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Genetics

Varicella zoster virus DNA exists as two isomers

(DNA cloning/restriction enzyme mapping/blot hybridization/electron microscopy)

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ABSTRACT **Fragments of varicella zoster virus DNA produced by *Eco*RI endonuclease cleavage were cloned in vector pACYC 184 and those produced by *Hin*dIII cleavage were cloned in pBR322. Restriction enzyme cleavage maps established by double digestion and blot hybridization showed that varicella zoster virus DNA has a M_r of 80 ± 3 × 10^6 and exists as a population of two isomers.**

Herpesvirus DNAs contain repeated sequences and exist as a population of isomers (1). Experiments have shown that ≈20% of herpes simplex virus (HSV) DNA contains repeated sequences and that HSV DNA is present as four isomeric forms (2). Analogous experiments for the DNA of the human herpesvirus varicella zoster virus (VZV), the causative agent of chicken pox and shingles, have not been reported. The paucity of data concerning VZV DNA is due, in part, to the very poor growth of VZV in cell culture and the fact that VZV is highly cell associated (3). To avoid the problems associated with growing VZV *in vitro* and yet undertake experiments concerning VZV DNA, we cloned VZV DNA in prokaryote host–vector systems. The *Eco*RI and *Hin*dIII fragments of VZV DNA were cloned in different plasmid vectors. The availability of two sets of cloned fragments allowed construction of restriction enzyme cleavage maps, which, in turn, yielded information on the presence of repeated sequences and the number of isomers.

MATERIALS AND METHODS

Viral VZV DNA. VZV (80-2) was obtained at The Milton S. Hershey Medical Center (Hershey, PA) from the vesicular fluid of an adult with herpes zoster. The virus was propagated by mixing infected and uninfected human embryo fibroblasts (Flow 5000 cells, Flow Laboratories, Rockville, MD) at an infected/uninfected cell ratio of 1:6 (4, 5). The VZV (80-2) used in these experiments was passed only four times in culture. ^{3}H-Labeled VZV DNA was extracted from virions purified by banding on a step CsCl gradient (6). Final purification of VZV DNA was accomplished by CsCl buoyant density centrifugation.

Recombinant VZV DNA. Plasmids pBR322 (7) and pACYC 184 (8) were propagated in *Escherichia coli* HB101 (9). Recombinant DNA studies were conducted under EK1-P2 containment conditions as described in the National Institutes of Health recombinant DNA research guidelines. *Eco*RI-digested VZV DNA was ligated to *Eco*RI-digested pACYC 184 DNA and *Hin*dIII-digested VZV DNA was ligated to *Hin*dIII-digested pBR322 DNA in (total vol, 20 μl) a mixture containing 3.0 μg of VZV DNA and 0.3 μg of plasmid DNA in 50 mM Tris base, pH 7.4/3.5 mM $MgCl_2$/10 mM dithiothreitol containing bovine serum albumin (nuclease free) at 50 μg/ml and T_4 ligase at 3 × 10^4 units/ml (New England BioLabs). The reaction was allowed to continue for 5 hr at 12°C. Ligated DNAs were then transfected into *E. coli* HB101 by the calcium-shock method (10). *E. coli* containing putative pACYC 184–VZV recombinant DNA were selected for resistance to tetracycline and sensitivity to chloramphenicol. *E. coli* containing putative pBR322–VZV recombinant DNA were selected for resistance to ampicillin and sensitivity to tetracycline. Determination of the size of the

