Different Sizes of Restriction Endonuclease Fragments from the Terminal Repetitions of the Herpes Simplex Virus Type 1 Genome Latent in Trigeminal Ganglia of Mice

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SUMMARY

Trigeminal ganglion DNA from mice latently infected with herpes simplex virus type 1 was analysed by restriction enzyme digestion, agarose gel electrophoresis and blot hybridization to \(^{32}\)P-labelled viral DNA. Viral DNA from parental virions and from virions obtained as a consequence of reactivation by ganglion neurectomy were similarly analysed. The \(\text{BamHI}\) restriction fragments of parental and reactivated virions were almost indistinguishable from each other, and several of the larger \(\text{BamHI}\) fragments of viral DNA were also found in latently infected ganglia at unaltered sizes. In contrast, fractionation of \(\text{EcoRI}\) fragments of latently infected ganglion DNA by reverse phase column (RPC-5) chromatography, followed by gel electrophoresis and blot hybridization to a viral DNA probe from the \(S\) component terminal repetition, revealed the presence of several terminal fragments at discrete sizes ranging from 1 kb to 15 kb, quite unlike the 5.7 kb terminal \(\text{EcoRI}\) \(K\) fragment of virion-derived DNA. These results indicate that structural changes occur in the viral genome concomitantly with the establishment of latency, such as may result from extensive gene rearrangement or integration into cellular DNA.

Herpes simplex virus (HSV) causes latent infections in the peripheral and central nervous systems of humans (Bastian et al., 1972; Baringer & Swoveland, 1973; Brown et al., 1979) and of experimentally inoculated animals (Stevens & Cook, 1971; Price et al., 1975; Puga et al., 1978; Cabrera et al., 1980; Fraser et al., 1981). In contrast with the wealth of information on the genome structure and temporal regulation of gene expression of herpesviruses in tissue culture, little is known about the molecular biology of the naturally occurring or experimentally induced latent HSV infection. The reasons for this lack of information stem from experimental drawbacks inherent in the system. Even under conditions where large amounts of virus are inoculated into the cornea, a mouse trigeminal ganglion, containing 5 to 10 \(\mu\)g DNA, harbours latent viral DNA copies at an average frequency of 0.05 equivalents per cell genome (Puga et al., 1978). This frequency, already at the limit of detection of present techniques, sets constraints on possible experimental approaches. However, information on the physical structure of the viral genome during latency may be essential to understanding the molecular basis of the virus-host interactions that lead to the establishment, maintenance and reactivation of the latent infection.

The focus of this report is on the structure of the latent viral genome by restriction endonuclease analyses. The findings described suggest that extensive genome rearrangements of integration into the cellular DNA occur during latency.

Six- to 8-week-old female BALB/cJ mice were inoculated with HSV-1 strain F (ATCC) by corneal scarification (Puga et al., 1978). Both trigeminal ganglia were removed 6 weeks after

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inoculation and tested for the presence of latent viral genomes as described previously (Puga et al., 1978; Cabrera et al., 1980). DNA was extracted from the latently infected ganglia by the sodium–trichloroacetic acid technique (Cabrera et al., 1980; Puga et al., 1982). The average size of the DNA preparations was 50 to 100 kb, as determined by comparison with native bacteriophage lambda DNA in 0.2% agarose gels. Trigeminal ganglia from 20 latently infected mice were removed by neurectomy and placed in culture, a procedure that has been shown to reactivate the latent ganglionic infection and cause a lytic infection in organ cultures of ganglion tissue (Stevens & Cook, 1971; Walz et al., 1974; Price & Schmitz, 1978). Three days after neurectomy, virus in the supernatant medium was amplified by one passage in primary rabbit kidney cells. An aliquot of the parental inoculum stock of HSV-1 was similarly passaged once in sister cultures of primary rabbit kidney cells. DNA was extracted from CsCl-banded virions (Puga et al., 1978).

