic fraction would include viral DNA within virions bound to the cell as well as that present in the cytoplasm, while the nuclear fraction would represent DNA in the nucleus or complexed with the nuclear membrane. Plaque assays were performed on HSV-1 and HSV-2 inoculums, as well as on washes of plates to determine the percentage of the virus adsorbed. In singly and mixedly infected cells 76% and 69% of the total infectious virions adsorbed to the cells. Although this analysis could not determine the proportion of HSV-1 or HSV-2 that attached to
the cell in mixed infections, there does not appear to be a
major reduction in viral adsorption. An aliquot of infected
RK cells was assayed to determine the number of cells that
were infected with HSV-1 and/or HSV-2. Infectious center
assays demonstrated that there was no difference in the
proportion of cells infected in either case. This aspect is
also examined in Figure 17 which demonstrates a 33\% reduc-
- \[ P \] labeled HSV-1 that
adsorbed to the cells in the presence of HSV-2 as compared
to singly infected cells. This difference alone is not
sufficient to account for the reduction in the level of
HSV-1 DNA in mixedly infected cells demonstrated in Figure 10.

A more striking difference in the distribution of
radioactivity was the localization of HSV-1 DNA in the cytoplasm
of mixedly infected cells. There was little change in the
radioactivity associated with the cytoplasmic fraction
throughout the 10 hour period. This characteristic was
paralleled by the failure of HSV-1 DNA to reach the nucleus to
the same degree found in singly infected cells. The pattern
of movement of HSV-1 DNA in singly infected cells is charac-
terized by a general decrease in radioactivity associated
with the cytoplasmic fraction as the DNA localizes in the
nuclear fraction. The period of most rapid movement appears
to be from 1 to 2 hours p.i. Ultimately only 10\% of the total
HSV-1 DNA available reached the nuclei of mixedly infected
Figure 17. The distribution of HSV-1 DNA in the nucleus and cytoplasm of singly and mixedly infected cells. Primary RK cells were infected singly with $32^P$ labeled HSV-1 (prepared as described in Materials and Methods) per cell (■—■) or mixedly with $15$ PFU of [$^P$] labeled HSV-1 and $3$ PFU of nonlabeled HSV-2 per cell (●—●). The virus was allowed to adsorb for 1 hour at $37\,\text{C}$ and after extensive washing incubation continued at $34\,\text{C}$. At the times indicated, the nuclear and cytoplasmic fractions were separated as described in Materials and Methods and the trichloroacetic acid insoluble radioactivity associated with each fraction determined.
cells, in contrast to 27% observed in singly infected cells (Table 6). The similarity in the amount of radioactivity associated with the nuclear fractions of singly and mixedly infected cells at 1 hour p.i., however, suggests that interference at this level may be exerted after this time period. The period from 1 to 2 hours p.i. appears to be the time of greatest change in the radioactivity associated with the nuclear fraction and could be the period critical to the interference process.

A comparison of the relative distribution of HSV-1 and HSV-2 DNA in the nucleus of infected cells is shown in Table 6. Although both HSV-1 and HSV-2 DNA reach the nucleus in maximum amounts at 2 hours p.i., a greater proportion of HSV-2 DNA localizes there. In both single and mixed infections, approximately 45% of the HSV-2 DNA reached the nucleus. This data suggests that interference is a specific phenomenon, since the presence of HSV-1 does not reduce the amount of HSV-2 DNA that reached the nucleus.
TABLE 6

RELATIVE DISTRIBUTION OF HSV-1 OR HSV-2 DNA IN THE NUCLEUS OF INFECTED RK CELLS

<table>
<thead>
<tr>
<th>CONDITION OF INFECTION a</th>
<th>TOTAL AVAILABLE RADIOACTIVITY IN THE NUCLEUS (%) b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 HR. p.i.</td>
</tr>
<tr>
<td>HSV-1 (15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.5</td>
</tr>
<tr>
<td>HSV-1 (15) + HSV-2 (3)</td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>10.2</td>
</tr>
<tr>
<td>HSV-2 (3)</td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>29.5</td>
</tr>
<tr>
<td>HSV-2 (3) + HSV-1 (15)</td>
<td></td>
</tr>
<tr>
<td>f</td>
<td>34.9</td>
</tr>
</tbody>
</table>

a Numbers in parentheses refer to the multiplicity of infection in PFU per ml.