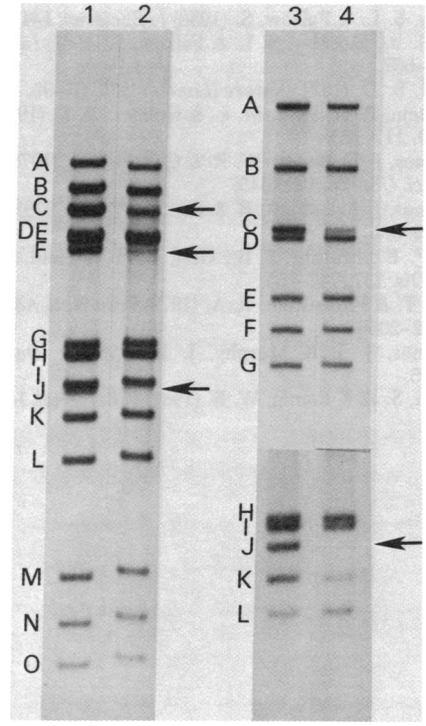

FIG. 1. Fluorographs of restriction enzyme cleavage patterns of VZV DNA. VZV DNA (1.0 μg) was digested with various concentrations (0, 0.08, 1.6, 3.2, 6.0 units) of λ exonuclease (New England BioLabs) in 100 μl of 67 mM glycine-KOH buffer, pH 9.6/2.9 mM $MgCl_2$/0.1 mM 2-mercaptoethanol for 25 min at 16°C (14). The reactions were stopped by heating to 65°C and freezing at −20°C. The DNAs were later thawed, extracted with phenol/chloroform/isoamyl alcohol, precipitated, suspended in buffer, and digested with either *Hin*dIII or *Eco*RI for 2 hr at 37°C. After electrophoretic separation of the DNA fragments in a 0.5% agarose gel (4), the gel was subjected to fluorography using sodium salicylate, dried at 37°C, and exposed to x-ray film at −70°C (15). For clarity, composites of different exposures are presented. Lanes: 1, VZV DNA cleaved with *Eco*RI; 2, VZV DNA digested with λ exonuclease (1.6 units) and then cleaved with *Eco*RI; 3, VZV DNA cleaved with *Hin*dIII; 4, VZV DNA digested with λ exonuclease (1.6 units) and then cleaved with *Hin*dIII. Arrows point to fragments that were missing or reduced in intensity. Fragment designations are to the left.

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Abbreviations: HSV, herpes simplex virus; VZV, varicella zoster virus; mDal, megadalton.

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cloned VZV *Eco*RI and *Hin*dIII DNA fragments was accomplished by *Eco*RI or *Hin*dIII (or both) digestion and electrophoresis. Final identification was accomplished by blot hybridization. For blot hybridization, VZV DNA was digested with either *Eco*RI or *Hin*dIII, and the fragments were separated by electrophoresis in a 0.5% agarose gel (4). The DNA was denatured and transferred from the gel to diazotized aminothiophenol paper (11, 12). After transfer, identical lanes were cut and used for hybridization (12) to VZV *Eco*RI- and *Hin*dIII-cloned DNA fragments and to VZV DNA. All probes were radiolabeled *in vitro* by nick translation (13).

RESULTS

Digestion of VZV DNA with *Eco*RI yields 17 fragments (Fig. 1, lane 1), whereas digestion with *Hin*dIII yields 16 fragments (Fig. 1, lane 3) of size >0.4 megadaltons (mDal). Smaller fragments were not studied. To identify the end fragments, VZV DNA was digested with various amounts of λ exonuclease and then cleaved with *Eco*RI (Fig. 1, lane 2) or *Hin*dIII (Fig. 1, lane 4). (To save space, Fig. 1 presents a single example of each.) Conclusions were drawn from data compiled from many experiments. *Eco*RI fragments C, F, and J were missing or considerably reduced in intensity. Based on the ability to clone *Eco*RI C (see below), we concluded that *Eco*RI F and J were end fragments, whereas *Eco*RI C was close to, but not, an end fragment. *Hin*dIII J and N fragments were missing, whereas *Hin*dIII C and M were reduced in intensity (Fig. 1, lane 4; data not shown). Because we were able to clone *Hin*dIII C and M (see below), we concluded that *Hin*dIII J and N were end fragments, whereas *Hin*dIII C and M were close to an end. Additional λ exonuclease digestion experiments showed sequential loss of *Hin*dIII J and K fragments (data not shown).

Restriction enzyme cleavage fragments were cloned in plasmid vectors pACYC 184 and pBR322, and the recombinant DNAs were designated by four letters: p for plasmid, V for VZV, H or E for *Hin*dIII or *Eco*RI, respectively, and the letter of the fragment (Fig. 1 and Table 1). The size of each fragment (Table 1) was determined from digestion of VZV DNA and of recombinant DNA followed by co-electrophoresis with appropriate DNA size standards (data not shown). Table 1 also gives the sizes of the *Eco*RI/*Hin*dIII double-digestion products.

