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Molecular cloning and physical mapping of varicella-zoster virus DNA*

(restriction map)

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ABSTRACT Varicella-zoster virus (VZV) DNA was cleaved with restriction endonuclease *Eco*RI, and most of the resulting fragments were successfully cloned in the phage vector λgtWES·λB. Double digestions of cloned fragments with *Eco*RI and *Bam*HI and hybridizations to blot-transferred *Bam*HI digests of VZV DNA were used to construct a physical map of the genome. The molecular termini of the DNA were identified by restriction enzyme analysis after exonuclease III digestion. The data indicate that VZV DNA exists in two isomeric forms that differ by inversion of one short terminal genome segment. Electron microscopic studies revealed that the short genome segment consists of a terminal sequence of about 3.4×10^6 daltons that is separated from an internal inverted repeat of itself by a 5.8×10^6-dalton unique DNA segment.

Varicella-zoster virus (VZV) is a human herpesvirus which causes varicella (chicken pox) and, after a highly variable latency period, may reactivate to produce zoster (shingles) (1). The poor growth of VZV in cell culture and the inherent difficulty in obtaining and purifying sufficient quantities of viral DNA have significantly impeded the detailed molecular characterization of the genome. Although early studies suggested that VZV DNA is a double-stranded molecule of 92–110 megadaltons (MDal) (2, 3), more recent estimates indicate that the genome is only about 80 MDal (4, 5). Initial restriction endonuclease-generated cleavage profiles of VZV DNA revealed the presence of submolar genome fragments, suggesting that this genome, like those of other herpesviruses, may exist in multiple conformations (2, 6–8). Recently, we have shown by quantitative densitometry of gel profiles that endonuclease digestion can generate up to four half-molar fragments, but no quarter-molar fragments. We postulated, therefore, that the genome may exist in either of two isomeric forms that differ by inversion of but one genome segment (5).

In this report we describe the molecular cloning of VZV DNA restriction fragments in a phage λ vector and present a physical restriction map of the VZV genome. This map plus electron microscopic analyses reported here demonstrate that VZV DNA exists in two isomeric forms.

MATERIALS AND METHODS

Cells and Viruses. VZV strain Ellen was obtained from the American Type Culture Collection (ATCC VR-586). Other VZV isolates used were recovered from vesicle fluid aspirates from patients with zoster infection. VZV was grown in human embryonic lung fibroblasts as described (5). Phage λgtWES·λB was grown and purified as described (9).

Preparation, Labeling, and Analyses of DNAs. Previously described methods were utilized for extraction of VZV DNA from viral nucleocapsids (5) and for purification of phage λ DNAs (10). DNAs were labeled *in vitro* with [^{32}P]dCTP by the nick-translation methods of Maniatis *et al.* (11) and Kelly *et al.* (12). Restriction endonucleases were purchased from Bethesda Research Laboratories and New England BioLabs and were used according to the manufacturers' recommendations. Exonuclease III was purchased from Bethesda Research Laboratories. Electrophoresis was carried out in slab gels of 0.5–1.2% agarose. DNA was stained with ethidium bromide and photographed with UV transillumination. The DNA in agarose gels was transferred to nitrocellulose membranes by the method of Southern (13). Blot hybridizations were performed as described by Wahl *et al.* (14). Autoradiography was performed with Kodak XR-5 film in cassettes containing intensification screens with exposures at −70°C for 2–240 hr.

Cloning of VZV DNA. Purified VZV DNA was digested with *Eco*RI and ligated to purified λgtWES·λB vector arms as described (9, 10). The DNA was packaged into phage particles *in vitro* (15), and the virus was assayed by plaque formation and amplified in *Escherichia coli* LE 392 (9). This procedure routinely yielded about 10^6 plaque-forming units/μg of vector DNA. Recombinant λ phage DNA was extracted and prepared as described (10).

Electron Microscopy of VZV DNA. Viral DNAs (0.5–1.0 μg/ml) were mixed with sufficient recrystallized formamide (16) to yield a final formamide concentration of 80% (vol/vol) and then denatured by heating at 60°C for 10 min. Sufficient 0.2 M Tris·HCl/0.001 M EDTA, pH 8.5, were added to reduce the formamide concentration to 66%. The DNA was allowed to self-hybridize at room temperature for 2–3 hr. Then simian virus 40 form II and either φX-174 or fd phage DNAs (all purchased from Bethesda Research Laboratories) were added as double- and single-strand-size markers, respectively. Finally, cytochrome *c* was added to a concentration of 0.3 mg/ml.