b Cells were fractionated as described in Materials and Methods and the radioactivity associated with the nuclear fraction compared to the total amount of label available in the cell prior to fractionation.

c Not done.

d HSV-1 labeled with [32P]-orthophosphate.

e HSV-2 labeled with [3H]-thymidine.
DISCUSSION

The importance of genetic recombination to the replicative cycle of HSV has been suggested from a variety of studies of genome structure, as well as by the ability of the virus to transform cells and establish a latent state. A connection between recombination and HSV DNA replication and isomerization may also exist and for this reason, the characterization of the process of recombination between HSV genomes is essential. Many of the models for HSV DNA replication suggest that recombination events mediate the isomerization of the genome prior to the formation of concatemers, or the major period of DNA replication (Ben-Porat, 1981; Smiley et al., 1981; Davison and Wilkie, 1983). It is important therefore to detect and quantify recombination events that occur early in the replicative cycle and to determine if they occur at a sufficient frequency to support genome isomerization.

The analysis of HSV recombination has been limited in the past to studies of genetic exchange between ts mutants (Schaffer, 1975; Brown et al., 1973; Schaffer et al., 1974). These studies have determined that HSV genomes recombine at a high frequency, but are limited in the ability to examine early recombination events because they can detect only
biologically active virus after several rounds of viral replication. It would be possible to detect recombinants by the analysis of infectious DNA, but like progeny virions, biologically active molecules cannot be detected even under the best circumstances until after DNA replication has been initiated (Figure 11 and 14).

Analysis of recombination has been facilitated greatly by the use of restriction endonucleases which have allowed the changes that accompany recombination events to be identified in a variety of genetic studies. This approach relies on the comparison of restriction endonuclease cleavage patterns of recombinant and parental genomes for the presence of characteristic DNA fragments, as well as for novel DNA fragments formed when parental restriction sites are lost by crossover events. The results presented in this study show that recombinants can be detected during productive replication by the analysis of a specific region of the genome for the appearance of novel DNA fragments. Because these fragments are detected by hybridization, the technique is sensitive and recombinants can be detected and quantified regardless of the biological activity of the viral DNA and may be present as parental, progeny or replicating DNA molecules. Within the region analyzed in this study, three novel fragments were predicted to be formed by crossover events that resulted in the loss of an
HSV-1 Bgl II cleavage site. Two of the three fragments were formed by reciprocal crossover events (R and R) and were most appropriate for analysis, because they appeared in a region least affected by background hybridization and had molecular weights that could be determined accurately. The fragments were present in equal amounts because they were formed by reciprocal crossover events, and therefore served as an additional control for quantitative measurements. The third novel fragment (R) had a molecular weight of 22.1 x 10\(^5\) daltons and could often be distinguished only on autoradiograms exposed for brief periods of time because it was obscured by the intensity with which the probe hybridized to HSV-2 fragment G (11.1 x 10\(^6\) daltons). The calculation of a participation index for the formation of R also was difficult because the parental HSV-1 fragment I (10.2 x 10\(^5\) daltons) was obscured by fragment G.

Despite these constraints, the analysis of restriction endonuclease cleavage patterns for the presence of novel DNA fragments is a useful approach for recombination studies. It is clear, however, that the regions of the genome to be studied must be carefully chosen to allow a sufficient area for recombination to occur, and to allow for discrimination of both parental and recombinant fragments. There are several other regions of the HSV genome that are appropriate
for this type of analysis and because they represent all areas of the genome, studies comparing recombination frequencies could be initiated.

Quantitation of both parental and recombinant genomes by densitometry makes it possible to identify the total pool of DNA molecules and to calculate participation indices as well as recombination frequencies. Such data may be used to determine the recombinational activity of HSV genomes throughout replication, and to determine how a variety of factors affect the recombination process.

Because this approach requires the exchange of DNA between the two serotypes of HSV, it was important to maintain the balance of input HSV-1 and HSV-2 genomes, in order to optimize the likelihood that an intertypic exchange would occur. Therefore the initial studies utilized equal input multiplicities of HSV-1 and HSV-2. Quantitation of parental and recombinant genomes made it possible to determine how the m.o.i.'s were maintained throughout the replicative cycle and demonstrated that HSV-2 consistently interfered with the ability of HSV-1 to replicate, thus reducing the relative amount of HSV-1 DNA maintained.