To determine which VZV *Eco*RI and *Hin*dIII DNA fragments contained common nucleotide sequences, each cloned DNA was nick translated and hybridized to blots of *Eco*RI- and *Hin*dIII-cleaved VZV DNA. As space is limited and the physical ends of the VZV DNA were the most interesting regions of the genome, only representative blot hybridizations relating to the ends are shown (Fig. 2). First, the left-hand end is presented. Hybridization of the cloned *Eco*RI C fragment, pVEC DNA, to a blot of *Eco*RI-cleaved VZV DNA showed homology only to itself (Fig. 2*A*, lane 2); in a parallel experiment, pVEC DNA showed strong homology to *Hin*dIII I, J, and K and limited homology to *Hin*dIII A (Fig. 2*B*, lane 2). *Hin*dIII digestion of pVEC DNA yielded four fragments: a 2.95-mDal fragment identical in size to *Hin*dIII I, two 2.5-mDal fragments identical in size to *Hin*dIII K, and a 1.1-mDal fragment (Table 1). From the blot hybridization, double-digestion, and λ exonuclease digestion information, a linkage arrangement of *Hin*dIII fragments was constructed as (left-hand end) J-K-I-A. Because end fragment *Hin*dIII J is 2.8 mDal, the *Eco*RI C fragment must be positioned <0.3 mDal from the left-hand end of the molecule. The remaining small *Eco*RI end fragment(s) would not be detected in these experiments.

The right-hand end of VZV DNA was more complicated. Hybridization of the cloned *Eco*RI A fragment, pVEA DNA,

Table 1. Molecular sizes and double-digestion products of *Eco*RI and *Hin*dIII fragments of VZV DNA

*Eco*RI* fragment	Molecular mass, mDal	*Hin*dIII digestion fragment(s), mDal	*Hin*dIII* fragment	Molecular mass, mDal	*Eco*RI digestion fragment(s), mDal
A†	10.8	5.7, 3.0, 1.25, 0.8	A	17.0	5.7, 3.4, 3.0, 2.2, 1.2, 1.1, 0.6
B	9.4	8.0, 1.4	B	11.0	8.1, 1.4, 1.3
C	9.0	2.95, 2.5, 2.5, 1.1	C	8.6	5.7, 2.9
D	8.3	8.3	D	8.0	8.0
E	8.25	ND	E	6.2	5.8, 0.4
F	7.7	End‡	F	5.5	4.3, 1.2
G	5.8	ND	G	4.6	3.8, 0.8
H	5.7	5.7	H	3.0	3.0
I	5.3	3.8, 1.2	I	2.95	2.95
J	5.25	End	J	2.8	End
K	4.9	4.2, 0.8	K	2.5	2.5
L	4.6	2.2, 1.3, 0.8	L	2.2	2.2
M	3.4	3.4	M	1.25	1.25
N	3.0	3.0	N	0.9	End
O	2.6	2.2, 0.4	O	0.7	0.7
P	1.2	1.2	P	0.65	0.65
Q	0.6	0.6			

Sizes of cloned and genomic VZV *Eco*RI and *Hin*dIII DNA fragments were determined graphically as described by Richards *et al.* (4) using as size markers HSV and λ DNAs cleaved with *Eco*RI and *Hin*dIII. Each recombinant plasmid DNA was double digested with *Eco*RI/*Hin*dIII, and the resulting fragments were sized as described. ND, not done.

* VZV *Eco*RI and *Hin*dIII DNA fragment designations have been revised since Richards *et al.* (4).

† The molar ratios of *Eco*RI fragments A, E, F, and J were determined (4) to be ≈0.5 M and all other *Eco*RI fragments were ≈1 M. *Hin*dIII fragment M was calculated at be ≈2 M and all other *Hin*dIII fragments were ≈1 M (data not shown).

‡ End, VZV DNA *Eco*RI and *Hin*dIII end fragments (Fig. 1) were not cloned by these methods.

