Comparison of Foldback Sequences of Herpes Simplex Virus Types 1 and 2 DNA

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SUMMARY

The DNAs of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) were separately denatured and allowed to renature briefly. The intrastrand foldback structures that resulted from base pairing of inverted repeated sequences on otherwise single-stranded (ss) DNA were visualized in the electron microscope. The two genomes were found to contain similar size classes of small duplex stem DNA sequences. However, HSV-2 DNA appeared to possess an additional, larger size class of foldback structures not found on HSV-1 DNA. Both HSV DNAs were found to contain stem-plus-loop structures; the larger stem-plus-loop structures of the two genomes had similar stem lengths but dissimilar loop lengths. Thus, a comparison of the genomes of HSV-1 and HSV-2 showed that they possessed similar size classes of foldback sequences.

INTRODUCTION

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) possess a genome of double-stranded (ds) DNA with a mol. wt. of $96 \pm 5 \times 10^6$ (Kieff et al. 1971; Wilkie, 1973; Grafstrom et al. 1975). The two genomes show approx. 50% DNA base sequence homology (Kieff et al. 1972; Bronson et al. 1972). About 80% of the HSV genome is unique sequence DNA and about 20% is repeated sequence DNA. The longest sequences of repeated DNA are internal inverted repeats of 4-3% of one end and 6% of the other (Sheldrick & Berthelot, 1974; Wadsworth et al. 1975). HSV DNA possesses a true terminal repetition of 0-5 to 1% at each end of the genome (Grafstrom et al. 1974, 1975; Wadsworth et al. 1976; Kudler & Hyman, 1979). The terminal repetition, in turn, contains within itself a small inverted repeat of the very end sequence (Hyman et al. 1976; Wadsworth et al. 1976). An additional type of repeated DNA, foldback sequences, has recently been described for HSV-2 DNA (Miller & Hyman, 1978). The presence of these sequences is demonstrated by denaturing duplex DNA and then allowing intrastrand reassociation to occur. On the original duplex HSV DNA where a sequence is immediately repeated in an inverted fashion, a duplex stem structure will form on the denatured HSV DNA. If the inverted repeated DNA is separated by a region of non-homology, a ssDNA loop will be present at the top of the duplex stem structure.

In this paper foldback sequences were identified on HSV-1 DNA and quantitatively...
compared to those of HSV-2 DNA. In our previous qualitative examination of the foldback sequences of HSV-2 DNA, the experimental conditions allowed 33% mismatch or less in the duplex stem structures (Miller & Hyman, 1978). In the present study, renaturation conditions allowed 25% mismatch or less in duplex regions. These more stringent renaturation conditions used with HSV DNA free of contamination by cell DNA allowed a quantitative identification and comparison of foldback sequences of the HSV-1 and HSV-2 genomes.

METHODS

DNA preparation. HSV-1 (Patton) and HSV-2 (333) stocks were plaque-purified three times. HSV-1 and HSV-2 DNAs were purified from Vero cells infected at a low multiplicity as previously described (Miller & Hyman, 1978). A sample of each HSV DNA preparation was banded isopycnically in CsCl. Fig. 1 shows a representative gradient of one of the HSV-2 DNA preparations. In any DNA preparation, the maximum amount of radioactivity banding at the buoyant position of cell DNA was 3% of the total ct/min. Even if the specific activity of the cell DNA was half that of HSV DNA, the HSV DNA was at least 95% pure. HSV-1 and HSV-2 DNAs in 0.025 M-EDTA (pH 8.4) were separately alkaline denatured, neutralized and renatured at room temperature for 20 min in 50% recrystallized formamide (Schmid et al. 1975). Cot values for both DNAs were $4 \times 10^{-4}$ mol s/l. After reassociation, the HSV DNA was dialysed against 0.01 M-tris and 0.001 M-EDTA (pH 8) at 4 °C. The DNA samples were mounted for electron microscopic examination using the procedure of Davis et al. (1971). Length standards included in the mount were single-stranded and duplex $\phi X_{174}$ DNAs. The base sequence of $\phi X_{174}$ DNA has been determined (Sanger et al. 1977). If it is assumed that the number of bases (or base pairs) per unit length is independent of sequence, then the lengths of the foldback structures can be expressed directly in units of bases (or base pairs). Electron micrograph photography and length measurements of the foldback structures were described previously (Miller & Hyman, 1978).