The phenomenon of interference between strains of HSV has been documented in a variety of studies by yield of progeny analysis. For example, both defective and non-defective HSV-1 strains interfer with the replication of
non-defective HSV-1 strains (Roizman, 1965; Bronson et al., 1973; Frenkel et al., 1975; Murray et al., 1975; Zelena et al., 1976; Purifoy and Powell, 1977; Jofre et al., 1981). Interference of HSV-1 replication by HSV-2 has also been documented (Purifoy and Powell, 1977). Despite the observations made in these studies, nothing is known about the mechanism of interference. The technique used in the study of intertypic recombination makes it possible to follow the effect of interference prior to the production of progeny virions, and has demonstrated that interference occurs early in the viral replicative cycle (Figures 15 and 16).

The results of this study suggest that interference is not due to obvious differences in the viral replicative cycle (Figure 10) or to simple competition at the level of viral adsorption, as increases in HSV-1 input could not increase the levels of HSV-1 DNA in mixedly infected cells above 10% that of HSV-2 (Figure 10). This interpretation is supported by the fact that superinfection with HSV-2 at time periods beyond those required for attachment and adsorption (3 hours p.i.) did not eliminate interference and that similar amounts of HSV-1 DNA were associated with the nucleus of singly and mixedly infected cells at 1 hour p.i. (Figure 16). Furthermore, interference is not due to differences in the rate at which HSV-1 and HSV-2 move from
the cytoplasm to the nucleus of singly infected cells (Figure 17). The interference exerted by HSV-2 in this system appears to affect HSV-1 prior to DNA replication, as evidenced by the protracted period prior to HSV-1 DNA replication in mixedly infected cells (Figure 15). This delay correlates with an alteration in the ability of HSV-1 to migrate from the cytoplasm into the nuclei of mixedly infected cells. These results indicate that interference may be overcome if HSV-1 DNA replication is initiated prior to the addition of HSV-2, perhaps because HSV-1 DNA has entered the nucleus and has begun to replicate by the time of HSV-2 addition.

The expression of interference by HSV-2 appears to be the localization of HSV-1 DNA in the cytoplasm of mixedly infected cells, and results in a limited amount of HSV-1 DNA in the nuclei. Whether this effect is due to a specific activity of HSV-2 on HSV-1 DNA altering its stability or ability to enter the nucleus cannot be determined by these experiments. The possibility exists that interference is mediated by a trans-acting function. Such a function has been demonstrated for an HSV-1 ts mutant ts P23 (Jofre et al., 1981) which is thought to affect the expression of an early gene product required for DNA replication. Coinfection of cells at the nonpermissive temperature with ts P23 and wild-type HSV-1 resulted in the reduced yield of the wild-type (Jofre et al., 1981). Although similar wild-type
interactions have not been demonstrated, it is conceivable that differences in HSV-1 and HSV-2 functions could be expressed in this manner.

Interference may also occur because of major differences in the events associated with the replicative cycle of HSV-1 and HSV-2. For example, HSV-2 disrupts host protein synthesis as early as 30 minutes p.i., or 90 minutes earlier than does HSV-1 (Fenwick and Clark, 1982; Hill et al., 1983). It has been proposed that HSV may require some host cell functions early in the replicative cycle (Powell and Courtney, 1975; Purifoy and Powell, 1977) and it is possible that HSV-1 relies on these cellular functions for a longer period of time than does HSV-2. Until these requirements are understood more completely, it is difficult to speculate on a role for such a function.

The nature of interference at the level of HSV DNA transport through the cytoplasm to the nucleus might be understood if the events occurring after cell penetration were defined. However, little is known about the release of DNA from the capsid, nuclear receptors, penetration into the nucleus, or initiation of events in the nucleus (Spear and Roizman, 1981), making it difficult to predict specific mechanisms involved. The more efficient localization of HSV-2 in the nucleus of the infected cell may suggest that the mechanism involves nuclear penetration. It is even more
difficult to speculate on how this affects the recombination process as genomes must align to exchange DNA, and the balance of the two genomes affects the observed RF. Additional studies on the interference exerted by HSV-2 on HSV-1 may lead to a definition of some of the events associated with this phase of the replicative cycle, and identification of points at which interactions may occur that will alter the cycle's completion.