The DNA was picked up from a buffered 10% (vol/vol) formamide hypophase (17) by using Parlodian-coated 200-mesh copper grids. The grids were stained with uranyl acetate, air dried after an isopentane rinse, and rotary shadowed with gold/palladium in an Edwards evaporator. The grids were examined in a Zeiss 10A electron microscope and photographed at a magnification of ×9800. Negatives were projected, and contour lengths of double- and single-strand regions were determined with a Keufel and Esser map measurer.

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Abbreviations: exoIII, exonuclease III; VZV, varicella-zoster virus; MDal, megadaltons.

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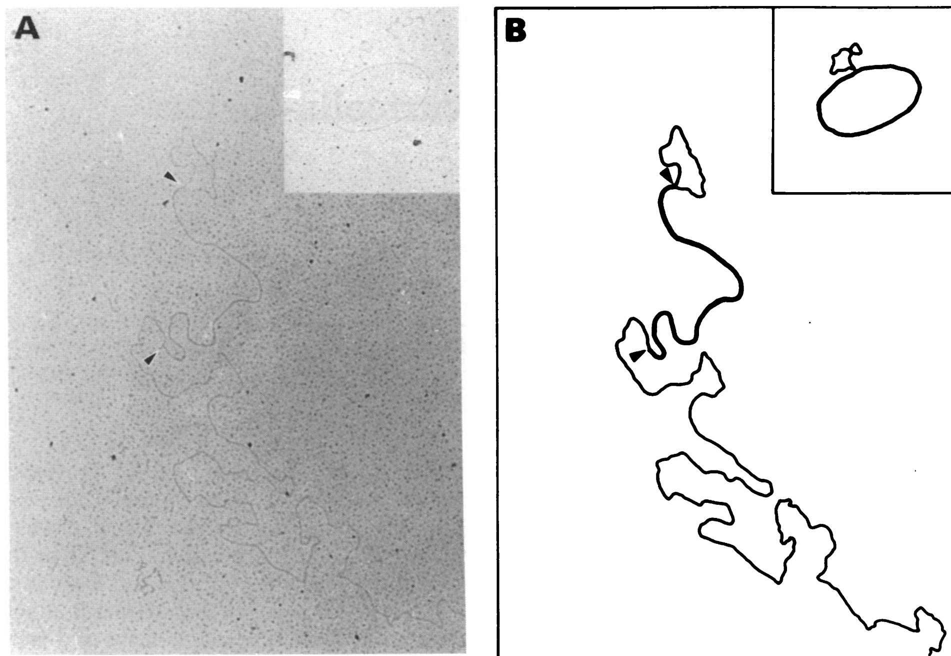

FIG. 1. Electron microscopy of the short, invertible segment of the VZV genome. (A) Electron photomicrograph of a self-hybridized VZV DNA molecule. (B) A tracing of the observed structures. In both A and B, the arrows delineate the double-stranded region corresponding to the inverted repeat; double-stranded regions are shown by the heavier lines, and the inserts depict simian virus 40 (double-stranded) and fd phage (single-stranded) DNA size markers.

RESULTS

Structure of the Invertible Genome Segment. We have shown (5) that *Eco*RI cleavage of VZV Ellen DNA generates four fragments (A, E, F, and J) with mole ratios of 0.5 with respect to the remaining 13 fragments. The sum of the molecular masses of fragments A and J (≈15.9 MDal) is very nearly equal to the sum of the molecular masses of fragments E and F (≈15.7 MDal). Similar observations were made with four half-molar fragments of VZV DNA that result from cleavage with *Bgl* II. These data led us to postulate that each of the pairs of half-molar restriction fragments encompasses the inverted repeat sequences of two isomeric forms of the VZV genome.