DNA preparations from latently infected ganglia, parental virions and reactivated virions were digested with BamHI, electrophoresed in 1% agarose gels, blotted onto nitrocellulose sheets and hybridized to HSV-1 DNA labelled with 32P by the nick-translation procedure (Puga et al., 1982). Results of this experiment are presented in Fig. 1. BamHI fragments of parental virion DNA (lane a) are almost indistinguishable from those of reactivated virion DNA (lane d), except possibly for a subtle difference in the size of the terminal TRs and TRl fragments (P and S), which may result from heterogeneity in the number of a sequences, a phenomenon known to occur in tissue culture-grown virus (Wagner & Summers, 1978; Locker & Frenkel, 1979). In contrast, only the larger BamHI fragments A to E can be distinguished in digests of 10 µg (lane b) or 5 µg (lane c) of latently infected ganglion DNA. Fig. 2(c) shows the map positions of these BamHI fragments in the viral genome. Identification of other viral DNA fragments in Fig. 1(b, c) cannot be made reliably because they become obscured by the background smear. Since the larger A to E fragments can be detected, it is unlikely that the smear results from random shearing of the viral DNA during extraction; more probably, this smear represents either non-specific hybridization to cellular DNA sequences or true hybridization to HSV DNA-related sequences present in a middle repetitive family of the mouse genome (Peden et al., 1982; Puga et al., 1982).

These experiments were repeated using other restriction enzymes (EcoRI and HindIII, data not shown) with qualitatively similar results: only a few of the restriction fragments of viral DNA could be identified in digests of latently infected ganglion DNA; the others were either absent or faded into the background smear. In order to increase the discrimination power of the techniques used, it became necessary to enrich latently infected ganglion DNA preparations for HSV sequences. Furthermore, we decided to use probes from the terminal repetitions of the viral genome, since fragments derived from them seemed to be undetectable using a total viral DNA probe. The 5.7 kb terminal EcoRI K fragment was chosen as a probe, since it maps entirely in TRs (Fig. 2a) and would allow the detection of the viral DNA fragments B and C mapping in the internal inverted repetitions (IRl/IRs) and, through the common a sequences, of fragments E and J, mapping in the left terminus (TRl) (Fig. 2a, b). In order to enrich latently infected ganglion DNA for viral DNA sequences, reverse phase column (RPC-5) chromatography of EcoRI digests was chosen. RPC-5 has been extensively used for large-scale separation of DNA restriction fragments of mammalian genomes (Hardies & Wells, 1976; Tilgham et al., 1977) and for the study of HSV-1 and HSV-2 DNA sequences in morphologically and biochemically transformed cell lines (Reyes et al., 1979, 1982). EcoRI digestion was selected because, as opposed to other restriction enzymes, such as BamHI, it gives a clearly resolved terminal fragment from TRs, and alterations in the size of this fragment could easily be distinguished.

Several control experiments were done. In one case, 1 µg of HSV-1 DNA was mixed with 2 mg of rabbit kidney DNA and the mixture was subjected to partial digestion with EcoRI. Following RPC-5 chromatography the fractions were electrophoresed in a 0.7% agarose gel, transferred to nitrocellulose sheets and the blots hybridized to a total 32P-HSV DNA probe. Fig. 3(a) shows the result of this control. The lane labelled HSV contains 500 ng of a complete EcoRI digest of HSV-1 DNA; lanes labelled 21 to 28 contain the corresponding fractions of the RPC-5 column. Partial digestion products can be seen at sizes as large as up to probably intact HSV
Fig. 1. Hybridization of $^{32}$P-labelled total HSV DNA to $BamHI$ fragments of: (a) 500 ng DNA from parental inoculum virions passaged once in primary rabbit kidney cells; (b) 10 μg trigeminal ganglion DNA from latently infected mice; (c) 5 μg of the same DNA as in (b); (d) 500 ng DNA from virions reactivated by ganglionic neuractomy and passaged once in primary rabbit kidney cells. DNA preparations were digested with a tenfold excess of $BamHI$, electrophoresed in a 1% agarose gel, transferred to nitrocellulose and the blots hybridized to $40 \times 10^6$ ct/min nick-translated $^{32}$P-HSV DNA. Hybridization conditions were as follows: blots were prehybridized for 24 h at 70 °C in $4 \times$ SET (1 x SET = 0.03 M-Tris-HCl pH 8.0, 0.15 M-NaCl, 2 mM-EDTA), $2 \times$ Denhardt's solution (1 x Denhardt’s solution = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 0.1% SDS, 0.1% sodium pyrophosphate, 50 mM-phosphate buffer pH 6.8 and enough uninfected mouse liver and kidney denatured DNA to have at least 25 μg DNA per cm$^2$ of paper. Hybridizations were carried out at 72 °C for 16 to 32 h in the same buffer as above. After hybridization, blots were washed twice for 30 min at 75 °C with each of $1 \times$, $2 \times$, $4 \times$, $8 \times$, $16 \times$ and $32 \times$ diluted hybridization buffer minus carrier DNA, and the washes monitored by Cerenkov counting for removal of unhybridized probe. If needed, washes were continued until no more $^{32}$P counts eluted from the blots. Lanes (a) and (d) were exposed overnight at $-70$ °C with Lightning plus intensifying screens. Lanes (b) and (c) were exposed for 6 days at $-70$ °C with Quanta III intensifying screens. The nomenclature of the fragments is according to Locker & Frenkel (1979).

