Distribution of G + C-rich Regions in Varicella-Zoster Virus DNA

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SUMMARY

The distribution of G + C-rich sequences in the genome of varicella-zoster virus (VZV) was investigated by partial denaturation, equilibrium sedimentation and Southern blot analyses. Portions of the IRs and TRs repeat sequences bounding the Us region of the DNA were found to have a G + C content 10 to 20% greater than the overall 47% G + C content of the VZV genome. A stretch of DNA (approx. 1500 base pairs) at the Ul-IRs junction and repeated at the terminus of the TRs sequences was found to be about 64% G + C, based on sedimentation equilibrium measurements. We also report the cloning of a novel fragment containing sequences from both the Ul and TRs termini of the VZV genome. Our ability to clone this fragment suggests that unusual forms of VZV DNA including closed circular molecules and molecules with an inverted Ul region can be packaged into nucleocapsids.

INTRODUCTION

The genome of varicella-zoster virus (VZV) is a linear double-stranded DNA molecule with a molecular weight of about 80 × 10^6. The genome is divided into two components designated L (long) and S (short). The S component consists of a set of quasi-unique sequences (4 × 10^6 to 5 × 10^6 mol. wt.) bracketed by a set of inverted reiterated sequences (3.5 × 10^6 to 4.5 × 10^6), whereas the L component (approx. 67 × 10^6) appears to consist only of quasi-unique sequences. The S component inverts relative to an invariant orientation of the L component (Dumas et al., 1981; Straus et al., 1981, 1982; Ecker & Hyman, 1982; Davison & Scott, 1983). Thus, the structure of the VZV genome is similar to the structure of the genomes of pseudorabies virus (PRV) and equine herpesvirus (EHV) types 1 and 3 (Stevely, 1977; Jean et al., 1977; Ruyechan et al., 1982; Atherton et al., 1982). The overall (46 to 47%) G + C content of the VZV genome (Ludwig et al., 1972; Straus et al., 1981) is 11 to 23% less than the G + C content of the genomes of Epstein–Barr virus (EBV), human cytomegalovirus (HCMV) and herpes simplex virus (HSV) types 1 and 2, as reviewed by Honess & Watson (1977).

Apparent clustering of G + C-rich sequences reported in studies on a number of herpesvirus genomes has been reviewed by Roizman (1980). Relatively G + C-rich regions have been found in the reiterated sequences flanking the quasi-unique regions of the S components of HSV-1 and HSV-2 DNA, HCMV DNA and EHV-1 DNA (Wadsworth et al., 1975; Kilpatrick & Huang, 1977; O’Callaghan et al., 1981). The internal tandemly repeated regions of EBV are significantly more G + C-rich than the remainder of the genome (Delius & Bornkamm, 1978; Given & Kieff, 1979). An even more striking difference in base composition is found between the tandem repeats in the DNAs of herpesvirus atetes and herpesvirus saimiri and the quasi-unique sequences which they bound (Bornkamm et al., 1976; Fleckenstein et al., 1978).

The significance of the existence of relatively G + C-rich regions in the DNAs of herpesviruses is not completely understood. It has been noted, however, that (i) the high G + C
content of these regions appears to have been maintained even though the remainder of the genomes has been subject to substantial genetic drift and (ii) genes expressed immediately after infection in the HSV, CMV and PRV systems map within and/or near the high G + C reiterated sequences (Clements et al., 1977; Jones et al., 1977; Roizman, 1980). In this paper we report on the distribution of G + C-rich sequences in the VZV genome.