In order to define more precisely the structure of the invertible genome segment, electron microscopic studies were undertaken. The DNAs of VZV Ellen and two low-passage clinical isolates (K. M. and Scott) were examined in the presence of both single-stranded [fd phage, 2.11 MDal (18); or ϕX174 phage, 1.78 MDal (19)] and double-stranded [simian virus 40 form II, 3.4 MDal (20)] marker DNAs. Thirty-three molecules, including several of the length expected for a complete VZV genome, were measured. All molecules showed intramolecular rehybridization at only one end (Fig. 1). By comparison to the lengths of the appropriate internal size markers, it was calculated that a terminal segment of 3.4 ± 0.3 MDal rehybridized to an internal sequence separated by a unique region of 5.8 ± 0.9 MDal (mean ± SEM). Within experimental error, no differences were noted in preparations with the three different VZV DNAs. This indicated that the invertible genome segment is about 12.6 MDal.

Assignment of Terminal Fragments. A comparison of the molecular masses of the four half-molar fragments with the size of the invertible genome segment obtained from electron microscopic observations suggests that the *Eco*RI fragments A + J or E + F encompass the entire segment and that no additional *Eco*RI fragments are likely to be derived from this region. Fragment A (≈10.8 MDal) is too large to be the terminal fragment, so in the isomeric form containing fragment A, fragment J must be terminal. Neither the endonuclease analyses nor the electron microscopic studies permitted us to determine whether fragment E or F is terminal. In addition, the data provided no clues as to the identity of the fragment that terminates the long, unique, noninvertible genome segment. In an effort to answer these questions, VZV DNAs (strains Ellen and Scott) were digested with exonuclease III, labeled *in vitro* by nick translation, and examined by electrophoresis in agarose gels. The autoradiograms showed quite clearly that the intensities of the bands in the positions of *Eco*RI fragments C, F, and J were significantly diminished by the exonuclease III treatment (Fig. 2). This indicates that fragment C occupies a terminal position in the long, noninvertible genome segment. *Eco*RI J is the terminal fragment of the invertible segment in one isomeric form

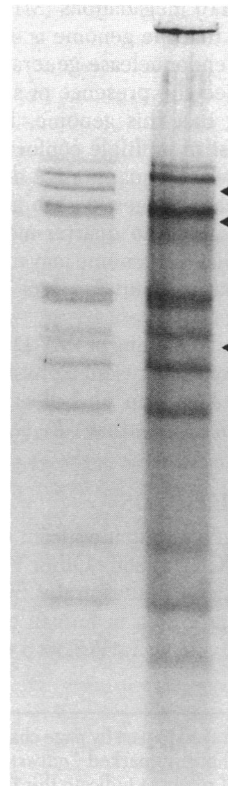

FIG. 2. Exonuclease III digestion of VZV DNA. VZV Scott DNA (100 ng) was digested with 30 units of exonuclease III in 50 mM Hepes, pH 7.9/1 mM 2-mercaptoethanol/1 mM $MgCl_2$ at 37°C for 10 min. The reaction was terminated by heating for 20 min at 70°C. *Eco*RI and salts conducive to its action were added. After DNA cleavage, the reaction products were labeled *in vitro* with [^{32}P]dCTP and analyzed by agarose gel electrophoresis (*Left*). VZV DNA not digested with exonuclease III. (*Right*) VZV DNA digested with exonuclease III. The arrows point to fragments C, F, and J, in which the bands are of reduced intensity after exonuclease III digestion.