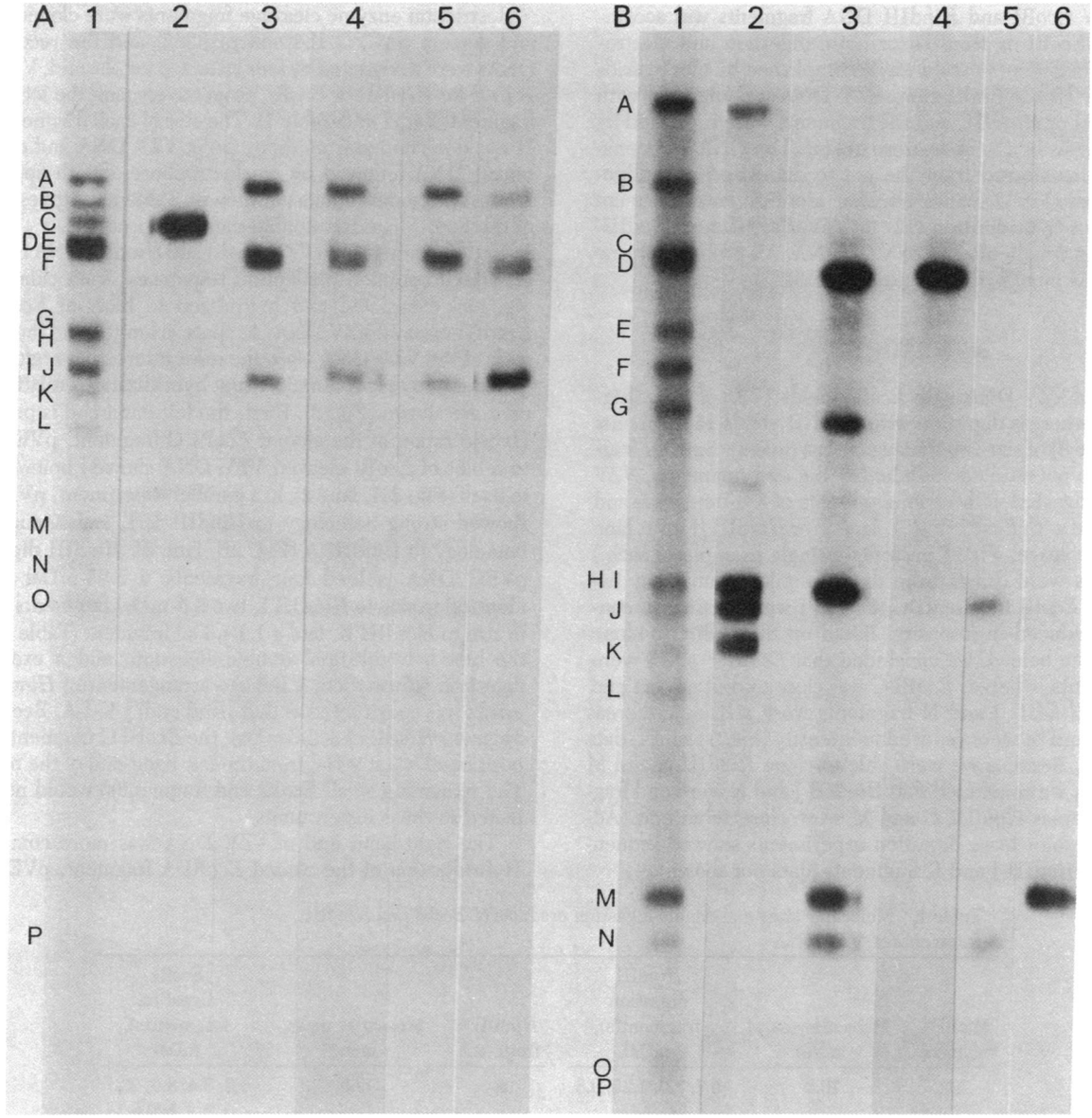

FIG. 2. Autoradiographs of blot hybridizations of cloned VZV DNA. VZV DNA was digested with either *Eco*RI (*A*) or *Hin*dIII (*B*); the fragments were electrophoretically separated, and the gel was blotted to prepared paper. Individual strips were cut and hybridized to total ^{32}P-labeled VZV DNA or ^{32}P-labeled cloned DNA. The probes were total VZV DNA (lanes 1), pVEC DNA (*Eco*RI C) (lanes 2), pVEA DNA (*Eco*RI A) (lanes 3), and pVHC DNA (*Hin*dIII C) (lanes 4), pVHH DNA (*Hin*dIII H) (lanes 5), and pVHM DNA (*Hin*dIII M) (lanes 6). The minor bands in *B* were caused by partial digestion products.