**METHODS**

**Purification and partial denaturation of VZV DNA.** Nucleocapsids were purified from human embryonic lung or human foreskin fibroblast monolayers infected with VZV strains Ellen, Oka or Scott, and DNA was purified using the method of Straus et al. (1981). Partial denaturation of purified VZV DNA and cloned VZV DNA fragments was carried out according to the method of Inman & Schnöls (1970) as modified by Kilpatrick & Huang (1977). Denaturation buffer (0.02 M-Na₂CO₃, 5 mM-EDTA, 3.7% formaldehyde) was titrated to pH 11.36 with 5 M-NaOH. VZV DNA at a concentration of about 10 μg/ml and single-strand marker phage fd DNA were added in 5 to 10 μl aliquots to 25 μl of denaturation buffer. The mixtures were then incubated at 25 °C for various times. After the desired time interval, denaturation was stopped by addition of 250 μl of ice-cold ammonium acetate spreading solution (0.77 M-ammonium acetate, 0.11 M-acetic acid, 0.11 mg/ml cytochrome c). Simian virus 40 (SV40) form II DNA was then added as a double-strand size marker. The DNA mixture was spread onto a 0.3 M-ammonium acetate hypophase which had been adjusted to pH 6-5 with acetic acid. The DNA was picked up with parlodion-coated 200-mesh copper grids. The grids were stained with uranyl acetate, rinsed with isopentane and air-dried (Davis et al., 1971). The partially denatured DNA was then examined and photographed with a Zeiss EM10A transmission electron microscope.

The sizes of single- and double-stranded regions were determined by projecting negatives onto a blackboard using a lantern slide projector and taking contour measurements with a Keuffel and Esser map measuring device. Molecular weights of single- and double-stranded regions were calculated by comparison with the contour lengths of the marker DNAs based on molecular weights of 2.11 × 10⁶ and 3.4 × 10⁶ for fd DNA and SV40 form II DNA, respectively (Beck et al., 1978; Fiers et al., 1978).

**Cloning and physical mapping.** EcoRI and BamHI cleavage fragments of VZV Ellen DNA were molecularly cloned either in phage λ strain Agt-Weß or the plasmid pBR325 and mapped as reported previously (Straus et al., 1982). The same mapping strategy, which involved double digestions and Southern hybridizations, was used to identify and orient Smal digestion fragments into a physical map for VZV Ellen. Similar procedures were used for cloning and mapping of selected VZV Ellen Smal fragments.

**Preparation of VZV EcoRI E and G fragment DNA.** VZV EcoRI E and G fragment DNAs cloned in plasmid pBR325 and phage respectively were prepared as outlined previously (Straus et al., 1982), cut with EcoRI enzyme and fractionated on 1% low melt agarose gels. The bands corresponding to the VZV E and G DNA fragments were excised from the gels after visualization with ethidium bromide and were extracted using the CTAB procedure of Langridge et al. (1980). Several VZV Smal fragments cloned in pBR325 were isolated by passive elution from acrylamide gels (Maxam & Gilbert, 1980).

**Sedimentation equilibrium measurements.** Fragments of VZV DNA prepared as outlined above were mixed with authentic whole HSV-2 DNA and whole BHK cell DNA. All DNAs were labelled by nick translation (Kelly et al., 1970; Maniatis et al., 1975) either with [³²P]dCTP (VZV) or [³²P]dTTP (cell and HSV-2). The DNA mixture (0.8 ml) was added to 3.4 ml CsCl solution (11 g in 65 ml) to give a final density of 1.700 g/ml. Samples were centrifuged at 95 000 g for 40 h at 20 °C in the TVL 865 Sorvall rotor. Fractions were collected from the base of the tube and precipitated on 3MM discs prior to scintillation counting.

**Blotting and hybridization.** DNAs were cut with the appropriate restriction enzyme(s), run on 0.6% or 0.7% agarose gels and transferred to nitrocellulose sheets according to Southern (1975). Probes were nick-translated with [³²P]dCTP and/or [³²P]dATP to a specific activity of about 10⁷ c.p.m./μg and hybridization proceeded at 55 °C in 30% formamide or 42 °C in 50% formamide. Blots were analysed autoradiographically.

**RESULTS**

**Physical mapping of VZV DNA**

EcoRI, BamHI and SmaI restriction endonuclease cleavage maps were generated as described in Methods. The EcoRI map and part of the BamHI map have appeared in recent publications from this laboratory (Straus et al., 1982, 1983a). These maps are presented in Fig. 1 and serve to orient the reader to the location of individual fragments and genomic features of VZV DNA relevant to experimental observations described below.
Partial denaturation of VZV DNA