DNA (160 kb). This control demonstrates that the moderately high pressures at which RPC-5 columns are run do not shear the DNA and produce spurious fragments. Fig. 3(b) shows another control experiment. Five-hundred ng of HSV-1 DNA were mixed with 1 mg of brain and trigeminal ganglion DNA from uninfected mice and the mixture was digested to completion with $EcoRI$. After electrophoresis and transfer onto nitrocellulose, the blots were hybridized to a $^{32}$P-labelled $EcoRI$ K DNA probe. The specificity of the probe is unambiguously demonstrated by its hybridization only to fragments B and C, from the IR$_L$/IR$_S$ joint, E and J from TR$_L$, and K from TR$_S$, known to appear as a doublet in many viral DNA preparations (Wagner & Summers, 1978; Locker & Frenkel, 1979). Fig. 3(c) shows the pattern of hybridization of the $^{32}$P-K DNA probe to RPC-5 fractions of $EcoRI$-digested trigeminal ganglion DNA from latently infected...
mice. Five mg of DNA were digested to completion with 16,000 units of EcoRI and after RPC-5 chromatography, one-half of each fraction (10 to 20 μg) was electrophoresed in 0.5% agarose gels, transferred to nitrocellulose and the blots hybridized to the same 32P-K DNA probe used for the experiment in Fig. 3(b). The marker lane, labelled HSV, contained a mixture of 25 ng of HSV-1 DNA, 1 μg of phage lambda DNA and a trace of 32P-labelled lambda DNA; this mixture was digested with EcoRI at the same enzyme : DNA ratio as the ganglion DNA, in order to test for the completion of the digestion; furthermore, the labelled lambda DNA provided autoradiographic markers for fragment sizes. Hybridization in this marker lane occurs only with the same B, C, E, J and K fragments of Fig. 3(b) (note that the three bands indicated by arrows are the 7.6 kb, 4.8 kb and 3.4 kb EcoRI fragments of 32P-lambda DNA). In contrast, hybridization to the RPC-5 fractions of latently infected ganglion DNA shows the presence of at least 19 discrete bands, encompassing fragment sizes ranging from 1 kb to 15 kb. Faint indication of fragments matching B, C, E, J and K can be seen, but clearly the majority of the hybridization bands occur at fragments unlike those of virion DNA. A third control experiment argues that these fragments are indeed of virus origin. Five mg of brain and trigeminal ganglion DNA from uninfected mice were subjected to the same enzymic digestion, RPC-5 chromatography, gel electrophoresis and hybridization to 32P-K DNA. Even after a 7-day autoradiographic exposure, no distinct bands of hybridization could be seen, and only the background smear was present (Fig. 3d).