to a blot of *Hin*dIII-cleaved VZV DNA (Fig. 2*B*, lane 3) showed strong hybridization to *Hin*dIII fragments C, H, M, and N and limited hybridization to *Hin*dIII G. Analogously, hybridization of the cloned *Hin*dIII G fragment to a blot of *Eco*RI-cleaved VZV DNA showed strong homology to *Eco*RI I and limited homology to *Eco*RI A and E (data not shown). Digestion of pVEA DNA with *Hin*dIII yielded four fragments with sizes 5.7, 3.0, 1.25, and 0.8 mDal (Table 1). From this information, it is clear that *Eco*RI A contained two internal *Hin*dIII fragments, H and M, a 5.7-mDal part of *Hin*dIII C and a 0.8-mDal part of *Hin*dIII G. Hybridization of pVEA DNA to a blot of *Eco*RI-cleaved VZV DNA (Fig. 2*A*, lane 3) showed sequence homology not only to itself but also to *Eco*RI E, F. and J. Therefore, *Eco*RI A, E, F, and J contained a large portion of their nucleotide sequences in common.

Additional data for determining the alignment of the right-hand end were obtained by hybridization with the cloned *Hin*dIII C fragment, pVHC DNA, as a probe. Hybridization of pVHC DNA to a blot of *Eco*RI-cleaved VZV DNA (Fig. 2*A*, lane 4) showed sequence homology to *Eco*RI fragments A, E, F, and J. Hybridization of pVHC DNA to a blot of *Hin*dIII fragments (Fig. 2*B*, lane 4) showed homology only to *Hin*dIII C. *Eco*RI digestion of pVHC DNA yielded two fragments, a 2.9-mDal fragment and a 5.7-mDal fragment identical in size to the *Eco*RI/*Hin*dIII double-digestion product of pVEA DNA.

As *Hin*dIII H and M were within *Eco*RI A and near the right-hand end, analogous blot hybridizations were undertaken with the cloned *Hin*dIII H and M fragments. pVHH DNA hybridized to four *Eco*RI fragments: A, E, F, and J (Fig. 2*A*, lane 5). pVHH DNA when hybridized to a blot of *Hin*dIII fragments hybridized to itself and to the *Hin*dIII end fragment N (Fig. 2*B*, lane 5). This important point demonstrated that end sequences (*Hin*dIII N) were repeated internally (*Hin*dIII H). Finally, *Hin*dIII M fragment, pVHM DNA, hybridized to *Eco*RI frag-