The ultimate effect of interference is a reduction in the availability of HSV-1 genomes to participate in recombination. Limiting the availability of one of two parental genomes would be expected to result in a reduction of the RF as has been observed for bacteriophage (Doermann and Hill, 1953) and adenovirus (Munz et al., 1983) and confirmed in these studies (Table 3).

In order to determine the effect of limiting input genomes on the RF observed for intertypic recombination, the number of input genomes in the cellular pool were manipulated. The results of these experiments demonstrate that the RF is limited when the input of HSV-1 was less than 15 PFU per cell. Although RF's could not be determined directly at HSV-1 input multiplicities of 20 and 25 PFU per cell, the area of the recombinant fragments did not change dramatically. This suggests that above critical genome concentrations (15 PFU per cell), recombination changes
little despite increasing availability of DNA (Figure 10).

The factor(s) limiting recombination at HSV-1 inputs above 15 PFU per cell is not known, but may involve viral or cellular enzymes involved in the recombination process. It is possible that the number of input genomes above an HSV-1 m.o.i. of 15 PFU per cell may be sufficient to saturate all available viral or cellular functions involved in HSV recombination. The specific viral or cellular functions involved in recombination have not been identified, although some have speculated that the activity of HSV encoded DNase may be involved (Hoffmann, 1981), particularly in light of its similarities in activity to *Escherichia coli* Rec BC nuclease, which has a role in bacterial recombination (Goldmark and Linn, 1972). The existence of viral specific enzymes for recombination may be supported by the ability of HSV to recombine in host cells deficient in ultraviolet repair systems (Selesky *et al.*, 1979; Hall *et al.*, 1980).

At nonlimiting DNA concentrations, the RF's were suprisingly high at 6.5% per Kb, particularly in view of the fact that HSV-1 and HSV-2 share only a 50% base sequence homology (Kieff *et al.*, 1972). It should be noted however, that heteroduplex analysis suggests that the region from 0.21 to 0.41 on the physical map has the highest base sequence homology along the genome (Kudler *et al.*, 1983). Other relatively high RF's have been obtained using
biological assays. For example, intratypic RF's of 3% per Kb have been reported for closely linked HSV-1 thymidine kinase mutants (Smiley et al., 1980) or ts mutants of pseudorabies virus (Ihara et al., 1982); and 5% per Kb was reported for HSV-2 ts mutants within a putative recombinational hot spot (Dixon et al., 1983). Nevertheless caution must be exerted in comparing RF's obtained in absolute terms with those reported for crosses between ts markers for several reasons, the most compelling of which is the substantial variability in RF's observed in repeated experiments even in the same laboratory (Honess et al., 1980) and discrepancies in the RF's per Kb reported between different ts markers (Brown et al., 1973; Schaffer et al., 1974; Esparza et al., 1976; Morse et al., 1977; Morse et al., 1978; Dixon and Schaffer, 1980; Honess et al., 1980; Smiley et al., 1981). An additional concern is that unlike the technique used in this study, biological activity is required to score recombination between ts markers and may result in an underestimate of actual physical exchanges.

Novel DNA fragments formed by recombination events may be detected by the analysis of restriction endonuclease cleavage patterns regardless of their biological activity. Thus, the RF measured in this study may reflect recombination events that occur between genomes resulting in the formation of DNA molecules that would never be
encapsidated, or containing lethal defects that would render
the recombinant inactive. Although the biological activity
of the recombinants is not known, it is likely that the
absolute recombinational activity of the genomes has some
significance for the replicative cycle and must be
understood. Regardless of the method used for their
determination, calculated RF's may be a conservative
estimate of the ability of HSV genomes to recombine because
each of these systems is limited by its ability to detect
only those recombinants produced by an odd number of
crossover events in a particular region. Furthermore studies
of intertypic recombination fail to detect intratypic
genetic exchanges which must occur as well.

The RF measured in these experiments may reflect more
than a single round of recombination per replicative cycle.
Recent evidence obtained from studies with pseudorabies
virus suggests that parental genomes may recombine several
times, (Ben-Porat and Tokalzewlski, 1977; Ben-Porat et al.,
1981), but that exchanges with progeny virions are
relatively rare (Ben-Porat et al., 1982). If HSV behaves in
a similar manner, it is possible that the RF would increase
with multiple rounds of recombination until it approached
50% at which time the apparent RF would decrease. This is
especially true if the parental DNA molecules do not enter
the same pool as progeny genomes, and intertypic and
intratypic exchanges continue to occur early in the replicative cycle. Thus, the actual RF for a single round of recombination cannot be determined by this technique.