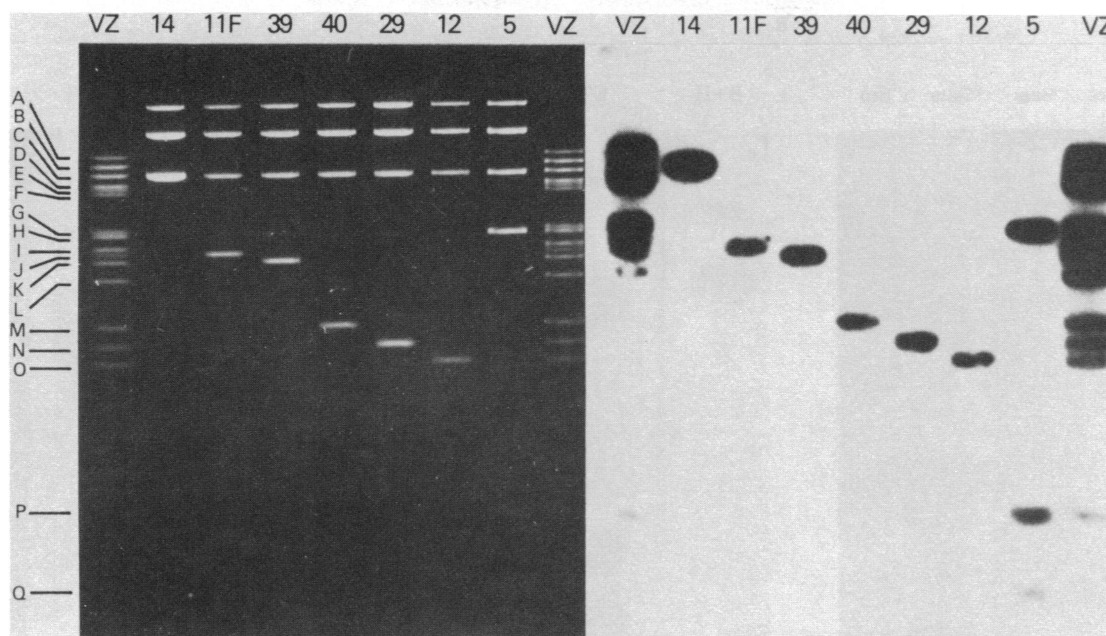

FIG. 3. Identification of VZV DNA clones. The DNAs of seven clones were cleaved with *Eco*RI and analyzed together with *Eco*RI-cleaved VZV nucleocapsid DNA by electrophoresis in horizontal slab gels of 0.5% agarose. The DNAs were blot-transferred to sheets of nitrocellulose and hybridized to *in vitro* ^{32}P-labeled VZV nucleocapsid DNA. (*Left*) The photograph of the UV transilluminated gel. (*Right*) The corresponding autoradiogram of the nitrocellulose sheet. Lanes contain *Eco*RI digests of the following DNAs: 1 and 9, VZV nucleocapsid DNA; 2, clone 14 containing *Eco*RI C; 3, clone 11F containing a fragment that fortuitously comigrates with *Eco*RI J but represents a portion of *Eco*RI B or G, or both; 4, clone 39 containing *Eco*RI K; 5, clone 40 containing *Eco*RI M; 6, clone 29 containing *Eco*RI N; 7, clone 12 containing *Eco*RI O; 8, clone 5 containing tandemly linked *Eco*RI fragments H, P, and Q. Omitted from this figure are the data from similar experiments performed with recombinants bearing *Eco*RI B, D, E, G, H, I, and L.

of the genome, whereas fragment F occupies that position in the second isomeric form.

Molecular Cloning of VZV DNA Fragments. To derive a complete physical map of the genome, VZV fragments were molecularly cloned in a phage λ vector. A total of 89 individual plaques were picked, reassayed by plaque formation, and amplified in 1-liter broth cultures. The phage was purified from each culture, and the phage DNA was extracted and then subjected to restriction endonuclease cleavage and agarose gel electrophoresis. Approximately 40% (36/89) of all clones contained VZV *Eco*RI H, whereas 20% (18/89) contained more than one VZV DNA fragment. Nearly all clones contained *Eco*RI fragments that comigrated precisely with corresponding VZV DNA fragments. However, several recombinants possessed aberrantly migrating VZV DNA fragments derived from the genome region in which *Eco*RI B and G map (see below).

Blot-hybridization experiments were undertaken to demonstrate that VZV DNA fragments had been successfully cloned. The DNAs of 14 clones believed to contain 15 VZV *Eco*RI fragments were cleaved with *Eco*RI, subjected to electrophoresis, transferred to nitrocellulose, and hybridized to *in vitro* ^{32}P-labeled VZV DNA. All inserted fragments hybridized to the VZV probe (data for seven clones are shown in Fig. 3). Later studies showed that the VZV DNA sequence in clone 11F represents a portion of *Eco*RI B or G rather than J with which it fortuitously comigrates.

Mapping of VZV DNA Fragments. Mapping of the VZV *Eco*RI fragments was facilitated by a series of blot-hybridization experiments. The DNAs of each of the 13 clones were individually labeled *in vitro* and hybridized to strips of nitrocellulose paper bearing blot-transferred lanes of endonuclease-cleaved VZV DNA.