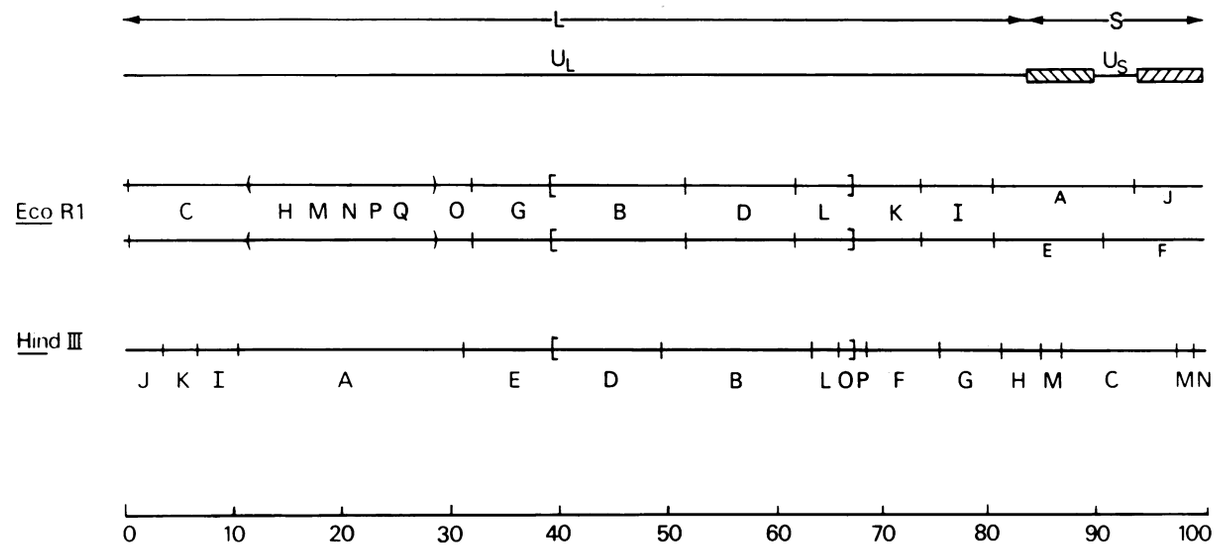

FIG. 3. Restriction enzyme cleavage maps of VZV DNA. The two orientations of the S region of the *Eco*RI map are shown. The S region of the *Hin*dIII map is symmetrically arranged. Letters represent individual fragments (Fig. 1 and Table 1); U_L and U_S, unique sequence regions. Parentheses enclose *Eco*RI fragments whose relative order is not known. *Hin*dIII and *Eco*RI cleavage sites are coincident, within experimental error, at map positions (about) 39 and 68. Inclusive fragments are enclosed in square brackets to indicate that their overall orientation has not been determined. ⊠, Repeated sequences.

ments A, E, F, and J (Fig. 2*A*, lane 6) but, when hybridized to a blot of *Hin*dIII fragments, showed sequence homology only to itself (Fig. 2*B*, lane 6). As shown, *Hin*dIII M was contained within *Eco*RI A; yet, from λ exonuclease digestion (data not shown), *Hin*dIII M must also be found very close to the *Hin*dIII end fragment N. Thus, *Hin*dIII M must have been present in two symmetrical positions on the VZV genome. From the combined blot hybridization (Fig. 2), double-digestion (Table 1), and λ exonuclease digestion (Fig. 1) data, a symmetrical linkage of *Hin*dIII restriction fragments was determined as (right-hand end) N-M-C-M-H-G. The linkage of *Eco*RI fragments was more complicated. No single linkage arrangement sufficed for the four fragments A, E, F, and J, especially as both F and J were end fragments (Fig. 1). Rather, two separate linkages were derived: (right-hand end) J-A and (right-hand end) F-E.

From analogous data and deductions, restriction enzyme cleavage maps of VZV DNA were produced (Fig. 3). The single most important point was that the right-hand end of the *Eco*RI map could only be described by two separate and distinct fragment alignments. The interpretation was that VZV DNA exists as a population of two isomers. The *Hin*dIII map of the right-hand end of VZV DNA was symmetrically arranged and, thus, did not demonstrate the presence of two isomeric forms.

If the arrangement of unique and repeated sequences of VZV DNA shown in Fig. 3 is correct, then an intact single strand of VZV DNA could fold back on itself to form a stem-plus-loop structure. VZV DNA was denatured, reassociated briefly, and examined by electron microscopy. One of many examples of the stem-plus-loop structure found at one end of the molecule is shown in Fig. 4. In an analogous experiment, the cloned *Hin*dIII C fragment was shown to form a stem-plus-loop structure (Fig. 3; data not shown). The duplex DNA region, 5.0 ± 0.4 mDal (size based on six genomic DNAs), represented inverted repeated DNA, whereas the single-stranded loop of 1.7 ± 0.15 mDal (size based on six genomic DNAs plus six cloned *Hin*dIII C DNAs) represented unique sequence DNA. These values were within 10% of those derived from the restriction enzyme cleavage maps.