Statistical analysis of the data. Davis et al. (1971) have demonstrated using fluctuation theory that the standard deviation ($\sigma$) of the length distribution of a homogeneous population of molecules is directly proportional to the square root of the length ($L$):

$$\sigma = k(L)^{1/2}, \quad (1)$$

where $k_D$ is the proportionality constant for duplex DNA and $k_s$ the constant for ssDNA. Both Davis et al. (1971) and ourselves express lengths in $\phi X_{174}$ units. Our experimentally determined values for $k_D$ and $k_s$ based on circular duplex and single-stranded $\phi X_{174}$ DNAs are 0.030 for $k_D$ and 0.055 for $k_s$. These values are in excellent agreement with the values given by Davis et al. (1971), where $k_D$ is 0.037 and $k_s$ is 0.049. For analysis of data in a histogram, where there appeared to be a population of foldback structures of uniform length, the theoretical standard deviation was calculated from equation 1. If the population fitted within two standard deviations, it was taken to be potentially of homogeneous length, and a least-squares analysis was used to calculate the average value and experimental standard deviation. If the experimental and theoretical standard deviations were equivalent within experimental error, the population of molecules was taken to be indistinguishable from a population with homogeneous length.
HSV foldback DNA

Fig. 1. Buoyant density analysis of HSV DNA. HSV-1 and HSV-2 DNAs were separately purified from virus infected Vero cells. Aliquots of each 14C-dThd-labelled HSV DNA preparation were banded isopycnically in CsCl. This representative gradient of 14C-dThd-labelled HSV-2 DNA demonstrates the absence of contaminating cell DNA from a typical HSV DNA preparation: less than 3% of the total radioactivity was found in the gradient at the buoyant position of cell DNA. Density increases from right to left and was determined through refractive index measurements.

RESULTS

HSV-1 and HSV-2 DNAs were separately denatured, neutralized and allowed to renature under conditions that favoured intrastrand reassociation and thus allowed the formation of foldback structures to occur. The renatured DNA was mounted for examination in the electron microscope. Foldback structures composed only of a duplex stem have been visualized on otherwise single-stranded HSV-1 and HSV-2 DNAs. Examples of electron micrographs are given in Fig. 2. Histograms of the lengths of these stem structures are shown in Fig. 3 for both HSV-1 and HSV-2 DNAs. Statistical analysis of the data for HSV-1 DNA (Fig. 3a) revealed that the observed population of foldback stem structures was indistinguishable from a homogeneous population with an average length of 180 ± 30 base pairs. The histogram of the lengths of HSV-2 DNA foldback stem structures (Fig. 3b) was modestly different from the analogous histogram for HSV-1 DNA (Fig. 3a). Statistical analysis of the data for HSV-2 DNA revealed that this histogram (Fig. 3b) can be represented as the sum of two distributions for two homogeneous stem sizes. The smaller stem structure for HSV-2 DNA corresponded, within experimental error, to the stem structure of HSV-1 DNA and had an average length of 190 ± 40 base pairs. However, an additional larger stem structure of 300 ± 30 base pairs was found on HSV-2 DNA. The two stem foldback structures of HSV-2 DNA have been seen together on the same molecule (Fig. 2b).

Stem-plus-loop structures have also been visualized on otherwise ssHSV-1 and ssHSV-2 DNAs. These foldback structures possessed a duplex DNA stem with a single-stranded DNA loop. Examples of electron micrographs of stem-plus-loop structures are given in Fig. 4. The data for stem-plus-loop foldback structures are presented in the form of coincidence diagrams (Fig. 5), where stem length is plotted against loop length. A comparison of the data for HSV-1 and HSV-2 revealed interesting similarities and differences in the sizes of the stem-plus-loop structures of the two genomes. Both HSV-1 and HSV-2 genomes gave rise to a population of small stem-plus-loop structures (Fig. 4b, c). For HSV-1 DNA (Fig. 5a), this population was statistically indistinguishable from a homogeneous population with an average stem length of 120 ± 40 base pairs and an average loop length of 860 ± 375 bases. The coincidence diagram of stem-plus-loop structures for HSV-2 DNA
was more disperse in the region of small stem-plus-loops. By statistical analysis it was determined that the distribution could be described as the sum of two small stem-plus-loop structures. One population had an average stem length of 135 ± 30 base pairs and an average loop length of 1215 ± 540 bases. These values corresponded approximately to the size of the small stem-plus-loop structure identified for HSV-1 DNA. The second population for HSV-2 DNA had a larger average stem length of 275 ± 50 base pairs but a smaller average loop length of 830 ± 240 bases. A stem-plus-loop structure of these dimensions has been seen only on HSV-2 DNA (Fig. 5).