Our model of the invertible short segment of the genome predicted that the 0.5 M *Eco*RI fragments A, E, F, and J should share sequences derived from the inverted repeat regions. To test this, the DNA of clone 11G, containing VZV *Eco*RI E, was labeled *in vitro* and hybridized to a nitrocellulose membrane bearing a blot-transferred VZV DNA *Eco*RI digest. This fragment annealed to VZV *Eco*RI fragments A, E, F, and J, as predicted (Fig. 4*A*). A very small amount of hybridization to *Eco*RI N was observed, suggesting that part of the *Eco*RI E DNA may be repeated in, or may be very similar to, a portion of VZV *Eco*RI N. Hybridization of the other *in vitro* labeled VZV *Eco*RI recombinants to nitrocellulose strips bearing VZV *Bam*HI digests indicated those *Bam*HI fragments that are homologous to the *Eco*RI fragments (Fig. 4*B*; Table 1). Except for the inverted repeat sequences, only neighboring *Eco*RI fragments should exhibit homology to the same *Bam*HI fragment.

The preliminary map prepared from the above data was verified and completed by electrophoretic analyses of the VZV DNA recombinants after digestions with *Eco*RI and *Bam*HI alone or in combination (Table 1). These studies permitted identification and in some cases precise localization of *Bam*HI fragments that lie entirely within the *Eco*RI fragments or overlap with adjacent *Eco*RI fragments. The combined data from these experiments, the blot hybridizations, the exonuclease III digestions, and the electron microscopic analyses provided a firm basis for constructing a physical map of the VZV genome (Fig. 5).

DISCUSSION

Of the five human herpesviruses, varicella-zoster virus has been most difficult to characterize molecularly because of the availability of only limited quantities of DNA. Detailed investigations have succeeded in defining the genomic structures, the major classes of transcribed messenger RNAs, and the virally encoded polypeptides of Epstein–Barr virus, cytomegalovirus, and herpes simplex viruses types 1 and 2. For these four viruses,