The combined restriction enzyme cleavage maps and data from electron microscopy led to the conclusion that VZV DNA is composed of two covalently linked segments, L and S, following the nomenclature for HSV DNA (2). The L segment consists of unique sequence DNA, U_L, and the S segment consists of a unique sequence region, U_S, bounded by inverted

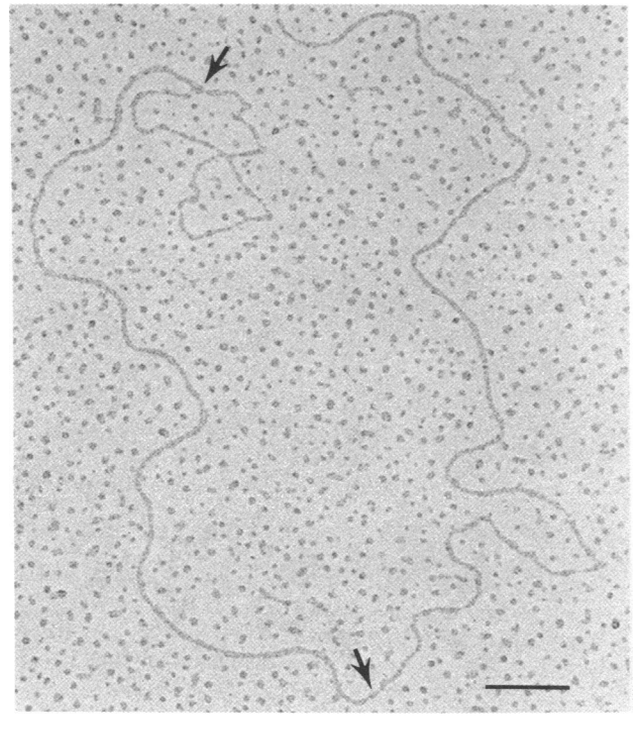

FIG. 4. Electron micrograph of the stem-plus-loop structure of VZV DNA. VZV DNA was denatured and briefly renatured (16), treated with glyoxal (17), and mounted for electron microscopic examination (18). Single-stranded and duplex φX174 DNAs were included as length standards. Arrows point to transitions from double-stranded to single-stranded DNA. Bar = 0.2 μm.

repeated DNA. U_L has a size of ≈67 mDal; U_S has a size of ≈3 mDal. Each repetition has a size of ≈5 mDal.

DISCUSSION

Two important conclusions can be derived from the restriction enzyme cleavage maps: (*i*) the size of VZV DNA is 80 ± 3 mDal (Fig. 3 and Table 1), in agreement with electron microscopy data published by Dumas *et al.* (19) but lower than the value based on sucrose gradient sedimentation data that we previously published (20, 21); and (*ii*) VZV DNA exists as two isomers (Fig. 3). The end of the long unique region is 1 M, within experimental error. The short unique region can be found in either of two orientations, and the ends of the short region are each ≈0.5 M. The number of cloned VZV DNAs studied was sufficient to account for the restriction enzyme cleavage products shown in Fig. 1. Thus, any possible additional isomers could only be present in minute amounts. Fragments of size <0.5 mDal were not positioned on the physical maps; e.g. *Hin*dIII Q DNA (0.4 mDal) hybridized only to itself and the *Eco*RI C fragment (data not shown). In addition, fine structure of the ends and of the joint region has not been addressed. Heterogeneities in both regions have been reported for HSV and Epstein–Barr virus DNAs (2, 22–25).

VZV DNA can now be compared with other human herpesvirus DNAs. HSV DNA, 95 mDal (26), exists as four isomers (2, 22, 27–29). Cytomegalovirus DNA, 150 mDal (30, 31), also exists as four isomers (32). Epstein–Barr virus DNA, 115 mDal (33), exists in one conformation (33, 34). Thus, VZV DNA is by far the smallest human herpesvirus DNA and assumes fewer isomeric forms than HSV and cytomegalovirus DNAs but more than Epstein–Barr virus DNA. In this last respect, VZV DNA resembles the DNA of pseudorabies virus, a herpesvirus of swine. Pseudorabies virus DNA exists as two isomers, wherein only the short, but not the long, unique region can be found in either of two orientations (35, 36).

The cloned restriction enzyme cleavage fragments of VZV DNA have been used to elucidate the basic structural organization of the VZV genome. The cloned fragments will also be valuable reagents for further experiments.

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