DNA extracted from VZV Ellen and VZV Scott nucleocapsids was partially denatured as described in Methods. Encapsidated VZV DNA is known to contain extensive nicks and gaps (Ludwig et al., 1972; Iltis et al., 1977); hence, partial denaturation would be expected to result in substantial fragmentation of the DNA. This was found to be the case using the classic Inman & Schnöls (1970) technique which involves heating of the DNA at alkaline pH. No molecules greater than one-third the length of intact molecules were found under conditions where an obvious denaturation pattern was seen. The milder conditions used by Kilpatrick & Huang (1977) resulted in less fragmentation. The 'best' conditions for partial denaturation were judged to be a 15 to 20 min incubation at pH 11.3 and 25 °C. Under these conditions it was possible to obtain molecules ranging from half- to full-size which displayed a reproducible denaturation pattern. Incubations at higher pH and/or for longer times resulted in an unacceptable amount of fragmentation. Incubations at lower pH and/or for slightly shorter times resulted in molecules which on average were slightly longer than those obtained under the 'best' conditions but did not result in an extensive or reproducible denaturation pattern. These observations confirm the existence of a large number of single-strand interruptions in encapsidated VZV DNA and indicate that the base composition of the DNA is relatively uniform throughout most of the genome.

VZV Oka and VZV Scott DNA molecules which showed a clear partial denaturation pattern were photographed at a magnification of 16000. Lengths of the single- and double-stranded regions were measured and these lengths were converted into units of molecular mass by comparison with the appropriate marker DNA. Representations of the partially denatured molecules were plotted on a scale of 10^6 daltons/2.5 mm on paper strips. Alignments were performed by arranging molecules according to maximum overlap with the same orientation of common A + T- and G + C-rich sequences. Fig. 2 is a composite representation of 24 partially denatured Oka and Scott molecules with molecular masses ranging between 40 × 10^6 and 84 × 10^6 daltons. The 0 to 40 Mdal region is based on all 24 molecules, the 41 to 50 Mdal region is based on 22 molecules, the 51 to 65 Mdal region is based on 16 molecules and the 65 to 85 Mdal region is based on nine of the 24 molecules. The basis of selection of this set of molecules was the presence of a common structural feature at their right-hand termini.

This feature consisted of an A + T-rich region bounded by two G + C-rich regions, one of which represents the terminus of the molecule. A full-length molecule of VZV Oka DNA
Fig. 2. Partial denaturation profile of VZV DNA. This figure represents a composite of 24 VZV Ellen, Scott and Oka DNA molecules which were partially denatured as described in the text. Note the characteristic region at the right-hand terminus which aligns with the S region elements shown in the schematic of the DNA sequence arrangement.

showing the characteristic A + T-rich region bounded by undenatured G + C-rich regions with substantial denaturation along the remainder of its length is presented in Fig. 3(a). The size of the S region of VZV DNA has been estimated to be $12 \times 10^6$ to $13 \times 10^6$ Mdal by electron microscopy and restriction enzyme analysis (Dumas et al., 1981; Straus et al., 1982; Ecker & Hyman, 1982). The size of the G + C-rich region mentioned above is about $4.1 \times 10^6$ Mdal and the size of the A + T-rich region is about $3.8 \times 10^6$ Mdal for the characteristic region. These sizes correspond reasonably well to the sizes of the inverted repeat and quasi-unique regions in the S segment of VZV DNA determined by restriction enzyme analyses in the studies cited above. The discrepancies between the size estimates for these regions obtained here and our current estimates obtained by restriction enzyme mapping (4-6 and 3-4 Mdal for IR$_S$/TR$_S$ and U$_S$ respectively) are most likely due to the marked differences in G + C content found between the IR$_S$/TR$_S$ and U$_S$ and U$_L$ regions (see below). Under the denaturation and spreading conditions used it is likely that IR$_S$/TR$_S$ sequences near the IR$_S$-U$_S$-TR$_S$ junctions would remain single-stranded, resulting in an underestimate of IR$_S$ and TR$_S$ and an overestimate of U$_S$. A similar argument can be applied to our initial electron microscopical measurements of the elements of the S region (Straus et al., 1982). A recent estimate of the size of U$_S$ obtained from sequencing studies (Davison, 1983) is 5232 base pairs (about 3-4 Mdal), although strain variation in the size of the U$_S$ has been identified in at least one case (T. A. Casey et al., unpublished observations). The implication from the partial denaturation data, therefore, is that the structure of the S region of VZV DNA is similar to the structure of S regions found in other herpesvirus genomes, i.e. a relatively A + T-rich quasi-unique region is bounded by relatively G + C-rich inverted repeats.