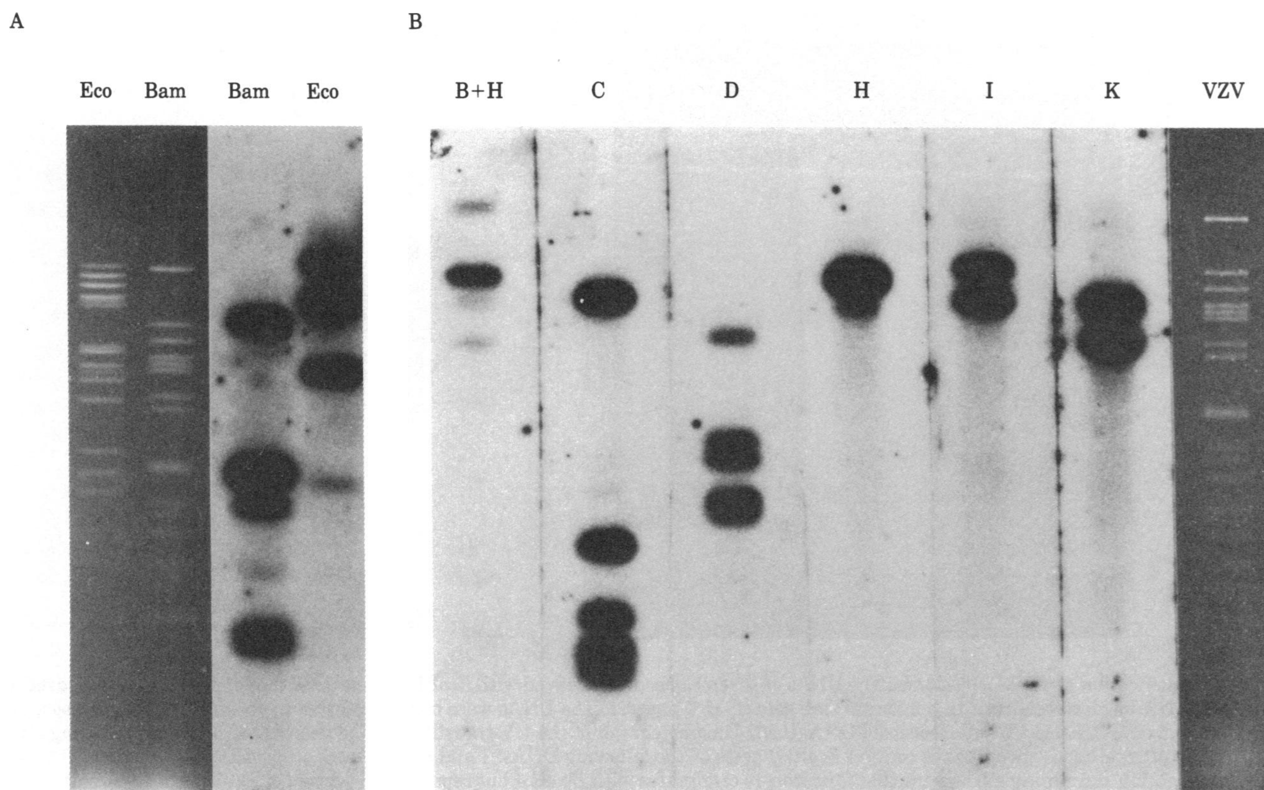

FIG. 4. Southern analyses of VZV DNA. (*A*) The phage λ recombinant (11G) containing *Eco*RI E was labeled *in vitro* by nick translation and was hybridized to a nitrocellulose strip bearing blot-transferred lanes of VZV DNA that had been cleaved with *Eco*RI or *Bam*HI. (*B*) VZV nucleocapsid DNA was cleaved with *Bam*HI, run in multiple lanes in agarose gels, and blot-transferred to nitrocellulose. The membranes were cut into strips and hybridized to individual *in vitro* labeled cloned DNAs. Lanes are identified by the *Eco*RI fragment(s) contained in the cloned DNAs. The photographs of the ethidium bromide-stained gels (*A Left* and *B Right*) are shown with the corresponding autoradiographs. Data have been omitted for similar experiments performed with recombinant DNA probes containing *Eco*RI fragments G, L, M, N, O, P, and Q.

molecular epidemiologic studies have been performed, and the sites of viral latency in the human host have been established. Varicella-zoster virus proteins only recently have been characterized (21, 22), and essentially nothing is known of the viral transcripts. VZV DNA is assumed to reside in a latent state in dorsal root ganglia (1), but direct evidence for this has not been obtained.

Table 1. Identification of *Bam*HI fragments that lie within or overlap with each cloned *Eco*RI fragment of VZV DNA

*Eco*RI fragment	*Bam*HI fragments*		
	Homologous	Internal	Overlapping
B	A,H,T,Y,Z	T,Y,Z	A,H
C	E,Q,W,X,AA,BB	E,Q,W,AA	X
D	H,L,M,O,P	M,O,P	H,L
E	B,J,K,N,R,S,U†	J	B,K
G	A,I	None	A,I
H	C,F	None	C,F
I	B,D	None	B,D
K	D,G	None	D,G
L	G,L,V	V	G,L
M	F,X	None	F,X
N	C,I,N	N	C,I
O	I	None	I
P	F	None	F
Q	F	None	F

* Homologies were determined by Southern-type experiments. Internal fragments were mapped by double digestions of recombinants with *Eco*RI/*Bam*HI. Overlapping *Bam*HI fragments demonstrated homology to two or more *Eco*RI fragments.

† *Eco*RI demonstrated homology to fragments lying elsewhere in the invertible genome region as expected.

Earlier restriction endonuclease analyses and electron microscopic investigations succeeded in defining the size of the VZV genome and hinted at the presence of inverting and noninverting segments (2, 4–8). The present report sheds additional light on the genomic organization. By a combination of methodologies, we have demonstrated that, similar to the DNAs of other herpesviruses including pseudorabies virus and equine abortion virus (EHV-1) (8, 23, 24), VZV DNA may exist in either of two isomeric forms. The genome contains an invertible terminal segment of about 12.6 MDal that is linked to a noninvertible segment of about 66–68 MDal. The invertible segment is comprised of a unique region of about 5.8 MDal that is bracketed by inverted repeat sequences of approximately 3.4 MDal each. Thus far, we have failed to uncover evidence for the invertibility of the longer genome segment. In addition, it is as yet uncertain whether small terminal redundancies exist in VZV DNA as they do in other herpesviruses.