If the changes in the size of the K fragment arose from random rearrangements of portions of TRs sequences, it might be expected that the size of subfragments contained within EcoRI K should also be altered. We tested one such subfragment, the 1.9 kb BamHI Y that maps entirely internally to EcoRI K. A pool of fractions 21 to 23 in Fig. 3(c) was digested with BamHI, electrophoresed in a 1.5% agarose gel, the DNA transferred to a nitrocellulose sheet, and hybridized to a 32P-labelled DNA probe of the recombinant plasmid pKL21 that contains the BamHI Y fragment (Denniston et al., 1981). Fig. 4 shows that the latent viral genome (lane 2) contains a single fragment of identical size to the Y fragment from marker virion DNA (lane 3). This would be expected if the changes in size of the EcoRI K fragments were not the result of random rearrangements in portions of TRs, and identifies the conservation of restriction sites distal to the termini. Furthermore, this experiment indicates that the changes in size of the K
Fig. 3. Hybridization of $^{32}$P-K DNA to RPC-5 fractions of latently infected trigeminal ganglia and various controls. Preparation of column packing for chromatography was according to instruction provided by Dr D. Novelli. After EcoRI digestion, DNA was extracted and bound to a 0.6 x 100 cm column of RPC-5 in 1 M NaOAc, 50 mM Tris-HCl pH 7-4, 1 mM EDTA. Fractions (1 ml) were eluted at 200 to 400 lbf/in$^2$ with a 120 ml gradient of 1.45 M to 1.68 M NaOAc in Tris-EDTA and continuously monitored for $A_{260}$. Elution pattern of DNA in a gradient of NaOAc varies for each batch of RPC-5. For the batch we used all the HSV-1 DNA EcoRI fragments elute between 1.475 and 1.525 M NaOAc. DNA in the fractions was precipitated with 2 vol. ethanol, the precipitates dissolved in 10 mM Tris, 1 mM EDTA and 10 to 20 pg DNA was electrophoresed in 0.5% agarose gels. After transfer to nitrocellulose sheets, blots were hybridized to 30 x 10$^6$ to 100 x 10$^6$ ct/min $^{32}$P-labelled HSV-1 DNA (a) or viral EcoRI K DNA (b to d) as described in Fig. 1. K DNA fragment was purified twice by electroelution from preparative gels of EcoRI digests of HSV-1 virion DNA. Lane numbers correspond to fraction numbers of RPC-5 columns, which were renumbered to show matching regions of the chromatograms. (a) Reconstitution mixture containing 1 pg HSV-1 DNA and 2 mg rabbit kidney DNA digested with 2000 units EcoRI for 30 min to produce partial digestion products. Lane labelled HSV contained 500 ng of a complete EcoRI digest of HSV-1 DNA. (b) Reconstitution mixture containing 500 ng HSV-1 DNA and 1 mg brain and trigeminal ganglion DNA from uninfected mice (mixture digested with 5000 units EcoRI for 2 h). (c) Five mg trigeminal ganglion DNA from infected mice digested with 16000 units EcoRI for 4 h. Lane labelled HSV contained 25 ng HSV-1 DNA, 1ug phage X DNA and a trace of $^{32}$P-X DNA (mixture digested with 4 units EcoRI for 4 h). (d) Five mg brain and trigeminal ganglion DNA from uninfected mice digested with 16000 units EcoRI for 4 h. The exposure times were: 18 h with Lightning plus screens for (a, b), 24 h with Quanta III screens for lanes 21 and 22 in (c), 7 days with Quanta III for the remainder of (c) and (d). Note that the bands marked with arrows at 7.6 kb, 4.8 kb, and 3.4 kb in (c) are EcoRI fragments of the tracer $^{32}$P-X DNA included in the mixture, and do not result from hybridization of the probe to DNA sequences on the blot.
Fig. 4. Hybridization of $^{32}$P-labelled BamHI Y DNA with trigeminal ganglion DNA from latently infected mice. Ten μg EcoRI-digested DNA from pooled RPC-5 fractions 21 to 23 (Fig. 3c) were digested with 20 units BamHI and electrophoresed in a 1.5% agarose gel (lane 2). A mixture of 1 ng HSV DNA and 2 μg uninfected mouse brain DNA was digested with EcoRI (lane 1) or with EcoRI and BamHI (lane 3) and electrophoresed in parallel. After electrophoresis, the DNAs were transferred to nitrocellulose and hybridized to $40 \times 10^6$ ct/min $^{32}$P-labelled BamHI Y DNA, purified from plasmid pKL21 (Denniston et al., 1981). Exposure was for 7 days using Quanta III intensifying screens. Positions of the EcoRI B, C and K fragments and of the BamHI Y fragments are indicated.

fragment are not an experimental artefact, since at least one other fragment, the BamHI Y, seems unaltered.