Partial denaturation of cloned VZV DNA fragments

The above-mentioned hypothesis implies that cloned fragments of VZV DNA known to contain an inverted repeat sequence should show a characteristic partial denaturation pattern,
Fig. 3. Partially denatured VZV molecules. (a) Full-length molecule of strain Oka DNA showing the S region as a single-stranded bubble flanked by the relatively G + C-rich undenatured repeat regions. (b) Cloned, excised EcoRI E fragments showing a characteristic partial denaturation pattern consisting of an undenatured relatively G + C-rich central region flanked by a fully open single-stranded fork at one end and a series of single-stranded bubbles at the other.

i.e. an undenatured G + C-rich stretch corresponding to a repeat region bounded by denatured A + T-rich regions corresponding to the quasi-unique regions of the L and S components of the genome. Based on the data described in the previous section, fragments taken from other portions of the genome should show substantial denaturation throughout their length under identical conditions.

The cloned EcoRI fragments E and G were isolated from recombinant plasmids as described in Methods. The EcoRI E fragment has a mass of about 8 Mdal, maps between 0.79 and 0.91 map units on the VZV genome and contains the left-hand repeat region (IR₅) bounded by sequences from the UL and US regions (Fig. 1). The EcoRI G fragment has a molecular mass of 5.8 Mdal, maps between 0.31 and 0.38 map units on the VZV genome and was taken to be a representative fragment from the UL region of the genome (Fig. 1). The procedure and conditions for partial denaturation of these fragments were identical to those used for DNA isolated from nucleocapsids.
Partial denaturation of the EcoRI E fragment resulted in molecules which were 45 (± 10)% single-stranded. The E fragment molecules shown in Fig. 3(b) displayed a characteristic denaturation pattern: one of the termini was completely denatured and was observed as a single-stranded fork. The other terminus was occasionally completely denatured but more often consisted of several closely spaced denaturation bubbles connected by short duplex regions. The central portion of these molecules remained duplex (Fig. 3b). Fig. 4 shows the relative percent denaturation of the EcoRI E fragment based on measurements obtained from 28 molecules. While it was not possible to orient the map absolutely with respect to the position of the Uₜ and Uₜ sequences, the least denatured and hence most G + C-rich region falls within the currently estimated limits of the repeat sequences in either orientation. These data indicate that approximately 2 Mdal of the repeat region are highly G + C-rich while the remaining sequences have a G + C content similar to that of the bulk of the genome.

In contrast, partial denaturation of cloned VZV EcoRI G fragment DNA under identical conditions resulted in molecules which were 66 (± 10)% single-stranded over their entire length (data not shown). This result is in agreement with the partial denaturation profile of this region shown in Fig. 2.

Equilibrium sedimentation analysis of cloned restriction fragments

In order to confirm and quantify the distribution of G + C-rich sequences within or near the inverted repeats, cloned VZV restriction enzyme fragments containing representative portions of this region were analysed by equilibrium sedimentation in neutral CsCl gradients. The fragments chosen for this analysis were the EcoRI E fragment and a series of SalI fragments derived from it. As noted above, the EcoRI E fragment contains the entire VZV internal repeat as well as flanking sequences from the Uₜ and Uₜ regions. The SalI cuts are contained within the internal repeat of VZV and are fortuitously distributed so as to allow determination of the G + C content of the repeat region at regular intervals along its length (Fig. 4). Equilibrium sedimentation of the EcoRI E fragment yields a density of 1.71 g/ml which corresponds to a G + C content of about 54%. This value is substantially higher than the 46 to 47% G + C content of the total VZV genome. The partial denaturation results described above indicate that the G + C distribution across the E fragment is not uniform and that sequences near its centre appear to have a high G + C content in comparison to the rest of the fragment. The G + C content results shown in Fig. 4 confirm this and allow orientation of the partial denaturation map of EcoRI E. Specifically, equilibrium sedimentation of a 1-1 kb SalI restriction fragment mapping between 0-45 and 0-57 units along the length of the EcoRI E fragment (i.e. in the left-hand portion of IRₜ in the EcoRI E fragment) (Fig. 4) show that this fragment has an overall G + C content of about 64%. A second (1-65 kb) SalI fragment which maps between 0-72 and 0-85 units was then analysed, since this fragment maps in a region of the EcoRI E fragment which exhibits a level of denaturation between that of the 1-1 kb SalI fragment and the majority of the genome. The equilibrium sedimentation results for this fragment indicate that it has a G + C content of about 54%. Thus, these data indicate that the inverted repeat regions flanking the Uₜ sequences in the VZV genome have a G + C content which is significantly higher than the overall G + C content of that genome.