The organization of the VZV genome defined by the present studies agrees very well with that proposed by Dumas *et al.* (25) while this manuscript was being completed. Her group succeeded in developing VZV DNA cleavage maps for enzymes *Bgl* II, *Pst* I, and *Xba* I using a cross-blot-hybridization system. Their map places *Bgl* II fragments C, E, G, and J in the invertible genome segment. Our data suggest a similar arrangement, but with *Bgl* II D rather than the slightly smaller-sized fragment E for strain Ellen DNA. The electron microscopic analyses of the invertible segment reported by Dumas *et al.* (25) are consistent with the general structure we observe.

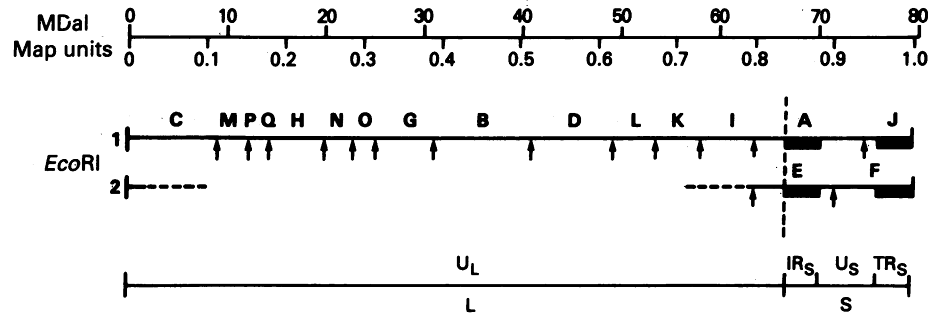

FIG. 5. The physical map of the two isomeric forms of VZV DNA cleaved by *Eco*RI. The drawing shows that the genome consists of a long (L) segment of ≈67 MDal covalently linked to a short (S) segment of ≈12.6 MDal. We have no current data to suggest that the genome contains inverted terminal repetitions, so that all of the long segment represents unique sequences (U_L). The short segment is composed of a 5.8-MDal unique sequence (U_S) that is bracketed by 3.4-MDal inverted repeat sequences in terminal (TR_S) and internal positions (IR_S).

Completion of an endonuclease cleavage map for the VZV genome helps to clarify a number of earlier observations. First, in some VZV DNA enzyme-digest profiles, there appeared to be fragments present in mole ratios of 0.8–0.9—for example, *Eco*RI fragments B and G. The physical map of the genome places these fragments in the center of the long unique segment. Corresponding fragments in other digests (*Bam*HI and *Bgl* II; unpublished observations) seem to be similarly underrepresented. Sequence rearrangements and deletions appear to be common in this region of the genome. Several recombinants were shown to contain aberrant VZV DNA fragments homologous to *Eco*RI B or G, or both.

Another observation that is now better explained with the physical map of the genome is the variability of restriction profiles among different clinical isolates (5, 7). In preliminary studies, we have examined the cleavage pattern of 10 different VZV isolates. Most of the differences in the cleavage patterns appear to derive from changes in the invertible segment and in the midportion of the long segment of the genome (unpublished observations).

One perplexing observation that questioned aspects of the physical map proposed here has been the cloning of terminal fragment *Eco*RI C. In general, the terminal fragments of a linear genome resist cloning because endonuclease cleavage does not produce the proper terminal overlapping base sequence required for successful ligation to the vector DNA. We believe that fragment C may be cloneable for either of two reasons. First, some portion of each pool of VZV DNA molecules is circular (5). If an *Eco*RI cleavage site exists in the circular form of the genome in the neighborhood of the junction of fragments C and J or F, all of the "terminal" fragments could be cloneable. Alternatively, because we have failed thus far to clone terminal fragments F and J, it is possible that *Eco*RI C lies internal to a very small, terminal fragment that we fail to observe in our gels.

The preparation of a library of DNA recombinants that contain sequences homologous to 94.7% of the VZV genome (4.2 MDal of the unique short sequence has not yet been cloned) has now made it easier to map viral mutants and transcripts and to explore further the important question of viral latency in human tissues.

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