Larger stem-plus-loop structures have been found for both HSV-1 and HSV-2 DNAs. Examples of electron micrographs are given in Fig. 4(a, b). Stem-plus-loop structures, such as those shown in Fig. 4(a, b), had an average stem length of approx. 6000 base pairs. The loop lengths of the structures for HSV-1 DNA, however, were shorter than those for HSV-2 DNA. For HSV-1 DNA, the loop size ranged from 4000 to 9200 bases, whereas, for HSV-2 DNA, the loop size ranged from 9700 to 12500 bases. Within each genome, by statistical
Fig. 2. Electron micrographs of stem structures on otherwise single-stranded HSV DNA. (a) Arrows point to three small stem structures of 180 ± 30 base pairs on the same molecule of HSV-1 DNA. Also seen in this and other micrographs are the internal length standards: single-stranded φX174 (φXss) and double-stranded φX174 (φXds) DNAs. (b) This molecule of single-stranded HSV-2 DNA contains five foldback structures: three small stem structures (I, III, IV) 190 ± 40 base pairs in length, a rabbit ear structure (RE) of stem length 120 ± 30 base pairs and ear lengths of 130 ± 30 base pairs, and a large stem structure (II) of 300 ± 30 base pairs. Other structures appearing to be stem structures on this molecule were determined to be single-stranded aggregations upon close examination of micrograph negatives.
Fig. 3. Histograms of the lengths of foldback structures composed only of a duplex stem. The lengths of stem structures on otherwise single-stranded HSV-1 (a) and HSV-2 (b) DNAs, such as those shown in Fig. 2, were measured as a function of duplex \$\Phi X174$ DNAs within the same frame. The lengths are plotted as histograms.

Fig. 4. (a) - for legend see page 58.
analysis, the range of loop sizes of the larger stem-plus-loop foldback structures was too wide to represent a homogeneous population of molecules. The key point in the comparison of the larger stem-plus-loop structures for HSV-1 and HSV-2 DNAs was that although the stems were approximately the same size, the loops were smaller for HSV-1 DNA (Fig. 5).

'Rabbit ear' structures are DNA foldback structures composed of a very short duplex DNA stem which forks into two very short duplex DNA stems ('ears'). Because of the small sizes of each of the three parts of the structure, it was impossible to determine whether each portion was composed of truly duplex DNA or collapsed ssDNA. Rabbit ear foldback structures previously have been seen on single-stranded simian virus 40 DNA (Hsu & Jelinek, 1977), RD-114 RNA (Kung et al. 1974) and mouse DNA (Cech & Hearst, 1975) but have not been reported for HSV DNA. Examples of electron micrographs of rabbit ear structures for HSV DNAs are shown in Fig. 2(b) and Fig. 6. Both HSV-1 and HSV-2 DNAs contain a population of rabbit ear foldback structures statistically indistinguishable from a homogeneous population: an average stem length of $120 \pm 30$ base pairs and average ear length of $130 \pm 30$ base pairs.

Of the approx. 1000 ssHSV-2 DNA molecules visualized in the electron microscope, about 20% possessed at least one foldback structure. Of these molecules, approx. one-half contained two or more foldback structures. In part because of the heterogeneous sizes of the ssHSV DNA molecules (Kieff et al. 1971; Wilkie, 1973), and in part because of the
Fig. 4. Electron micrographs of stem-plus-loop structures on otherwise single-stranded HSV-1 DNA. Small arrows indicate the transition from ss to ds DNA. (a) This micrograph shows a representative large stem-plus-loop foldback structure with a stem length of about 6700 base pairs and a loop length of about 6600 bases. (b) The large arrow points to a small stem-plus-loop structure which appears on the single-stranded loop portion of a large stem-plus-loop foldback structure. The stem and loop lengths of the small foldback structure are $120 \pm 40$ base pairs and $860 \pm 375$ bases, respectively; the larger foldback structures are about 4700 base pairs and about 7400 bases, respectively. (c) Two small stem-plus-loop structures of stem length $120 \pm 40$ base pairs and loop length $860 \pm 375$ bases appear together on the same HSV-1 DNA molecule. Both single-stranded ($\phi X_{ss}$) and duplex ($\phi X_{ds}$) $\phi X_{174}$ DNAs are present as length standards.

major inversions of the HSV genome (Hayward et al. 1975; Clements et al. 1976; Delius & Clements, 1976; Skare & Summers, 1977), no overall pattern composed of all the identified HSV DNA foldback structures has been detected. However, the smaller stem structure of HSV-2 DNA (190 $\pm$ 40 base pairs) has been seen in multiple copies on the same ssDNA molecule. One pattern (Fig. 2b) showed three copies of the 190 $\pm$ 40 base pair stem structure and one copy of the 300 $\pm$ 30 base pair stem structure. A rabbit ear structure was also present. Various parts of this pattern have been seen on other molecules. Patterns involving the other identified foldback structures of HSV-2 DNA have been seen at low frequency. As no overall pattern could be determined, further analysis is not reported for either HSV-1 or HSV-2 DNAs.
**DISCUSSION**