Identification of minor bands in restriction enzyme digests of VZV DNA

In the course of mapping studies using cloned fragments of VZV DNA, we observed that when whole VZV DNA was cut with EcoRI or BamHI and probed with cloned EcoRI E fragment, minor bands (one visible in each digest) appeared in the autoradiogram in addition to the four major bands (half-molar) predicted from the genome structure (Straus et al., 1982). These minor bands co-migrated with EcoRI N and BamHI N and we subsequently showed that EcoRI N and BamHI N share extensive sequence homology, mapping to about 0-27 map units in Uₜ. Based on these data, we explored the possibility that homology between EcoRI E and BamHI N and EcoRI N was due to the presence of sequences in Uₜ homologous to part of EcoRI E, which includes the repeat (IRₜ/TRₜ) sequences. To test this hypothesis, we cloned what we
believed to be the *BamHI* N fragment into bacteriophage λ, using whole VZV DNA cut with *BamHI*. This putative *BamHI* N fragment, as expected if the above hypothesis were valid, hybridized to *EcoRI* E. Digestion of both this *BamHI* fragment and *EcoRI* E with *SmaI* gave four common bands which mapped in the IRs sequences close to the UL/IRs junction. The bands amounted to about 1 kb and their common nature was confirmed by Southern blotting experiments (Fig. 5). Thus, these experiments were consistent with the presence in VZV DNA of sequences common to UL and IRs. However, when we carried out additional studies with *EcoRI* N cloned in pBR325 (which should behave like *BamHI* N) we failed to observe any homology between it and *EcoRI* E and, subsequently, between it and the ‘*BamHI* N’ λ clone described above (data not shown). This paradox was resolved when we examined the ‘*BamHI* N’ clone in more detail. Using it as a probe against *BamHI* digests of whole VZV DNA, it failed to hybridize with authentic *BamHI* N but did have homology with *BamHI* Y, R and B (Fig. 6). Thus, the ‘*BamHI* N’ λ clone appears to be not *BamHI* N, but an unusual fusion of sequences normally found at the termini of linear VZV DNA molecules (Fig. 1, 7). In order to confirm this result, the putative fusion fragment was double-digested with *EcoRI* and *HindIII*. If the fusion fragment is represented by the cloned ‘*BamHI* N’ construct, a new *EcoRI/HindIII* fragment of 2.11 kb should be generated by the digestion (Fig. 7). When the experiment was carried out, a novel fragment of 2.18 kb was found, confirming the identity of the ‘*BamHI* N’ fragment as a fusion of the normal VZV DNA molecular termini.
DISCUSSION

The results described above indicate that the distribution of G + C-rich sequences in the VZV genome is reminiscent of the distribution of such sequences in other herpesvirus DNAs. Even in this low G + C DNA, sequences containing a high percentage of G + C residues can be found in the inverted repeats bounding the quasi-unique sequences contained in the Us region.
VZV G + C-rich regions

Sedimentation equilibrium studies of cloned restriction enzyme fragments indicate an overall G + C content of the repeat regions of not less than 54%. This minimum value has been confirmed by DNA sequencing studies in our laboratories (T. A. Casey, unpublished observations) and in the laboratory of A. J. Davison (Davison, 1984). Specifically, Davison has shown that the G + C content of VZV DNA at the U_L–IR_S junction undergoes a sharp transition from 45% G + C in the U_L region to 65% G + C just inside the IR_S sequences and that the average G + C content of the U_S region is 43%. These results are in keeping with our results obtained through sedimentation equilibrium studies and electron microscopy.