The participation of parental and progeny genomes in recombination has been analyzed by Ben-Porat et al. (1981). The results of density labeling studies suggested that recombination occurred between parental molecules, but not between parental and progeny genomes. This supports the observation that recombination is primarily an early event in the replicative cycle, but that multiple rounds of recombination occurred in a single cycle. That recombination is an early event also is supported by lack of detectable intertypic recombination when infection by one genome is followed by superinfection with the other (Figure 16).

It is appropriate to consider if the frequency of recombination measured in this study is sufficient to support genome isomerization. The frequency of HSV recombination appears to be extremely high; at least 6.5 % per Kb under optimal conditions in intertypic crosses. There is no evidence to suggest that the area analyzed in this study is an area characterized by extremely high recombinational activity involving site-specific events, particularly since the crossovers occurred within two DNA fragments in relatively close proximity. It appears that these recombination events are supported only by base
sequence homology and generalized recombination, although it is impossible to rule out broad base sequence specificity based on palindromic or other structurally unique sequences. Similar RF's may occur along the length of the genome in both L and S, so that a RF of 50% would occur over approximately 8 Kb of DNA. Since an RF of 50% represents a 100% chance of genetic exchange, then a segment of DNA the size of the short region would be capable of at least 2 physical exchanges in a single round of viral replication, and could result in isomerization. The RF might be expected to be even higher, particularly in areas where site-specific recombination signals exist. Evidence for such sequences exist in the junction and terminal regions of the HSV genome (Mocarski et al., 1980; Mocarski and Roizman, 1981; Smiley et al., 1981; Davison and Wilkie, 1981; Davison and Wilkie, 1983). Recombinational models for genome isomerization require inter- or intramolecular exchange within the terminal and internal repeat regions of the molecule (Sheldrick and Berthelot, 1974; Skare and Summers, 1977; Ben-Porat, 1981; Ihara et al., 1982; Davison and Wilkie, 1983). The terminal and junction region of L and S each contain 9.0 Kb of DNA, while the same region of S contains 6.5 Kb. Recombination events occurring between at least a portion of these sequences alone may mediate isomerization of one segment of the genome in a single round
of replication if an RF of 6.5% per Kb occurs, and it is possible that the RF in these regions may be even higher. Therefore, it appears that the recombinational activity of HSV-1 and HSV-2 genomes as measured in this study is sufficient to support isomerization, even considering only 2 rounds of generalized recombination within 1 cycle of replication.

Skare and Summers (1977) attempted to determine the frequency of recombination that would have to occur to support genome isomerization in a different manner. Calculations were made to follow the growth of a viral stock from 1 PFU of a single isomer to $1 \times 10^8$ PFU containing equimolar quantities of all four isomers over four lytic cycles of growth. It was determined that 35% of the virions would have to isomerize in each round of replication to reach equimolar quantities at the end of the four cycles. It was argued further that the frequency of recombination within the $1 \times 10^6$ dalton junction region would be equivalent to an RF of 70% (7% per Kb) since one crossover event would produce 1 isomerization in 2 genomes. In a similar analysis of data, Smiley et al. (1980) determined that an RF of 3% per Kb as determined in genetic studies would be sufficient to support isomerization. They reasoned that the actual RF was at least twice the calculated value because only half of the recombination events occurred
between heterotypic genomes and therefore could be detected. The RF for the formation of $R_1$ and $R_2$ (6.5% per Kb) found by the studies presented here is very similar and confirms the ability of recombination to support isomerization.

The rationale for analyzing observed RF's to determine if they are sufficiently high to support isomerization must include a consideration of whether they represent inter- or intramolecular events. All systems employed to date for analyzing recombination measure only intermolecular recombination events, but the frequency of intramolecular exchange particularly in the junction region is probably even higher (Smiley et al., 1980). The type of recombination involved in genome isomerization will be understood only when the physical conformation of recombining molecules is determined.