The amount of base pair mismatch possible in the duplex stem structures can be calculated if it is assumed that the stem structures have the same guanine-plus-cytosine content as HSV DNA. Using the equation of Frank-Kamenetskii (1971), it was calculated that the melting temperature ($T_m$) of HSV-2 DNA in a sodium ion concentration of 0.05 M was 90 °C. Since 1% formamide lowered the $T_m$ by approx. 0.65 °C (Hyman *et al.* 1973) and the renaturation solution was 50% in formamide, the $T_m$ was lowered by 33 °C. At room temperature (22 °C) the combined conditions were equivalent to $T_m - 35$ °C. Since the $T_m$ is lowered by about 1 °C for every 1% base sequence mismatch (Hutton & Wetmur, 1973), a duplex stem with 25% or less base pair mismatch would form stably during renaturation.

In addition to the problem of possible base mismatch within the duplex stem structures, there were several other problems in the interpretation of these data. A population of foldback structures that was indistinguishable statistically from a homogeneous population of molecules may actually be the sum of two or more populations that vary in size to an extent that is below our detection limits. A population of foldback structures, though homogeneous with respect to size, may be composed of groups of foldback structures that contain different DNA base sequences. Lastly, the foldback structures of HSV-1 and HSV-2 DNAs, although of comparable size, may not represent structures with the same base sequence.
Fig. 6. Electron micrographs of rabbit ear structures on single-stranded HSV DNA. (a) A rabbit ear (RE) foldback structure is seen on the same single-stranded HSV-2 DNA as a small stem-plus-loop foldback structure (arrow). Both single-stranded (φXss) and double-stranded (φXds) φX174 DNAs are present as internal length standards. (b) In this micrograph a rabbit ear (RE) structure is shown on the loop of a large stem-plus-loop structure of HSV-1 DNA. The arrow points to the single- to double-stranded DNA transition. All rabbit ear structures have a stem length of 120 ± 30 base pairs and ear lengths of 130 ± 30 bases.
Nevertheless, the most important point in this paper was the comparison of foldback structures for HSV-1 and HSV-2 DNAs. The two genomes, which share 50% base sequence homology (Kieff et al. 1972; Bronson et al. 1972), have been found to contain comparable foldback structures. HSV-1 DNA gave rise to a foldback stem structure of 180 ± 30 base pairs and a stem-plus-loop structure with a 120 ± 40 base pair stem and a 860 ± 375 base loop. HSV-1 DNA also gave rise to a class of larger stem-plus-loop structures composed of an average stem length of about 6000 base pairs and loop lengths in the range of 4000 to 9200 bases. HSV-2 DNA gave rise to two foldback stem structures of 190 ± 40 and 300 ± 30 base pairs, respectively. HSV-2 DNA also yielded two stem-plus-loop structures with average stem lengths of 135 ± 30 and 275 ± 50 base pairs and loop lengths of 1215 ± 540 and 830 ± 240 bases, respectively. A disperse group of larger stem-plus-loop structures was also seen with an average stem length of 6000 base pairs and loop lengths in the range of 9700 to 12 500 bases.

For the large stem-plus-loop structures, it is probable that the loop represents the short region of unique sequences of HSV DNA and the stem represents the terminal repetition pairing with its inverted repeat (Sheldrick & Berthelot, 1974; Wadsworth et al. 1975). Our values for the lengths of these sequences are slightly shorter than the current numbers derived from restriction enzyme mapping (Roizman, 1979); however, the modest differences may only reflect the method of measurement. The implication from the fact that the stem lengths are indistinguishable between HSV-1 (Patton) and HSV-2 (333) DNAs is that the lengths of the terminal and inverted repeats are indistinguishable between the two strains. The lengths of the loops are different for the two DNAs. This observation implies that the previously observed strain differences in the length of the short segment of HSV DNA (Knipe et al. 1979; Roizman, 1979) can be attributed to differences in length of the unique sequences. The relative heterogeneity of the loop sizes probably reflects the known heterogeneity in length of the junction between the unique and repeated sequence regions of HSV DNA (Wagner & Summers, 1978; Roizman, 1979).

Using electron microscopy we have described several populations of foldback sequences on HSV-1 and HSV-2 DNAs. In a recent study of the reassociation kinetics of HSV-1 (Angelotti) DNA, Ott et al. (1979) found that about 7% of the total HSV-1 DNA renatured rapidly with first-order kinetics, indicative of foldback sequences. Therefore, using two different experimental techniques, Ott et al. (1979) and ourselves have identified foldback sequences of HSV DNA.

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