The high G + C inverted repeat regions of HSV-1, HSV-2, PRV and HCMV code for immediate-early functions. In the case of HSV-1, HSV-2 and PRV, one of these functions is a high molecular weight (M, approx. 175K) protein which, in concert with other polypeptides, will bind to DNA (Hay & Hay, 1980; Wilcox et al., 1980; Freeman & Powell, 1982). We have found that a protein of mol. wt. about 170K to 185K is synthesized at early times in VZV-infected cells. Analysis of sequence data (A. J. Davison, personal communication) from the repeat regions shows that these sequences could potentially code for a polypeptide of molecular weight 150000 with an amino acid composition similar to the amino acid composition of the HSV-1 175K protein. Thus, the repeated sequences bounding the U_S region of the VZV genome...
Fig. 7. Diagram of the novel BamHI joint fragment cloned in λgt-WesB. The heavy horizontal bar indicates the extent of the 2647 bp fragment. The thin horizontal bars indicate the ends of the λ arms. The locations of BamHI, HindIII and EcoRI cuts are shown and the sizes of the resulting fragments are indicated in base pairs.

may have a functional role similar to that of the inverted repeat sequences found in other human herpesviruses.

These studies have also resulted in the resolution of the question regarding homology between sequences in UL and the inverted repeats, and in the correct identification of minor bands present in restriction enzyme digests of VZV DNA. Our preliminary observations on the nature of the ‘BamHI N’ clone (Straus et al., 1983b) suggested homology between UL and IRs in VZV DNA. Our subsequent work and that of Davison (1984) leads us to the conclusion that ‘BamHI N’ is, in fact BamHI (R + Y) (Fig. 7) and represents a novel VZV DNA joint fragment resulting either from the presence of circular VZV DNA molecules or head-to-tail concatemers. The existence of such a fragment in digests of intact nucleocapsid VZV DNA explains our earlier result from blotting with an EcoRI E probe (Straus et al., 1982), since BamHI (R + Y) co-migrates with BamHI N. Similarly, a novel band seen in HindIII digests of VZV DNA (Straus et al., 1983a) seems likely in the light of the above finding to be HindIII (J + N). On the other hand the presence of joint fragments cannot explain the presence of the minor band in EcoRI digests of VZV DNA which blots to EcoRI E and which co-migrates with EcoRI N; the EcoRI fusion fragment would be J + R, too large to be mistaken for EcoRI N (Straus et al., 1982). The most likely explanation for the minor ‘EcoRI N’ band is that it represents not a novel joint fragment, but a novel end fragment derived from sequences normally seen in EcoRI E. Examination of the maps for VZV DNA shows that such a novel EcoRI end fragment would be approximately the same size as EcoRI N, and would hybridize to EcoRI E. If this is so, it would be predicted that the EcoRI and BamHI digests would also contain novel joint and novel end fragments, respectively. In fact, they probably do, but their location on gels (calculated from maps) would be obscured in blots by major EcoRI and BamHI fragments.

The presence of both types of novel fragment suggests that there exist unusual DNA structures in VZV DNA preparations from nucleocapsids. One of these would be formed if the L sequences of the DNA could invert relative to the S sequences. Such inversion would generate both novel ends and novel joints in equal amounts. In addition, circular molecules formed by linking the normal termini of linear molecules would generate novel joints, but no novel ends. It is not possible at present to make a quantitative assessment of the relative amounts of novel ends and joints present in VZV DNA preparations and, thus, VZV DNA preparations may contain both linear molecules with L inverted and covalently closed circular molecules. In DNA preparations that we have examined, the occurrence of these unusual DNA forms appears to be a few percent of total VZV DNA; we have previously suggested that a few percent of VZV DNA molecules are circular, based on electron microscope observations (Straus et al., 1981) and a recent report by Ecker et al. (1984) indicated the presence of HindIII (J + N) joint fragments.
and circular DNA molecules in preparations of VZV strain Oka DNA. Davison (1984) has suggested that the L region of VZV DNA is able to invert relative to the S region and that this may be due to the presence of an 88:5 base pair repeat at the ends of the L region, which allows intramolecular recombination. Our data confirm his observations, and suggest that in several different strains of VZV DNA there is a similar small quantity of unusual DNA forms, probably generated as a by-product of DNA replication.

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