Clearly, the latent HSV DNA fragments containing terminally repeated sequences are unlike the corresponding fragments in virion DNA. Several trivial explanations for these results may be ruled out. For example, shearing during RPC-5 chromatography, hybridization to partially digested DNA fragments or hybridization to HSV DNA-related mouse genomic sequences. The controls shown in Fig. 3(a, b, d) indicate that no random DNA shearing occurs during chromatography, that no partial digestion products appear at sizes between fragments K and J, that the probe is specific for the viral B, C, E, J and K DNA fragments, and that it does not hybridize to discrete fragments of uninfected mouse DNA. The latter might be particularly surprising, since results from our and other laboratories (Puga et al., 1982; Peden et al., 1982) have shown the presence of middle repetitive DNA sequences in the genomes of mice and humans related to the TRs region of HSV DNA. In fact, these cell-related sequences are
distributed in 40 to 80 nucleotide stretches throughout the length of TRs (A. Puga et al., unpublished results), which precluded the use of probes with no known homology to mouse DNA sequences for the present experiments. Since the object of these experiments was to examine the terminal repetitions of the latent viral genome, we used a hybridization regime that would minimize detection of TRs-related host cell sequences. Hence, we included in all prehybridizations and hybridizations at least 25 µg of denatured mouse DNA carrier per cm² of nitrocellulose (typically, 1 mg/ml in the reaction mixture). During a 24 h prehybridization, this amount of DNA reassociates in solution to an equivalent C₀t of about 3000 moles of nucleotide x s x 1⁻¹, sufficient to compete out at least 95% of the middle repetitive DNA families in the blot. It is to this competition effect that we ascribe the success in greatly reducing the hybridization of our probes to uninfected mouse DNA.

Random shearing during DNA extraction cannot be ruled out completely as an explanation for these findings. However, since the average size of the DNA preparations was 50 to 100 kb and intact HSV DNA is approx. 160 kb, random shearing will only cause an average of two to three double-stranded breaks per viral genome, unlikely to affect the size of the viral DNA fragments studied. In agreement with this prediction is the fact that the large BamHI fragments A to E (10 to 6 kb) can be found at unaltered sizes (Fig. 1). Furthermore, random shearing would cause a random distribution of the sizes of the resulting pieces, which would not resolve into discrete fragments.

The findings presented in this report point to major alterations in the molecular structure of the latent HSV genome, absent in both the parental inoculum virus stock and in the virions reactivated from latently infected ganglia. The difference may possibly be attributed to the latent state of the viral genome. Three possible explanations may account for these alterations. (i) Intramolecular rearrangements of free viral genomes may occur such that regions of the DNA exchange physical positions within the genome. As a consequence, the restriction fragments obtained would be unlike those of virion DNA, and, if several of these putative rearrangements occurred, the same DNA sequence would appear in an increasingly larger number of different fragments. (ii) The latent viral genome could be integrated into the host genome, and the novel sizes of the terminal sequences may reflect the presence of virus DNA–cell DNA junction fragments. In this case, the new fragments would have different sizes, depending on the position of the restriction sites in the cellular part of the junction. The presence of discrete fragment sizes would indicate that integration is not random, but occurs at a number of preferred sites. (iii) The majority of the DNA fragments detected are part of defective viral DNA molecules and hide a minority of fragments from non-defective viral genomes of unknown structure.

Recently, Rock & Fraser (1983) have been able to detect most of the HSV-1 genome in central nervous system tissues of latently infected mice, conclusively ruling out the presence of defective viral genomes in these tissues. In addition, they found that the latent viral DNA seems to lack the terminal fragments. They concluded that the latent viral genome has undergone biochemical changes, and that it persists in a form other than linear, unit length DNA.

Our results are in good agreement with this hypothesis. The evidence that we present argues against a genome structure like the one found in virions, and also against circularization of the genome via the termini, since circular molecules would have an overrepresentation of IRₗ/IRₘ fragments and absence of terminal fragments. We were, however, able to detect the terminal fragments, but this discrepancy with the work of the above authors is most likely only apparent, and probably results from the enrichment given by RPC-5 chromatography. This fractionation procedure allowed us to probe for terminal fragments in 5 mg of DNA from latently infected ganglia, and thus find them in an array of different sizes, each one of which would have been undetectable in the unfractionated DNA samples (20 µg) that Rock and Fraser examined.

Final conclusive evidence as to the structure of the latent HSV genome can only be derived from molecular cloning experiments. We have constructed recombinant DNA libraries of latently infected ganglion DNA and have found a variety of clones containing cellular DNA sequences related to HSV DNA (Puga et al., 1982). Since these cellular sequences correspond to a family of moderately repetitive DNA (50 to 100 copies per genome), they are present in a 1000- to 2000-fold excess over viral DNA. All attempts at obtaining clones of bona fide latent HSV
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DNA fragments must deal with the bleak prospect of screening tens of millions of recombinant clones and purifying and analysing several thousand putative ones.

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REFERENCES


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