Among the major questions that remain to be answered before the relationship between genome isomerization and recombination can be determined is the time at which recombination events occur. This is particularly important to elucidate the role of DNA replication in this process. It is clear that recombination plays a role in isomerization (Ben-Porat, 1981; Smiley et al., 1981), but the relative time at which recombination events occur with respect to DNA replication has not been determined. Several models for isomerization propose that recombination occurs prior to the
formation of concatemers (Ben-Porat, 1981; Smiley et al., 1981; Davison and Wilkie, 1983) and because it has been possible to detect input genomes as early as 30 minutes p.i. by hybridization analysis, it seemed appropriate to use this approach to examine recombination events throughout the HSV replicative cycle. The data in Figures 12 and 15, together with Tables 4 and 5, demonstrate that recombination can be detected by 4 hours p.i. and that the percentage of genomes participating in these events increases from 12% at 4 hours p.i. to 21% at 6 hours p.i. At 8 and 18 hours p.i. the PI increased slightly to 28 and 32% respectively. DNA replication begins for HSV-2 after 2 hours p.i., although for HSV-1 DNA replication it is limited even through 6 hours p.i. in mixedly infected cells (Figure 15). Although it is difficult to interpret this data in light of the difference between the HSV-1 and 2 replicative cycles, it appears that intertypic recombination events can occur early in the cycle, and for HSV-1, before significant amounts of DNA replication has occurred. It is clear, however, that recombination occurs prior to the completion of the replicative cycle in contrast to the proposal of Roizman (1979), and may begin to occur prior to the formation of concatemers as has been suggested by several other models (Ben-Porat, 1981; Smiley et al., 1981; Davison and Wilkie,
1983). The observation also supports the results of Ben-Porat et al. (1982), that recombination occurs primarily early in the replicative cycle.

It is not known whether DNA replication and recombination are interdependent events, although if genome isomerization is part of the process, they may be interrelated. Recombination may be required to initiate the replicative cycle, perhaps as has been proposed by Ihara et al. (1982) by controlling the formation of replicative intermediates such as circles of greater than unit length. In this case recombination would control entry of molecules into the replicative cycle, and inhibition of DNA replication might not restrict recombination. The observed RF's might be expected to increase over time to 50% even in the absence of replication. Alternatively, replication could be required to either physically alter molecules to allow them to recombine or to stimulate exchanges by putting the genomes in close proximity or increasing the number of genomes available to recombine. Recombination also may be characterized by both types of phenomenon if exchanges occur throughout the cycle, between parental and progeny genomes.

The determination of the interdependence of DNA replication and recombination will be important not only to an understanding of the isomerization process, but also to the determination of the mechanism of DNA replication.
itself. The analysis of recombination by the detection of novel DNA fragments lends itself to several approaches that may be used to investigate this relationship. Because recombinants can be detected early, and without the constraints of biological activity, the inhibition of DNA replication should not restrict detection. Furthermore, replicative intermediates, that may not be infectious can be analyzed using this approach. Viral DNA synthesis can be inhibited with phosphonoacetic acid which is a specific inhibitor of HSV DNA polymerase (Mao et al., 1975). DNA isolated during the replicative block can be analyzed for the presence of R<sub>1</sub> and R<sub>2</sub>, as indicators of recombination. The calculation of RF's may suggest the role of replication in the process of recombination.

The objective of this study was to understand the role recombination in the replicative cycle of HSV. This was accomplished by the quantitation of intertypic recombinant genomes by analysis of restriction endonuclease cleavage patterns. The criterion for detection of recombinants was the presence of novel DNA fragments formed by the loss of a characteristic restriction site. This approach was complicated by the unexpected finding that HSV-2 consistently interfered with the replication of HSV-1. Due to interference by HSV-2, the number of HSV-1 input genomes was raised to 15 PFU per cell, and resulted in an increase
in the level of HSV-1 DNA to 10% that of HSV-2 DNA. The mechanism of interference involved the localization of HSV-1 DNA to the cytoplasm of the infected cell, and may involve nuclear receptors or other as yet undefined interttypic interactions.

This study demonstrated that up to 35% of parental genomes participated in recombination. This high degree of participation represents an RF of 6.5% per Kb and is sufficient to support isomerization of the genome. Recombination occurred by 4 hours p.i., and the intensity of hybridization to recombinant fragments increased through 8 hours p.i., but remained relatively constant thereafter. Thus, recombination occurs early, prior to the completion of viral DNA synthesis. These findings are consistent with those models for genome isomerization that call for recombination events to occur between genomes early in the replicative cycle, prior to concatemer formation.
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